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CELL ACTIVATION THROUGH THE AGGREGATION OF CELL SURFACE RECEPTORS. Byron Goldstein, Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM 87545.

Receptors diffusing over the surface of a cell allow the cell to sense its environment and respond to it. Often the aggregation of these receptors is crucial to the triggering of cellular responses. The model system we use to study ligand induces receptor aggregation consists of bivalent haptens that reversibly bind to monoclonal antibodies (IgE) anchored to high affinity Fc receptors on the surface of rat basophilic leukemia (RBL) cells. The formation of IgE aggregates on RBL cells triggers numerous cellular responses (calcium influx, degranulation, etc), but not all aggregates trigger all responses. We briefly review a mathematical model that is used to predict the time course of IgE aggregate formation. We then show how the model predictions are used to help understand the role IgE aggregation plays in initiating cellular responses.

Poster Sessions: Sperm and Spermatogenesis II (577-580)

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Bacterially Expressed Abalone Sperm Lysin Shows Species Specificity. T.A. Critchlow and V.D. Vacquier, 0202 UCSD, La Jolla, Ca 92093-0202.

Sperm of the abalone, Haliotis, have a large acrosomal granule containing two major proteins that are released by the acrosome reaction. One of these is lysin(16,000 MW) that dissolves the egg vitelline envelope(VE) by a stoichiometric mechanism. Lysin exhibits species specificity; lysin from the red abalone will not dissolve pink abalone VE. A bacterial expression system was developed using the pETlld vector. An additional Met-Gly were inserted before the amino-terminal arginine. Following induction with IPTG, lysin is expressed in inlusion bodies and comprises approximately one percent of the total bacterial protein. Recombinant lysin can be dissolved in 5M guanidine-HCl and then renatured by dilution. When isolated egg VE's are treated with the recombinant lysin only VE's, of the same species as the lysin are dissolved. This confirms the earlier work that lysin is, by itself, sufficient to dissolve isolated egg VE. Development of this expression system will allow analysis of the residues responsible for the species specificity of abalone sperm lysin.

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Further Studies of a Putative Sperm Membrane Protein Required for Sperm Differentiation in C. elegans. P. Michele Arduengo, O.K. Appleberry and S. W. L'Hernault, Dept. of Biology, Emory University, Atlanta, GA 30322

Spermatogenesis in the nematode C. elegans uses unusual organelles, called the fibrous body-membranous organelle (FB-MO) complexes to prepackage and deliver macromolecules to spermatids during their formation. Mutations in spe-4 disrupt FB-MO's and cytokinesis, and consequently, spe-4 mutants arrest morphogenesis as terminal spermatocytes that contain four haploid nuclei; spermatids never form. Microinjection of a plasmid containing the putative spe-4 gene was used to create fertile, transgenic worms and confirm that we have cloned this gene. The spe-4 gene transcribes a 1.5 kb spermatogenesis-specific mRNA, and the gene sequence suggests that it encodes a 465 a.a. integral membrane protein. This suggests that maturation of the FB-MO complexes is required in order to form spermatids, and mutation of spe-4 disrupts these complexes. However, the spe-4 encoded protein might also reside within other sperm membranes. We hope to soon have antibodies to the spe-4 encoded protein. So far, we have cloned a near full-length gene fusion in either pET or pGEX, which are inducible expression vector systems. Unfortunately, expression of either of these constructs appears to be lethal for bacteria, as is commonly seen for integral membrane proteins. We have recently cloned a hydrophilic region (lacking putative transmembrane spanning regions) of spe-4 protein into pGEX, and this fusion expresses well. Immunofluorescence and E.M. localization of gold-labeled Ab's should reveal the position of spe-4 in sperm.

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An Insertional Mutation Sheding Light on the Role of the Manchette and Acrosome on Head Shaping in Mouse Spermiogenesis. L. D. Russell, P. A. Overbeek* and Li Ying, Department of Physiology, Southern Illinois University, Carbondale IL. 62901 and *Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX 77030

The transgenic family OVE 219, generated by microinjection of the Ty811C tyrosinase minigene, has a single multicopy integration site. Transgenic mice in this family are all pigmented but coat color intensity is highly variable. All homozygous males are infertile, indicating that integration of the transgene has caused a recessive insertional mutation. Histological evaluation of the sterile males has revealed two specific testis phenotypes in which spermiogenesis is altered. *In the first testis phenotype*, there is incomplete spread of the acrosome over the spermatid head in spermiogeneais (steps 4-8). The nuclear envelope is modified in the usual maner (Tiss. & Cell 13:615), although in many areas the acrosome does not overlie the modified nuclear envelope. Thus, acrosomal development is not required for modification of the nuclear envelope. The manchette, although sometimes ectopic, generally orginates near the modified nuclear envelope and not the edge of the underdeveloped acrosome, indicating that the topography of manchette origination is related to the modified nuclear envelope and not the develpment of an acrosome. *In the second testis phenotype*, there is a flagellar abnormality. Most spermatid heads are elogated but abnormally shaped. Manchettes are often ectopic. In a minority of spermatids, *no* manchettes are formed. In these spermatids the caudal head does not elongate and remains rounded indicating that the influence of the manchette on elogation and shaping of the caudal sperm head has been lost. In these same spermatids, the anterior head, covered by the acrosome is distorted from a rounded shape, implicating the acrosome and/or the ectoplasmic specialization of the Sertoli cell in anterior sperm head shaping. These data are consistent with the role recently proposed for the manchette in elongation and shaping of the caudal sperm head (Am. J. Anat. 92:97-120). Supported by NIH HD25340.

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Expression of Boar Proacrosin in Transgenic Mice Disrupts Spermatogenesis. D.A. O'Brien,[1,2] J.E. Welch,[2] E.H. Goulding,[2] A.A. Taylor, Jr.,[2] T. Baba,[3] N.B. Hecht,[4] and E.M. Eddy,[2] [1]Laboratories for Reproductive Biology, University of North Carolina, Chapel Hill, NC; [2]Gamete Biology Section, NIEHS, Research Triangle Park, NC; [3]Institute of Applied Biochemistry, University of Tsukuba, Tsukuba, Japan; [4]Department of Biology, Tufts University, Medford, MA.

A 2.3 kb transegne was constructed by ligating the mouse protamine 2 promoter (859 bases of 5' flanking region, Stewart et al., 1988, Mol Cell Biol 8:1748) to the cDNA for boar proacrosin (Baba et al., 1989, J Biol Chem 264:11920). Six founder mice that incorporated the transgene were identified by PCR and Southern blot analysis. The two male founders (Ac.2 and Ac.5) and male progeny from three female founders (Ac.3, Ac.4, Ac.6) expressed transegne mRNA as determined by Northern blot analysis of testicular poly (A)$^+$ RNA. Spermatids in these mice synthesized boar proacrosin, as determined by immunohistochemistry with an affinity-purified antibody to boar acrosin. Both male founders and all Ac.4 and Ac.6 males were infertile, assayed by multiple matings for at least two months. Ac.3 males were either infertile or rarely transmitted the transegne to their offspring. Only one transgene-positive mouse has been produced in a total of 124 offspring from three fertile Ac.3 males mated with wild-type females. Although the infertile Ac.3, Ac.4 and Ac.6 males produced copulatory plugs and had normal seminal vesicle weights, they had lower testis weights and significantly fewer epididymal sperm compared to controls. Histological examination indicated that the testes of mice in these lines and the infertile male founders contained many seminiferous tubules which were vacuolated and disorganized. Although early spermatogenesis appeared to proceed normally, condensing spermatids were missing from many tubules. Numerous round cells and cytoplasts were seen in the lumen of the epididymis of these mice. We hypothesize that boar proacrosin may be actived in these transgenic mice and that its proteolytic activity disrupts spermatogenesis during spermatid formation.

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Chromosome Pairing and Transcription in Cultured Pachytene Spermatocytes T. Wiltshire, K.A. Caldwell and M.A. Handel; Graduate Program in Cellular, Molecular and Developmental Biology and Department of Zoology, Univ. of Tennessee, Knoxville, TN, 37996,

In order to understand both pairing of homologous chromosomes and the maintenance of pairing during meiotic prophase, it will be advantageous to have a system that permits experimental manipulation of meiocytes. We have assessed parameters of chromosome pairing and transcription in short-term cultures of pachytene spermatocytes of the mouse. Germ cells were isolated from adult testes after enzymatic digestion and pachytene spermatocytes enriched by sedimentation on BSA gradients. Cells were cultured at 32°C in MEM supplemented with 5% fetal bovine serum, lactate and pyruvate. Cell viability was assessed by trypan blue dye exclusion; there was greater than 90% viability through the first 24 hours of culture. Cells that had been spread on a hypophase for visualization of synaptonemal complexes were used to determine meiotic prophase substages, by the criteria of Moses (1980). The distribution of cells in substages at the time of enrichment closely reflected the distribution in the total germ cell population and the distribution of cells across substages during the first 24 hours of culture was that predicted given the estimated duration of each substage. During this period, the cells maintained typical pachytene nuclear morphology, chromatin condensation patterns, and chromosome pairing as assessed by light and electron microscopy. Uridine incorporation was linear in this time period and, as monitored by autoradiography, reflected *in vivo* spatial distribution patterns. Grains were located over the axes of autosomal chromosomes but not over the sex chromatin domain of the nucleus. Thus features of chromosome pairing and sex chromatin inactivation are maintained in culture. (Supported by a University of Tennessee Faculty Research Award to MAH).

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Molecular Cloning and Expression of mRNAs Encoding Proteins of Perforatorium, Outer Dense Fibers and Fibrous Sheath of Rat Spermatozoa. R. Oko, and C. Morales, Department of Anatomy and Cell Biology, McGill University, Montreal, Canada.

We have screened a testicular lambda-gt 11 phage cDNA library with three affinity purified polyclonal antibodies to the major 16 kDa perforatorial polypeptide (P16) of the sperm head and to the major 27 kDa outer dense fiber (O27) and 75 kDa fibrous sheath (F27) polypeptides of the sperm tail. The respective cDNAs from positive clones were cleaved with EcoR 1, and then isolated and purified on agarose gels. The cDNA inserts, ranging between 0.7-1.0 kb, were radiolabeled by nick translation and hybridized to equivalent concentrations of total testicular RNA from 10-45 days old rats. The Northern blot analysis revealed a single mRNA band of 1.0 kb for P16, two bands of 1.3 kb and 1.6 kb for O27 and a single mRNA band of 1.7 for F75. The mRNAs were first detected in testes of 25 (P16) and 30 (O27 & F75) day old rats with an increase in corresponding mRNA concentrations after day 30. These data suggest that the proteins are developmentally expressed during spermiogenesis and that the appearance of the O27 and F75 mRNAs corresponds to the round spermatid stage whereas the P16 mRNA may correspond to a slightly earlier stage of development. (Supported by NSERC, MRC of Canada and WHO).

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Distribution of snRNPs in Germ Cells During the Cycle of the Seminiferous Epithelium of the Rat. F. Mousse, R. Oko, and L. Hermo, Department of Anatomy, McGill University, Montreal, Canada. (Spon. by P. Walton.)

Anti-Sm antibodies from the serum of patients with Systemic Lupus Erythematosus and monoclonal anti-Y12 antibodies recognize a 6 protein core common to U1, U2, U4/U6 and U/5 small nuclear ribonucleoprotein particles (snRNPs). These snRNPs are essential for splicing mRNA precursors. LM immunoperoxidase staining, with both antibodies on paraffin embedded rat testicular sections and with an anti-U1 antibody on frozen LM sections, was found predominantly in the nuclei of germ cells up to step 10 spermatids. The absence of immunoreactivity in spermatids after step 11 is concomitant with nuclear condensation and transcriptional arrest (Monesi, 1964). Cytoplasmic reactivity in pachytene spermatocytes increased progressively from stage VII to reach a peak at stage XII, coincident with maximal rates of RNA synthesis in these cells. Cytoplasmic reactivity in spermatids was concentrated over the chromatoid body (CB). EM immunogold labeling performed on Lowicryl and freeze-substituted testes confirmed the LM observations. Immunogold particles arranged as speckles accumulated over the condensed nuclear chromatin. The CB of mid and late spermatocytes and spermatids (steps 1-14) were intensely labeled over the dense fibrillar regions. These results indicate that at the time of nuclear condensation, snRNPs are lost from the nucleus but are still abundant in the CB of spermatids. Because protein synthesis persists after nuclear condensation, it is possible that the snRNPs in the CB may replace some mRNA biosynthetic activity normally found in the nucleus. (Supported by NSERC, MRC, FCAR of Canada.)

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Both Retinoic Acid and Retinoid X Receptors are Expressed in Rat Testis: cDNA Cloning of Rat Retinoic Acid Receptor-α and -γ. K.M. Akmal and K.H. Kim, Department of Genetics and Cell Biology, Department of Biochemistry and Biophysics, Washington State University, Pullman, WA 99164.

Vitamin A is essential for the development of germ cells during spermatogenesis. Although the mechanism of vitamin A action on the testis remains unknown, it is postulated that the effects of vitamin A on spermatogenesis may be mediated by two nuclear retinoid receptor families, retinoic acid receptors (RAR-α, -β and -γ) and retinoid X receptors (RXR-α, -β and -γ). These receptors may act as heterodimeric transcription factors in the regulation of gene expression. To determine whether the newly discovered RXR-α is expressed in testis and to study in depth the expression of RARs, we performed Northern blot analyses using either human cDNAs or rat cDNAs encoding various receptors. We found that RXR-α is detected exclusively in Sertoli cells with a transcript size larger than the 4.8 kb reported in other tissues. Further studies of the RARs required rat-specific probes, so the rat cDNAs for retinoic acid receptor-α and -γ were isolated from a testis cDNA library. Analysis of RAR-γ transcripts using the rat cDNA revealed two Sertoli cell-specific mRNAs, the previously detected 3.4 kb and a second transcript of apparent 5.0 kb size. In addition, we confirmed using rat cDNA in Northern blot analysis that RAR-α is present in both Sertoli cells and germ cells. In situ hybridization utilizing cRNA probe derived from the rat cDNA verified the stage-specific expression of RAR-α in stage VIII of the spermatogenic cycle. Previously, we have shown that RAR-β is detected only in Sertoli cells. These results indicate that RAR-α, -β, -γ and RXR-α are utilized in the mediation of retinol action on spermatogenesis (supported by grant HD25094).

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Expression of LINE-1 RNA and Protein in Mouse Testis. D. Branciforte and S.L. Martin, Department of Cellular and Structural Biology and Program in Cell and Developmental Biology, University of Colorado School of Medicine, Denver, CO 80262.

L1 is a moderately repetitive DNA family that is interspersed throughout mammalian genomes, presumably by a duplicative transposition mechanism that involves an RNA intermediate. The gene(s) encoding the active transposable L1 in the mouse is at least 6.5 kb in length and contains two long open reading frames, ORF 1 and ORF 2. The protein products of these ORFs are thought to be involved in the process of transposition, acting on a full-length, sense-strand RNA transcript. Transposition must occur in germ cells or in early embryos, hence, we have examined the expression of L1 RNA and protein in the testis. Sense-strand transcripts of L1 RNA are readily detectable on Northern blots of total RNA prepared from testis. This RNA is found mainly in the round spermatid fraction when the cells of the testis are dissociated and separated in BSA gradients. ORF 1 protein is also detectable on Western blots of testis protein extracts. Immunolocalization studies on paraffin sections of the testis reveals a cytoplasmic structure in round spermatids that stains intensely with the anti-ORF 1 antibody. Results of efforts to co-localize this structure with known structures in germ cells will be discussed.

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Amplification and Sequence Comparison of the Human and Rhesus Monkey Testicular Histone H1t Genes Using PCR. D.A. Koppel, S.A. Wolfe, L. Fogelfeld, and S.R. Grimes, Research Service, VA Medical Center, Department of Biochemistry and Molecular Biology, and Department of Medicine, LSU Medical Center, Shreveport, LA 71101.

The testis-specific H1t gene is transcribed exclusively in pachytene primary spermatocytes during spermatogenesis. Studies of the rat H1t gene revealed a unique sequence element between the SP1 element and the H1/CCAAT box. We have shown that proteins in crude nuclear extracts of rat testis bind specifically to this unique sequence element. Furthermore, there is a temporal correlation between the appearance of these DNA binding proteins and the onset of transcription. These discoveries led us to initiate a search for the H1t gene in other species. In this report we present a method for amplification of the human and monkey H1t genes from genomic DNA using the polymerase chain reaction. The amplified genes were cloned and the plasmid clones were sequenced using linear PCR. A comparison of the two genes revealed a 90% homology between the predicted amino acid sequences. Both proximal promoters contained all four of the highly conserved sequence elements found in nongerminal H1 genes. These included the H1/AC box, SP1 element, H1/CCAAT box, and TATA box. The promoters also contained the unique sequence element between the SP1 and H1/CCAAT box previously seen only in other H1t promoters. These findings reveal that both the coding regions and promoters of the human and monkey H1t genes are highly conserved.

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Evolution of Protamine P2 genes in Primates. J.D.Retief and G.H.Dixon, Department of Medical Biochemistry, University of Calgary, Calgary, T2N 4N1, Alberta, Canada.

The sperm protamine P2 genes comprise a rapidly diverging gene family with very high nucleotide substitution rates. These genes present a good opportunity to examine recent evolutionary events, such as the divergence of the primates. The P2 protamine genes of the common chimp(Pan troglodytes), pygmy chimp(Pan pygmaeus), gorilla(Gorilla gorilla), oran-utan(Pan paniscus), gibbon(Hylobates lar), red howler(Alouatta seniculus) and pigtailed macaque(Macaca nemistrina) have been sequenced, using PCR amplification and direct sequencing, and compared with the known human P2 sequence(Domenjoud et al. Genomics **8** pp127-133 (1990)). The sequenced genes are clearly orthologous, with a high degree of homology and maintenance of the overall gene structure. The genes are all approximately 473 bases long with a single intron of approximately 162 bases, the junctions between the introns and the exon being particularly well conserved. An 80 base sequence, 3' of the gene, is repeated in all the primates except the gorilla and the red howler, where it only occurs once. Parsimony and distance trees of the nucleotide and deduced protein sequences confirm the close evolutionary relationships between the humans, gorillas and chimps.
(Supported by M.R.C. of Canada)

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Identification of novel RNA-binding proteins which interacts with the coding region of protein D sense RNA In Vitro. N.G. Reddy, J.C. Hall, J.G. Kochins, and E.M. Perez, Department of Molecular & Cell Biology, Biochemistry Program, The Pennsylvania State University, University Park, PA.

In a previous study (Hall, J.C and Reddy, N.G. Biochem. Biophys. Res. Comm. 183: 1109-1116, 1992), we demonstrated that the level of expression for corpus protein D mRNA was significantly reduced (>82%) when compared to that of the caput and cauda regions of the rat epididymis. We postulated that the reduced level of expression of corpus mRNA for protein D was the result of insufficient primer binding to the mRNA. In the present study, we provide evidence that the reduced expression of corpus mRNA for protein D may be due to proteins that bind at or near the primer binding site. Ultraviolet (UV)-cross-linking and sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis were used to identify proteins of nulcear and cytosolic (S100) origin that specifically bind to an *in vitro* transcribed mRNA sequence for protein D. The coding region of the protein D cDNA was subcloned, *in vitro* transcribed to [^{32}P]RNA, and incubated with proteins of nuclear and cytosolic (S100) origin from extracts of enzymatically dispersed epididymal epithelial cells. These procedures identified two proteins, having a molecular weight mass of ~2.5 and ~35 Kd, respectively. These proteins specifically recognize and bind tightly to the *in vitro* transcribed mRNA sequence. Our finding suggest that the regulation of protein D gene expression in the rat epididymis may involve novel RNA-binding proteins. This work was supported by NSF grant # DCB-871441 (awarded to Dr. Hall). Dr. Hall is a recent (1991) recipient of a Presidential Young Investigator Award (PYI) from NSF.

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Characterization of Specific Retinol-regulated Transcripts during Regeneration of Rat Spermatogenesis. Z.Q. Wang and K.H. Kim, Department of Genetics and Cell Biology and Department of Biochemistry and Biophysics, Washington State University, Pullman, WA. 99164. (Spon. by C.C. Linder).

Avitaminosis in male rats arrests spermatogenesis leading to a complete loss of advanced germ cells. Replenishment of retinol into these vitamin A-deficient (VAD) rats regenerates synchronized spermatogenesis mostly from the type A1 spermatogonia. We have shown in previous studies that the type A1 spermatogonia undergo mitosis within 12 hours after retinol injection indicating a VAD block at the S/G2 phase transition of the cell cycle. To characterize the mechanisms by which retinol releases the block and synchronizes spermatogenesis, we studied two types of transcripts that may have potential roles in the reinitiation of spermatogenesis in VAD rats. Polymerase chain reaction (PCR) was utilized on a testis mRNA to amplify two fragments using primers derived from the conserved regions of known cdc25 sequences which encode tyrosine phosphatases or cdc2 kinase sequences. Northern blot analyses using the cdc25 fragment revealed a 1.8 kb and two smaller transcripts, distinct from the known 2.7 kb to 3.0 kb cdc25 transcripts. The two lower transcripts increased in a time-dependent manner, whereas the 1.8 kb transcript seemed to be cycling after retinol injection into VAD rats. In addition, we isolated a 2.3 kb cDNA from a rat testis library using the cdc2 PCR fragment. Preliminary analyses of this cDNA indicate that it encodes a potential protein kinase, but not cdc2 kinase. Northern blot analyses detected a 2.6 kb and a 3.4 kb transcript, in both stage-synchronized and VAD rat testes. They decreased after retinol replenishment of VAD rats and the 2.6 kb transcript cycled, increasing after 72 hrs (supported by grant HD25094).

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Elevated steady-state levels of c-*fos* mRNA in isolated spermatogenic cells after treatment with fetal bovine serum or phorbol ester. J. K. Tsuruta and D. A. O'Brien, The Laboratories for Reproductive Biology, Departments of Pediatrics and Cell Biology and Anatomy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

Activation of receptor-mediated signal transduction pathways often results in an increase in the transcription of the immediate-response gene c-*fos*. In order to investigate the possibility that receptor-mediated signal transduction plays a role in the differentiation of spermatogenic cells, c-*fos* mRNA levels were examined in intact mouse testes and cultured germ cells. In contrast to intact testes, germ cells contained significant levels of c-*fos* mRNA immediately after isolation from adult testes by enzymatic dissociation with collagenase and trypsin. C-*fos* mRNA levels returned to baseline levels after 5 hours of culture at 32°C in Eagle's MEM supplemented with 1 mM pyruvate and 6 mM lactate. Germ cells were then treated with either 10% fetal bovine serum (FBS) or with 1 μM phorbol 12-myristate 13-acetate (TPA). Steady-state levels of c-*fos* mRNA were increased by treatment with FBS or TPA and were maximal after 60 minutes of treatment. Cultured Sertoli cells also responded to treatment with FBS or TPA by increased levels of c-*fos* mRNA. In contrast to germ cells, the maximal c-*fos* mRNA levels in Sertoli cells occured after 30 minutes of treatment. The ability of isolated germ cells to respond to FBS or TPA treatment by increased steady-state levels of c-*fos* mRNA is initial evidence supporting the hypothesis that signal transduction pathways may be involved in regulating the development of spermatogenic cells.

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Mouse Male Germ Cells Contain Proteins Homologous to Germ Cell-Specific RNA- and DNA-Binding Proteins of Xenopus Oocytes. Y.K. Kwon[1], M.T. Murray[2], B.S. Nikolajczyk[1] and N.B. Hecht[1], Dept. of Biology, Tufts University, Medford MA 02155[1] and German Cancer Research Center, Heidelberg, Germany[2].

During male germ cell differentiation, the cessation of transcription in post-meiotic spermatogenic cells necessitates storage of many mRNAs encoding proteins expressed late in spermatogenesis. Here we report that antiserum against two cytoplasmic mRNA-binding proteins of Xenopus oocytes (p54/56; mRNPs 3 & 4) or antiserum against FRG Y2, a Xenopus sequence-specific DNA-binding protein, recognize two RNA-binding proteins present in mouse testis cytoplasm. Immunologically similar proteins are recognized in testis nuclear extracts. The mouse proteins, with estimated molecular weights of 48 and 52 kDa, form RNA-protein complexes with either translationally regulated or non-expressed RNAs indicating they are sequence independent RNA-binding proteins. Heparin reduces the RNA protein binding *in vitro*. The expression of the 48 and 52 kDa mouse proteins appears to be germ cell-specific and developmentally regulated in the testis. The highest levels of the two proteins are detected in round spermatids (where many mRNAs are stored) and in the non-polysomal mRNP fractions of testis extracts. In elongating spermatids (where many stored mRNAs are translated), the 48 and 52 kDa proteins do not bind RNA in Northwestern blots. We conclude that stored mRNPs in male germ cells contain two sequence independent RNA-binding proteins that appear to be homologues of Xenopus female germ cell-specific RNA and DNA-binding proteins.

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Endogenous Nicks in Elongating Spermatid DNA: Involvement of DNA Topoisomerase II and Protamine. S.M. McPherson and F.J. Longo, Department of Anatomy, University of Iowa, Iowa City, IA 52242.

Chromatin of rat elongating spermatids (steps 12, 13) is distinguished by the replacement of histones with transition proteins and by the presence of endogenous nicks within its DNA, which are formed by an endogenous nuclease, possibly DNA topoisomerase II (topo II). Topo II was localized in rat testicular cells using an affinity purified antibody, which recognized a ~187 kDa protein in pachytene spermatocytes, while in round and elongating spermatids, it reacted with a ~150 kDa protein. In cryosections of the testis, topo II was localized to the meiotic chromosomes of pachytene spermatocytes, and to nuclei of elongating spermatids. Extracts prepared from isolated testicular nuclei and from sonication resistant spermatid nuclei (steps 12-19) demonstrated topo II activity as determined by the decatenation of kinetoplast DNA. The potential relation between nucleoprotein changes during spermatogenesis and the formation of DNA nicks was also examined. Heterogeneous testicular and sonication resistant spermatid nuclei were treated with protamine, followed by nick translation in the absence of DNase I. In both cases incubation with protamine resulted in a dramatic decrease in DNA polymerase I dependent label incorporation when compared to nuclei incubated with BSA or buffer alone. Since association of protamine masked endogenous nicks in elongating spermatids, it was possible that endogenous nicks were present in mature sperm, but were inaccessible due to protamine-DNA interactions. Epididymal sperm were extracted with high salt-DTT, followed by nick translation in the absence or presence of DNase I. Extracted sperm nuclei did not nick translate in the absence of DNase I, however, incorporation increased with increasing concentrations of DNase I, indicating that endogenous nicks were repaired prior to the completion of spermiogenesis. These results suggest that a novel, spermatid-specific, topo II may alter elongating spermatid DNA, permitting chromatin rearrangements characteristic of spermatogenesis. The temporal appearance and disappearance of endogenous nicks may reflect changes that elongating spermatid DNA must undergo as a consequence of alterations in nucleoprotein composition to establish the condensed state of the mature sperm.

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Loss of ß-COP and ER Markers During Spermiogenesis Correlates with Golgi Apparatus Unstacking but Retention of Active Saccular Elements in Cytoplasmic Droplets of Rat Spermatozoa. L. Hermo, R. Oko, P. Chan, F. Moussa, A. Fazel, and J.J.M. Bergeron, Department of Anatomy and Cell Biology, McGill University, Montreal, Canada.

During spermiogenesis LM immunocytochemistry revealed the loss of ß-COP in step 17 spermatids. At the EM level this loss coincided with the unstacking of the saccules of the Golgi apparatus and their eventual translocation into cytoplasmic droplets of late step 19 spermatids just before spermiation. The saccular elements within the cytoplasmic droplet were loosely arranged and often in close proximity to the plasmalemma of spermatozoa during epididymal transit. LM and EM immunolabeling of caput epididymal spermatozoa identified sialyl transferase, galactosyl transferase and TGN38 exclusively within these elements while antibodies to specific ER markers showed no immunoreactivity. The saccular elements were isolated and found to be enriched in sialyl and galactosyl transferase activities. They were also found to be capable of glycosylating endogenous glycoprotein substrates from CMP^{3}H-NeuAc or UDP^{3}H-gal in the absence of detergent. Lectin labeling (RCA I, HPL) of epididymal spermatozoa revealed a high concentration of their cognate ligands over these saccular elements. We propose that the cytoplasmic droplet of spermatozoa contains a developmentally modified Golgi apparatus. Saccular unstacking was correlated with the loss of ß-COP. Intermittent associations with the plasmalemma may lead to its modification and sperm maturation. (Supported by MRC of Canada and NSERC.)

Mammalian Development (594-597)

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Characterization of the Unusually Rapid Cell Cycles during Rodent Gastrulation. A. MacAuley[1], Z. Werb[1] and P. Mirkes[2]. [1]Lab. of Radiobiology, University of California, San Francisco, CA 94143, and [2]Dept. of Pediatrics, University of Washington, Seattle, WA 98195.

Most adult cells regulate their growth exclusively in G1. In Drosophila and Xenopus G1, S and G2 are regulated by developmental programs during early embryogenesis. Developmental regulation of S and G2 has not been demonstrated in mammals. The early cleavages of mammalian embryos are relatively slow in comparison to those of Drosophila and Zenopus. The onset of gastrulation in rodents has been associated with shortening the cell cycle to 7-7.5 hr. One study suggested that regional variations in cell cycle length in the embryo proper appear at gastrulation. To examine the cell cycle during rodent gastrulation, rat embryos were explanted into culture shortly after the onset of gastrulation (d8.5 of gestation) and the cell cycle structure determined in the embryonic ectoderm and mesoderm. The data support the idea that there are at least two types of cell cycle in the embryo. The cycle time for cells of the primitive streak was 3-3.5 hr, while the cycle for mesodermal and ectodermal cells was 7-7.5 hr. The cell cycle structure appears to be:

	G1	S	G2
primitive	0 hr?	2.5-3 hr	≤20 min
ecto- and mesoderm	1.5-2 hr	3-3.5 hr	≤40 min

S=DNA synthesis; G1 and G2=growth intervals; M=mitosis=0.75 hr (assumed) The profound alterations in the cell cycle from that of adult cells show that all phases of the cell cycle are subject to developmental regulation in rodents. The rapidity of the cell cycles suggests that normal G1 and G2 regulatory events are substantially modified. Experiments are underway to test for the presence and robustness of cell cycle checkpoints during rodent gastrulation. Supported by NIH grants HD 26732 (Z.W.), HD 16287 and HD 22095 (P.M.), and DE-AC03-76-SF01012 (Z.W.).

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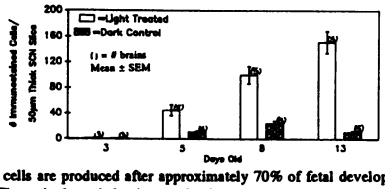
Development of Light Induction of Fos in the Hamster Suprachiasmatic Nucleus. C.M. Kaufman and M. Menaker. NSF Center for Biological Timing, Dept. of Biology, Univ. of Virginia, Charlottesville, VA 22901.

The immediate early gene *c-fos* is transcribed in response to light pulses given at those phases in the rodent circadian cycle at which light shift the locomotor activity rhythm. Within 30 minutes following such pulses, Fos protein assayed by immunocytochemistry is highly concentrated in the hypothalamic suprachiasmatic nucleus (SCN) -- the locus of the mammalian circadian pacemaker. Fos may or may not be causally related to the light-induced phase shifts in circadian rhythms; nevertheless, it is clearly a robust marker of light input to the clock. In an ongoing investigation of this input pathway we examined the ontogeny of light-induced Fos in the SCN of the golden hamster. Fos could be induced in the SCN at postnatal day 5 (10 days before the eyelids open) by 1-3hr light pulses beginning 3 hours after lights off in 14hr light:10hr dark cycle, but not at 3 days postnatal.



Rodent SCN cells are produced after approximately 70% of fetal development is completed. The retinohypothalamic tract begins connecting the retina and SCN a day before birth in the rat. The ability of light to induce Fos in the SCN occurs nearly one week prior to light-inducible changes in pineal melatonin synthesis, an output pathway of the SCN. Our results suggest input pathways to the clock may become functional well before output pathways.

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Gap Junction Formation in the Fetal Epidermis. B. Risek, F.G. Klier, and N.B. Gilula, Department of Cell Biology, The Scripps Research Institute, La Jolla, CA 92037.

During early fetal development (embryonic period) the mammalian epidermis is composed of two cell layers, a basal germinative layer and a superficial layer, the periderm. The intermediate cell layers appear between the periderm and the basal layer with advancing gestation (epidermal stratification), which subsequently differentiate into spinous and granular layers (epidermal keratinization). In order to understand the role of gap junctions (GJs) during epidermal development, fetal rat skin was analysed by ultrastructure and immunohistochemistry at different prenatal stages. During the embryonic period (E12-E14), both basal and periderm layers coexpressed α_1 (Cx43) and β_2 (Cx26) GJ proteins. A developmentally specific down-regulation of β_2 occured in the periderm during the period of epidermal stratification (E14-E16). At the same time, the coexpression of α_1 and β_2 was high in the intermediate and basal layer. Ultrastructurally, GJs were identified between the apical border of peridermal cells, at the periderm/basal layer interface and between the basal cells. Junctional size (as measured by length in thin-sections or areas in freeze-fracture) varied from very small plaques (0.05 µm²) in the periderm, medium size (1 µm²) in the intermediate layer, to very large plaques (25 µm²) in the basal layer. Also, periderm GJs were interspersed with desmosomes, while no desmosomes were observed in the basal layer. The coexpression of α_1 and β_2, as well as the number and size of GJs in all epidermal layers, suggests a large communication compartment in developing skin. (Supported by NIH Grant GM 37904 and J&J Grant J-188.)

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Developmental Expression and Targeted Gene Inactivation of an Epithelial Cell-Surface Mucin, Muc-1. V.M.M. Braga, A.P. Spicer, L. Pemberton, S.J. Gendler. Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, U.K.

The Muc-1 mucin is a highly glycosylated integral membrane glycoprotein which is expressed at the apical surfaces of glandular epithelia. To provide insight into possible functions, we have assessed the timing of expression and distribution of the Muc-1 protein during mouse embryogenesis using RT-PCR, northern blots and immunohistochemistry. Our results indicate that Muc-1 expression correlates with epithelial differentiation in stomach, pancreas, lung, trachea, kidney and salivary glands. Although Muc-1 is present in different organs, its expression is not induced systemically, but according to the particular onset of epithelial polarization and branching morphogenesis of each individual organ. There is pronounced polarity in the subcellular distribution, with the protein detected only on membranes of cells lining lumens. We speculate that Muc-1 may participate in epithelial sheet differentiation/lumen formation during early development of the organs known to express it. This speculation is based on: (1) the detection of Muc-1 expression early during organogenesis, (2) the defined apical localization in different epithelia, (3) the decrease in cell-cell interactions when Muc-1 protein is highly expressed and (4) the interaction of its cytoplasmic tail with the actin cytoskeleton. To test our hypothesis and gain direct information about the function of Muc-1, we have disrupted the gene in embryonic stem cells using homologous recombination and are presently generating mice with decreased and no Muc-1 expression. These mice should enable us to delineate more precisely the function of this molecule during development and differentiation.

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Expression of the Paired-Box Containing Gene HuP48 in the Developing Human Vertebral Column. C. A. Smith and R. S. Tuan, Dept. of Biochemistry, Thomas Jefferson University, Philadelphia, PA. 19107

Vertebrate segmentation is first apparent with the formation of the somites, which differentiate into dermamyotome and sclerotome; the sclerotome then resegments to contribute to the axial skeleton. The molecular regulation of segmental development is well characterized in Drosophila, and involves the sequential action of numerous segmentation genes, including the pair-rule genes. These genes contain the highly conserved paired-box sequence. To elucidate the function of putative segmentation genes, we have analyzed the expression of the paired-box containing gene HuP48 (EMBO J. 8:1183, '89) in the fetal human vertebral column. Using *in situ* hybridization we observed that HuP48 is expressed in a segmented pattern in the developing spine; specifically, in the cells of the intervertebral discs of 7-8 week old fetuses. Histologically, the spine at this stage is composed of alternating intervertebral discs and chondrifying pre-vertebrae. HuP48 is expressed in a uniform manner throughout the apparently homogeneous population of mesenchymal cells in the intervertebral discs, while the chondrocytes of the pre-vertebrae do not express HuP48, resulting in a segmented pattern of expression. There is an apparent rostral to caudal gradient of expression of HuP48; being highest in the older, more rostral discs. HuP48 expression at this stage in development appears to be restricted to the vertebral column, since an 8 week old limb with developing digits and cartilage elements shows no positive signal. HuP48 is homologous to the mouse Pax 1 pair-rule gene, which has been shown to be necessary for proper segmentation of the vertebral column. The observed expression pattern of HuP48 suggests that it may play a role in establishing the segmented pattern of the vertebral column, perhaps by helping to establish and/or maintain a border between these two different populations of cells. (Support: NIH HD15822/ OREF/ March of Dimes).

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The Embryonic Pattern of Rat Insulin-Like Growth Factor-I Gene Expression Suggests a Role in Induction and Early Growth of the Liver. R. D. Streck and J. E. Pintar, Department of Anatomy and Cell Biology, Columbia Physicians and Surgeons, New York, NY 10032 (Sponsored by R. Fishman).

The vertebrate liver develops from interactions between cells of distinct lineages: endodermally-derived liver bud cells proliferate and differentiate into the parenchymal liver component under the influence of mesodermally-derived mesenchymal cells within the neighboring septum transversum. LeDouarin ('75, Med. Biol. 53: 427) demonstrated the septum transversum's critical role in this process; chick liver bud cells cocultured with septum transversum proliferated and differentiated into hepatocytes, while liver bud cells cultured alone failed to grow or differentiate. The molecular basis for this tissue interaction is not known. We have used in situ hybridization to test whether this inductive activity correlates with the expression in rat embryos of insulin-like growth factor-I and -II (IGF-I and -II) and/or proteins that may mediate IGF action (IGF binding protein-2 and IGF-I receptor). We found that the onset of expression of the transcript for IGF-I in the septum transversum correlates precisely with this induction. In fact, the septum transversum is the earliest site of IGF-I mRNA expression detectable in the rat embryo by in situ hybridization. LeDouarin also demonstrated that this inductive activity was present in a subset of additional chick mesodermal tissues. In rat embryos we found that the corresponding tissues express IGF-I mRNA. IGF-II mRNA is also expressed in the septum transversum, although its expression begins much earlier and it is found in most other mesodermal tissues as well. Transcripts for two proteins that may mediate IGF action (IGF binding protein-2 and IGF-I receptor) are expressed in liver endodermal cells, consistent with the possibility that the IGFs may act on these cells. Taken together, these results suggest that IGF-I produced in the septum transversum acts in a paracrine fashion to stimulate division of neighboring endodermal cells, thereby triggering the initiation of liver development. Supported by NS 21970.

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Analysis of Glutaminase and Glutamate Dehydrogenase mRNA Expression in Preimplantation Mouse Embryos by RT-PCR. C.L. Chatot and W.Kamper. Biology Department, Ball State University, Muncie, IN 47306.

Glutamine is utilized as an energy substrate in pre-implantation mouse embryos. To study expression of enzymes involved in glutamine metabolism in embryos, RT-PCR was used to detect mRNA for glutaminase (GA) or glutamate dehydrogenase (GDH). RNA was isolated from mouse brain, 1-, 2-, 8-cell and blastocyst embryos grown in vivo and 2-, 4-, 8-16-cell and blastocyst embryos after culture in CZB medium from the 1-cell stage. RNA was reverse transcribed using a 3' antisense primer. Products were amplified by PCR and analyzed by Southern blotting. A GA specific 341 bp band was observed in plasmid control, brain and embryo samples suggesting that mRNA is present even at the 1-cell stage. Densitometric analysis showed that GA RNA that is present at the 1-cell stage in vivo declines at the early 2-cell stage and then continues to rise from the late 2-cell through the blastocyst stage. RNA from cultured embryos demonstratedsimilar increases through the blastocyst stage. Control without RNA or DNA yielded no detectable 341 bp product. PCR using alkaline phosphatase specific primers that span an intron-exon junction demonstrated no genomic DNA contamination of RNA samples. Preliminary RT-PCR data using primers specific for GDH suggest that this enzyme is expressed at all in vivo stages of preimplantation development. (Supported by NIH agreement HD21942 to C. Ziomek and an Indiana Academy of Sciences grant to CLC.)

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Expression of a Secretory Glycoprotein in Intestinal Crypt Undifferentiated Cells during Development. R. Calvert, G. Millane and J.-F Beaulieu, Groupe de recherche en biologie du développement, Faculté de médecine, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4.

The role of intestinal crypts in cell proliferation and in the renewal of the epithelium along the crypt-villus axis has been studied extensively. Meanwhile, although many morphological observations indicated that undifferentiated crypt cells appeared to have a secretory activity, much less attention was given to this postulated role because of the absence of specific markers. We have produced a monoclonal antibody (MIM-1/39) that defines an antigen localized by immunocytochemistry in the secretory granules of undifferentiated crypt cells of the duodenum. By indirect immunofluorescence, this antigen is expressed in the duodenal epithelium of the intervillous area of the mouse two days before the formation of crypts and is localized only in crypt cells in the adult. By immunoblotting the mAb revealed a single band of about 350 kDa at 15 days of gestation. Three days later, a second band of about 330 kDa was also revealed. At that stage, the second band appeared quantitatively more important. After birth, a maturation process appears to take place since after weaning the major band was observed at 350 kDa. The role of this new glycoprotein remains unknown. (Supported by the MRC of Canada).

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Coordinate Gene Expression During Heart Development Gary.L. Engelmann, Department of Medicine, Loyola University-Stritch School of Medicine, Maywood, IL 60153.

Growth of the mammalian heart during the fetal-neonatal-adult transitions is coordinately regulated. Fetal development is the period of most active myocyte proliferation; whereas neonatal development is a period of "transitions" from proliferative to non-proliferative myocytes and active ventricular remodeling. Growth factors (GFs) and/or their receptors appear to play an essential role in such processes and have been found to be temporally limited in their expression and regionally localized to distinct cell types. We report that embryonic/fetal rat myocytes are sources of/and responsive to members of the FGF and IGF family of peptides as cell mitogens through specific cell surface receptors. Primary myocyte cell cultures have detailed specific changes in cell responsiveness. Late fetal/neonatal myocytes are sources of and responsive to TGF-ßs such that terminal proliferative events occur during the first postnatal week. Autocrine/paracrine methods of growth regulation have been proposed. Fetal/neonatal non-myocyte cell types are responsive to myocyte-derived GFs such as TGF-ßs, SPARC, and FGFs in ECM biosynthesis (Types I, III, and IV collagens, vimentin) and capillary angiogenesis. A hypothesized paracrine method of growth and regulation of ECM biosynthesis and capillary angiogenesis has been proposed. FGF-receptor Flg expression and localization shows developmental changes and cell-type localization by immunohisto-chemical and *in situ* studies. A working paradigm is presented to illustrate the temporal and developmental changes in GFs, GF-receptors, ECM, capillary angiogenesis and heart development. (Support HL-42218)

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Gamma-glutamyl Transpeptidase Activity in Rat Lung Is Expressed Late in Development and the Pattern of Expression of mRNA Transcripts Appears to Reflect A Differential Sensitivity to Oxygen. M. Joyce-Brady, Y. Takahashi, S.M. Oakes, W.V. Cardoso, A.K. Rishi. Pulmonary Center, Boston University School of Medicine, Boston, MA 02118

We have reported that the hydrophobic form of gamma-glutamyltranspeptidase (GGT) is found within the lung alveolus of the adult rat. The differentiated alveolar type 2 (T2) cell expresses the GGT mRNA, synthesizes the protein, then activates and secretes the enzyme into a surfactant-associated pool where it is redistributed throughout the gas exchange portion of the lung. In T2 cells GGT mRNAIII, a liver-specific transcript, predominates over GGT mRNAI, a kidney specific transcript. In order to explore the developmental expression of GGT we studied gene expression by RT-PCR and enzyme activity with a sensitive fluormetric assay in two models of lung development. Whole lung GGT activity is not detectable in lung development until 18 days of gestation and increases up to the time of birth parallelling the ontogeny of lung surfactant. The activity of the fetal lung is 15% of the adult level and remains constant for 1 week into postnatal life whereupon whole lung activity again rises to 68% of adult levels by 3 weeks and 76% by 8 weeks of age. When embryonic lung or fetal epithelial cells isolated from the peripheral lung at 18 days gestation are cultured under normoxic (21%) conditions GGT mRNA can be detected (using primers specific for coding domain) and GGT activity is induced over time. However when the fetal cells are cultured for 4 days under hypoxic (3%), normoxic (21%) or hyperoxic (30-40%) conditions, RT-PCR with GGT transcript specific primers suggests that the mRNAI transcript predominates in hypoxia while the mRNAIII transcript is induced in hyperoxia. This developmental data suggests that the pattern of GGT mRNA expression in the adult T2 cell could result from the differential sensitivity of the two GGT transcripts to oxygen in the developing lung.

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Distribution of Hyaluronan in Postimplantation Mouse Embryos. B.A. Fenderson[1], I. Stamenkovic[2], and A. Aruffo[3], [1]Department of Pathology and Cell Biology, Thomas Jefferson University, Philadelphia, PA 19107, [2]Department of Pathology, Massachusetts General Hospital, Boston, MA 02114, and [3]Department of Cellular Interaction, Bristol-Myers Squibb Research Institute, Seattle, WA 98121.

Hyaluronan was localized in postimplantation mouse embryos (Days 6 - 13) using CD44 receptor globulin (CD44 Rg). The specificity of CD44 Rg labeling was confirmed using *Streptomyces* hyaluronidase, anti-chondroitin sulfate antibody, and three selectin receptor globulins (GMP-140, Leu-8, and ELAM-1). Following implantation, hyaluronan is rapidly lost from the anti-mesometrial side of the uterus as stromal cells undergo decidualization. Within the egg cylinder-stage embryo (Day 6), hyaluronan appears as a basement membrane between the primitive ectoderm and primitive endoderm. During gastrulation (Days 6.5 - 7.5), hyaluronan is present, intracellularly, in a distinct subset of mesodermal cells leaving the primitive streak. In contrast, chondroitin 6-sulfate is present on the surface of all mesodermal cells at this stage. In the forelimb-bud embryo (Day 9.5), hyaluronan is associated with head mesenchyme, cranial neural crest, and para-axial mesoderm. Differentiation of somites is accompanied by the loss of hyaluronan from most, but not all, cells. In the hindlimb-bud embryo (Day 11.5), hyaluronan is associated with neural crest and mesenchymal cells in the head, neck, and vertebral column. On Day 13, hyaluronan is found within the endocardial cushions of the heart, and is associated with cartilage, skeletal muscle, and connective tissue throughout the body. Also at this stage, E-selectin (GMP-140 Rg) labeled blood cells and macrophages, as well as neuronal or glial cells within the central nervous system. The results establish hyaluronan and chondroitin 6-sulfate as markers of early mesoderm in the mouse embryo, and provide evidence for heterogeneity of mesodermal cell types in the primitive streak and somites.

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Comparison of Leukocyte Subpopulations in Murine Decidua and Deciduomata. K.A. Knisley and H.M. Weitlauf. Department of Cell Biology and Anatomy, Texas Tech Health Sciences Center, Lubbock, TX 79430.

During the process of implantation the blastocyst initiates local changes in the uterus that are characterized by proliferation and swelling of uterine stroma resulting in formation of decidual tissue that completely surrounds the conceptus. The goal of this proposed study was to characterize decidual leukocyte subpopulations based on phenotypic markers using monoclonal antibodies; M1/934 (anti-common lekocyte antigen), M17/4 (anti-LFA-1, alpha subunit), M1/70 (anti-Mac-1, alpha subunit), or PC-61 (anti-IL2 receptor). Uterine tissues were recovered from mice on day 8 of pregnancy or at an equivalent time after induction of deciduomata in a ligated pseudopregnant horn. M17/4, M1/70, and PC-61 defined three different patterns of reactivity in the mesometrial region of the decidual mass. There was no obvious effect of the embryo on the selection or distribution of these leukocyte subpopulations. It appears therefore, that after the initial nidatory stimulus, whether it be blastocyst, oil, or scratch, the ensuing leukocyte infiltration of the decidual mass is the result of a preprogrammed maternal response.

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Effect of Retinoic Acid on Mouse Lung Branching Activity. *L. Schuger, R. Mitra, J. Varani, and K. Gilbride. Dept. of Pathology, Boston University School of Medicine, Boston, MA 02118.*

Retinoic acid (RA) stimulated branching morphogenesis in organ cultures of mouse embryonic lung. In growth-arrested lungs stimulation was maximal with concentrations of 1μM and progressively decreased with either lower or higher concentrations. An increase in mitotic activity was observed in both the epithelium and the mesenchyme during RA exposure. The effect of RA on lung cell monocultures was examined next. Direct cell counting and [3H]-thymidine incorporation were used to determine cell proliferation. When isolated from each other, only mesenchymal cells proliferated in presence of RA, whereas epithelial cells remained quiescent. This suggested an indirect effect of RA on epithelial cell proliferation which required the presence of mesenchyme. RA is known to stimulate production of laminin, a glycoprotein implicated in lung morphogenesis, however no changes in laminin synthesis were detected by SDS-PAGE or ELISA. While TGF-α and EGF induced branching, unlike RA, these growth factors stimulated proliferation in both epithelial and mesenchymal cell monocultures. Since TGF-α and its receptor are present in embryonic lungs and their expression is controlled in part by RA, the latter might affect branching development through a mechanism involving modulation of TGF-α and/or its receptor. This hypothesis is currently being investigated.

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The Expression and Role of Cytokines in Human Choriocarcinoma Cell Lines. R.R. Adler, C.A. Brenner* and N.S. Rote, Department of Microbiology and Immunology, Wright State University School of Medicine, Dayton, OH 45435 and *Department of Biology, University of Dayton, Dayton, OH 45469.

The choriocarcinoma cell lines BeWo and JEG provide an excellent system for the study of placental cytotrophoblast different *in vitro*. It has been shown that BeWo cells will undergo differentiation characterized by increased expression of hCG and hPL and syncytium formation, when treated for 48-72 hr in 100 nM forskolin, but this has not been documented in JEG cells. In this study, the expression of cytokines by these cells was observed by cytokine MAPPing. Both controls and forskolin treated cells were observed to express the genes for IL-3, IL-4, IL-5, IL-6, TNFα, TNFβ, CSF-1 and GM-CSF. Forskolin treatment appeared to stimulate expression of IL-1α as well. The potential role of cytokines was investigated incubating BeWo cells in IL-4 or GM-CSF and these two factors both stimulate syncytium formation in either serum containing and serum-free medium. In addition, it was observed that JEG cells would undergo syncytium formation when treated with forskolin, IL-4 or GM-CSF. These results suggest that cytotrophoblast differentiation may be stimulated by cytokines. (Supported by NIH HD23697.)

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The *In Vivo* and *In Vitro* Phosphorylation of BiP Occur at Different Sites but Have Similar Consequences on Function. J.R. Gaut and L.M. Hendershot, Department of Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN 38101.

When stressed by glucose starvation, mammalian cells respond, in part, with the induction of BiP synthesis and the dephosphorylation of existing BiP. Our previous work revealed that this *in vivo* phosphorylation was found only on the dimeric form of BiP which is not associated with immunoglobulin heavy chains. Consequently, this suggested a regulatory role for phosphorylation in BiP function. We report here that *in vivo* phosphorylation of BiP functionally blocks its ability to bind ATP. Recently, others have shown that BiP is autophosphorylated *in vitro*. We find that similar to *in vivo* phosphorylation, *in vitro* phosphorylated BiP cannot bind ATP and migrates as a dimer free of bound heavy chain. Serine mutations were made in BiP replacing Thr residues at position 37 and 229. Autophosphorylation of these BiP mutants followed by phosphoamino acid analysis revealed that Thr229 is the site autophosphorylated *in vitro*. Thr229 corresponds in position to Thr204 in HSC71, which was proposed by Flaherty et al., to interact with the γ-phosphate of ATP. It follows that autophosphorylation at this site could thwart subsequent ATP binding. Comparison of proteolytic fingerprint analysis of *in vitro* and *in vivo* phosphorylated BiP indicated that Thr229 is not a major site of phosphorylation *in vivo*. However, since the *in vivo* site of phosphorylation is affecting BiP function in a manner similar to *in vitro* phosphorylation, it may also occur within the ATP binding domain, but on a Thr residue other than Thr229.

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Supernumerary Ribs (SNR): A Manifestation of a Basic Alteration(s) in the Fate of the Axial Skeleton. N. Chernoff, C. Y. Kawanishi, J. M. Rogers, S. Branch, and B. M. Francis. U.S. E.P.A., R.T.P., NC 27711.

Treatment with retinoic acid (RA) in CD-1 mice on gestation d7 results in a vertebral pattern of 7 cervical (C), 14 thoracic (T), and 5 lumbar (L) as compared to the normal C7, T13, and L6. RA given on d8 results in C7, T14, and L6. These data may reflect the general lability of the thoracic-lumbar border on d7 and the lumbar-sacral border on d8. An increase in the size of the 13th rib in fetuses with induced lumbar SNR (irrespective of fetal weight) was noted indicating that the presence of lumbar SNR is associated with an increase in the size of all ribs. An increase in the number of vertebrae may occur along with SNR. Others have reported RA-induced cervical SNR after d7 exposure in a strain with a high incidence of this anomaly in controls while our strain has a high incidence of lumbar SNR. SNR are anomalies caused by maternal stress and/or toxicity in specific strains of laboratory animals. Compound-induced SNR occur at lower doses than required to elicit other defects. In the CD-1 mouse, the peak embryonic period of susceptibility for lumbar SNR is the 8-15 somite stage. We conclude that patterns of SNR and/or vertebral changes may reflect strain-specific instabilities in the early developmental fates of portions of the axial skeleton.

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Effect of Cadmium on Placental Calcium Transport: An *in vitro* study using JEG-3 Human Choriocarcinoma Cells. R.S. Tuan and C.A. Iannotti, Department of Orthopaedic Surgery, Thomas Jefferson University, Philadelphia, PA 19107 (Spon. By C.W. Lo)

Placental calcium transport is crucial for embryonic development and growth. Exposure to heavy metals, such as cadmium, is toxic to placental functions and fetal development. To analyze the molecular basis of cadmium toxicity, we report here findings using JEG-3, an *in vitro* trophoblast-like, choriocarcinoma cell line. We have previously shown that JEG-3 cells, which express hCG and alkaline phosphatase, also express a trophoblast-specific calcium-binding protein, and exhibit active calcium uptake (*J. Cell Sci.* 98:333,'91). Tolerance to cadmium is induced in JEG-3 cells in a time and dose dependent manner by cadmium pre-treatment, as measured by retention of cellular LDH. The development of cadmium tolerance is accompanied by by a 4-8 fold increase in metallothionein expression, analyzed at both mRNA (slot blot) and protein (^{109}Cd binding) levels. Because of the similarity between cadmium and calcium ions, we have examined the effect of cadmium exposure on calcium uptake activity in JEG-3 cells. Interestingly, cadmium tolerance is accompanied by a concomitant decrease in calcium uptake activity by the JEG-3 cells, e.g. 4 μM cadmium pretreatment results in over 50% decrease in calcium uptake. These results are highly suggestive that cadmium toxicity to placental function involves perturbation of trophoblast calcium handling, perhaps as a result of over-expression of metallothionein. (Supported in part by NIH HD 15822)

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Complement Lysis Inhibitor (CD59) on Cellular Interfaces Between Mother and Fetus. O.A.Vanderpuye, C.A. Labarrere, and J.A.McIntyre Center for Reproduction and Transplantation Immunology, Methodist Hospital of Indiana, Indianapolis, Indiana 46202

The fetus uses extraembryonic tissues (placenta and amniochorion) to interact with its mother. Extraembryonic tissues foreign to the mother are at risk from complement-mediated damage by the maternal immune system. We assessed extraembryonic expression of CD59, an inhibitor of the membrane attack complex of complement to identify molecules contributing to the survival of these tissues. Immunofluorescence with Mab MEM-43 to CD59 showed strong expression of CD59 on syncytiotrophoblast brush border (STM), but limited reactivity with its basal plasma membrane. Extravillous trophoblast and amniotic epithelia bound MEM-43 in a membranous non-polarized distribution. CD59 was present from the 1st to 3rd trimester of pregnancy in STM. By ELISA, STM contained CD59 at levels equal to or greater than membranes from platelets, kidney and erythrocytes. The lectin-binding profile of STM CD59 was similar to that of erythroid CD59 and indicated the presence of bi/tri antennary glycans. CD59 is unusual among STM proteins in binding to Erythrina cristagalli lectin. Our results demonstrate expression of CD59 on extraembryonic tissues at sites of contact with complement in maternal blood, tissue interfaces and amniotic fluid. CD59 is thus situated to protect extraembryonic cells from complement-mediated effects of maternal immunity throughout pregnancy.

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ESTROGEN AND TAMOXIFEN REGULATION OF PROTOONCOGENE, GROWTH FACTOR, GROWTH FACTOR RECEPTOR AND STEROID HORMONE RECEPTOR EXPRESSION IN RODENT UTERINE TISSUES

Nandan Bhattacharyya, Roger Ramsammy, Vincent W. Hollis, Winston A. Anderson*, Elaine Eatman**, and James Wyche***, *Department of Biology, Howard University, Washington, D.C., * *Department of Biology, Hampton University, Hampton, Va. and ***Department of Biology, Brown University, Providence, R.I.

Complex hormonal, growth factor and complimentary receptor interactions regulate proliferative and differentiative changes in normal cycling uterine tissues and in estrogen-dependent tumor cells and tissues. Estrogen induction, repression and derepression of various nuclear genes are known to mediate these proliferative and differentiative events. How antiestrogens, like Tamoxifen, regulate early events in estrogen induction is totally undetermined. Consequently, the major objective of this study was to investigate the regulatory effects of estrogen and Tamoxifen on specific genes known to play key roles in proliferation and/or differentiation in rodent uterine tissues. Specifically, the probes used in these studies to evaluate proliferation and differentiation included growth factors (EGF, IGF-1, IGF-2 Exxon I, IGF-1 Exxon II, IGF-2 Exxon IV,TGFB-types 1, 2, 3), growth factor receptors (EGFR, IGF-1R, IGF-2R, TGFB-2,3R), hormone receptors (ER, hPR) and protooncogenes (*c-myc; c-fos; c-ras; c-jun*). In this temporal study, polyA$^+$ RNA was isolated from estrogen and Tamoxifen-treated uteri at 0', 15', 30', 1hr, 6hr, 24hr, 48hr, and 72hr. Expression studies were analyzed by dot/Northern blot hybridization with the above mentioned probes. Nuclear runoff transcriptional assays were also performed. Autoradiograms were further analyzed by densitometric scans. Results from these studies indicate significant changes in protooncogene, growth factor, receptor and steroid hormone receptor expression relative to estrogen induction of uterine growth and Tamoxifen-mediated antiuterotrophic effects. These results will be discussed in detail at the time of prosentation. (Supported by the MBRS/NIH grant).

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Fate and Formation of Pre-Blastula Dorsal Animal Blastomeres in the Gastrula of Xenopus laevis. M. A. Vodicka and J. C. Gerhart, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720.

Dorsal animal blastomeres from early cleavage stage *Xenopus* embryos possess greater axis inducing ability and responsiveness to inducers than the corresponding ventral animal cells. The dorsal identity of animal blastomeres on the future dorsal side of the *Xenopus laevis* embryo is established as early as the 8-cell stage. This dorsal identity, or dorsal bias, may be due to segregation of cytoplasmic components at cortical rotation, cell-cell interactions, induction, or any combination of these. The fates of the cleavage stage dorsal animal cap cells in blastula and gastrula embryos has not previously been examined; more is known about the position of dorsal axis inducing and organizing cells in these stages. Because this dorsal identity in animal cap cells is apparent in embryos between the 8- and 32-cell stages, it is thought that following the distribution of early dorsal animal cytoplasm through the beginning of gastrulation may provide a clue about the nature of this dorsal bias. With the use of fluorescent lineage tracers, we have followed the fates of dorsal animal blastomeres of the 32-cell *Xenopus* embryo through blastula and early gastrula. Single dorsal tier 1 or tier 2 cells were injected with either rhodamine or fluorescein conjugated dextran at the 32-cell stage. Embryos were fixed immediately after injection (stage 6), at early blastula (stage 8), late blastula (stage 9), and early gastrula (stage 10). Fixed embryos were viewed in whole mounts using epifluorescence and confocal microscopy. Our analysis reveals that the cells descendent from the dorsal animal cap at the 32-cell stage gradually spread vegetally throughout blastula stages. By the beginning of gastrulation, labelled cells appear in the dorsal marginal zone of the embryo.

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Thyroid Hormone-Induced Changes in Gene Expression Preceding Amphibian Tail Resorption. Z. Wang and D.D. Brown, Department of Embryology, Carnegie Institution of Washington, 115 W. University Parkway, Baltimore, MD 21210.

The changes that tadpoles undergo at metamorphosis are controlled by thyroid hormone (TH). Since TH is known to exert its action at the transcriptional level, we have begun a study of tail resorption, one of the dramatic events of metamorphosis, by studying TH-induced changes in gene expression in Xenopus laevis tails. In order to do this, we developed a "gene expression screen" (Wang, Z. and Brown, D.D., 1991, Proc. Natl. Acad. Sci. USA, Vol. 88, pp. 11505-11509), a PCR-based subtractive hybridization method that has the special advantage of estimating the number of differentially expressed genes. This method has been applied to genes in tail that are up- or down-regulated 2, 24, and 48 hours after TH treatment. We estimate that there are about 30 TH up-regulated genes (>6-fold up-regulation, >15 mRNA/cell) and less than 10 TH down-regulated gene (>6-fold down-regulation, >200 mRNA/cell) in Xenopus tadpole tails. Sixteen up-regulated and four down-regulated genes were isolated from the tail. Characterization of the isolated genes suggests that TH induces a single wave of down-regulation. There are two waves of up-regulation of gene expression. The first wave begins at 4 to 8 hours; the second is maximal by 30 hours. The first wave of up-regulated genes respond directly to TH since induction of their mRNA is insensitive to cycloheximide. The second wave of up-regulated genes are in fact weakly activated within the first 8 hours. This weak activation is also resistant to protein synthesis inhibition. Tails amputated from tadpoles after 48 hours of TH treatment undergo resorption irreversibly, even when protein synthesis is inhibited. Thus, one wave of down-regulation and two of up-regulation comprise the major changes in TH-induced gene expression preceding amphibian tail resorption.

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The Regulation of Gastrointestinal Metamorphosis in Xenopus laevis by Thyroid Hormone. Y.-B.Shi[1] and D.D. Brown[2]; [1]Laboratory of Molecular Embryology, Building 6, Rm B1A13, National Institute of Child Health and Human Development, NIH, Bethesda, MD 20892; [2]Carnegie Institution of Washington, 115 W. University Pkwy, Baltimore, MD 21210.

During metamorphosis, the gastrointestinal track (GI) of an herbivorous Xenopus laevis tadpole is completely remodeled to one suited for a carnivorous frog. A tadpole intestine is a long tube with a single layer of epithelium. This simple structure is changed during metamorphosis to a frog gastrointestinal track with a stomach,and small and large intestines. This remarkable change, like other processes in metamorphosis, is entirely controlled by thyroid hormone (TH). We presume that TH acts by influencing gene expression by way of thyroid receptors (TR). Consequently, to understand the process of GI development, we have begun to characterize genes whose expression is regulated by TH using a differential screening method. We will present data on characteristics of up- and down-regulated genes induced by TH in tadpole intestine. This includes their tissue specificity, and developmental regulation. The response of the GI track will be compared to that of limb and tail.

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Lineage and Migratory Patterns of Neural Crest Cells in Living Xenopus laevis Embryos. A. Collazo, S. E. Fraser, and M. Bronner-Fraser*. Division of Biology, Beckman Institute 139-74, California Institute of Technology, Pasadena, CA 91125, and *Developmental Biology Center, University of California, Irvine, CA 92717.

We used two techniques to study the lineage and migratory patterns of neural crest or dorsal neural tube cells in the frog *Xenopus laevis*: 1) small injections of DiI or lysinated rhodamine dextran (LRD) to follow the migration of populations of neural crest cells and 2) injections of LRD into single cells to follow cell lineage. We followed the movement and phenotypes of these cells as a function of time in living embryos, which were later sectioned to determine the precise position and morphology of labelled cells. In the rostral to mid-trunk levels, we confirmed that neural crest cells migrate along two paths, as reported previously: a dorsal pathway into the fin and a ventral pathway along the edges of the neural tube and notochord. In the caudal trunk, two additional paths were noted. The first originates from the dorsal neural tube, progressed ventrally to the anus, and subsequently populated the ventral fin. The second path emerges from the neural tube, progressing caudad on a superficial pathway around the tail and into the ventral fin. Lineage analysis of individual neural crest cells demonstrated that neural tube and neural crest cells often share a common precursor. The majority of clones produced labelled progeny cells in the dorsal fin. The remaining clones were all multipotent, containing spinal ganglion cells, pigment cells, enteric cells, fin cells and/or neural tube cells in all possible combinations. This suggests that many premigratory *Xenopus* neural crest precursors can be multipotent. Progeny of a single neural tube cell often were located bilaterally and could be spread along many segments of the rostrocaudal axis. Although the pathways of neural crest cell migration differ in *Xenopus* and avian embryos, their lineage relationships are remarkably similar. (Funded by N.I.H. and M.D.A.)

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Disruption of Cell-Cell Interactions Reduces the CNS Contribution of Tier-3 Blastomeres of the 32-Cell *Xenopus* Embryo. D.V. Bauer and S.A. Moody. Department of Anatomy and Cell Biology, University of Virginia, Charlottesville, VA 22908, and Department of Anatomy, George Washington University, Washington, DC 20037.

Normal fate maps of the 32-cell *Xenopus* embryo indicate that the nervous system primarily derives from the three animal-most tiers of cells (Moody, 1987, *Devel. Bio.* 119:560; Dale and Slack, 1987, *Development* 99:527). The vegetal tier (tier-4) rarely contributes to two primary neurons of the spinal cord (Rohon-Beard neurons and primary motoneurons), although their animal neighbors (tier-3) almost always contribute to these cells (Moody, 1989, *J. Neurosci.* 9:2919). Since the tier-3 cells are on the border of the "neurogenic" region of the cleavage stage embryo, we tested whether their ability to produce their normal CNS progeny relies upon interactions with their animal neighbors prior to neural induction. Using lineage tracing techniques, we determined the fates of tier-3 blastomeres following a variety of manipulations that disrupted their contacts with tier-2 neighbors:

A) single tier-2 blastomeres were removed,

B) embryos were dissociated within the vitelline membrane for one hour at various time points prior to neural induction, and

C) Nucleopore barriers were placed between individual tier-2/tier-3 pairs of blastomeres.

These manipulations resulted in significant reductions in tier-3 contribution to primary neuron populations indicating that the tier-3 blastomeres require direct contact with their tier-2 neighbors during cleavage stages in order to produce their normal neuronal progeny. This work is supported by NIH grant NS23158.

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The protein encoded by the localized maternal mRNA An3 from *Xenopus* has RNA dependent ATPase activity. R. Gururajan and D.L. Weeks. Department of Biochemistry, University of Iowa, Iowa City, Iowa, 52242.

Our aim has been to study the role of localized maternal mRNA in directing events in early development in *Xenopus*. An3 is a maternal mRNA localized to the animal pole of *Xenopus* oocytes and embryos. We have determined that An3 mRNA encodes a protein that is similar to the protein encoded by the mouse testis specific mRNA PL10. Both proteins belong to the family of ATP dependent RNA helicases. To understand the role of the localization of the mRNA and the function of the protein, we have determined the distribution of the mRNA and the enzymatic activities associated with the protein. We find that An3 mRNA is uniformly distributed in the later stages of embryogenesis and is present in various adult tissues unlike mouse PL10 mRNA. Different forms of the An3 protein have been expressed in bacteria and we show RNA dependent ATPase activity for partially purified An3 proteins. We have also studied the RNA helicase activity of the recombinant An3 protein. Monoclonal antibodies have been used to determine the expression of An3 protein in the early stages of development.

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An1a and An1b: Two Related mRNAs Encoding Ubiquitin-like Fusion Proteins are Localized to the Animal Pole of *Xenopus* Eggs. J.M. Linnen and D.L. Weeks, Department of Biochemistry, University of Iowa, Iowa City, IA 52242

An1a and An1b are two mRNAs localized to the animal pole of *Xenopus* eggs. We have deduced from their cDNA sequences that these messages encode 77 kD proteins which are 88% similar. Both of these novel proteins have an N-terminal ubiquitin-like region (50% similarity to the 76 amino acids Xenopus ubiquitin) and a possible zinc finger near their C-termini. The two cDNA isoforms of An1 are about 89% similar in their open reading frames and about 81% similar in their untranslated regions. RNase protection assays demonstrate that both An1a and b mRNAs are present at approximately equal levels in the maternal pool and during development. Both isoforms are abundant during early embryogenesis (egg to stage 9) and lower levels are detected throughout embryogenesis with increased levels at stages 22 and 24. RNase protection assays of total RNA isolated from various adult tissues show that both isoforms are found at low levels in most tissues tested. In an effort to understand the role of the N-terminal ubiquitin-like region found in the An1 protein, we have studied the stability of *in vitro* synthesized and endogenously synthesized An1 protein. Additionally, we report that the An1a and b mRNAs are essential for normal embryonic development as shown by antisense oligonucleotide mediated depletion of these mRNAs during development.

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Identification of Brachyury (T) Homologues in Chicken Tissues. C.C. Wendler, J.-X. Huang, L.A. McKinney, and R.B. Runyan, Department of Anatomy, University of Iowa, Iowa City, IA 52242. (Spon. by R.J. Tomanek)

Considerable interest has been raised in the brachyury (T) gene due to its apparent role in gastrulation in mouse and Xenopus embryos. It was previously seen that T/T mutant mice had defects in structures formed by mesoderm cells including the face, limbs, heart, and tail. Our own interest has focused upon the potential role of brachyury in mesenchymal cell formation and migration in the heart. In Xenopus, brachyury is an early response gene to Activin, TGFß, or FGF. As we have previously shown a functional role for TGFß3 in the formation of mesenchyme in the heart, we are interested in the potential function of brachyury in this developmental pathway. To obtain an avian homologue of the brachyury gene, we aligned the Xenopus and murine sequences and produced degenerate oligomers (36 fold and 24 fold) for a conserved 5' region of the two sequences. These primers were used for RT-PCR amplification and produced an amplified sequence of the predicted size of ~ 430 bp from RNA of stage 17 whole chicken embryos. A 432 bp product was cloned and sequenced and found to have 79% nucleic acid sequence identity with Xenopus brachyury and 86% identity with the murine sequence. At the amino acid level, the sequence shares 94% and 96% identities with Xenopus and murine sequences, respectively. As there were large areas of identity between the murine and avian sequences, new non-degenerate primers were prepared for PCR analysis. Subsequent PCR analysis has shown that brachyury can be found in both the embryonic heart and the rest of the stage 17 embryo. These data show that a regulatory gene heretofore associated primarily with gastrulation is expressed in older tissues and is likely to function in additional developmental processes. The rest of the brachyury gene is currently being cloned from our avian heart library. Supported by NIH HL 42266 and HL 38649.

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Characterization of novel receptor tyrosine kinases expressed during *Xenopus* gastrulation. M Servetnick and RM Grainger, Dept. of Biology, Univ. of Virginia, Charlottesville, VA 22901, USA.

Xenopus development is characterized by a series of inductive interactions, including the induction of mesoderm, neural tissue, and the lens of the eye, all from tissue that would otherwise form ectoderm. During embryonic development, ectoderm undergoes a series of precisely timed changes in competence, the ability to respond to induction, beginning with the acquisition of mesodermal competence during blastula stages, and neural competence by gastrula stages. Our previous work has shown that ectoderm loses competence to respond to neural induction by mid-gastrula stages, then gains competence to respond to lens induction. Lens competence persists for only a brief period during mid-gastrula stages. As a first step toward analyzing the molecular basis of the ectodermal response to inductive signals, we have screened a *Xenopus* mid-gastrula stage cDNA library at low stringency, using probes for receptor tyrosine kinases (RTKs), which are known to act as receptors for inductive signals in other systems. Partial sequence analysis shows that four of the isolated clones encode amino acid domains diagnostic of RTKs, and that these RTKs have not previously been described. Experiments to define the expression patterns of these cDNAs, by Northern analysis and *in situ* hybridization, are in progress.

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Examination of Brachyury (T) Gene Expression in the Developing Avian Heart During Valvular Mesenchyme Formation. J.-X. Huang, L.A. McKinney, C.C. Wendler, and R.B. Runyan, Deptment of Anatomy, University of Iowa, Iowa City, IA 52245.

The brachyury (T) gene product is thought to be involved in mesoderm induction in vertebrate embryos but the role of brachyury in later stages of development has not been well addressed. Endothelial-to-mesenchymal cell transformation (EMT) during heart development is similar to gastrulation in that the primary event is formation of three dimensional (cushion) tissue from a two dimensional layer of cells. Further, cardiac cushion tissue defects were found in homozygous T/T mutant mice. To investigate the role of brachyury in EMT, degenerate primers were designed based on a homologous region between mouse and Xenopus and used to identify a similar sequence from the chick (see abstract by Wendler et al.). Non-degenerate primers were subsequently used for RT-PCR analysis of avian heart tissue. Expression of brachyury was detected throughout stages14-20, a period extending from before EMT till after mesenchymal cell formation in the atrioventricular (AV) canal is complete. Brachyury message was also found in both AV canal (transforming) and ventricular (non-transforming) portions of the heart. Cardiac cell types were separated in culture and RT-PCR analysis suggests that brachyury is present in both myocardial and endothelial cells. Preliminary in situ data support this widespread expression of brachyury in the developing heart. Additionally, several bands of larger than expected size were detected by PCR in both embryo and heart RNA under high stringency conditions. Sequence data for one of these bands shows a ≈150 bp insert within the expected sequence. Genomic contamination in the RNA preparation was ruled out by use of actin primers which differentiate cDNA from genomic DNA by a size difference. Nor did PCR amplification of a chik genomic library produce the same signal. This additional band may represent an alternatively spliced form of brachyury. The apparent widespread expression of brachyury is unexpected considerng a supposed temporally specific mesoderm-inducing function. However, both myocardium and endothelium are mesodermally-derived cells. The role of brachyury expression in heart development and its interplay with other signal molecules is being investigated by use of an antisense oligonucleotide knockout technique. Supported by NIH HL 42266 and HL 38649.

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Characterization of the conditioned medium which induces beating in hearts of cardiac nonfunction mutant axolotls. N. Erginel-Unaltuna, D.K. Dube, S. M. LaFrance and L.F. Lemanski. Department of Anatomy and Cell Biology, SUNY, Health Science Center, Syracuse, NY.

Homozygous mutant (c/c) axolotl embryos lack sarcomeric myofibrils and their hearts fail to beat. This defect can be corrected by culturing mutant hearts with normal tissue from the anterior endoderm region or in a medium which is conditioned by this tissue. RNAase digestion of the conditioned medium completely abolishes its inducing activity whereas proteinase K digestion has no significant effect; these studies suggest that the active material is RNA from the anterior endoderm region. A cDNA library has been constructed using total RNA from conditioned medium which showed positive induction activity utilizing random priming. The transfected clones are amplified and sequenced employing the dideoxy chain terminator method. The sequences are compared to other known sequences in all available databases utilizing the Genetic Computer Group (GCG) DNA analysis package. Six out of twelve clones are found to have homology with 28S rRNA from a variety of eukaryotic species. Two are homologous to 18S rRNA. No significant homology is detected with the remaining four clones suggesting the possibility that they may be products of distinct coding genes. The four unique clones contain open reading frames of 2$1, 186, 186 and 132 bases respectively and show no overlapping regions among themselves. RTPCR analysis with primers from the sequence with the largest open reading frame showed that this gene is differentially expressed in brain, skeletal muscle, heart and lungs, but the expression is not detectable in liver. Further studies are in progress to specify the active material. (Supported by NIH Grants HL-32184, HL-37702 and an AHA Grant to L.F.L.)

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RNA promotes terminal differentiation in cardiac mutant axolotl hearts. S. M. LaFrance, N. Erginel-Unaltuna, M. E. Fransen, D. K. Dube*, T. K. Ray, L. F. Lemanski. Dept. of Anatomy and Cell Biology, Dept. of Medicine*, SUNY Health Science Center, Syracuse, N.Y.

In the axolotl, *Ambystoma mexicanum*, a recessive cardiac lethal mutation causes incomplete differentiation of the myocardium. Mutant hearts do not contain sarcomeric myofibrils nor do they beat. We have previously shown that tissue from the normal (+/+) anterior endoderm region, medium conditioned by this tissue, or total RNA isolated from intact tissue of the same region stimulated differentiation of mutant hearts in culture as indicated by the presence of organized myofibrils and rhythmic contractions of the "rescued" mutant heart tube. In order to isolate the inducing RNA with minimal contamination from other RNA species (that are present in total RNA from intact tissue), RNA was extracted from endoderm/mesoderm-conditioned medium. Mesoderm was included in the explants to facilitate the dissection.The RNA was assayed for the ability to promote myofibrillogenesis in mutant hearts. Mutant heart mesoderm responded to conditioned medium RNA in a dose-dependent manner. Proteinase K treatment of the RNA did not significantly affect inductive activity while digestion with RNase A completely abolished the ability to rescue mutant hearts. Since mesoderm had been included in the explants for conditioned medium, we tested the effectiveness of medium conditioned by endoderm or mesoderm alone. We found that medium conditioned by either tissue was equally effective in correcting mutant hearts.This result is consistent with data on other developmental regulators where the inducing molecule is present in the inducing and responding tissues. Further studies will be designed to examine the pathway of the inducing RNA as it is produced in one embryonic tissue (inducing) and promotes differentiation of another (responding). (Supported by NIH Grants HL-32184, HL-37702 and a Grant-in-aid to LFL.)

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Growth factor-mediated cardiogenesis in chick mesodermal explants. Y. Sugi and J. Lough, Department of Cell Biology and Anatomy, Medical College of Wisconsin, Milwaukee, WI 53226

It has previously been shown that heart development is promoted by anterior endoderm from H-H stage 5/6 chick embryos (Lough et al., 1990). Polypeptide growth factors are likely candidates as initiators of this inductive interaction during embryonic development. In this study, the cardiogenic effects of selected growth factors on precardiac mesoderm from stage 5/6 embryos have been examined. Explanted anterior lateral plate mesoderm, which includes the heart forming region, rarely formed beating cardiac tissue when cultured in serum-free defined medium (SFM), with or without 50 ng/ml transferrin. However, when SFM including 5-50 ng/ml insulin, bFGF, or activin A was used, the incidence of explants that exhibited synchronous contractions increased markedly. Cardiogenic differentiation was also characterized by the appearance of cardiac alpha-actin mRNA, as assessed by PCR. Whereas freshly explanted stage 5/6 lateral plate mesoderm contained only cytoplasmic beta-actin, explants cultured for four days with insulin, bFGF or activin A exhibited both cytoplasmic beta-actin and cardiac alpha-actin. These results indicate that avian cognates of insulin, bFGF and activin A regulate the initial stages of vertebrate heart development. Supported by NIH grant HL39829.

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Delayed Somite Formation and Muscle-specific Gene Activation in a Genetic Line of Japanese Quail with Extensive Muscle Hyperplasia. R. Ivarie, L.L. Coutinho, J. Morris, and [1]H.L. Marks. Department of Genetics, University of Georgia and [1]USDA, ARS, SEPRL, Athens, GA., 30602

A line of fast-growing quail (P) has been genetically selected from a control line (C) for heavy body weight at 4 weeks. The P line has undergone 87 generations of selection; it is three times heavier and examined muscles had a higher number of muscle fibers and myonuclei than the C line. Because myofiber number is determined early in development and muscle cells originate from somites, we have analyzed somite formation in P and C embryos (n>10). After 30 and 40 hr of incubation (1 and 5 somite pairs, respectively), there was no detectable difference in somite number between the lines. At 44 hr, C line had 12.9 ± 1 and P line 11.2±1 somite pairs. However by 47 hr, C had 18.3 ± 2 somite pairs and P had 13 ± 2. These differences were significant at P<0.05. At 65 and 120 hr, both lines appear to be at the same stage of development. To determine whether activation of muscle specific gene expression was also delayed, we used whole mount *in situ* hybridization. The use of digoxigenin-labeled probes and immunological detection with alkaline phosphatase-conjugated antibody to digoxygenin allowed signal development in 6-10 hours. Whole mount *in situ* hybridization studies revealed that at 48 hr of incubation, C embryos were expressing *qmf1* in the first 12 somites, while P embryos expressed *qmf1* in only the first 7 somites. At 64 hr, C embryos had activated MHC in the first 15 somite pairs and P embryos in first 10. The phase delay in somite formation, *qmf1* and MHC activation could be associated with the observed hyperplasia in P line quail.

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Axolotl Pronephric Duct, Cranial and Trunk Neural Crest Cells Follow Different Molecular Guidance Systems. G. Thibaudeau, A.W. Dollarhide, T. Haque, J. Drawbridge, and M. S. Steinberg, Department of Molecular Biology, Princeton University, Princeton, N.J. 08544.

The aim of this study is to elucidate any differences in the ability of different embryonic migratory cell populations to utilize the same guidance system. Previous studies on the migration of the pronephric duct (PND) of *Ambystoma mexicanum* have suggested that the information guiding this migratory event is distributed in a dorsocaudally increasing gradient along the embryonic flank mesoderm ventral to the developing somites. Not only PND primordia but also cranial neural crest cells (CNC), which migrate concurrently with the PND during development, are able to follow this guidance information when transplanted to the flank mesoderm. In order to determine whether PND and neural crest cell populations utilize similar or different molecular systems to guide their migrations, we used the wild type-to-albino cell marking system to observe the migratory behavior of PND, CNC and trunk (TNC) neural crest cell populations when transplanted to one another's migration pathways. Neither PND nor TNC cells followed the guidance system utilized by either of the other two cell populations. Our findings indicate that the three cell populations normally utilize different molecular systems to guide their migrations despite the fact that CNC cells are capable of following the PND's normal pathways. These observations have bearing upon the issue of conservation vs. diversification of embryonic cell guidance systems.

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The Location of the Alkaline Phosphatase Functional in Pronephric Duct Cell Migration. G. Thibaudeau, and M.S. Steinberg, Department of Molecular Biology, Princeton University, Princeton, N.J. 08544.

Experimental studies of the migratory behavior of the embryonic pronephric duct (PND) of the axolotl (*Ambystoma mexicanum*) have suggested that its migration may be guided by a gradient of adhesiveness between the duct's advancing tip and its substratum, the dorsal flank mesoderm. During studies designed to identify the molecule(s) governing this directed migration, the glycosyl phosphatidylinositol (GPI)-linked cell surface protein, alkaline phosphatase (AP), was found to be distributed in a pattern consistent with a potential role as a PND cell guidance molecule. This possibility was reinforced by the finding that removal or blockage of AP [by cleavage from the cell surface with phosphatidylinositol-specific phospholipase-C (PI-PLC) or by reaction with anti-AP monoclonal antibodies] prevented PND migration. In a related study, we learned that cranial neural crest (CNC) cells, which migrate along the same trajectories as PND cells on the flank, can do so even after enzymatic removal of AP. Both PND cells themselves as well as the cells of their migration substratum display AP on their surfaces, but CNC cells do not. This led us to question whether the requirement of AP for PND migration resides on the migrating PND cells themselves or on the cells of its pathway. To determine the location of the AP functional in PND migration, we have utilized PI-PLC to remove GPI-linked proteins, including AP, selectively from either the PND or its pathway. We find that AP-positive PND cells are capable of migrating on an AP-stripped flank, but that AP-stripped PND cells do not migrate even on an AP-positive flank. These results indicate that the AP required for PND migration resides on the duct cells themselves and not on their substratum. This site suggests a role for AP in the PND system related to the capacity of the duct cells to migrate in response to guidance information rather than as a component of the guidance information itself.

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Immunocytochemical Localization of Tumor Necrosis Factor-alpha (TNFα) During Early Chick Embryo Development. M. A. Wride and E. J. Sanders, Department of Physiology, University of Alberta, Edmonton, Alberta, T6G 2H7, Canada.

Tumor Necrosis Factor-α (TNFα) is a cytokine with a role in the pathophysiology of inflammation and cellular immune responses. It is becoming apparent, however, that this protein can also mediate normal tissue remodelling through apoptotic cell deletion and by induction of other growth factors. In this study we describe the spatial and temporal distribution of TNFα from 3 to 6 days of chick embryo development using a polyclonal antibody to mouse TNFα. Immunoreactivity is detected in the notochord and sclerotome of 3 day embryos. At 4 days, a more extensive distribution is observed: notochord, sclerotome and myotome are reactive, as well as heart trabeculae and areas of head mesenchyme, including pre-scleral mesenchyme. The lens fibres of the eye are positive, implicating TNFα in the nuclear disintegration preceding crystallin deposition. In the brain, intense immunoreactivity is observed close to the optic recess, while in the neural tube staining is associated with ventral nerve roots. By days 5 and 6, sclerotome/myotome labelling has diminished and staining is now associated with mesenchymal cells outside the myotome. Staining persists in the lens, brain and notochord and is increased in ventral nerve roots and peripheral nerve ganglia. These observations suggest a fundamental role for TNFα in early avian development. (Supported by the Medical Research Council and the Alberta Heritage Foundation for Medical Research).

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Migration of Medial Edge Epithelia (MEE) In Vitro Stimulated by Epidermal Growth Factor. C.F. Shuler, Y. Guo, K. Dalrymple, G. Ackermann, Ctr. for Craniofacial Molec. Biol. University of Southern California, Los Angeles, CA 90033.

The fate of the MEE during palatogenesis has been determined by cell lineage analysis with DiI labeling. The MEE both *in vivo* and *in vitro* have been shown to undergo transdifferentiation from an epithelial to a mesenchymal phenotype. *In vivo* the transdifferentiated MEE migrate through the palatal mesenchyme during subsequent fetal development. *In vitro* the transdifferentiated MEE remain localized in the midline fusion zone. In this study we examined the effects of exogenous EGF on the process of palatal fusion and MEE migration *in vitro*. Fetal mouse palatal shelves (14d 3h p.c.) were labeled with DiI (0.025%), dissected, and placed in organ culture with the MEE approximated in a chemically-defined medium (BGJb). EGF was added to the medium of the experimental cultures in concentrations of either 20ng/ml, 200ng/ml or 2000ng/ml. The cultured palatal shelves were examined 24, 48, 72, and 96 hours after initiation of the organ culture. The tissues were frozen for DiI detection and immunohistochemistry. The localization of keratin, vimentin, laminin and epidermal growth factor receptor was determined by immunohistochemistry. In the control and 20ng/ml EGF-treated cultures the MEE underwent phenotypic transdifferentiation but remained in the midline fusion region. Treatment of the cultures with 2000ng/ml EGF inhibited palatal fusion and the MEE retained their epithelial phenotype. The 200ng/ml EGF treated palatal shelves fused and the MEE transdifferentiated in a sequence identical to the control cultures however the DiI labeled MEE migrated into the surrounding mesenchyme. The data suggest that MEE migration following phenotypic transdifferentiation is dependent on epigenetic factors. (Supported by P50 DE-09165).

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Guanylate Depletion is an Early Metabolic Event in Dictyostelium Development Triggered by Nutrient Deprivation. D.G. Wright and A.R. Kimmel, Department of Hematology, WRAIR, Washington, DC, and Laboratory of Cellular and Developmental Biol, NIDDK, NIH, Bethesda, MD.

Depletion of intracellular GTP has been shown to be a critical metabolic event in the starvation-induced sporulation of Saccharomyces cerevisiae and Bacillus subtilis. It has also been shown that GTP depletion, as well as an increased ratio of [GDP]:[GTP] and a down-regulation of IMP-dehydrogenase (IMPD) activity, which mediates the rate-limiting step of guanylate synthesis from IMP, are features of both the terminal maturation of normal hematopoietic progenitor cells and the induced maturation of leukemia cell lines. Furthermore, inhibitors of IMPD have been shown to induce the maturation of human myeloid leukemia cells by causing guanylate depletion. In order to determine whether the early development of Dictyostelium discoideum (Dd) is also associated with changes in guanylate metabolism, we measured [GTP], [GDP]:[GTP] ratios, and IMPD activity in Dd during early development initiated by nutrient deprivation. Vegetative Dd were grown to $2x10^6$ cells/mL and then resuspended at $1x10^7$ cells/mL in shaking cultures with nutrient deprivation medium both with and without added cAMP as described previously (Dev Biol 122:163-171, 1987). [GTP] and IMPD activity were found to be decreased by 50-70% within 8 hrs of initiating nutrient deprivation cultures and [GDP]:[GTP] ratios were increased >3 fold. Maximal changes in [GTP] and IMPD activity were similar in Dd cultures with and without cAMP, but occurred more rapidly when cAMP was added. These studies demonstrate that guanylate deprivation is a metabolic feature of the early development of Dd and indicate that Dd may be a useful experimental model for examining the role of guanylate deprivation in triggering cellular maturation.

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Generation of Developmental Mutants in Dictyostelium by Transformation with an Antisense cDNA Library. T.Spann, and R. Gomer, with technical support from R. Ammann and D. Brock, Howard Hughes Medical Institute, Dept. of Biochemistry & Cell Biology, Rice University, Houston, TX 77251.(Spon. by J.W. Campbell.)

A small genome and a simple life cycle make Dictyostelium discoideum an ideal eukaryotic system to study mechanisms of cell differentiation. Dictyostelium normally exists as free amoebae which feed on bacteria; however, when starved these vegetative cells begin a developmental program. The cells aggregate and form a slug which migrates to a suitable site to then form a fruiting body which is made up of two major cell types, spore and stalk. After dispersal, spore cells can germinate to form vegetative cells. Although transformation procedures are well established in Dictyostelium, determining the genes responsible for morphological mutants has proven to be exceedingly difficult. In this report, we describe a mutagenesis technique that uses antisense cDNA to identify genes required for normal development. We have generated developmental mutants by transforming Dictyostelium cells with a cDNA library produced from the mRNA of vegetative and developing cells. The cDNA is cloned in an antisense orientation immediately downstream of an actin promoter. Individual transformants contain 1-4 different antisense cDNAs. We have concentrated on two types of mutants, those which, although motile, fail to aggregate and those which aggregate but do not form fruiting bodies. The individual cDNA molecules from the mutants were identified and cloned using PCR and retransformed into Dictyostelium. The cDNAs are currently being further characterized and sequenced. The phenotype of Dictyostelium cells transformed with a specific cDNA matches that of the original mutant from which the cDNA was obtained. We feel that this technique will prove useful in analyzing the genes involved in cell motility, differentiation and morphogenesis.

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Cloning and Characterization of Sea Urchin Homologues of *Snail* and *Twist*. C. Illingworth, J. Cheetham and J. Hardin, Zoology Department, University of Wisconsin, 1117 W. Johnson St., Madison, WI 53706

The sea urchin embryo is a convenient system for studying pattern formation by mesenchyme cells during gastrulation. We have sought molecular probes specific to secondary mesenchyme cells in order to determine whether distinct populations of these cells respond to different guidance cues as they differentiate. Using low stringency library screening and/or PCR, we have isolated two transcription factors known to be mesoderm-specific in other organisms: *u-snail* and *u-twist*. Conceptual translation of a 1.2 kb *u-snail* cDNA yields a sequence of 338 amino acids with large regions of identity to both the *Drosophila* and *Xenopus snail* proteins, including five canonical zinc-finger loops, and large non-canonical regions between the loops. PCR amplification resulted in the cloning of the basic helix-loop-helix domain of *u-twist*; canonical b-HLH amino acids are 100% conserved, and overall *u-twist* is 86% identical with the *Drosophila* sequence at the amino acid level. We have used whole mount in situ hybridization to localize *u-snail* transcripts in *Lytechinus variegatus*. Interestingly, at the late gastrula stage a group of 6-7 cells at the tip of the archenteron are *u-snail* positive. At the early prism stage, two clusters of 5-6 cells in the right and left ventrolateral ectoderm are *u-snail* positive. Staining is not detectable at the pluteus stage. These results suggest that transient expression of *u-snail* may be important for the differentiation of a subset of secondary mesenchyme cells that recognize pattern information in the ventrolateral ectoderm. We are currently performing additional in situ hybridizations and are generating polyclonal antibodies to *u-snail* and *u-twist* fusion proteins to examine their spatial distribution in the embryo.

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Cell Motility and Involution During the Initial Phase of Gastrulation in Sea Urchins. R.D. Burke and R.L. Myers

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We have previously reported that the cells lateral to the vegetal plate move into the archenteron during the initial phase of gastrulation and have proposed that tractoring of cells on the hyaline layer is responsible for these movements (Burke et al. 1991 Dev. Biol. 146:542-557). Using time lapse video microscopy we have documented the movements of these cells during a brief phase coincident with the buckling of the vegetal plate. Using irregularities in the hyaline layer or silica particles attached to the hyaline layer as reference points, it can be shown that the cells move relative to the hyaline layer. During these movements the apical ends of the cells lateral to the vegetal plate become skewed towards the vegetal plate. The movements and skewing of the cells is associated with the extension and retraction of apical pseudopodia in the direction of the vegetal plate. Treatment of embryos with monoclonal antibodies to the apical lamina, a component of the hyaline layer inhibits the initial phase of gastrulation and reduces the apical skewing of the cells lateral to the vegetal plate. It is suggested that the movements of these cells produces a compressive force at the vegetal pole of the embryo which is in part responsible for the inward movement of the vegetal plate.

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Localization and Characterization of an Extracellular Matrix Antigen Involved in Sea Urchin Gastrulation: Evidence for Its Generation by Proteolysis. O. Vafa, D. Yen, C. Thomas, R. Ward, and D. Nishioka Dept. Biol., Georgetown Univ., Washington, DC 20057.

Monoclonal antibody (mAb) LG11C7 was generated against testicular cells of the sea urchin, Strongylocentrotus purpuratus. Indirect immunofluorescence and immunogold electron microscopy reveal that it recognizes a component of the basal lamina lining the blastocoele of early embryos. Exposure of embryos to mAb LG11C7 causes exogastrulation, presumably by blocking antigenic sites in the basal lamina. Western immunoblots indicate that mAb LG11C7 recognizes embryonal antigens with relative molecular weights of 158, 68, and 37 kDa. Developmental studies employing immunoprecipitations and Western blot analyses show that the 68 kDa antigen appears between 18 and 24 hr after fertilization, closely preceding gastrulation. The appearance of the 68 kDa antigen may be blocked by exposing embryos to 2 mg/ml soybean trypsin inhibitor (SBTI) beginning 9 hr following fertilization, suggesting that the 68 kDa antigen is generated by proteolysis. Additionally, gastrulation is inhibited in embryos treated with SBTI. These results suggest that the 68 kDa antigen is a component of the basal lamina required for normal gastrulation and that it is generated by developmentally regulated proteolysis. (Supported by NIH Grant HD-19054).

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Origin and Characterization of Fertilization Envelope Proteins in the Sea Urchin Egg. M. Laidlaw and G.M. Wessel, Division of Biology and Medicine, Brown University, Providence, RI 02912.

An egg synthesizes and stores the organelles and proteins required to prevent the entry of multiple sperm during fertilization. In the slow block to polyspermy, a mass secretion of cortical granules inflates and modifies the vitelline layer (VL) to form the fertilization envelope (FE), physically excluding multiple sperm from the fertilized egg. To understand the mechanism of protein targeting to the FE we have begun by isolating cDNAs that encode FE proteins. A crude preparation of FE protein from *Strongylocentrotus purpuratus* was injected into rabbits and a polyspecific polyclonal serum was obtained. This serum was used to screen a cDNA expression library made from poly A RNA isolated from *Strongylocentrotus purpuratus* ovaries. Nine independent clones resulted from a screen of over 250,000 plaques. Two clones, SFE1 and SFE6 were used to make fusion peptides in *E. coli* that were then injected into rabbits to produce monospecific polyclonal antibodies. Immunolocalization with these sera showed SFE1 to be present in cortical granules in mature oocytes and then concentrated in the FE after the fertilization reaction. In contrast, SFE6 was diffuse throughout the cytoplasm of immature oocytes and was subsequently concentrated in an extracellular layer of mature oocytes, likely to be the vitelline layer. Western blot analysis of FE proteins with these sera has shown that SFE1 and SFE6 represent different polypeptides that migrated at 200 kDa and 26 kDa respectively. Northern blots of these clones showed enrichment of both mRNA in eggs as compared to later stages in development. Thus, these clones can be used as tools to study the ontogeny of cortical granules and the fertilization envelope in the developing sea urchin oocyte.

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Mapping Cell Surface Sugar Receptors in Early Cleavage Stage Sea Urchin Embryos Using Lectin Derivatized Beads. J. Ducut, M. Lopez, V. Latham, C. Herrera and S.B. Oppenheimer, Department of Biology and Center for Cancer and Developmental Biology, California State University, Northridge, Northridge, CA 91330.

Strongylocentrotus purpuratus eggs were treated with dithiothreitol to prevent formation of fertilization membranes. After fertilization, some samples were also treated with calcium-magnesium-free sea water to remove the hyaline layer. One and two cell embryos treated as described were rotated with lectin derivatized agarose beads (Sigma) with or without sugars. No cell binding to any lectin beads was observed when the hyaline layer was intact. When removed, however, one and two cell embryos bound to Dolichos biflorus, Vicia villosa and Triticum vulgaris derivatized beads but not to Ricinus communis 60, Tetragonolobus purpureas , Pisum sativum or Phytolacca americana derivatized beads. This method provides a means of identifying cell surface sugar receptors that may be developmentally important and lectin beads could be used to affinity purify specific cell surface sugar receptors in investigations on their function in specific developmental events.(supported by grants and fellowships from NIH MARC, Thomas Eckstrom Trust, Joseph Drown Foundation and California State University, Northridge).

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Cell Cycle Of Artemia Epidermal Cells During Growth and Differentiation. J.A. Freeman. Department of Biological Sciences, University of South Alabama, Mobile, 36688.

The form of an organ is established through cell replication and differentiation during early morphogenesis. Cell differentiation may involve a change in the cell cycle such that the cell leaves the cycling population or replicates at a much slower rate. In the developing segment of instar IV brine shrimp, the medial population of epidermal cells on the dorsal surface differentiate to general epidermal cells by increasing the apical surface area and cycle of at a greatly reduced rate. The lateral population continues to replicate. Similar changes occur in ventral cells forming the limb bud. Under low nutrient conditions, the medial cells remained in G1 and did not expand the apical surface area. Few of the lateral epidermal cells that were in early G1 entered S phase (BrdUrd incorporation) or divided. To characterize the nutrient-dependent G1 transition step, the thorax epidermis of starved and fed larvae was stained for DNA (Hoechst 33258) and nuclear protein (FITC). In larvae reared under low nutrient conditions early G1 cells in both populations demonstrated low nuclear protein levels and did not pass the G1a-G1b transition point, defined as the level of nuclear protein content associated with the commitment to progress through the cell cycle or differentiate to a general epidermal cell. There was no nutrient-dependent control point in G2. The results suggest that epidermal cells must pass a G1 restriction point by acquiring a minimum level of protein or a specific nutrient before entering S phase or differentiating.

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A Novel Member of the Drosophila Nuclear Receptor Family Implicated in the Regulation of Fushi Tarazu. C. Ohno and P.M. Petkovich
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The Drosophila melanogaster gene censor (cnr), encoding a novel member of the steroid/thyroid hormone receptor gene superfamily, was isolated by cross-hybridization with a complementary DNA for the Drosophila nuclear receptor, FTZ-F1. The cDNA deduced protein sequence for cnr displays significant amino acid identity in both the DNA and ligand binding domains with vertebrate and invertebrate nuclear receptors. The DNA binding domain of cnr is most similar in sequence to that of FTZ-F1. Bacterially expressed cnr protein binds to a FTZ-F1 binding site found in the zebra stripe promoter element of the segmentation gene fushi tarazu (ftz). Northern blot analysis with the cnr cDNA detected expression of a 5.1 kb cnr transcript at all stages of the Drosophila life cycle except in early embryos. Maximal expression of this 5.1 kb cnr transcript was observed at the prepupal stage of development. Early embryos express a single 3.5 kb RNA species, possibly representing a maternal transcript. In situ hybridization in whole mounted embryos detected transcripts for cnr evenly expressed throughout the blastodermal layer in early embryos. At later stages of development strong cnr expression is observed in both the brain and ventral chord structures as well as in the hindgut. Temporal and spatial expression patterns of the cnr gene suggest that cnr may have multiple roles in early embryogenesis, neurogenesis, and in the adult. Furthermore, the identification of cnr as a nuclear receptor family member suggests that an as yet undiscovered cnr specific ligand is involved in Drosophila development.

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Quantitative Studies of *Wolbachia pipientis* in inter- and intra-specific transfer lines of *Drosophila*. L. Boyle[1], S. O'Neill[2], H. Robertson[1], and T. Karr[1], 1) Departments of Biochemistry and Entomology University of Illinois, Urbana, IL 61801, 2) Yale University School of Medicine, New Haven, CT 06510.

Cytoplasmic incompatibility(CI) is a phenomenon in which matings between uninfected females and infected males fail to produce progeny. The probable causative agent is *Wolbachia pipientis*, a rickettsia-like endosymbiont. To better understand how this endosymbiont causes CI and how it is transmitted through the generations; we microinjected the bacteria from one of it's natural hosts *Drosophila simulans* Riverside(DSR) into a tetracycline treated DSR line (DSR_T) and into *D. melanogaster* (DM). Isofemale lines were established and tested for CI. The DSR_T line showed a stable infection level(as seen via PCR and DAPI fluorescence of fixed embryos) and a high level of CI (although not as high as for a wild type CI cross). The DM lines however, show little or no significant level of CI. PCR and DAPI fluorescent staining shows that the level of infection in these lines is highly variable. This raises the question of whether or not a "threshold" level of infection is necessary for CI expression. Using laser scanning confocal microscopy (LSCM) bacterial distribution and quantification for the control (DSR) and all transinfected lines was obtained. The results lend credence to our theory of a threshold level being required for CI.

Protein Folding and Assembly (641-644)

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Aspartyl Residue-10 is Essential for ATPase Activity of Rat Hsc70. S-P. Huang, M-Y. Tsai, Y-M. Tzou, W-g. Wu, and C. Wang Institute of Molecular Biology, Academia Sinica, Taipei and Institute of Life Sciences, Tsing Hua University, Hsinchu, Taiwan.

Site-directed mutation was utilized to substitute Asn for Asp-10 of rat hsc70. The mutatant, hsc70(D10N), lost not only its constitutive but also peptide-stimulated ATPase activity. Furthermore, the N-terminal ATPase domain of rat hsc70, Nt-hsc70, has been expressed in *E. coli* and has been purified to homogeneity. The ATPase activity at 37°C of the Nt-hsc70, 270 pmol/hr/μg of protein, is comparable to that of peptide-activated hsc70. This N-terminal ATPase core also loses its capability to hydrolyze ATP after Asp-10 has been replaced by Asn. These site-directed mutants are capable of binding ATP; and, based on the results of fluorescence spectroscopy, the three dimensional structure of the protein remains largely unchanged after mutation. Therefore, Asp-10 of hsc70 appears essential for ATP hydrolysis. Purified hsc70 and its mutants can be phosphorylated *in vitro* at a substoiciometric level. In average, less than 1% of the hsc70 and Nt-hsc70 proteins are phosphorylated. Furthermore, the amount of phosphates incorporated into hsc70(D10N) and Nt-hsc70(D10) is reduced; however, significant level of phosphorylation can be achieved in these two site-directed mutants. Hence,*in vitro* phosphorylation of hsc70 and its mutants is not correlated with their ability to hydrolze ATP.

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The Threshold Induction Temperature of a 90-kDa Heat Shock Protein is Subject to Acclimatization in Eurythermal Goby Fishes. T.J. Dietz and G.N. Somero, Department of Zoology, Oregon State University, Corvallis, OR 97331.

Heat shock or stress proteins have roles in stressed and unstressed cells. Some stress proteins function as molecular chaperones, mediating such events as protein folding and translocation while others are implicated as having structural roles. The examination of two extremely eurythermal goby fishes, Gillichthys mirabilis and Gillichthys seta, which encounter habitat temperature variations of ca. 30°C, showed seasonal acclimatization of endogenous levels and of onset temperatures for enhanced synthesis of a 90-kDa stress protein (HSP90) in brain. Summer-acclimatized fishes had higher levels of HSP90 in brain tissue than winter-acclimatized specimens, as shown by Western blot analysis. For winter-acclimatized fishes, increased synthesis of HSP90 was observed when the temperature was raised from a control temperature (18°C) to 28°C. For summer-acclimatized fish, no significantly increased synthesis of HSP90 occurred until the experimental temperature was raised to 32°C. These data suggest that the threshold temperature at which enhanced expression of HSP-encoding genes occurs is not hard-wired genetically but may be subject to acclimatization. The ability of an organism to acclimatize the set point for stress protein synthesis may lie in its ability to adjust the steady-state levels of stress proteins in its cells under non-stress conditions.

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Role of BiP in folding and transport of secretory polypeptides in *S. cerevisiae*., J. Rocco and R. Green, Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, N.Y. 10029 (Spons. by R. Green).

The precise function of the ER chaperone protein BiP in monitoring the structural maturation of nascent secretory proteins remains to be elucidated. We describe here a model system to study the role of yeast BiP in the intercompartmental transport and processing of a triple glycosylation mutant of preprodactor (ΔN123). Expression of ΔN123 in yeast results in a 75-80% reduction in the secretion of mature α factor, accompanied by increased intracellular degradation of the mutant prohormone (Caplan et al., J. Bacteriol. 173, 627; 1991). A role for BiP in this phenomenon is indicated by the following observations: 1) Overexpression of ΔN123, but not wild type prodactor, leads to a 5-fold increase in the steady-state level of BiP mRNA; and 2) Treatment with tunicamycin (TM), an inhibitor of N-linked glycosylation, *rescues* the secretion of α factor from ΔN123-expressing cells. Since ΔN123 prodactor is itself never glycosylated, the surprising *trans* effect of TM could result from its documented ability to increase intracellular BiP, which in turn could promote appropriate folding and transport of the mutant precursor. Alternatively, TM treatment, which also leads to the accumulation of bulk unglycosylated (and presumably misfolded) proteins in the ER lumen, could saturate the available BiP and allow the escape of ΔN123. To distinguish between these postulated positive or negative roles for BiP in ΔN123 biogenesis, yeast strains have been constructed in which expression of BiP can be regulated independently of TM. Overexpression of BiP relative to ΔN123 results in a further reduction in α factor secretion, which can be partially reversed by treatment with TM. These data suggest that, in this case, BiP acts to retain the mutant precursor in the ER and possibly targets it for degradation, and that other unglycosylated proteins can compete with ΔN123 for BiP binding. ΔN123 prodactor would thus appear to be one of the first candidates for a direct in vivo substrate for yeast BiP. This system should allow a detailed analysis of the molecular recognition events that regulate intercompartmental transport and processing in the yeast secretory pathway.

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Transcriptional regulation of yeast BiP, J. Rocco and R. Green, Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, N.Y. 10029 (Spons. by J. Rocco).

In *S. cerevisiae*, expression of the ER chaperone BiP is induced by a variety of stressors, including heat shock and treatment with tunicamycin (TM), an inhibitor of N-linked glycosylation. A working hypothesis is that accumulation of misfolded proteins in the ER lumen initiates a signalling pathway that ultimately increases transcription from the BiP promoter. The precise nature of the primary signal(s), however, remains to be determined (i.e. general or specific misfolded proteins, free BiP, BiP:substrate complexes, etc.). To investigate this phenomenon, we constructed centromeric and high-copy plasmids containing BiP cDNA under the control of the copper-inducible yeast CUP1 promoter, and introduced them into wild-type yeast; in some strains, additionally, the chromosomal BiP gene was disrupted by homologous recombination. First, we observed that induction of plasmid-encoded BiP prior to exposure of cells to TM protects cells from the cytotoxic effects of this agent. This result provides the first direct evidence that, in analogy to thermotolerance conferred by cytosolic heat shock proteins, induction of BiP by TM represents a functional response to secretory pathway stress. Second, using hybridization probes that discriminate between chromosomal- and plasmid-encoded BiP mRNA, we tested the whether plasmid-directed BiP overexpression could reduce the TM-mediated induction of BiP promoter activity. In contrast to what is observed in mammalian cells, 10-fold overexpression of BiP mRNA and protein had little effect on TM-stimulated transcription. This observation suggests that, in this system, the primary signal for transcriptional induction by TM is not a simple decrease in the intraluminal concentration of free (i.e. uncomplexed) BiP protein. Furthermore, maximal induction of BiP mRNA by TM is observed under conditions in which inhibition of N-linked glycosylation is sub-maximal. We speculate that TM induction of BiP results from the appearance of a specific unglycosylated "sensor" protein, or, alternatively, from earlier effects of TM treatment (e.g. the accumulation of unglycosylated dolichol phosphate in the ER membrane).

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Requirement of a Factor for Uncoating of Clathrin Baskets by Uncoating ATPase. K. Prasad, W. Barouch, L. Greene, and E. Eisenberg, Lab of Cell Biology, NHLBI, NIH, Bethesda, MD 20892

Uncoating ATPase, a constitutively expressed member of the hsp70 family of heat shock proteins, dissociates clathrin from clathrin coated vesicles in an ATP dependent reaction. Clathrin baskets made from purified clathrin and mixed assembly proteins are uncoated by uncoating (UC) ATPase in the same way as coated vesicles. However, we now find that baskets made from clathrin and the purified assembly protein, AP-2, which is associated with the plasma membrane, are not uncoated by UC ATPase, alone. Addition of the flow-through fractions from the hydroxylapatite column used in the purification of AP-2 is necessary to restore the uncoating activity to its original level, demonstrating the existence of a new factor essential for uncoating. This factor is insensitive to heat, GdHCl, and NEM, but is sensitive to proteolysis. After partial purification, we find that it has a subunit MW of about 55 kDa based on its mobility on SDS gels. It appears to work at a stoichiometry of less than one 55 kDa subunit per clathrin triskelion in the baskets. Although we do not yet understand its mechanism of action, it does not appear to be a kinase. We conclude that the UC ATPase, alone, is not sufficient to uncoat clathrin from coated vesicles, but requires another factor for the uncoating process. This factor may play an important role in regulating the uncoating of clathrin coated vesicles in vivo. The interaction of this factor with the UC ATPase may be analogous to the interaction of the cofactors, DnaJ and GrpE, with DnaK, the 70 kDa protein of E Coli.

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Analysis of a Series of Deletion and Site-Directed Mutations in Yeast *PDI*: Effects on Viability, Cellular Localization and *in vitro* Catalytic Activity. M.L. LaMantia and W.J. Lennarz, SUNY at Stony Brook, Dept. of Biochemistry and Cell Biology, Stony Brook, NY 11794.

Protein disulfide isomerase (PDI) is a multifunctional protein residing in the ER lumen of a diverse variety of organisms. *In vitro*, PDI catalyzes the oxidation, reduction, and isomerization of disulfide bonds in other protein substrates; *in vivo*, PDI is believed to indirectly facilitate proper protein folding by catalyzing disulfide bridge formation in nascent secretory proteins, although this function has not been addressed directly. In all systems thus far studied, PDI contains two active sites, consisting of the tetrapeptide sequence, Cys-Gly-His-Cys. These sequences are thought to be directly involved in PDI catalysis. We are interested in the role PDI plays in the yeast, *Saccharomyces cerevisiae*, and have previously shown it to be essential for viability in this organism. However, our preliminary evidence suggested that certain C-terminal deletions in PDI could be tolerated without affecting cell viability. We have confirmed and extended these results through the analysis of a series of *PDI* deletion and active site- directed mutant strains created and subsequently isolated by the plasmid shuffling technique. In this study, we have determined that removal of up to 1/3 of the amino acid sequence from the C-terminus of PDI does not affect cell viability, but has profound effects on its cellular localization. The lack of any observable growth defect in strains containing extensive C-terminal deletions suggests that the second PDI active site and the ER-retention signal (which have been removed in these mutants) is not required for cell viability. We are in the process of comparing the viability and localization data obtained from these mutants with their catalytic activity *in vitro*. The mutant proteins have been expressed and isolated from *E. coli*, and their ability to refold and reactivate reduced, denatured trypsin inhibitor is currently being investigated. Supported by NIH grant GM33185 to WJL.

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Molecular Requirements for the Cell Surface Expression of Multisubunit Ion Transporting ATPases. C.J. Gottardi and M.J. Caplan, Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT 06510.

The oligomerization of individual membrane protein subunits to form a multisubunit assembly takes place in the ER and is required for the delivery of a functionally competent complex to the cell surface. The ion transporting H,K-ATPase and Na,K-ATPase enzymes are each comprised of an α and β subunit. It is known that assembly between α and β subunits of the Na,K-ATPase is necessary for the cell surface delivery of the active enzyme. We have examined the molecular domains involved in the assembly of the H,K-ATPase and Na,K-ATPase α and β subunits by expressing individual subunits and subunit chimeras in transiently transfected COS-1 cells. Our results demonstrate that the H,K-ATPase α subunit requires its β for efficient cell surface expression, as determined by indirect immunofluorescence. We find that transfection of a chimera encoding the NH_2-terminal half of the H,K-ATPase α and the carboxy-terminal half of the Na,K-ATPase α can assemble with endogenous Na,K-β subunit and reach the plasmalemma. Transfection of the complementary α chimera requires co-expression with H,K-β in order to attain surface delivery. Thus, it is the carboxy-terminal half of the α subunit which specifies assembly with a particular β subunit. Interestingly, the H,K-β subunit appears to be able to get to the cell surface unaccompanied by any alpha-like subunit, and appears to localize to a population of intracellular vesicles. (Supported by NIH GM-42136 and the David and Lucille Packard Foundation)

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In vivo dimeric association of class I MHC heavy chains. G.G. Capps, B.E. Robinson, K.D. Lewis, and M.C. Zúñiga, Department of Biology, The University of California, Santa Cruz, CA 95064.(Spon by J. Feldman)

Class I MHC molecules are believed to occur in vivo as heterodimers of the class I MHC heavy chain and β_2-m light chain which are or are not associated with antigenic peptide, and as free class I MHC heavy chains. We have now found that class I MHC molecules also occur as heavy chain-heavy chain dimers. Biochemical studies show that heavy chain dimers are disulfide-linked via a conserved cytoplasmic domain cysteine. H-2L^d, H-2D^b, and H-2D^d class I dimers are not associated with β_2-m and fail to react with certain α_1 and α_2 domain-specific antibodies. In vitro studies show that newly synthesized H-2L^d dimers can be dissociated by the addition of specific antigenic peptide and β_2-m. Concomitant with heavy chain dimer dissociation H-2L^d molecules become reactive with mAb 30.5.7, an antibody which is sensitive to the conformation of the peptide binding domains. These findings indicate that exogenously supplied β_2-m and peptide reduce the amount of H-2L^d dimer by forming an H-2L^d-antigenic peptide-β_2-m complex. Biosynthetic analyses of heavy chain dimer formation in β_2-m$^+$ and β_2-m$^-$ cell lines indicate that the availability of β_2-m influences the time at which heavy chain dimers appear: In β_2-m$^+$ mouse cells H-2L^d heavy chains form late in biosynthesis, whereas in β_2-m$^-$ cells they form immediately. We conclude that dimer formation occurs as a consequence of loss or unavailability of β_2-m and antigenic peptide. (Supported by NSF grants DCB-9196051 and DCB-9096241)

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Dependence of stable peptide binding by the H-2L^d class I MHC molecule on its association with β_2-microglobulin early in biosynthesis. M.C. Zúñiga, H. Wang, and B.E. Robinson, Department of Biology, The University of California, Santa Cruz, CA 95064.

In β_2-m$^-$ HCT-L^{d}3 cells, cell-surface H-2L^d is detectable only by the α_3-domain specific mAb 28.14.8. In contrast, pulse-chase experiments show that H-2L^d is transiently detectable early in biosynthesis by the α_2-domain specific mAb 30.5.7 (whose binding is influenced by association with β_2-m and peptide). The addition of an H-2L^d-specific peptide (L^d peptide) to HCT-L^d cell lysates greatly enhances both the quantity and half life of mAb 30.5.7-reactive β_2-m$^-$ H-2L^d molecules. The stability of the H-2L^d-peptide complex, however, is not as stable as the same complex made in β_2-m$^+$ cells, even if exogenous β_2-m is added to the β_2-m$^-$ cell lysates. Kinetic analyses of peptide binding shows that when either mouse or human β_2-m is present at the time of biosynthesis of H-2L^d, L^d peptide binding increases continuously over a 16 hour incubation period. However, if β_2-m is not present at the time of H-2L^d biosynthesis, but is added at the time of addition of peptide, L^d peptide binding by H-2L^d is four-fold lower and is unstable, falling off considerably by 16 hours after addition of peptide. The loss of L^d peptide is not due to dissociation from the exogenous β_2-m, which remains stably associated with H-2L^d throughout the entire incubation period. Biosynthetic studies with β_2-m$^+$ cells show that exogenous L^d peptide appears to be bound by Endo H-sensitive and not by Endo H-resistant H-2L^d molecules. Taken together with the above studies on H-2L^d molecules from β_2-m$^-$ cells, these results suggest that stable association of H-2L^d with antigenic peptide requires that β_2-m be available at the time of synthesis of the H-2L^d heavy chain. (Supported by NSF grants DCB-9196051 and DCB-9096241)

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The Use of Brefeldin A to Localize the Intracellular Association of Class I MHC with Naturally Processed Peptide. C.Lapham, J. Yewdell, and J. Bennink, VIS, LVD, NIAID, Bethesda, MD.

Indirect evidence has suggested that class I Major Histocompatibility Complex (MHC) molecules associate with peptide antigens in the endoplasmic reticulum (ER) and that association with the "correct peptide" is required for stabilization and subsequent transport of class I out of the ER. We isolated MHC-specific nucleoprotein peptides from cells that were infected with influenza virus in the presence of BFA. Since BFA blocks protein trafficking to the Golgi complex, and the association of peptide with class I MHC is necessary for its isolation from whole-cell lysates, this is the first direct evidence that naturally processed peptides associate with class I MHC molecules in an early transport compartment.

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The role of inter-chain disulfide bonds in fibrinogen assembly and secretion. J.Z. Zhang, and C.M. Redman, New York Blood Center, NY 10021. (Spon. by D. Banerjee.)

Fibrinogen is a dimer with each half-molecule composed of 3 different chains (Aα, Bβ,γ). The 2 half-molecules are linked by 3 symmetrical disulfides between adjacent Aα 28 and γ 8 and 9. There are 26 other inter- and intra-chain disulfide bonds. Fibrinogen has a trinodal structure with the central N-terminal domain linked to globular C-terminal domains by a "coiled-coil" region. Flanking the coiled region, in each of the chains, are 2 pair of cysteines which form inter-chain disulfide rings. To study the role of inter-chain disulfides in fibrinogen assembly various cysteines in Bβ and γ were changed to serine by site-directed mutagenesis. The 3 chains were transiently co-expressed in COS cells and assembly and secretion measured. Changing Bβ 76 and 80, or γ 19 and 23, which flank the N-terminal end of the coiled region, markedly inhibited assembly and secretion. Substituting Bβ 193 and 197 or γ 135 and 139 at the C-terminal side also inhibited secretion but the fibrinogen chains were assembled. Changing both pairs of cysteines at either side of the coiled region abolished assembly and secretion. Other substitutions, Bβ 65, Bβ 76 or Bβ 65 and 76 had less, or no, effect on assembly and secretion. Significantly, changing the Aα 28 and γ 8 and 9, which link the 2 half-molecules, did not affect assembly or secretion.

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Unusual Structural Features in Spider Dragline Silk. M.B. Hinman, Z. Dong, and R.V. Lewis, Department of Molecular Biology, University of Wyoming, Laramie, WY 82071

Spider dragline silk is a unique protein fiber possessing both high tensile strength and high elasticity. A partial cDNA clone for one dragline silk protein (Spidroin 1) was previously isolated. However, the predicted amino acid sequence could not account for the amino acid composition of dragline silk. We have isolated a partial cDNA clone for another dragline silk protein (Spidroin 2), demonstrating that dragline silk is composed of multiple proteins. The amino acid sequence exhibits an entirely different repetitive motif than Spidroin 1. Spidroin 2 is predicted to consist of linked β-turns in proline-rich regions which alternate with β-sheet regions composed of polyalanine segments. When combined with biophysical studies done on synthetic peptides derived from Spidroin 2, a model for dragline silk structure and function emerges.

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The Putative Role of a Thioredoxin-like Molecule in Growth Cones. M.R. Wood and K.H. Pfenninger, Department of Cellular and Structural Biology, University of Colorado School of Medicine, Denver, CO 80262. (Spon. by M.-F. Maylie-Pfenninger)

Saxitoxin (STX) binding to sodium channels in growth cones (GCPs) isolated from fetal rat brain shows high affinity (↑NaCh) binding sites mainly on the plasmalemma and low-affinity (↓NaCh) sites on an internal membrane compartment. High K^+ stimulation of intact GCPs externalizes the internal NaCh, increasing the numbers of both ↑NaCh and ↓NaCh on their surfaces. This suggests a precursor-product relationship and could be due to channel oligomerization involving the formation of S=S bonds between the channel α and β2 subunits (J. Neurosci., in press). Membranes treated with reducing agents show a shift from ↑NaCh to ↓NaCh. We now have evidence that the dithiol-disulfide oxidoreductase THIOREDOXIN (TRX) has a similar effect on STX binding affinities. An antibody to E. coli TRX shows (a) intense immunofluorescence in neurites and especially growth cones of cultured cortical neurons and (b) a major band at 23.8kD in Western blots of GCPs. This may be a dimer of TRX (MW12,000) or may be a larger, related molecule with oxido-reductase/isomerase activity. We hypothesize that this molecule may play a part in the assembly of disulfide-linked protein subunits of the NaCh and consequent conversion from the immature ↓NaCh to the mature ↑NaCh. Such plasmalemmal oligomerization processes seem to occur for other membrane proteins as well. (Supported by NIH NS24676).

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Characterization of Protein-Disulfide Isomerase Secreted from Activated Platelets. Kui Chen and Thomas C. Detwiler, Department of Biochemistry, SUNY HSCB, Brooklyn, NY 11203

Intramolecular and intermolecular disulfide bond isomerizations have been observed with proteins released by activated platelets (Danishefsky, Alexander, Detwiler: *Biochemistry*, 23:4984, 1984; Speziale, Detwiler: *J. Biol. Chem.*, 265:17859, 1990; Speziale, Detwiler: *Arch. Biochem. Biophys.* 286:546, 1991). These observations led to the hypothesis that activated platelets release a protein-disulfide isomerase (PDI). PDI activity, measured by renaturation of scrambled RNase (RNase with mismatched disulfide bonds) was detected in the releasate of platelets. PDI was purified from platelets. It showed an apparent mass of about 62 kDa on SDS-PAGE and a pI of about 4.5 on isoelectric focusing, both consistent with other PDIs. Sequence analysis of the purified protein indicated that the first 33 residues were identical to those predicted by Tasanen *et al.* (*J. Biol. Chem.*, 263:16218, 1988) from the sequence of human genomic DNA for the β-subunit of prolyl 4-hydroxylase, a protein believed to be an identical gene product to PDI. In Western blots, antisera against human platelet PDI or bovine liver PDI reacted with a protein in the platelet releasate as well as with purified PDI.

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Multiple Pathways in Cultured Epithelial Cells for the Transcytosis of Basal Membrane-Bound Peroxidase-Polylysine Conjugates. M.E. Taub and W.-C. Shen, University of Southern CA School of Pharmacy, Los Angeles, CA 90033.

A conjugate of horseradish peroxidase (HRP) to poly(L-lysine) (PLL) is used as a non-specific adsorptive probe to study transcytosis in MDCK strain I and Caco-2 epithelial cells. As shown previously, HRP-PLL transcytosis proceeds via an intracellular, non-lysosomal proteolytic compartment; yet, this compartment is utilized for transcytosis only in the basal-to-apical direction. Using size exclusion chromatographic techniques, we demonstrate that the PLL moiety of the conjugate is effectively cleaved during transport, releasing free HRP from the apical surface of the cells. Pulse-chase studies indicate that ~6% of basal surface-associated HRP-PLL conjugate in Transwell-grown cell monolayers enters the basal-to-apical transcytotic pathway. Brief (1 hr) treatment with phorbol ester (PMA), a protein kinase C activator, elicits a marked increase in the rate of transcytosis, but only following a 1 hr lag time. Pretreatment with brefeldin A (BFA) inhibits the transcytosis of conjugate by ~30%, yet completely abolishes the PMA effect. Removal of BFA causes a re-establishment of the normal rate of transcytosis within 2 hr, but does not restore the increased transcytosis noted for PMA-treated cells. Thus, PMA most likely elicits an increase in the amount of basally-internalized conjugate delivered to BFA-sensitive transcytotic compartments. Leupeptin, a protease inhibitor which we have previously shown to inhibit transcytosis of HRP-PLL conjugate by 50%, fails to abolish the PMA effect, indicating that the protease involved in the PMA-enhanced pathway is either insensitive or inaccessible to basolaterally internalized leupeptin. These results suggest that there exists more than one plausible intracellular mechanism for processing of endocytosed HRP-PLL conjugate.

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Apical and Basolateral Distribution of HB-EGF-like Growth Factor/DT-receptor in Polarized MDCK, BeWo and CaCo Cells. K. Shailubhai, C. Sharp and D. M. Neville, Jr. Lab. of Mol. Biol. NIMH, NIH, Bethesda, Maryland 20892.

The polarized distribution of epithelial plasma membrane proteins is maintained by specific cell sorting machinery. In most epithelia the apical surface is towards the external milieu, while the basolateral surface faces the blood supply. In contrast, in the placental trophoblast, the apical layer is opposed to the maternal bloodstream, whereas the basolateral layer is in direct contact with the foetal blood supply. Diphtheria toxin (DT) enters the mammalian cells by specific receptor-mediated endocytosis. The DT-receptor gene has recently been cloned and shown to be identical with the gene for HB-EGF-like growth factor. We provide evidence that the DT-receptor is mainly distributed, on the basis of DT-toxicity, on the basolateral surface in MDCK cells, whereas it is almost evenly distributed on apical and basolateral layers in BeWo cells. In contrast, in CaCo cells the DT-receptor appears to be concentrated on the apical layer. These results were supported by ^{125}-I-CRM197 cell surface binding on apical and basolateral surfaces in the Transwell grown MDCK cells. In Vero cells the DT-receptor-ligand complex recycles continuously. The current experiments are designed to probe the interrelationship between DT endocytosis, receptor recycling and translocation in polarized epithelial cells.

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Differential Role of Albumin Binding Proteins in Transcytosis and Endocytosis. J. E. Schnitzer and P. Oh Cellular & Molecular Medicine; Univ. Calif.- San Diego, La Jolla, CA 92093

Plasmalemmal vesicles appear to involved in both transcytosis and endocytosis. Albumin binding to the luminal surface of continuous endothelium initiates its transcytosis via plasmalemmal vesicles whereas binding of various modified albumins results in endocytosis and accumulation in lysosomal compartments. Several albumin binding proteins (ABP) called gp60, gp30, and gp18 have been identified; however, the functional relationship of these ABP to each other is unclear. Our efforts in characterizing these ABP have revealed several functional differences. Gp30 and gp18 interact much more avidly with various modified albumins (albumin-gold particles; maleic anhydride or formaldehyde treated albumin) than with native albumin. These modified albumins bind the surface of cultured endothelial cells via gp30 and gp18, are internalized and degraded within lysosomes, and do not compete with native albumin binding. Antibodies to gp60 inhibit native albumin binding to the cell surface and to purified gp60. Modification of albumin eliminates albumin's ability to compete with ^{125}I-BSA binding to the cell surface and to gp60. Apparently, the induced conformational change in albumin is sufficient to prevent recognition by gp60 while increasing its affinity for gp30 and gp18. These results bear a striking resemblance to the known interaction of LDL with its receptor wherein chemical modification of LDL (ie., acetylation) prevents its interaction with the native LDL receptor but induces binding to another set of receptors on the same cell surface, namely scavenger receptors such as the acetylated LDL receptor. Cumulatively, our results indicate that endothelial cells can distinctly recognize modified and native albumins via different surface receptors. Gp60 mediates the binding of native but not modified albumins to the cell surface apparently for transcytosis whereas gp30 and gp18 primarily mediate modified albumin binding, endocytosis and degradation. (NIH Grant HL43278 and Grants-In-Aid from AHA, National and California affiliates.)

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PATHWAYS IN TRANSCYTOSIS OF IMMUNOGLOBULINS BY PORCINE ENTEROCYTES. J.P. Heath and L.G. Kőműves. Children's Nutrition Research Center, Baylor College of Medicine, Houston, TX 77030.

Maternal immunoglobulin (**mIg**), a major component of colostrum, is transported as intact protein across the small intestinal epithelium in suckling piglets. We are using quantitative postembedding colloidal gold immunocytochemistry to examine the intracellular pathways taken by **mIgs** as they are endocytosed and transported to the basolateral surfaces of jejunal enterocytes. **MIgs** entered the apical tubulo-vesicular endosomal complex via deep uncoated invaginations and accumulated in apical granules, whose **mIg** content was 1.7 times enriched as compared to the colostrum. Stereo views of thick sections showed the granules were connected to many tubules, which may provide a route for membrane recycling. Granule density was variable indicating possible sorting of components. The granules migrated basally where their fusion led to the development of a large basal granule (*lbgr*), located beneath the nucleus within 1 h of the onset of suckling. The **mIg** content in the *lbgrs* was 3.6 times higher than in the colostrum. **MIgs** were detected in the lamina propria within 1 hour of suckling. **MIgs** were also detected in the Golgi apparatus which suggests partial mixing of the exocytotic and transcytotic routes in neonatal enterocytes.

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Intracellular processing of immune complexes formed on the surface of glomerular epithelial cell (GEC). A.K. Singh and M.A. Rahman, Department of Medicine, Loyola-Hines Medical Center, Maywood, IL 60153.

Immune complexes formed on the surface of GEC resolve slowly and therefore, result in inflammation of the glomerular capillary wall as in the case of human membranous glomerulonephritis and experimental Heymann nephritis of rat. The metabolic defect in the processing of these complexes has not been identified. Immune complexes of cationic bovine gamma globulin (BGG) and anti-BGG were formed on cultured GEC, and their intracellular processing was followed by tracing the fate of radioiodinated and colloidal gold labelled anti-BGG in the endosomal, lysosomal and extracellular compartments. It was determined that the complexes were rapidly internalized in endosomes (86% of saturation achieved in 30 minutes). The rate of expulsion of complexes was however much slower (50% of internalized complex exteriorized in ~2 hrs). Of the internalized anti-BGG, less than 5% were proteolytically degraded, suggesting a defect in the endosomal-lysosomal fusion. This aspect was studied further by separating anti-BGG colloidal gold loaded vesicles by low-speed centrifugation and quantitating the lysosomes in them by determination of lysosomal enzymes acid phosphatase and β-galactosidase. It was observed that at equilibrium approximately 10% of vacoules containing internalized immune complexes fused with lysosomal granules.

It was concluded that immune complexes are rapidly internalized by the GEC. However, only a fraction of endosomes fused with lysosomes at any time, making the process inefficient. The majority of accumulated endosomes apparently route back to the plasma membrane at a slower rate and discharge their contents in the medium in the form of free antigen and antibody.

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Intracellular Distribution of Major Histocompatibility Complex (MHC) Class I Antigens in Rat Trophoblasts. D.N. Misra, A. Kanbour, M.J. Becich, T.A. Howard, H.W. Kunz, and T.J. Gill III, Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261.

In mammalian pregnancy, the placenta provides an immunoprotective barrier between the mother and fetus. In several species, however, subpopulations of trophoblasts that are in direct contact with maternal circulation express MHC class I antigens. Our previous studies in the rat have demonstrated that in the allogeneic WF (RT1^u: female) x DA (RT1^a: male) placenta the classical class I antigen RT1.A^a and a nonclassical, pregnancy-associated, class I antigen Pa are both expressed in the cytoplasm of basal trophoblasts, and only the Pa antigen is expressed on the cell surface. In the syngeneic DA x DA, however, both antigens are expressed on the surface. In order to understand this difference, we have compared the intracellular distribution of these antigens by immunogold electron microscopy using specific monoclonal antibodies. The antigen distribution has been sub-divided into three different groups: secretory (rough endoplasmic reticulum and secretory vesicles), endocytic (coated pits/vesicles, multivesicular bodies, endosomes and lysosomes) and indeterminant (cytoplasm, plasma membranes and golgi). In WF x DA trophoblasts, the two antigens display an almost identical distribution in all three groups: A^a/Pa: secretory, 46%/48%; endocytic, 24%/24%; and indeterminant, 30%/28%. In contrast, the DA x DA trophoblasts show a distinct difference: A^a/Pa secretory, 59%/39%; endocytic, 16%/22%; and indeterminant, 25%/39%. The results indicate shifts between allogeneic and syngeneic animals in the amounts of label observed in the rough endoplasmic reticulum, plasma membrane and all endosomal/lysosomal compartments, suggesting a direct regulation of expression of these antigens in this system between secretory and endocytic pathways. Further studies should provide deeper insight into MHC class I antigen regulation in trophoblasts. (Supported by NIH grant HD-08662).

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Confocal Microscope Studies Of Endocytic Sorting Of Low Density Lipoprotein And Transferrin In Hep2 Cells. R. N. Ghosh and F. R. Maxfield, Pathology Department, College of Physicians & Surgeons, Columbia University, New York, NY 10032.

We are studying the sorting and intracellular transport of endocytosed macromolecules in Hep2 human carcinoma cells, using low density lipoprotein (LDL) and transferrin (Tf) to probe the lysosomally directed and recycling pathways respectively. Fluorescent LDL (diI-LDL) and Tf (BODIPY-Tf) are used together with laser scanning confocal microscopy to simultaneously visualize both probes' sorting and subsequent post-sorting behaviour in live cells.

We find that both ligands initially appear in the same punctate sorting endosomes (SE), and small fingers of Tf extend from these SEs. Within a few minutes, Tf leaves the SE and enters an extensive tubular post-sorting recycling compartment, which is similar to the tubular endosomes reported by Hopkins *et al.* (*Nature* 346, 335-339 (1990)). Most of the Tf is recycled out of the cell with a half time of 10 minutes. LDL remains in punctate SEs that eventually mature into late endosomes. A 30 minute BODIPY-Tf labelling followed by a 7 minute chase fills the tubular recycling compartment, and a subsequent 4 minute diI-LDL pulse identifies the SEs. A majority of these diI-LDL labeled SEs also contain BODIPY-Tf, suggesting that backflow of Tf from the tubular recycling compartment back to SEs occurs. We propose that the recycling compartment is a dynamic but long-lived organelle that exports membrane back to SEs as well as to the plasma membrane. Evidence for these mechanisms and the rate constants for each step will be presented.

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Multivalent Transferrin Reveals a Sorting Function of the Recycling Compartment of TRVb-1 Cells. E. W. Marsh, A. P. Ozols, D. L. Gelman, and F. R. Maxfield. Department of Pathology, Columbia University. New York, NY.

The cross-linking of surface receptors has been shown in many systems to result in the redistribution of the receptors within cells, suggesting that cross-linking of surface receptors may form a sorting signal that alters receptor trafficking with respect to well described monovalent pathways. To better understand the cellular and molecular mechanisms by which receptor cross-linking affects the intracellular trafficking of both ligand and receptor we have prepared multivalent transferrin (M-Tf) by chemically cross-linking native transferrin (Tf) whose intracellular trafficking is well described. We have found that, while ^{125}I-Tf was released intact with a half time of 15 minutes by the TRVb-1 cells, ^{125}I-M-Tf was retained by TRVb-1 cells 4 to 5 times longer. Furthermore, a portion of the internalized ^{125}I-M-Tf was slowly degraded. After three hours, degradation accounted for 20 to 30% of the original cell associated ^{125}I-M-Tf. The intracellular localization of M-Tf was investigated using fluorescence confocal microscopy. M-Tf was not delivered to the pathway followed by LDL to late endosomes or lysosomes, as M-Tf sorted from LDL with kinetic comparable to that of Tf. Moreover, following a 45 minute chase much of the M-Tf remained accessible to Tf indicating that the M-Tf was in a transferrin accessible recycling compartment. The presence of M-Tf within the recycling compartment does not appear to affect trafficking of subsequently added Tf, since the Tf recycled to the cell surface with a half time of approximately 15 minutes. Together the data suggests that the cross-linking of transferrin receptors by a multivalent form of Tf results in the retention of the M-Tf within the recycling compartment where, by its retention, it is sorted from the rapidly recycling native transferrin.

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PRESENCE OF TGN38, AN INTEGRAL *TRANS*-GOLGI PROTEIN IN THE SUPRANUCLEAR VACUOLE IN THE ABSORPTIVE CELLS OF SUCKLING RAT ILEUM. L.G. Kőműves, B.L. Nichols, J.P. Heath and J.P. Luzio. Children's Nutrition Research Center, Baylor College of Medicine, Houston, TX77030 and Department of Clinical Biochemistry, University of Cambridge, Cambridge UK.

Neonatal development in suckling rats depends, in part, on vesicular transport of biologically active macromolecules from maternal milk. We have studied this process in 7-d-old newborn suckling rats. Cryosections, LR White- and LR Gold-embedded sections from the jejunum and ileum were labelled with rabbit anti-rat IgG or rabbit anti-TGN38 (*Luzio et al., 1990, Biochem J., 270:97*) followed by goat anti-rabbit IgG-Au_{10}. In the jejunum, TGN38 was localized in the *trans*-Golgi network (TGN), whereas in the ileum, the highest amount of TGN38 immunoreactivity was seen in the large supranuclear vacuole (LSNV). Components of the apical endocytic pathway (early tubulo-vesicular endosomes, late endosomal vesicles) and basolateral endosomes, however, were not labelled with anti-TGN38. Furthermore, we found that the LSNV contained endocytosed IgG. The LSVN also contains lysosomal markers (as shown by *Fujita et al., 1990, J. Cell Sci., 97:385*). This study indicates that LSNV is a unique, multifunctional organelle at the crossroad of the endo- and exocytic (secretory and/or transcytotic) pathways.

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Fluorescence Lifetime Imaging Microscopy (FLIM) of Endosome Fusion in Single Cells. Takatoku Oida, Yasushi Sako, and Akihiro Kusumi. Dept. of Pure and Applied Sciences, College of Arts and Sciences, The University of Tokyo, Meguro-ku, Tokyo 153, Japan

Time-resolved microscope fluorimetry has recently been greatly improved to enable the measurement of fluorescence lifetimes in submicrometer regions[1]. Its initial applications indicate that fluorescence lifetime measurement under a microscope is a powerful, noninvasive method for obtaining molecular information in single living cells. We now report the first microscopic imaging of cells based on spatial variations of (nanosecond) fluorescence lifetime, which we name fluorescence lifetime imaging microscopy (FLIM). This technique allows the extent of resonance energy transfer (which reduces the lifetime of the energy donor) to be visualized in single living cells, and is free from variations due to path length, light scattering, and the number of fluorophores that necessitate complex corrections in steady-state microspectrofluorimetry. The total data acquisition time needed to obtain a FLIM image has been reduced to only 10 sec for ≈25 fluorescent molecules/pixel. By using the FLIM technique, the extent of fusion, and fused and unfused endosomes were clearly visualized in single normal rat kidney (NRK) cells in culture. The results indicate extensive fusion between primary endocytic vesicles and/or between sorting endosomes. These data are consistent with maturation models for biogenesis of sorting and late endosomes, and necessitates modification of the model for pre-existing sorting endosomes (stable compartment model), so that it includes extensive fusion between primary endocytic vesicles, and possibly between sorting endosomes.

[1]Kusumi, A., Tsuji, A., Murata, M., Sako, Y., Yoshizawa, A. C., Kagiwada, S., Hayakawa, T., & Ohnishi, S. Biochemistry 30, 6517-6527, 1991.

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Differing Patterns of Uptake and Intracellular Localization of Fluorescently Labelled Liposomes in Rat Granular Pneumocytes. W.J. Muller, A.B. Fisher, and H. Shuman, Institute for Environmental Medicine and Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104

Lipid uptake and trafficking in cultured rat alveolar type II cells was investigated by fluorescence microscopy. Small (0.1 um) unilamellar liposomes of a composition reflecting lung surfactant lipids (DPPC:PC: PG:cholesterol = 50:25:10:15) were prepared by extrusion through polycarbonate membranes. The fluorescent PC analogues 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine (NBD-PC) and 1-palmitoyl-2-(7-methyl-BODIPY-1-dodecanoyl)-L-a-phosphatidylcholine (BODIPY-PC) were substituted for different amounts of DPPC, with NBD-PC replacing 15 mol% DPPC and BODIPY-PC replacing 2 mol% DPPC. In addition, surfactant-like liposomes with the lipophilic fluorescent tracer 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI) added as 2 mol% were studied. Isolated cells cultured on plastic for 24 hours and observed at room temperature in a microperfusion set-up showed patterns of uptake and intracellular localization which differed for differently labelled liposomes. Cells which were perfused with NBD-PC containing liposomes showed a localization of fluorescence into discrete vesicles within 3 min, with a bright punctate pattern of staining observable by 7 min of exposure. Cells exposed to DiI labelled liposomes were observed at first to exclude fluorescence. By 8 min, fluorescence was observed to concentrate into small vesicles near the plasma membrane, though intracellular localization was not seen up to 30 min. For cells perfused with BODIPY-PC labelled liposomes, fluorescence began to appear in small, bright vesicles within 2-4 min of exposure, but did not localize to larger intracellular vesicles until 15-20 min after beginning the perfusion. The different uptake and distribution characteristics, combined with evidence for intact uptake of liposomes, imply the existence of mechanisms for cell-liposome recognition and lipid sorting after internalization.

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Bulk Membrane Flow and Transferrin Receptor Recycling in an Endocytosis Mutant. J. F. Presley, S. Mayor, L. Johnson, T.E. McGraw, and F. R. Maxfield, Department of Pathology, College of Physicians and Surgeons, Columbia University, 630 West 168th Street, New York, NY, 10032.

We have isolated a CHO cell line (12-4) by selection with a Tf-diptheria toxin A chain conjugate. These cells show slowed externalization of ^{125}I-Tf after loading to steady state (Johnson, L. S., and McGraw, T. E. 1990. J. Cell Biol. 111, 461a.). These cells are members of the *end2* endocytosis defective complementation group. We have used quantitative digital microscopy to further define the defect in these cells. In TRVb-1 (parental) cells, the rate limiting step in the recycling of Tf appears to be its exit from a recycling compartment situated near the MTOC. By using quantitative digital microscopy and image processing, we show that the slowed step in Tf recycling in 12-4 cells is a delayed exit from this compartment. Tf leaves the recycling endosomes of wild type cells with a rate constant of 0.061 min^{-1} as contrasted to 0.025 min^{-1} for 12-4 cells. In wild type cells, NBD-C_6-sphingomyelin leaves this compartment at rates similar to Tf, indicating that Tf may exit *via* a bulk membrane flow pathway from this compartment. We are comparing the rate of exit of NBD-C_6-sphingomyelin to the rate of exit of Tf from the recycling compartment in mutant cells to determine whether the defect in trafficking in these cells is due to a defect in bulk membrane flow or to specific retention of Tf in the recycling compartment. In both cell lines, the rates of trafficking in the exocytic pathway from the Golgi apparatus to the cell surface are similar, as measured by the rate of delivery of Golgi-derived NBD-ceramide metabolites to the plasma membrane.

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Characterization of the Yeast mutant *end2* which is defective for endocytosis. D. Hamburger, M. Egerton* and H. Riezman, *ICI Pharmaceuticals, Cheshire SK10 4TG, UK; Department of Biochemistry, Biocenter of University of Basel, CH-4056 Basel, Switzerland.

The pheromone α-factor and the fluorescent dye Lucifer Yellow Carbohydrazide (LY) are taken up and delivered to the lysosome-like vacuole in *Saccharomyces cerevisiae* via endocytosis. Endocytosed α-factor gets degraded in the vacuole in a *PEP4*-dependent manner. The temperature sensitive (ts) yeast mutant *end2* does not accumulate LY in the vacuole at the restrictive temperature (37°C). At this temperature α-factor gets taken up by *end2* with the same initial rates as in wt cells but it is not degraded. That means further delivery to the vacuole is blocked. In contrast, secretion of invertase and maturation of CPY occur with approximately the same kinetics as in wt cells at 37°C. This indicates that *end2* is the first mutant in yeast which is blocked in the endocytic pathway later than uptake and where vacuole biogenesis is not affected. α-factor trapped by *end2* at 37°C is protected by a membrane delimited compartment. Cells with the disrupted *END2* gene were not viable, therefore this gene is essential. It encodes a 70kD protein which is poorly transcribed. End2p has several hydrophobic stretches, leading us to predict that End2p is a transmembrane protein.

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A Role for Clathrin in Spermatogenesis. C. Bazinet,[1] A. L. Katzen,[2] M. Morgan,[3] A. P. Mahowald,[3] and S. K. Lemmon,[1]Dept. of Molecular Biology and Microbiology, Case Western Reserve University, [2]University of California, San Francisco, [3]Dept. of Molecular Genetics and Cell Biology, University of Chicago.

The identification of genes encoding clathrin and associated proteins in a complex metazoan makes possible the genetic analysis of clathrin functions in the specialized cell types of differentiated tissues and organs. We have identified a genetic complementation group encoding the clathrin heavy chain (Chc) of *Drosophila melanogaster* by germline transformation with a modified P transposable element carrying the cloned Chc gene. The P[Chc] element complements a group of four mutations mapping to the Chc locus on the X chromosome. Three of these mutations are lethal, blocking development late in embryogenesis. Development probably proceeds this far due to the presence of substantial quantities of maternal clathrin transported into the oocyte from heterozygous nurse cells. A fourth allele, EM9, is leaky in the sense that EM9 hemizygous males survive to adulthood at low but significant frequencies. These individuals appear morphologically and behaviorally normal but are sterile. Examination of their testes reveals a defect in sperm motility and the presence at many sites on individualized sperm of a widening or dilation of the cell membrane. The defects are completely rescued by a wild-type copy of the Chc gene, arguing that the are due to the Chc mutation and not some other mutation in the genetic background we are examining. The observations suggest a role for clathrin in the individualization of sperm, during which spermatids become invested in their own cell membrane after having developed in a syncytial cyst cell. Sperm individualization is an extraordinarily active, coordinated process of membrane morphogenesis and reorganization likely to be especially sensitive to defects in cellular transport.

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Cerulenin blocks the cytotoxicity of ricin, modeccin, *Pseudomonas* toxin and diphtheria toxin in brefeldin A-resistant cell lines. T. Oda and H. C. Wu, Department of Microbiology, Uniformed Services University of the Health Sciences, MD 20814-4799.

We have found that cerulenin, an antibiotic that inhibits *de novo* fatty acid and cholesterol biosynthesis and fatty acylation of proteins, inhibited the cytotoxicity of ricin, modeccin, *Pseudomonas* toxin and diphtheria toxin in a brefeldin A(BFA) resistant mutant of Vero cells (BER-40). The protective effect of cerulenin against ricin was also observed in two BFA resistant cell lines, MDCK and Ptk1 cells of which the Golgi complex was shown to be intrinsically resistant to BFA. However, no significant effect of cerulenin was observed in Vero cells, even though this cell line was completely protected by BFA against ricin and other toxins. It appears that there is an apparent correlation between BFA resistance and protection of toxins by cerulenin. Although cerulenin did not affect the binding of ricin to the receptors, endocytosis of ricin was reduced in BER-40 cells, whereas no change in the endocytosis of ricin was obseved in Vera, Ptk1 and MDCK cells. In BER-40 cells, endocytic uptake of fluid-phase markers such as horseradish peroxidase and Lucifer yellow was inhibited by cerulenin but the endocytosis of transferrin, which was shown to enter cells by the coated pit/coated vesicle pathway, was increased. These results suggest that in addition to the slight modification of endocytosis, cerulenin interferes with the intracellular processing or trafficing of toxin molecules, which is a common pathway of above protein toxins. Since fatty acids were not able to reverse the effects of cerulenin, its protective effect against protein toxins seems not to be a consequence of the inhibition of *de novo* fatty acids biosynthesis. The relationship between BFA resistance and inhibition of ricin cytotoxicity by cerulenin remains to be elucidated.

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EM Immunogold Localization of the EGF and Prolactin Receptors in Cryosections of Rat Liver following Ligand-mediated Receptor Internalization. S. Dahan*, J.P. Ahluwalia*, L. Germain@, W.H. Lai*, M. DeBanne#, B.I. Posner*, and J.J.M. Bergeron*. *Depts. of Anatomy and Cell Biology & Medicine, McGill University, Montreal; @Hôpital du St-Sacrement, Québec, Québec; #BRI, NRC, Montreal, Québec, Canada.

In an attempt to define the intracellular compartments harboring endocytosed prolactin receptors (PRL-R) and EGF receptors (EGF-R), we have evaluated by cryoimmune EM the distribution of receptors using receptor-specific monoclonal and polyclonal antibodies. In uninjected males, EGF-Rs were randomly distributed over the sinusoidal microvilli. Five and 15 minutes after the injection of EGF, progressively greater numbers of multivesicular endosomes were labeled largely over the intraluminal content. These vesicles were probably late endocytic compartments as vesicles of identical morphology were positive for MPR, and HRP injected 30 min prior to fixation. Smaller endocytic vesicles (ca. 150-400 nm diam) close to the bile canaliculus were also labeled for the EGF-R. Interestingly, unlike the polyclonal anti-EGF-R antibody, the monoclonal antibody did not recognize the cell surface EGF-R under all conditions examined, possibly due to a conformation-specific EGF-R epitope that only became accessible to the antibody after the EGF-R was internalized. We have also studied the ligand-dependent internalization of PRL-R in rat liver. The endocytosed PRL-Rs, unlike the EGF-Rs, were mostly concentrated at the periphery of small endocytic vesicles (ca. 150-400 nm) that were morphologically similar to those labeled for pIgR. The different intracellular distributions of PRL-R and EGF-R following ligand stimulation may reflect differences in their intracellular signalling mechanisms.

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Ligand-Induced Endocytosis of Nerve Growth Factor (NGF) Receptors in PC12 Cells. M. Grimes and W.C. Mobley, Department of Neurology, University of California, San Francisco, CA 94143.

To compare constitutive and ligand-induced internalization of plasma membrane receptors, endocytosis of NGF and transferrin were compared in PC12 cells. Radiolabeled ligands were bound to cells in the cold for one hour, washed and warmed to 37°C for 0 or 10 min. Cells were then mechanically permeabilized and vesicles that escaped permeabilized cells in the presence or absence of ATP were fractionated on sucrose velocity gradients. Three classes of vesicles could be distinguished. These vesicles, in order of decreasing velocity, are tentatively classified as 1) clathrin coated, 2) uncoated plasma-membrane derived, and 3) early endosome-derived vesicles. Transferrin was found immediately after binding in the first vesicle class; these escaped permeabilized cells in the absence of ATP and could be chased into the lighter vesicles. In contrast, NGF was present in vesicles only after warming. We conclude that NGF receptors must be bound to ligand to be clustered into coated pits. At the plasma membrane, labeled NGF was crosslinked to $p75^{NGFR}$ and $p140^{trk}$. After cells were warmed to 37°C for 10 min, the amount of $p140^{trk}$ available for crosslinking diminished. Experiments are underway to determine the phosphorylation state and enzymatic activity of trk kinase in different membrane fractions. These experiments may help elucidate the role of receptor internalization in NGF signal transduction.

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Endocytosis of Oxidized LDL and Reversibility of Migration Inhibition in Macrophage-Derived Foam Cells In Vitro. H. Robenek and M. Pataki, Institute for Arteriosclerosis Research, Department of Cell Biology and Ultrastructure Research, University of Münster, Domagkstraße 3, yD-4400 Münster, FRG.

The ability of macrophage-foam cells to migrate from atherosclerotic lesions represents one potential mechanism for regression of atherosclerosis. It is, however, generally recognized that the transformation of macrophages to foam cells results in greatly reduced migrational ability. In the present study, we set out to investigate the factors affecting migratory capability in foam cell-like cells using an in vitro assay. Foam cell-like cells were prepared by incubating macrophages in the presence of oxidized LDL. The transformation to a typical foam cell morphology was demonstrated by Nile Red staining (light microscopy) and the mechanisms of binding, uptake and intracellular processing of oxidized LDL established by colloidal gold labeling on the ultrastructural level. Using the in vitro assay, the migration of these foam cell-like cells was found to be markedley inhibited compared with untreated, control macrophages. However, zymosan activated mouse serum restored migration in oxidized LDL-treated cells to levels similar to those of controls. Restoration of migratory capacity was accompanied by alterations in the cytoskeleton system, especially in actin arrangement. Supported by the Deutsche Forschungsgemeinschaft (SFB 310).

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Receptor Mediated Endocytosis of 39kDa α_2MR/LRP-Associated Protein by Rat MH_1C_1 Hepatoma Cells. S.P. Iadonato*, G. Bu*, and A.L. Schwartz*#. Depts. of Pediatrics* and Molecular Biology and Pharmacology#, Washington University, St. Louis, MO 63110.

Recombinant tissue-type plasminogen activator (t-PA) undergoes rapid liver-dependent clearance *in vivo*. Recent studies in our lab have identified a PAI-1 independent t-PA import pathway on the rat MH_1C_1 hepatoma cell line. Subsequent studies have implicated the α_2-Macroglobulin/Low Density Lipoprotein Receptor Related Protein (α_2MR/LRP) as a mediator of PAI-1 independent t-PA import. We now describe the function of a 39kDa α_2MR/LRP receptor associated protein which co-purifies with the receptor and which modulates PAI-1 independent t-PA binding. At 4°C, binding of ^{125}I-39kDa protein to the rat MH_1C_1 hepatoma cell line was rapid, specific, and saturable, with an average of 252,000 high affinity binding sites (K_d=3-4 nM) per cell. In addition, binding did not appear to be mediated through an association with accessory proteins, as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed no high molecular weight ^{125}I-39kDa protein complexes. Subsequent studies showed that although t-PA and the 39kDa protein independently bind to α_2MR/LRP with similar kinetics, the 39kDa protein strongly inhibits t-PA binding (K_i=0.5 nM) in competition experiments involving both proteins. Furthermore, crosslinking of ^{125}I-39kDa protein to rat MH_1C_1 cells generates a high molecular weight protein complex which is immunoprecipitable with both anti-α_2MR/LRP and anti-39kDa protein antibodies. Subsequent experiments involving the crosslinking of unlabeled 39kDa to [^{35}S]-methionine labeled rat MH_1C_1 cells gave a similar high molecular weight protein complex which was also immunoprecipitable with both anti-α_2MR/LRP and anti-39kDa protein antibodies and which contained the 500kDa subunit of the α_2MR/LRP receptor. Endocytosis experiments demonstrated a rapid uptake of ^{125}I- 39kDa protein from the MH_1C_1 cellular plasma membrane. The kinetics of 39kDa protein endocytosis were almost identical to those of t-PA, with much of the ligand-associated radioactivity being ultimately exported to the overlying buffer as trichloroacetic acid soluble counts. Finally, metabolic labelling of MH_1C_1 cells with [^{3}H]-leucine revealed a low level of 39kDa protein synthesis by this rat hepatoma cell line. In short, these studies suggest a prominent role for the 39kDa protein in the regulation of PAI-1 independent t-PA metabolism.

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Cytometric Quantitation of Attached vs. Internal PMN Phagocytic Beads. [1]Y. NUMABE, [2]M. RYDER, and [1]K.KAMOI. [1]Department of Periodontology, Nippon Dental University, [2]Division of Periodontology, University of California, San Francisco.

Alterations in phagocytic function of PMNs play an important role in the pathogenesis of several diseases. Flow cytometric quantitations (FCM) using uniform-sized fluorescent beads are a useful tool since the phagocytic capacity of each cell (number of beads/per cell) as well as % phagocytosis can be rapidly determined. However, this method cannot distinguish between attached and internalized beads. We present a novel double labelling technique and quantitation analysis to distinguish these two bead populations. Human PMNs extracted from peripheral blood were incubated with serum-opsonized goat IgG conjugated fluoresceinated microspheres. After fixation in paraformaldehyde, cells were incubated with Texas-Red conjugated rabbit anti-goat IgG. Fluorescent micrographic examination revealed all external and internal PMN associated beads stained green while only external beads stained red. Flow cytometric quantitations of 20,000 cells were compared with manual microscopic counts of 100 cells per sample. Calculation of attached only or total beads/cell were based upon the fluorescein or Texas Red stain values of a single bead after correction for background stain. From 1 to 10 minutes of incubation, % Phagocytosis increased (FCM: 9.99 to 65.53, manual count: 4.00 to 55.00). Total beads/cell also increased (FCM: 2.56 to 5.02, manual count: 4.00 to 4.39). Attached beads/cell values decreased from 1-3 min., then remained stable for 4-10 min. (FCM and manual count values showed similar tendency). These studies demonstrate the value of double labelling and dual laser cytometry in distinguishing particle attachment vs ingestion in PMN phagocytosis.
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Fluid-Phase Endocytosis - A Possible Source of High Atrial Granule Ca Content. E. Page, G. E. Goings, J. Upshaw-Earley, and Dorothy A. Hanck. Department of Medicine, The University of Chicago, Chicago, Illinois 60637.

We tested the hypothesis that the membrane-impermeant fluid phase marker ^{14}C-sucrose (chromatographically repurified) is internalized by in situ rat atrial myocytes due to recycling of plasma membrane fused with atrial granule membrane during exocytosis of atrial natriuretic peptide. By measuring simultaneously equilibrium distributions of ^{14}C-sucrose and ^{3}H-methoxy-inulin at 37°C in isolated atrial preparations rendered quiescent by 0.2 mM $[Ca^{2+}]_{ext}$ +10 µM ryanodine (unstretched or stretched by 5mm Hg distending pressure, Am. J. Physiol. 259:C801, 1990), we identified S*, an inulin-inaccessible component of sucrose space whose volume = V_s* (in ml/g dry atrium). The existence and magnitude of S* were independently confirmed by compartmental analysis of ^{14}C-sucrose efflux and by morphometry of interstitial volume. V_s* (mean±SE,n) at 37°C was not statistically different in unstretched controls (.31+.02, 12), stretched controls, 10mM caffeine treated unstretched atria, and stretched atria cooled to 18°C. V_s* significantly increased in unstretched atria cooled to 18°C (.52±.02, 12) and in stretched atria whose secretory rate was stimulated 2.3-fold by 3mM neomycin (.52±.04, 8). If free $[Ca^{2+}]$ in S* is 1.5mM (as expected for internalized interstitial fluid), the calculated Ca^{2+} content in S* was .464 µmoles/g dry atrium, which is similar to the total Ca content of atrial granules in atrial myocytes (0.5 µmoles/g dry atrium) (Somlyo et al, PNAS 85:6222, 1988). We suggest that the high granule Ca^{2+} content may derive from interactions of the endocytic vesicles that make up S* with nascent atrial granules. Thus fluid-phase endocytosis via S* could be the inward loop and ANP secretion via granules the outward loop of a novel ryanodine-insensitive vesicular Ca^{2+} flow in atrial myocytes. Supported by USPH-NHLBI HL-10503.

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Reversible Caveolar Enlargement And Increase In Caveolar Surface Density In Myocytes Of Atria Exposed To Hypertonic Solution. L. Kordylewski, G. E. Goings, and E. Page. Department of Medicine, The University of Chicago, Chicago, Illinois 60637. Sponsored by S. Jakovcic.

Caveolae of rat atrial myocytes in situ take up the fluid-phase endocytosis probe horseradish peroxidase (HRP) but, unlike rat atrial myocytes in primary culture, do not internalize it. To investigate whether access to caveolae via caveolar necks is physiologically variable, we studied the effects of physiological or pharmacological perturbations on the ability of caveolae to take up HRP, or on washout of HRP from preloaded caveolae. Neither caveolar uptake nor caveolar release of HRP was affected by perturbations which stimulate or inhibit atrial natriuretic peptide secretion (alpha-1-adenergic stimulation, raising cytosolic cAMP concentration with 10 µM caffeine or raising cytosolic $[Ca^{2+}]$ with 1 µM ionomycin at $[Ca^{2+}]_{ext}$ = 0.2 mM). Incubation of intact unstretched atrial preparations (Page et al., Am. J. Physiol. 259, C801, 1990) for 5 min. at 37°C in modified Krebs-Henseleit solution made hypertonic with 150 mM sucrose followed by glutaraldehyde fixation at the identical osmolarity caused striking reversible caveolar enlargement within 1 min. (detected by TEM of thin-sectioned or cross-fractured caveolae). Image processing of electron micrographs of replicas from two halves of the same atria incubated in isotonic and sucrose-containing hypertonic media, respectively, yielded caveolar neck surface densities (number of caveolae/16 µm^2 plasmalemmal fracture face, n=30 areas) of 178 ± 16 and 273 ± 14 (P = .0001). Caveolar enlargement suggests either osmotic caveolar swelling (possibly associated with neck closure) or recruitment of additional caveolar membrane. Increased caveolar surface density indicates that caveolae can be rapidly inserted into or assembled in plasmalemma from an as yet unidentified reservoir of caveolar precursors. Supported by USPHS-NHLBI HL-10503.

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Effect of acidic pH on fragments of *Bacillus anthracis* protective antigen bound to J774A.1 Cells. M.P. Stein, J.M. Novak, and A.M. Friedlander, Bacteriology Division, USAMRIID, Fort Detrick, MD 21702.

Recently, we described methods for *in vitro* limited digestion of *Bacillus anthracis* protective antigen (PA), the common cellular binding component of the anthrax toxins. Limited digestion of PA (83-PA) with trypsin generated two major fragments, 20-kDa and 63-kDa (T-PA). Digestion with chymotrypsin generated a 37-kDa and a 47-kDa fragment (Ch-PA). Treatment of PA with both trypsin and chymotrypsin resulted in three major fragments, 20-kDa, "17"-kDa and 47-kDa (Ch/T-PA). All three of these conditions yielded PA preparations that bound to the cell-surface receptor with the same affinity as uncleaved PA and facilitated binding and internalization of ^{125}I-labelled lethal factor (LF). Only trypsin-treated PA retained biological activity when combined with LF in a J774A.1 cytolysis assay. The defect in biological activity of Ch-PA appeared to be at an intracellular step such as toxin processing in an acidic compartment. In order to determine if the defect was due to differences in Ch-PA interaction with cellular membranes we tested the effects of low pH on surface-bound PA fragment preparations. At pH <5.0, 75% of 83-PA and 50% of Ch-PA could be removed from the cell surface, while only 25% of the T-PA and Ch/T-PA preparations were removed. SDS-PAGE autoradiography demonstrated that 83-kDa PA and the 37-kDa fragment of PA were most susceptible to removal by low pH treatment, while the 63-kDa, 47-kDa and "17"-kDa fragment remained cell-associated. Almost all of the cell-associated 83-kDa PA and 37-kDa fragment were removed in a citric acid buffer at pH 6.0. Interestingly, treatment with sodium acetate buffer at pH 5.8 resulted in only a slight loss of 83-kDa or 37-kDa PA. Thus, there appears to be a differential sensitivity of cell-bound PA fragments to acid conditions.

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Cytotoxicity of Folate-Momordin Conjugates in Cultured Human Cells. C.P. Leamon and P.S. Low, Dept. of Chemistry, and J. J. Turek, Dept. of Vet. Anatomy, Purdue University, West Lafayette, IN.

We have shown previously that macromolecules can be nondestructively delivered into cultured cells via folate receptor-mediated endocytosis if the macromolecules are conjugated to folic acid prior to addition to the cells (C.P. Leamon and P.S. Low (1991) *Proc. Natl. Acad. Sci. USA* 88, 5572-5576). Although an intracellular destination of the folate-linked proteins could be easily documented, the spatial resolution of the earlier data was insufficient to evaluate whether any endocytosed material was delivered into the cytosol. To determine whether cytosolic deposition was achieved, a folate-toxin conjugate was constructed using the impermeable ribosome-inactivating protein, momordin. Diminution of ^{3}H-leucine incorporation into newly synthesized protein was then employed as a quantitative measure of the entry of the toxin into the cytosol. In studies with both HeLa and KB cells, cellular protein synthesis was found to be inhibited in a time and concentration dependent manner by the momordin-folate conjugate, but not by the underivatized toxin. IC_{50} values centered around 10^{-9}M for the folate-linked samples. Furthermore, analysis of the intracellular location of folate conjugates linked to 15nm gold particles revealed that while most of the conjugate was found in multivesicular bodies, part of the material was free in the cytoplasm. These observations provide direct evidence that folate conjugates not only reach the cytosol, but do so in a functionally active form.

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Identification of Intracellular Asbestos Fibers in Mesothelial Cells Using Fluorescence Confocal Microscopy. A.M. Boylan, D.A. Sanan, and V.C. Broaddus, Department of Medicine, Lung Biology Center, and Gladstone Institute of Cardiovascular Disease, San Francisco General Hospital, Cardiovascular Research Institute, University of California, San Francisco 94110.

Internalization of asbestos by cells may be an important determinant of fiber toxicity. The main technique used to identify intracellular fibers has been electron microscopy, a labor-intensive technique necessarily limited to small numbers of cells. To study fiber internalization quantitatively and distinguish intracellular from extracellular fibers, we used fluorescence confocal microscopy. Since fibers are phagocytosed enclosed within plasma membrane, we reasoned that the lipid-soluble fluorescent dye, dioctadecyl-d,d,d',d'-tetramethylindocarbocyanine (DiI) could be used to label them. Confluent rabbit pleural mesothelial cells were incubated with crocidolite asbestos (30 g/ml) and DiI (0.1-0.2 g/ml) for 6 h, fixed using glutaraldehyde (2.5% v/v) in cacodylate buffer (0.1M), and then examined using a confocal microscope (Bio-Rad MRC 600). We observed asbestos fibers that were either fluorescent, nonfluorescent (phase contrast imaging) or fluorescent along part of their length. In 3 experiments, we counted 1.1 ± 0.3 fluorescent fibers (SE) per cell (range, 0-6). To confirm that fluorescent fibers were intracellular, we optically sectioned 100 cells vertically and found that fluorescent fibers co-localized with intracellular organelles, defining their intracellular location. When cells and asbestos were incubated at 4°C to prevent fiber internalization, we found no fluorescent fibers. To show that extracellular fibers required a lipid coating to fluoresce, we coincubated fibers with dipalmityl phosphatidylcholine and DiI at 37°C: all fibers were strongly labeled. We conclude that we can use this method to identify and quantify intracellular fibers and distinguish them from extracellular fibers. We anticipate using this method to study the mechanism of fiber internalization and the relationship of internalization to toxicity. [Supported by HL 19155 (Pulm Vasc SCOR), NRSA HL08280, Clin Invest Award HL01893, & ALA of Calif].

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Dynamin is Not Required for VSV G Protein Transport in *shibire* Tissue Culture Cells. H. Radhakrishna and T.F. Roth, Department of Biological Sciences, University of Maryland Baltimore County, Catonsville, MD 21228.

The protein encoded by the *shibire* gene of *Drosophila* is thought to be the homologue of the 100 kD GTPase, dynamin. Evidence for the involvement of dynamin in endocytosis has come from the characterization of *shibire*^{ts1} mutants. Endocytosis occurs normally at 22°C but is rapidly inhibited at 30°C. The conversion of clathrin coated pits to coated vesicles ceases within 1-2 min at 30°C, but rapidly reinitiates upon lowering the temperature to 22°C. To determine if the *shibire* protein is required for vesicular transport along the secretory pathway, we examined the transport of VSV G protein (G protein) in transfected *shibire* tissue culture cells at 22°C and 30°C. G protein was inducibly expressed from a plasmid in *shibire* cells at 22°C and 30°C, and localized at various times thereafter by indirect immunofluorescence. G protein was synthesized and transported to the cell surface at both 22°C and 30°C. Within 30 min of induction at 22°C and 30°C, G protein localized to large perinuclear vesicles which also stained with fluorescent *lens culinaris* agglutinin, a lectin that labels glycoproteins found in the Golgi. Cell surface labeling of G protein was observed within 1 hr of induction at both 22°C and 30°C. Transfected *shibire* cells internalized fluorescent dextran at 22°C but not at 30°C indicating that transfection did not affect the ability of the *shibire* mutation to inhibit endocytosis at 30°C. These results suggest that the formation of vesicles used to transport proteins from the RER to the plasma membrane does not depend on the presence of a functional *shibire* protein.

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Metabolism and Intracellular Distribution of C_6-NBD Ceramide in *shibire* Tissue Culture Cells. H. Radhakrishna,* R. E. Pagano§, and T.F. Roth*, *Department of Biological Sciences, University of Maryland Baltimore County, Catonsville, MD 21228 and §Department of Embryology, Carnegie Institution of Washington, Baltimore, MD 21210.

The temperature sensitive *shibire*^{ts1} mutant of *Drosophila* inhibits endocytosis at non-permissive temperatures by blocking the formation of clathrin coated vesicles from coated pits. Endocytosis occurs normally at 22°C, but is rapidly inhibited at 30°C. Fluid phase endocytosis is reinitiated upon lowering the temperature to 22°C. Some of the membrane internalized during endocytosis is delivered to the Golgi from late endosomes, a process which should be inhibited in *shibire* cells at 30°C. To determine if membrane retrieval is essential for the processing and transport of membrane lipids through the Golgi complex, we examined the metabolism and intracellular distribution of a fluorescent analog of ceramide (C_6-NBD-Cer) in living *shibire* tissue culture cells at 22°C and 30°C. In animal cells, exogenously added C_6-NBD-Cer localizes to the Golgi apparatus where it is processed to C_6-NBD glucosylceramide (GlcCer) and C_6-NBD sphingomyelin (SM). These fluorescent sphingolipids are then delivered to the plasma membrane by a vesicle-mediated process analogous to the movement of proteins along the secretory pathway. When *shibire* cells labeled with C_6-NBD-Cer at 2°C were warmed to either 22°C or 30°C for 30 min, large perinuclear vesicles, tubulo-vesicular structures, and the plasma membrane were brightly labeled at both temperatures. Lipid analyses of labeled cells indicated that the fluorescent ceramide was metabolized to fluorescent GlcCer. These results suggest that membrane retrieval to the Golgi apparatus during endocytosis is not essential for a) the metabolism of C_6-NBD-Cer to C_6-NBD-GlcCer, or b) the subsequent transport of fluorescent sphingolipids to the plasma membrane.

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Differential Accumulation of Cholesterol in Golgi Compartments of Normal and Niemann-Pick Type C Fibroblasts Incubated with LDL: A Cytochemical Freeze Fracture Study. R.A. Coxey*, P.G. Pentchev**, G.Campbell***, and E.J. Blanchette-Mackie*. *LCDB, NIDDK, **DMNB, NINDS, ***LSM, DCRT, National Institutes of Health, Bethesda, MD 20892.

Cholesterol accumulation in the Golgi of normal and Niemann-Pick Type C (NP-C) cultured fibroblasts incubated with low density lipoproteins (LDL) for 24 hours was studied with fluoresence light microscopy and freeze fracture electron microscopy. NP-C disease is a human cholesterol storage disorder in which cells do not show the normal homeostatic responses to LDL uptake. We used filipin as a probe for unesterified cholesterol because fluorescent filipin-cholesterol complexes, visible with light microscopy also form membrane deformations (pits and protuberances) that are visible in freeze-fracture replicas. Fluorescent studies showed that, after incubation with LDL, normal fibroblasts accumulated cholesterol in Golgi whereas NP-C fibroblasts accumulated cholesterol in both lysosomes and Golgi. Quantitative analysis of freeze-fracture replicas showed that LDL uptake induced accumulation of filipin-cholesterol membrane deformations in some Golgi compartments in both normal and NP-C fibroblasts. The findings indicate that fibroblasts traffick exogeneously derived cholesterol from lysosomes through Golgi to other intracellular sites. In normal cells, filipin-cholesterol deformations accumulated in trans Golgi vacuoles and cis/medial cisternae suggesting a route of transport for cholesterol from trans Golgi vacuoles to plasma membrane and cis Golgi to endoplasmic reticulum. In NP-C cells, filipin-cholesterol deformations accumulated in trans Golgi cisternae suggesting impaired cholesterol transport through the Golgi to trans Golgi vacuoles and cis Golgi cisternae. The ability of cells to process endocytosed cholesterol may in part depend on modulation of cholesterol enriched membrane traffic through the Golgi, a function which appears to be defective in NP-C fibroblasts.

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Receptors and PKC can Regulate Constitutive Secretion and Vesicular Coat Protein Cycling. G. Santini, G. Di Tullio, A. Luini, and M.A. De Matteis Consorzio Mario Negri SUD, 66030 S.Maria Imbaro (CH) Italy.

In RBL cells the activation of the IgE receptor and protein kinase C (PKC) by antigen and PMA, respectively, markedly stimulated constitutive secretion (assayed by the release of ^{35}S-glycosaminoglycans, GAG immediately after labeling). Both basal and stimulated GAG release were inhibited by Brefeldin A. PKC down-regulation strongly reduced the antigen effect, suggesting that IgE receptors regulate constitutive membrane traffic through PKC. Vesicular traffic involves the membrane-cytosol cycling of arf and β-COP. We find that the cycling of these coat proteins is modulated by the IgE-receptor and PKC. Both proteins dissociated from Golgi membranes in permeabilized cells in the absence of Mg^{++} and GTP, and reassociated upon addition of Mg^{++}GTP, $Mg^{++}AlF^{4-}$ and Mg^{++}GTPγS. Reassociation in response to Mg^{++}GTP and $Mg^{++}AlF^{4-}$, but not Mg^{++}GTPγS, was lost in cells depleted of the β isoforms of PKC and was potentiated by stimulation of PKC. IgE receptor stimulation, which involves PKC, also potentiated the reassociation of both proteins in response to Mg^{++} GTP and $Mg^{++}AlF$. In addition to PKC, other transduction systems are coupled with IgE receptors and appear to be involved in the regulation of coat protein cycling. The identities of these signals and of their molecular targets are under investigation.

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Isolation and characterization of Chinese Hamster Ovary cell lines resistant to Brefeldin A. J. P. Yan, L. Beebe and P. Melancon, Department of Chemistry and Biochemistry, University of Colorado, Boulder CO 80309.

Following mutagenesis with EMS, Chinese Hamster Ovary cells were challenged with Brefeldin A (BFA). Colonies resistant to BFA appeared after 1-2 weeks at a frequency of ≅ 1 x10^{-6}. Several colonies were cloned and stored for further characterization. The response of growth rate to BFA concentration was determined for each mutant clone and was found to vary widely. Several clones show no growth inhibition at concentrations of up to 1μg/ml. Resistance appears stable in most cases; one cell line, BFY-1, retained resistance even following 3 months of continuous culture in the absence of BFA. This clone was further characterized to establish that its Golgi apparatus remains functional in the presence of BFA. The distribution of Golgi-associated proteins β-COP and p-200 was studied using indirect immunofluorescence. Treatment with BFA causes rapid redistribution of both proteins from Golgi structure in the wild-type parent cell line but has no effect in mutant BFY-1 cells. Pulse-chase studies with VSV-infected BFY-1 cells further demonstrate that the rate at which VSV-G becomes endo-H resistant is not affected by BFA. Resistance does not result from changes in drug import or export since the distribution of β-COP in cells permeabilized with Streptolysin-O remains unaffected by treatment with 10μg/ml BFA. In conjunction with cell-free assays, these cell lines provide a novel approach to elucidate the mechanism of action of BFA.

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Interaction of Recombinant Sec4p with a Mammalian GTPase Activating Factor(s). S. G. Miller, Y.-A. Yoon, Y.-T. Chen, and H.-P.H. Moore. Dept. of Molecular & Cell Biology. University of California, Berkeley, CA 91320.

Sec4p is the prototypic member of the Rab family of *ras*-related low molecular weight GTP-binding proteins. It is required for vesicular transport between the *trans* Golgi and the plasma membrane in the budding yeast *S. cerevisiae*. We are using recombinant Sec4p, expressed using the baculovirus system, to identify proteins that are involved in constitutive exocytosis in mammalian cells. When recombinant Sec4p is expressed in baculovirus-infected Sf9 cells, it is found in both soluble and membrane-associated forms. Sec4p incorporates [^{3}H]-mevalonate in infected Sf9 cells, indicating that it is modified by isoprenylation, but does not appear to incorporate either [^{3}H]-palmitate or [^{3}H]-myristate. Recombinant Sec4p exhibits a low endogenous rate of GTP hydrolysis, but this rate is stimulated by factors (GTPase activating proteins, GAPs) present in crude rat liver homogenates. Subcellular fractionation demonstrated that Sec4p-GAP activities are present in both soluble and membrane-associated fractions from rat liver. The soluble rat liver Sec4p-GAP activity can be separated into two components (approximately 90-130 kDal and 350-400 kDal) by gel filtration chromatography.

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Distinct GAP Activities in Rat Pancreas Specific for Yeast Low M_r GTP-Binding Proteins. B. P. Jena, P. Brennwald, M. D. Garrett, P. Novick, and J. D. Jamieson, Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06510.

In the last few years, a number of low M_r GTP-binding proteins have been implicated in distinct vesicular transport processes. Sec4, a 23.5 kDa GTP-binding protein associated with post-Golgi secretory vesicles, is required for exocytosis in yeast. Ypt1, another low M_r GTP-binding protein (23 kDa), has also been implicated in protein transport from ER to the Golgi complex in yeast.

In a search for mammalian homologues to Ypt1 and Sec4, rab1 has been identified as the equivalent of yeast Ypt1 (EMBO [1989] 8:1427-1432). In the present study, we provide evidence for distinct and specific Ypt1 and Sec4 GAPs (GTPase activating proteins) in rat pancreas. Using radiolabeled GTP-bound to purified recombinant Sec4 and Ypt1 proteins, we have studied GAP activities in rat pancreatic fractions. Our studies demonstrate that while 65% of the Sec4-GAP activity was associated with the 150,000 x g particulate fraction, only 35% was found in the cytosol; greater than 95% of the Ypt1-GAP activity was found associated with the particulate fraction. The data indicate the presence of separate pancreatic GAPs for Ypt1 and Sec4. Results from Sec4 and Ypt1 competition assays further demonstrated the specificity of the pancreatic Sec4 and Ypt1 GAPs. Our study demonstrates for the first time the presence of distinct GAPs in rat pancreas, each specific for the yeast Sec4 and Ypt1 proteins. This supports earlier findings for the presence of a Ypt1 homologue in mammalian cells (rab1) and also suggests that mammalian cells may possess a Sec4 homologue. [Supported by USPH grant DK17389 (JDJ) and GM35370 and CA46218 (PN)]

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A *Drosophila* Homolog of Bovine *smg* p25a GDP Dissociation Inhibitor Undergoes a Shift in Isoelectric Point in the Developmental Mutant, *quartet*. J. E. Zahner and C. M. Cheney, Dept. of Genetics, Washington University School of Medicine, St. Louis, MO 63110.

The *Drosophila* developmental mutant *quartet* causes late larval lethality, small imaginal discs and has a maternal effect on early embryogenesis. Developmental defects in *quartet* mutants include defective eggshell membranes, accumulation of large vesicles in late larval brains and movement coordination defects. *quartet* mutants also show a low mitotic index in larval brains and incomplete separation of chromosomes in mitosis in the early embryo. In addition to these developmental problems, *quartet* mutations have a biochemical phenotype, which is a basic shift in isoelectric point in three proteins. We have purified one of these proteins, raised an antibody to it, isolated its cDNA and have sequenced the cDNA. At the amino acid level, the sequence shows 68% identity and 81% similarity to bovine *smg* p25a GDP dissociation inhibitor (GDI), a regulator of *ras*-like small GTPases of the *rab/SEC4/YPT1* subfamily. The *Drosophila* GDI (dGDI) is a unique gene, located at chromosomal location 30B, a location different from the *quartet* chromosomal location. Antibodies raised to dGDI and to a dGDI fusion protein show a general cytoplasmic staining in early *Drosophila* embryos. Cellular fractionation studies show that dGDI is present in the cytoplasmic, and not nuclear, fraction and dGDI is present in the non-membranous, and not the membranous, fraction. dGDI is triton-soluble and is not associated with the triton-insoluble cytoskeleton. Western analysis shows that dGDI is present at all stages of development examined and in all tissues examined. The correlation between a basic shift in isoelectric point in dGDI in *quartet* mutant tissue and the *quartet* developmental phenotype raises the possibility that *quartet* codes for an enzyme which catalyzes a post-translational modification of dGDI. This modification may be necessary for dGDI function.

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Identification of Seven Additional Members of the rho Family of Small Molecular Weight GTP Binding Proteins and One Gene Highly Homolgous to TC4 in the Eukaryote D. discoideum. J. Cardelli, K. Franek, and J. Bush, Dept. of Microbiology, LSUMC, Shreveport, LA 71130.

The simple eukaryote D. discoideum has proven to be a useful system to investigate the molecular mechanisms regulating the organization and function of the cytoskeleton, vesicular traffic, and secretion of proteins comprising the lysosomal/endosomal system, because of the relative ease of application of molecular genetics and biochemical approaches. Small molecular weight GTP-binding proteins of the rho family have been implicated in cytoskeleton organization and regulation of vesicular traffic along the exocytotic pathways of eukaryotic cells. We report here the identification of 7 additional members of the rho group of genes. An oligonucleotide coding for the amino acids DTAGQE (a region highly conserved between ras, rho and rab proteins) was used to probe a cDNA library generated with mRNA from cells developing for 4 hours. The largest members of each group (classified by Southern blot analysis) were sequenced and the predicted amino acid sequences derived from the longest open reading frames were used to scan the NBRF protein data base. Seven of the groups contained rho-like genes while one group contained genes that were >80% identical to the human TC4 gene, hypothesized to represent a new family. Three of the rho members (rac1A, rac1B, and rac1C) were >90% identical to each other and >80% identical in amino acid sequence compared to the human rac1 gene over the entire gene while the other members (racA, racB, racC, and racD) were 50-65% identical to rac1, rhoA and to each other. Interestingly, the Dictyostelium rac-like genes revealed different patterns of expression during growth and development. For instance, Rac1 mRNA accumulated to high concentrations during early development and then disappeared while mRNAs coded for by the other rac like genes decreased in concentration at different rates from the peak levels observed during growth suggesting these proteins play unique roles during the different stages of the Dictyostelium life cycle. This study forms the foundation of future investigations to determine the role of these rac-like GTP binding proteins in organization of the actin cytoskeleton and vesicular trafficking during growth and development.

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Identification of Nine New Members of the Rab Family of Small Molecular Weight GTP Binding Proteins in D. discoideum. J. Bush, J. M. Daniels, G. B. Spiegelman, G. Weeks, K. Franek, and J. Cardelli, from the Dept. of Micro., LSUMC, Shreveport, LA 71130 and Dept. of Micro., UBC, Vancouver, Canada

D. discoideum has proven to be a useful system to investigate the molecular mechanisms regulating the transport, trafficking, and secretion of proteins comprising the lysosomal/endosomal system, because of the relative ease of application of molecular genetics and biochemical approaches. Small molecular weight GTP-binding proteins of the rab/ypt1/sec4 family have been implicated in regulation of vesicular traffic along the exocytotic and endocytotic pathways of eukaryotic cells, and to date, only 2 rab-like genes (sas1 and sas2) have been identified in the slime mold. We report here the identification of 9 new members of the rab family. Two approaches were used to clone the genes: 1) An oligonucleotide coding for the amino acids DTAGQE (a region highly conserved between ras, rho and rab proteins) was used to probe a cDNA library generated with mRNA from cells developing for 4 hours, and 2) oligonucleotides corresponding to conserved GTP binding domains I and III were used in PCR with cDNA made from mRNA from different developmental stages. DNA inserts of each group (segregated by Southern blot analysis) were sequenced and the predicted amino acid sequences derived by conceptual translation of the open reading frames were used to scan the NBRF protein data base. In addition to groups containing new members of the ras and rho family, we identified nine genes belonging to the rab family of GTP binding protein genes. Two of the members, clone 3 and clone 64 are >80% identical to the canine and human rab1 genes and are considered D. discoideum homologues. Two other clones, 44 and 47 are also proposed to be homologues to the human rab11 and rab2 genes. The other 5 genes are more closely related to rab1 (40% to 70% identity) than to the other rab genes, however, we propose they represent new members of the rab family. For instance, the amino acid sequence of the effector domain of the predicted rabA gene (clone 29) product is FAPTLGVD which is distinctly different from the Rab1 effector domain, YISTIGVD. Interestingly, Northern blot analysis indicated that these rab gene family members showed different patterns of expression during growth and development suggesting their gene products play important roles during multiple phases of the Dictyostelium life cycle.

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Subcellular compartmentalisation of proenkephalin within astroglia, a novel neuroendocrine cell type. D.S. Holt, U. Weinhart, A.R. Prescott, B.A. Spruce, Department of Biochemistry, The University, Dundee DD1 4HN, U.K.

Proenkephalin, the precursor to Met-and Leu-enkephalin, is synthesised within and released from astrocytes as the unprocessed pro-hormone (Melner et al., 1990; Spruce et al., 1990). Immunolocalisation by confocal scanning laser microscopy, using a panel of monoclonal antibodies to proenkephalin, has been carried out on astrocytes in dissociated primary culture and C6 glioma cells stably transfected with human preproenkephalin cDNA. Endogenous proenkephalin (in primary cultures of cortical astrocytes) and transfected proenkephalin within the stable glioma cell lines appear to be packaged similarly, within vesicle-like organelles in the cell body and processes. In PC12 cells (a neuroendocrine chromaffin cell line) stably transfected with human preproenkephalin, a similar pattern of intracellular compartmentalisation is seen. Ultrastructural analysis and co-staining with vesicle markers are underway, to determine the nature of the glial organelles. Their association with the cytoskeleton is also under investigation. In cultures of primary astrocytes, proenkephalin-containing organelles are apparently associated with a subset of intermediate filaments co-tracking with a stable microtubule subpopulation, which may represent a novel mode of vesicle transport. We propose to perform cytoskeletal disruption experiments to assess the importance of these cytoskeletal elements in proenkephalin localisation within this cell type.

References: Melner et al. (1990) EMBO J 9, 791-796; Spruce et al. (1990) EMBO J 9, 1787-1795.

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Apical secretion of apolipoproteins from enterocytes. E. Michael Danielsen, Gert H. Hansen & Mona D. Poulsen. Dept. of Biochemistry C, The Panum Institute, University of Copenhagen, 3 Blegdamsvej, DK-2200 Copenhagen N, Denmark.

Synthesis and secretion of apolipoproteins in pig small intestine was studied by pulse-chase labeling of jejunal segments, kept in organ culture. Apo A-1 and apo B-48 were the two major proteins released, constituting about 25 % and 10-15 % of the total amount of labeled proteins in the medium after 3 h of chase, respectively, and their appearance in the medium occurred with a half-time of less than 60 min. In the fasting state, more than 80 % of newly made apo A-1 was released to the medium, and the secretion was shown to occur from the mucosal surface. During culture, the enterocytes and their tight junctions largely remained intact as evidenced by the diffusible marker Ruthenium red. Our results therefore show that enterocytes release the majority of their newly made apolipoproteins by exocytosis via the brush border membrane into the intestinal lumen. Unless apolipoproteins carry a sorting signal for apical targeting, this implies that the 'default' pathway in mature enterocytes goes to the apical rather than to the basolateral cell surface. Fat absorption reduced apolipoprotein secretion to the medium and induced the formation of chylomicrons, containing apo A-1 at their surface, as evidenced by immunogold electron microscopy. The chylomicrons were localized in the Golgi complex and near the basolateral plasma membrane, but not in the apical region of the enterocytes, indicating that only free apolipoproteins are secreted to the intestinal lumen.

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Two Microsomal Esterases May Regulate the Exit of the Acute Phase Plasma Protein, C-Reactive Protein, From the ER of the Hepatocyte. S.S. Macintyre and P.A. Kalonick. Department of Medicine, Case Western Reserve University at MetroHealth Medical Center, Cleveland, OH 44109.

Hepatic synthesis and secretion of the acute phase protein, C-reactive protein (CRP) increases by as much as several hundred-fold during the acute phase response (APR) to tissue injury. In addition, the intracellular transit time of newly synthesized rabbit CRP decreases dramatically from 18 h in normal cells to about 45 min in hepatocytes isolated from rabbits stimulated *in vivo* to undergo the actue phase response. Changes in transit time were found to be due to specific retention of CRP within the ER of unstimulated hepatocytes. Studies of the binding of CRP to permeabilized microsomes indicated the presence of both low and high affinity binding sites specific for CRP and downregulation of the high affinity site in microsomes from animals undergoing the APR correlated with the observed decrease in ER retention of CRP during the APR. In the present studies, we have identified and purified two 60 kD ER lumenal glycoproteins (gp60a and b) which bind CRP in a nitrocellulose blot assay. These two proteins appear to represent the low and high affinity binding sites previously identified as judged by their relative abundance, affinities for CRP, and downregulation during the APR. Partial amino acid sequence data indicate that gp60a and b are 60k microsomal esterases (forms 1 and 2), previously identified by others from complete protein sequence analysis. These two homologous proteins are members of a highly conserved family of proteins whose function within the ER remains largely unknown. Though both gp60a and b have demonstrable esterase activity, binding of CRP by these two proteins appears not to directly involve the esterase site in that CRP binding is unaffected by treatment with PMSF, an inhibitor which abolishes their esterase activity. The interaction between these proteins and CRP may represent a novel mechanism which can regulate the intracellular trafficking of a secretory protein under differing physiological conditions. Supported by NIH grant AR34313.

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The propeptide of fibronectin retards exit from the endoplasmic reticulum in AtT-20 cells. R.L. Wright[1], A.M. Castle[1], J.E. Schwarzbauer[2], and J.D. Castle[1]. [1] Dept. Anatomy and Cell Biology, Univ Virginia, Charlottesville, VA 22908, and [2] Dept. Molecular Biology, Princeton University, Princeton, NJ 08544.

Recombinant fibronectin polypeptides - deminectins - have been used for studies of structure/function relationships of different domains. We have been interested in the possible role of the putative fibronectin propeptide (11-13 amino acids) in the intracellular transport of deminectins expressed in pituitary AtT-20 cells. Deminectin containing the fibronectin propeptide (pro-DN) exited the ER with a $t_{1/2}$ of 2 hrs whereas deminectin lacking the propeptide (DN) exited with a $t_{1/2}$ of only 20 min. The slower drainage of pro-DN from the ER did not reflect the rate of dimerization as dimerization was rapid for both pro-DN and DN ($t_{1/2}$ = 7-10 min). These results suggest possible interactions of the propeptide with the resident proteins in the ER, thereby retarding the exit of the deminectin from the ER. The effect was specific for the fibronectin propeptide since its replacement with another basic propeptide (the parathyroid hormone pro hexapeptide) did not decrease the rate of ER exit ($t_{1/2}$ = 20 min). We are currently evaluating whether fusion of the fibronectin propeptide to other polypeptides will similarly retard their exit from the ER. These findings suggest that the propeptide may regulate the transport of fibronectin in the ER.

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Overexpression of a Carboxyl Terminal Peptide of Alzheimer β-Amyloid Protein Precursor Results in Accumulation within an ER Compartment. T.J.Raub, S.L.Kuentzel, P.A.Gonzalez-DeWhitt, R.A.Altman, S.M.Ali, and B.D.Greenberg, Upjohn Labs, Kalamazoo, MI.

A hallmark of Alzheimer's disease is the formation of lesions in human brain that contain βA4 derived from the β-amyloid protein precursor (βAPP). To understand the role of β-protein better, transgenic mice expressing all or fragments of the βAPP are being used. We have studied the stable expression of a 103 amino acid peptide (residues 593-695) of the βAPP-695 in transfected HeLa cells. The peptide consists of the cytoplasmic and transmembrane domains of βAPP including the amyloidogenic βA4 region. When butyrate-treated transfected cells are permeabilized and immunostained with a COOH-terminal specific antibody, numerous and discrete cytoplasmic inclusions are observed. These inclusions react with Con A, but not wheat germ agglutinin, nor do they immunostain for the ER-Golgi intermediate compartment specific protein p53 or cathepsin D. That βAPP peptide accumulates in an ER compartment is confirmed by immunostaining for an ER-specific antigen. Western blots of whole-cell extracts reveal major 14 and 16 kD protein species which are dramatically induced in these cells, as are several higher mol wt minor species. Butyrate-treated control cells and untreated transfectants do not accumulate these proteins. Pulse-chase experiments using [^{35}S]met show that newly synthesized peptide is not readily degraded. Further, endogenous full-length βAPP and fibronectin are processed normally suggesting that secretion is unaffected by this accumulation. Small amounts of full-length βAPP accumulate in the inclusions after 20 h in butyrate as shown by immunofluorescence microscopy with a N-terminal specific antibody. These data show that overexpression of this peptide containing the amyloidogenic fragment results in intracytoplasmic inclusions within the ER as a result of aberrant processing. It remains to be determined how this relates to processing of this peptide by cells of CNS origin, especially neurons, and the relationship to processing in the transgenic mouse model.

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Isoprenoid Requirement for Intracellular Transport and Processing of Murine Leukemia Virus Envelope Protein. J.H. Overmeyer and W.A. Maltese, Weis Center for Research, Geisinger Clinic, Danville, PA 17822.

Lovastatin blocks the biosynthesis of cellular isoprenoids, including those required for posttranslational modification of low molecular mass GTP-binding proteins. When Friend murine erythroleukemia (MEL) cells are cultured in medium containing lovastatin, the murine leukemia virus envelope glycoprotein precursor ($gPr90^{env}$) fails to undergo proteolytic processing, which normally occurs in the Golgi complex, and its oligosaccharide chains remain sensitive to digestion by endoglycosidase H. Consequently, viral particles shed from lovastatin-treated cells lack the mature envelope protein, $gp70^{env}$. The inhibition of $gPr90^{env}$ processing is prevented by addition of the isoprenoid precursor, mevalonate, to the medium. Low molecular mass GTP-binding proteins encoded by the *rab* genes (*e.g., rab*1p and *rab*6p) are believed to function in early steps of the exocytic pathway. These proteins are normally modified by geranylgeranyl isoprenoids, and are localized to endoplasmic reticulum (ER) and Golgi membranes. However, in MEL cells treated with 1 μM lovastatin, *rab*1p and *rab*6p fail to undergo isoprenoid modification and accumulate in the cytosol prior to arrest of $gPr90^{env}$ processing. These observations suggest that lovastatin may prevent viral envelope precursors from reaching the Golgi compartment by blocking the isoprenylation of *rab* proteins required for ER to Golgi transport. Supported by NIH Grant CA34569.

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Coupling of Furin activity and the proteolytic processing of a precursor protein targetted to the regulated pathway of secretion. L. Paquet, C. Lazure, N.G. Seidah, M. Chrétien and M. Mbikay. IRCM, 110 Pine Ave West, Montréal, Québec, Canada. H2W 1R7. (Spon. by L. Gaspar).

One of the key steps in the maturation of biologically active peptides involve proteolytic processing at basic amino acids. To date, three enzymes, PC1, PC2 and Furin, have been shown to possess this convertase activity. While PC1 and PC2 colocalize with hormonal precursors in endocrine and neuronal cells, Furin distribution is more ubiquitous. The presence of Furin in cells which do not express prohormones nor possess a regulated pathway of secretion suggest a coupling of Furin with the constitutive pathway of secretion. A neuroendocrine protein named 7B2 is targetted to the regulated pathway of secretion in many cell lines and is processed at a site rich in basic residues (FEBS 294:23). Using a recombinant vaccinia virus, mouse 7B2 was overproduced in various cell lines which express PC1 (AtT-20), PC2 (GH4C1) or Furin (AtT-20, GH4C1, PC12 and Ltk-). Immunological forms of 7B2 detected in all these cell lines included the precursor form and several processed ones, suggesting that many enzymes or a single but ubiquitous one could process pro-7B2. However, co-expression of PC1 or PC2 with 7B2 in Ltk- cells did not enhance the processing index of pro-7B2. Only Furin was able to convert all this precursor to the shorter forms. These results indicate that processing of pro-7B2 can be uncoupled from the activities of PC1 and PC2 and suggest that proteins found in secretory granules can be substrates for a convertase such as Furin not exclusively associated with these organelles.

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Evidence for a Secretory Pathway originating from the Maturation of Granules in the Cannulated Pig Pancreas under Atropine Treatment. GP-2 is its Distinctive Component. G. Viau, J. Lainé, T. Corring[†], J. Morisset and D. LeBel. Centre de Recherche sur les Mécanismes de Sécrétion, Université de Sherbrooke, Canada, J1K 2R1, and [†]Centre de Recherche de l'INRA, Jouy-en-Josas, F-78352, France.

Pigs were fitted with a permanent pancreatic cannula. After an overnight fast, the unanesthetized pigs were sequentially infused intravenously with 1°) Saline, 2°) Secretin at 36 pmole/kg/h, and 3°) Secretin + Atropine (10 to 200 μg/kg/h). Pancreatic secretions were collected every 30 min. Amylase, trypsin, chymotrypsin, elastase, and lipase were determined, as well as GP-2, the major zymogen granule membrane protein. All doses of atropine induced an almost complete inhibition of secretion of the 5 zymogens assayed. A parallel inhibition of the secreted GP-2 was observed. While the activity per mg of protein of the 5 zymogens stayed constant, the specific activity of GP-2 increased almost exponentially in the residual secretion. Under atropine infusion, the pattern of proteins secreted was dramatically different from that under normal conditions with secretin alone. GP-2 and amylase were the two major constituents. The identity of GP-2 was confirmed by immunoblots. Triton X-114 phase partitioning showed that GP-2 was soluble and had lost its diacylglycerol anchor. GP-2 did not show any evidence of proteolytic digestion. Presence of inositol 1,2-(cyclic) monophosphate was confirmed, and suggests that GP-2 was released by cleavage of its glycosyl phosphatidylinositol anchor by a phospholipase C as previously shown under basal secretions in the rat. These results confirm the existence in the exocrine pancreas, of a secretory pathway different from the regulated pathway. This pathway would correspond to the constitutive pathway and/or to a pathway originating from the maturation of secretory granules. Since GP-2 is highly concentrated in the zymogen granule membrane and its content, its high concentration in the atropine-treated animals let us believe that this secretion would more likely originate from a diversion of the regulated pathway, a pathway that could then be called "maturative".

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A chimeric Insulin-CD5 protein expressed in AtT-20 cells is directed to the cell-surface mostly via the constitutive pathway. M. Mbikay, N. Rondeau and M. Chrétien. Institut de Recherches Cliniques de Montréal, Montréal, Québec, Canada H2W 1R7.

A chimeric gene encoding mouse insulin fused to the transmembrane-spanning and the cytoplasmic domains of the CD5 antigen of human T lymphocytes was transfected in the endocrine cell line AtT-20. Its expression was confirmed at the mRNA and the protein levels, by Northern and Western blots, respectively. Its localization was examined by immunofluorescence histochemistry and flow cytometry. The fusion protein was localized on the cell surface, largely as a precursor or partially processed form recognizable by an anti-C peptide antibody. Secretory stimuli by KCl or 8-Br-cAMP did not increase the surface density of insulin. However, when the antigen was depleted by protease pre-treatment, a slight increase of surface immunofluorescence could be detected following stimulation, suggesting that a small fraction of the chimeric protein was stored in the cells. These results indicate that, unlike normal proinsulin, most of this chimeric molecule was not transported in AtT-20 cells by the regulated storage granules, but was routed to the cell surface via the constitutive pathway. They also suggest that the signals that direct a hormone towards storage granules are not always dominant and can be altered by fusion to sequences derived from a constitutively secreted molecule. (Funded by MRC Canada).

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The Release of Parathyroid Hormone and the Constitutive Secretion of a Proteoglycan Are Modulated by Calcium in a Similar Manner in Parathyroid Cells. Z. Muresan. and R.R. MacGregor. Department of Anatomy and Cell Biology, The University of Kansas Medical Center, Kansas City, KS 66160

Parathyroid (PT) cells secrete parathyroid hormone (PTH) in response to catecholamines via a regulated secretory pathway. In response to hypocalcemia, however, PTH secretion has the characteristics of a constitutive pathway: rapid and continuous release of newly synthesized hormone, without depletion of cellular PTH. The decreased rate of PTH release in high Ca is accompanied by an augmentation of degradation of hormone. To test the hypothesis that Ca modulates the constitutive secretion in PT cells, Ca effects on the release of a sulfated proteoglycan (PG), a marker for constitutive pathway, were examined. Fresh bovine PT cells labeled with $^{35}SO_4$ released a chondroitinase-sensitive PG that bound tightly to Q Sepharose and migrated as a smear with M_{app} of 90-150 kD on SDS-PAGE. The discharge of PG from PT cells had the characteristics of constitutive secretion: $^{35}SO_4$-PG appeared in the medium within 15 min after a 5 min pulse, 80-90% of labeled PG was cleared from the cells in 4-6 hrs, and isoproterenol, given 6 hrs after the pulse, stimulated release of PTH but not that of PG. In chase incubations at 0.5 or 2 mM Ca, following 5 min pulse with $^{35}SO_4$, similar kinetics of PG secretion were observed, but more PG was released in the medium, and more PG was recovered in cell+medium at 0.5 mM than at 2 mM Ca. These results indicate that Ca negatively modulates constitutive secretion of PG in PT cells. The similar effect of Ca on PTH and on PG release suggests a Ca-modulated constitutive component in PTH secretion.

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Cell Surface Expression of Chimeric Proteins Consisting of Variants of Alpha-1-Proteinase Inhibitor Fused to the Moloney Murine Leukemia Virus Envelope Protein is a Function of the Transport Competence of the Alpha-1-Proteinase Inhibitor Variant. O. Pfenninger, T. Yohannes, and J.L. Brown. Department of Biochemistry, Biophysics and Genetics, University of Colorado Health Sciences Center, Denver CO 80262

Chimeric proteins constructed to study aspects of protein targeting and secretion are frequently retained in the endoplasmic reticulum rather than entering the exocytic pathway. To test the idea that fusion of complete or essentially complete cDNAs specifying proteins containing the signals of interest may be more reliably transported to the cell surface we have constructed cDNAs (A1PiEnv cDNA) specifying the completed alpha-1-proteinase inhibitor (A1Pi) linked to the mature form of the Moloney murine leukemia virus envelope protein (mmlvEnv) via a 2 amino acid spacer. cDNAs specifying the normally secreted A1PiM and the poorly secreted A1PiZ attached to mmlvEnv have been constructed (A1PiMEnv and A1PiZEnv, respectively) and expressed in transiently transfected COS 1 cells. The fates of the chimeric proteins have been followed by immunofluorescence using antibodies specific for A1Pi and mmlvENV and by analyses of metabolically labeled products using immunopre-cipitation, SDS-PAGE, and phosphorimaging. Our results show that a protein of appropriate size for the chimer is precipitated by antibodies to both A1Pi and mmlvEnv. Neither A1PiMEnv nor A1PiZEnv is secreted, however, A1PiMEnv, but not A1PiZEnv, is transported to and anchored into the plasma membrane in the orientation normally assumed by mmlvEnv. These results show that A1PiMEnv, but not A1PiZEnv, is efficiently transported to cell surface and suggest that chimeric proteins produced by fusion of complete, transport competent forms may be generaly useful for studies of the exocytic pathway.

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Okadaic Acid disrupts synaptic vesicle clusters in frog motor nerve terminals. W.J. Betz. Department of Physiology, University of Colorado Medical School, Denver, CO 80262.

Synaptic vesicles are clustered in nerve terminals near sites of exocytosis. We found that the phosphatase inhibitor okadaic acid (OA; 2 μM) disrupts vesicle clusters.

Motor nerve terminals in acutely dissected *Rana pipiens cutaneus pectoris* muscles were first stained with FM1-43, a fluorescent dye that labels the membranes of recycled synaptic vesicles, producing a series of fluorescent spots along the length of nerve terminals. Each spot is a cluster of several hundred labeled vesicles.

OA (2 μM) caused the fluorescent spots to blur, and the neighboring regions of cytoplasm to brighten, as if synaptic vesicles were dispersing in the cytoplasm. Spots did not reform during a 6 hr wash in OA-free medium. Nerve stimulation after FM1-43 staining and OA treatment still caused destaining, suggesting that dye loss via exocytosis was not abolished. Pretreatment with OA interfered with FM1-43 staining: dye was internalized, but staining was diffuse, not punctate. Nerve stimulation in these cases did *not* cause destaining, suggesting that OA interferes with vesicle recycling. Electrophysiologically, OA-treated muscles were remarkably normal. The amplitude of end plate potentials was moderately reduced after OA washout. Synaptic facilitation, depression, and post-tetanic potentiation were all present.

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Association of Cytosolic Lipids with Fatty Acid Synthetase from Lactating Mammary Gland. B.H. Keon, D. Ghosal, and T.W. Keenan, Department of Biochemistry and Nutrition, Virginia Polytechnique Institute and State University, Blacksburg, VA 24061

A > 10,000 M, fraction of cytosol from lactating mammary gland is necessary, in vitro, for precursor lipid droplet formation and fusion. Studies were performed to characterize this complex and define its role in the synthesis of milk lipid globules. Membrane-free cytosol contained over 4% of both the total lipids and phospholipids which were present in homogenates of lactating rat mammary gland. Most of this lipid was associated with a high molecular weight complex isolated from cytosol by gel exclusion chromatography or by density gradient centrifugation. This complex principally consisted of polypeptides with apparent molecular weights of 220 and 116 kilodaltons. Lipids associated with this complex were transferred to endoplasmic reticulum and to intracellular lipid droplet precursors of milk lipid globules upon incubation in a cell-free system. This lipoprotein complex was abundant in cytosol from lactating mammary gland, but was diminished in amount in cytosol from involuted mammary glands. The 220 kilodalton constituent of this complex was identified as the monomer of fatty acid synthetase. These results suggest that fatty acid synthetase complex in lactating mammary gland may function in transfer of lipids necessary for formation or growth of lipid droplet precursors of milk lipid globules.

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Distribution of the Complement Membrane Attack Complex and its Regulators in Myocardial Infarction. P. Laurila, A. Väkevä, and S. Meri. Department of Pathology, and Department of Bacteriology and Immunology, University of Helsinki, Finland.

Recent studies have the suggested that the complement (C) system is involved in the development of tissue injury of myocardial infarction. It is not known why the C system starts to react against autologous heart tissue. We have therefore analysed the expression of the membrane regulators of C (CD59, CR1, DAF, MCP) and the pattern of C component and plasma C regulator (C4-binding protein and S-protein) deposition in normal (n=7) and infarcted (n=13) human myocardium. Indirect immunofluorescence methods were applied using specific monoclonal and polyclonal antibodies. Both cryosections and paraffin embedded sections were used. Normal myocardial cells showed a strong expression of the CD59-antigen (protectin), whereas DAF, MCP and CR1 were only weakly or not at all expressed in the sarcolemmal membranes. The staining for CD59 was clearly diminished or totally absent from the large areas of infarcted myocardial tissue. The CD59-negative areas appeared as distinct zones or multiple patch-like lesions within individual heart specimens. Depositions of the C membrane attack complex (MAC) were observed in infarcted myocardial lesions. The MAC deposits colocalized with the protectin negative areas. Depositions of S-protein (vitronectin), and of C4-binding protein were also seen within the same areas of infarcted myocardial tissue. The results provide further evidence for the role of the MAC in the pathogenesis of tissue injury in myocardial infarction. Furthermore, the loss of the CD59-antigen from the heart muscle cells may be the main regulatory event rendering the cells susceptible to the complement-mediated membranolysis during myocardial infarction.

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Characterization of a Heparin-Binding Site in Lipoprotein Lipase. D. Berryman and A. Bensadoun. Division of Nutritional Sciences and Division of Biological Sciences. Cornell University, Ithaca, NY 14853.

Lipoprotein lipase (LPL) binds to heparin and heparan sulfate proteoglycans. We have employed site-directed mutagenesis to dissect one of the proposed heparin-binding domains of avian LPL which contains the sequence Arg-Lys-Asn-Arg (aa 281-284). We have constructed various single, double, and triple mutants of chicken LPL to alter the positive charge of this region. The mutant and wild-type cDNAs were subcloned into an expression vector pRC/CMV and transfected into CHO-K1 cells. LPL mutants with a decrease in positive charge showed a decrease in affinity for heparin. The greatest effect was seen with the triple mutant (LPL MUT5) in which all of the positively charged amino acids were altered to neutral. On the heparin sepharose column, this mutant eluted at 0.96 M NaCl compared with 1.35 M for wild-type LPL. This mutant also had the lowest specific activity. The specific activity for LPL MUT 5 was 1.7 ueqfa/ug/hr compared with 6.7 ueqfa/ug/hr for wild-type cells. Finally, the CHO-K1 cells transfected with this mutant had a ten fold decrease in the amount of lipase associated with the cell surface when corrected for medium enzyme concentration. In conclusion, this region of LPL does appear to be involved in heparin-binding, however, additional regions must also be involved since binding was not completely abolished. In addition, the interaction between LPL and heparin-like molecules appears to stabilize the active form of the enzyme.

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Elimination of the O-linked Oligosaccharide at Thr 104 Results in the Generation of a Soluble Transferrin Receptor. E.A. Rutledge and C.A. Enns Department of Cell Biology and Anatomy, Oregon Health Sciences University, Portland, OR 97201.

The transferrin receptor (TfR) is the plasma membrane protein that is responsible for the binding and internalization of the major iron transport protein, transferrin. To understand the effect of the O-linked carbohydrate on TfR function, cDNA encoding human TfR was altered to replace Thr 104 with Asp, and thus eliminate the site of O-linked carbohydrate attachment. The mutated cDNA was expressed in a cell line that lacks endogenous TfR. Elimination of the O-linked carbohydrate at Thr 104 results in a form of the receptor which is more susceptible to cleavage and which releases an 80 kDa form that can bind transferrin into the growth medium. The intact mutant TfR is not grossly altered in its structure and does not differ from the wild type human receptor in many respects: 1) It shows the same distribution between the plasma membrane and intracellular compartments. 2) The binding constant for transferrin is similar to that of the wild type TfR. 3) After synthesis it shows similar kinetics of processing and transport to the cell surface. 4) It is not rapidly degraded. Preliminary protein sequence analysis of the soluble form indicates that the sequence begins at amino acid 101 of the intact receptor. This is the same cleavage site reported for a soluble form of normal receptor found in human serum, and suggests that elimination of the O-linked carbohydrate at position 104 enhances the susceptibility of TfR to cleavage and may mimic a naturally occuring process in the body.

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Mutation of Cys_{442} of the $Alpha_2$- Adrenergic Receptor Eliminates ^{3}H-Palmitate Incorporation but does not Perturb Receptor G-protein Coupling. M.E. Kennedy and L.E. Limbird. Department Of Pharmacology, Vanderbilt University, Nashville, TN

The porcine brain α_{2A}- adrenergic receptor (α_{2A}-AR) is coupled to a variety of effectors via pertussis-toxin sensitive GTP-binding proteins. Like most members of this receptor superfamily, the primary structure of the α_{2A}-AR possesses a concensus sequence for palmitoylation in the C-terminus at Cys_{442}. Previous studies in our laboratory have indicated that the α_{2A}-AR indeed incorporates ^{3}H-palmitic acid in metabolic labeling studies and that mutation of Cys_{442} to Ala_{442} or Ser_{442} eliminates detectable ^{3}H-palmitoylation but does not alter adrenergic ligand specificity or allosteric modulation by amiloride analogs. The present study was undertaken to examine whether mutation of Cys_{442}, and parallel loss of detectable palmitoylation, would alter α_{2A}-AR-G-protein coupling, as reports in the literature suggest that a homologous mutation in the β_2-AR attenuates coupling to G_s and and chemical removal of palmitate from bovine rhodopsin enhances coupling to G_t. Several independent cell lines of MDCK cells expressing WT(Cys_{442}) or mutant (Ala_{442};Ser_{442}) α_{2A}-ARs were cloned. Metabolic labeling of MDCK cells expressing WT (Cys_{442}) or Ala_{442} α_2-ARs with ^{3}H-palmitic acid indicated that only WT: Cys_{442} containing receptors incorporated ^{3}H-palmitate, monitored following isolation of the α_{2A}-AR from detergent extracts using yohimbine-agarose chromatography. Receptor-G-protein coupling was assessed by evaluating sensitivity of receptor agonist interactions to guanine nucleotides both in competition for ^{3}H-yohimbine antagonist binding and in sensitivity for detection of ^{125}I-p-iodo-clonidine binding. Using either approach, no detectable change in α_{2A}-AR-G-protein coupling was apparent, in contrast to apparent effects on β_2-AR-G_s and rhodopsin-G_t coupling reported previously by others. One interpretation is that this highly conserved cysteine may play differing roles at different R-G interfaces. Alternatively, this shared structural motif may play a role in not yet investigated pathways, such as receptor expression, localization and turnover. (HL25182, HL43671, GM07628)

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Purification and Parial Characterization of Outer Membrane Iron Transport Receptors from Escherichia coli: FepA and FecA. X.H. Zhou and D. van der Helm, Department of Chemistry and Biochemistry, The University of Oklahoma, Norman, OK 73019 USA

The E. coli plasmid UT5600/pBB2 produces two outer membrane iron transport receptors in the 80K molecular weight range. The receptors can be purified with difficulty using FPLC with DEAE-Sepharose Fast Flow, PBE-94 and Q-Sepharose Fast Flow. Both Proteins were demonstrated to have the specific binding activity to ^{55}Fe-enterobactin, the most powerful siderophore to date. Each purified protein displayed a single amino acid sequence by determination of the N-terminus. With the help of a computer search from GeneBank one was identified as genuine ferrienterobactin receptor (FepA), while another protein was not the degradation product of FepA and identified to be FecA, a ferric decitrate receptor. Competition studies showed that Fe-MECAM was competitively bound to both receptors and Fe-parabactin only partially competed with ^{55}Fe-enterobactin to bind these proteins. It was found that ferrichrome A had not any inhibitory effect on the binding between the receptors and ^{55}Fe-enterobactin. The similarity of FepA and FecA also was investigated by a computer matching program, indicating that the middle part of FepA is similar to the former half of FecA. It may, in part, reveal the reason that both receptors have a similar character except for the binding activity levels.

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Alternative Splicing of Exons Encoding the Calmodulin Binding Domains and C-termini of Rat Plasma Membrane Ca2+-ATPase Isoforms 1, 2, 3, and 4. T. P. Keeton and G. E. Shull, Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0524 (Spon. by N. Ratner)

Plasma membrane Ca2+-ATPase mRNAs were analyzed to determine the tissue specific splicing patterns involving exons encoding the alternative Calmodulin Binding Domains and C-termini of isoforms 1, 2, 3, and 4. Poly(A)+ RNAs from various rat tissues were examined by S_1 nuclease protection and polymerase chain reaction analyses, and the intron/exon organization of the corresponding region of each gene was determined. Five forms of PMCA1 (PMCA1e and the previously reported PMCA1a, b, c, and d) arise by alternative splicing of a single 154 base pair exon. PMCA1e, which is expressed at highest levels in brain but is also present at low levels in all tissues examined, has the same calmodulin binding site as PMCA1a, c, and d but has a C-terminus that differs from previously identified forms of the enzyme. Three forms of PMCA2 were identified, which arise by inclusion of a 172 bp exon (PMCA2c), inclusion of the 172 bp exon and a 55 bp exon (PMCA2a), or exclusion of both the 172 and the 55 bp exons (PMCA2b). PMCA2a and c have the same calmodulin binding site, which differs from that of PMCA2b, but each form has a different C-terminus. Four forms of PMCA3, termed PMCA3a, b, c, and d, arise by alternative splicing of a 154 bp exon, in a pattern that is directly analogous to the formation of the PMCA1a, b, c, and d mRNAs. Two forms of PMCA4 were identified which arise by inclusion or exclusion of a 175 bp exon. Our goal with this research is to study the consequences of Calmodulin Binding Domain alternative processing by analyzing the relative sensitivity of each isoform to varying amounts of calmodulin and cAMP-dependent protein kinase activity using for the first time the entire pump expressed in tissue cultures. Studies of the phosphorylation of the human PMCA1 product have demonstrated a lowering of the Km of the pump for Ca2+, although a study comparing the relative Km values for each Isoform of the pump has not been done. Affinity studies with peptide fragments have shown the alternative Calmodulin Binding Domains of human erythrocyte PMCA1 differ in their affinity for calmodulin, but detailed studies of the interaction with calmodulin upon the activity of the intact protein have yet to be done. We are currently working on the expression and purification of these pumps both from COS and Sf9 cells.

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Evidence for a H^+/Nitrate Symporter in Aspergillus nidulans. R.J. Downey* and C. Gedeon, Ohio University, Athens, OH 45701

The nitrate transport system system (NTS) in Aspergillus was examined in terms of its kinetic behavior and its relationship to the nitrate reductase enzyme (NaR) during nitrate assimilation. When shifted to a minimal nitrate medium, mycelia with no history of nitrate required about 4 hours to achieve the maximum rate of 155 umol nitrate taken up/hr x g dry wt. The apparent Km for the NTP as determined from Lineweaver-Burke reciprocal plots of velocity vs. varied concentrations of external nitrate was calculated to be 0.24 mM. The NTP exhibited K kinetics since the velocity of nitrate transport was proportional to the external hydrogen ion concentration but the maximal velocity was independent of the nitrate concentration. A dual label procedure with ^{14}C-inulin and ^{3}H-water was used to compute internal volume and the net influx of hydrogen ions in the presence and absence of nitrate. In wild type mycelia where NaR activity was eliminated by vanadium treatment during growth on nitrate and in nitrate-nonutilizing mutants where NaR activty is abolished due to alterations in it's structural gene (niaD) or the regulatory gene governing its formation (nirA) nitrate was actively transported up to an internal concentration of 85 uM. At this point both hydrogen ions and nitrate are effluxed. Total inhibition of NTS activity occurred in cultures of all of the above cell types when shifted to minimal nitrate medium containing ammonium, the protein synthesis inhibitor cycloheximide or the chitin synthesis inhibitor polyoxin D abolished nitrate transport. In such cases, the half life of the NTS was determined to be about 3 hrs. We concluded that NTS activity in Aspergillus occurs independent of NaR activity and exhibits properties which suggest that it is a hydrogen-nitrate cotransporter [symporter].

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Cloning and Characterization of the Putative Distal Colon H^+,K^+-ATPase cDNA and Gene. M.S. Crowson and G.E. Shull Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267 (Spon. by Dr. Michael Lieberman)

The mammalian colon plays a major role in K^+ homeostasis. There is evidence that active absorption of K^+, which occurs in distal colon, is mediated by an H^+,K^+-ATPase (HKA) of the P-type family of ion-transport ATPases. Biochemical and pharmacological evidence indicate that this protein is closely related to gastric HKA and Na^+,K^+-ATPase (NKA). Both of these proteins pump K^+ into the cell and display a high degree of similarity in their amino acid sequences and general organization. A series of Northern blot hybridization experiments using probes derived from the rat gastric HKA and the human ATP1AL1 gene revealed the presence of a 4.3-kilobase mRNA in colon that seemed likely to encode the distal colon HKA, the enzyme responsible for K^+ absorption in mammalian colon. A rat colon library was then screened using a probe from the ATP1AL1 gene, and cDNAs containing the entire coding sequence of a new P-type ATPase were isolated and characterized. The deduced polypeptide is 1036 amino acids in length and has an *Mr* of 114,842. The protein exhibits 63% amino acid identity to the gastric HKA α-subunit and 63% identity to the three NKA α-subunit isoforms, consistent with the possibility that it is a K^+-transporting ATPase. Northern blot analysis show that the 4.3-kilobase mRNA is expressed at high levels in distal colon; at much lower levels in proximal colon, kidney, and uterus; and at trace levels in heart and forestomach. The high mRNA levels in distal colon and the similarity of the colon pump to both gastric HKA and NKA suggest that it is the distal colon HKA. In order to characterize the distal colon HKA gene, a rat cosmid library was screened using the distal colon HKA cDNA, and three clones where isolated which contain the entire gene. Analysis of the intron/exon organization and 5'-flanking region is currently in progress.

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Transcriptional upregulation of the 56 kD "brain" isoform of the V-H+ ATPase during monocytic differentiation. B.S. Lee, D.M. Underhill, and S.L. Gluck, Departments of Medicine and Cell Biology and Physiology, Washington University Medical School, St. Louis, MO 63110.

We have used the phorbol ester-inducible monocytic cell line THP-1 as a model for studying regulation of the vacuolar H+ ATPase during monocyte/macrophage differentiation. Immunocytochemistry and immunoprecipitation show increased expression of the assembled pump in mature macrophages over monocytes. Examination of the transcripts for various subunits of the V-H+ ATPase reveals little or no increase at the RNA level for most subunits; however, the 56 kD "brain" isoform exhibits a large increase of steady-state message. Nuclear run-off shows this increase to be transcriptionally mediated. One transcriptional start site is utilized for both differentiated and undifferentiated THP-1's. Sucrose gradient fractionation shows the 56 kD "brain" subunit to be the predominant isoform in lysosomes. Its increase correlates with known upregulation of the lysosomal compartments in differentiating macrophages. Regulation of expression of this isoform may be key to acquiring such lineage-specific functions as antigen processing.

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Evidence for expression of V-type proton ATPase in plasma membranes of cultured human cells. RJ Gillies and R Martinez-Zaguilan, Dept. Biochemistry, Univ. Arizona, Tucson AZ 85724

It is commonly believed that intracellular pH (pH^{in}) is regulated by a combination of Na^+/H^+ exchange and HCO_3^- transport systems. Recently, it has become apparent that some mammalian cells possess a plasma membrane-bound H^+ ATPase of the vacuolar, or V-type. We have investigated the functional occurance of these ATPases in cultured human cells using pharmacological and ion-substitution approaches. Cytosolic pH of some naturally occuring human tumor cells, such as leiomyosarcoma, mesothelioma and myeloma, is acidified upon treatement with bafilomycin A_1, a specific inhibitor of V-type H^+ ATPases. These fluorescence-based measurements were carried out with simultaneous measurement of endosomal pH. Furthermore, all cells tested exhibited a Na^+- and HCO_3^- -independent recovery to NH_4Cl-induced acid loads. This recovery activity occurs immediately in the bafilomycin-sensitive cells, and occurs after a 2-5 min lag in the bafilomycin-resistant cells, and is inhibitable by N-ethyl maleimide, ATP depletion and by colchicine. These data suggest that most cells can recruit V-type H^+ ATPase activity to their plasma membranes in response to acid stress and that some human tumor cells constitutively express these pumps in thier plasma membranes. (Supported by GM43046 and ACS CN-53).

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The Structure and Biosynthesis Analysis of Vacuolar H^+ATPase (V-ATPase) in LLC-PK1 Cells. J-Y. Fu, K. Zhang, Z-Q. Wang, and S. Gluck. Depts of Medicine and Cell Biology, Washington Univ. Sch. of Medicine, and Jewish Hospital, St. Louis, MO 63110.

We have examined the biosynthesis of the V-ATPase in LLC-PK1 cells using a monoclonal antibody specific to the assembled V-ATPase. Continuous labeling of the cells with ^{35}S-methionine and immunoprecipitation of the enzyme showed a $k_{\frac{1}{2}}$ of 2.4 hr for synthesis of the 31 kD subunit, and a $k_{\frac{1}{2}}$ of 5.8 to 9.5 hr for other subunits of the V_1 sector. Degradation rates of individual subunits of the V-ATPase in cells labeled with ^{35}S-methionine gave a $t_{\frac{1}{2}}$ of 14 hr for the 31 kD subunit 37 to >50 hrs. for other subunits of the V_1 sector. Pulse-chase labeling showed that the 31 kD subunit appeared on membranes concomitantly with synthesis. No assembled V-ATPase V_1 sector was found in the cytosol by immunoprecipitation; on immunoblots of cytosol, no free 31 kD subunit was detected, but free 70 kD and 58 kD subunits were found.

The results indicate that 31 kD subunit may play a role in limiting and initiating V1 assembly on the vacuolar membrane by inducing nucleation of free 58 kD and 70 kD subunits in the cytosol.

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Multidrug Resistance in the Echiuroid Worm *Urechis caupo*: Dealing with Toxic Compounds in the Marine Environment. B. Holland Toomey, and D. Epel. Department of Biological Sciences, Hopkins Marine Station, Stanford University, Pacific Grove, CA 93950.

The ability of certain organisms to live in polluted environments may be related to the presence of cellular defense or detoxification mechanisms that provide protection from toxic compounds. We have characterized a novel defense mechanism, related to multidrug resistance (MDR) in mammalian cells, in the marine worm *Urechis caupo* that may enable this worm to live in mudflats which contain both natural and anthropogenic pollutants. MDR transport activity was measured in *Urechis* eggs using a dye assay in which the fluorescence of eggs exposed to dye alone (rhodamine B) or to dye + inhibitor (rhodamine B + verapamil) was compared. Eggs in inhibitor typically accumulated 3-8 times more dye than control eggs. This assay was also used to identify competitive substrates for the protein (by substituting a drug for verapamil). Similar to the properties of mammalian MDR activity, drugs such as cyclosporin A, vinblastine, emetine, and forskolin inhibited MDR activity and caused dye accumulation in the eggs. Compounds extracted from the mud in which the worm lives also competed for dye transport indicating that this mechanism protects the eggs from compounds in their environment. Western blot analysis of *Urechis* eggs identified a major protein of 150-160 kd using three different antibodies to the mammalian MDR protein. In addition the egg protein was labelled with a photoactivatable derivative of forskolin, and the labelling was inhibited by verapamil, rhodamine B, forskolin, and 1,9 dideoxyforskolin. This labelled protein was then immunoprecipitated by an antibody to the mammalian MDR protein. These results show that the eggs of *Urechis caupo* contain a protein similar to the mammalian MDR transport protein, and it may have a role in expelling hydrophobic compounds to which the worm is exposed in its natural environment, thus providing protection from their toxic effects.

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Evidence that the COOH-terminal Region of GLUT-4 Confers Suceptibility to Inhibition of Glucose Transport by Nucleotides.

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Insulin stimulates glucose transport by triggering movement of GLUT-4 from intracellular vesicles to the plasma membrane (PM). In fat cells, cAMP-derivatives, such as dibutyrl cAMP (dbcA) inhibit insulin-stimulated glucose transport without affecting the GLUT-4 concentration in the PM. These agents also stimulate the phosphorylation of GLUT-4 at a site (Ser488) in the C-terminal tail . We have expressed a set of mutant GLUT-4 transporters with a recombinant Sindbis virus system to study the mechanism of glucose transport inhibiton by dbcA. In Sindbis virus-infected CHO cells, GLUT-4 was expressed at high enough levels so that GLUT-4 mediated transport could be measured even though only a fraction of GLUT-4 was in the PM. dbcA inhibited GLUT-4 mediated transport by 60%, but did not affect the concentration of GLUT-4 in the PM as assessed by immuno-electron microscopy. Inhibiton was not due to phosphorylation since replacing Ser488 with Ala abolished GLUT-4 phosphorylation but did not prevent inhibition by dbcA. Furthermore, dbcA-inhibiton did not seem to be due to activation of cAMP-dependent protein kinase (cAdPK). 8-Bromo cAMP (which activates cAdPK) did not inhibit transport, while 8-Bromo-AMP (which does not activate cAdPK) was as effective as dbcA in inhibiting transport. dbcA did not inhibit transport mediated by a homologous isoform, GLUT-1. We therefore used chimeric GLUT4/1 transporters, which retained functional acitivty, to map the dbcA-responsive inhibitory domain of GLUT-4. Our data suggest that dbcAMP may inhibit GLUT-4 by a direct interaction which involves the COOH-terminal 226 amino acids.

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Structural and Functional Characterization of Soybean Nodulin 26 Phosphorylation by the Calcium-Dependent Protein Kinase.

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During the infection and nodulation of soybean roots by rhizobia bacteria, the bacterium becomes enclosed in a specialized organelle known as the symbiosome. The symbiosome membrane is proposed to be involved in the control of metabolic flux between the bacteroid and the plant cytoplasm, including dicarboxylate transport, which is essential to support nitrogen fixation. Nodulin 26 is a nodule-specific protein associated with the symbiosome membrane of soybean root nodules. Based on the fact that nodulin 26 shares homology with a number of transport/channel proteins, it has been suggested that nodulin 26 may be involved in symbiosome membrane transport activities. Nodulin 26 is an endogenous substrate for a novel, symbiosome membrane-associated, calcium-dependent protein kinase (CDPK) and, it is the major phosphoprotein detected in symbiosome membranes. By phosphopeptide mapping as well as manual and automated peptide sequence analysis of endoproteinase Lys-C digested nodulin 26 we have identified the site phosphorylated on nodulin 26 by CDPK in vitro and in vivo as ser^{262}. Ser^{262} is located in the hydrophillic carboxyl-terminal region of nodulin 26. In order to test the potential significance of nodulin 26 phosphorylation on symbiosome membrane transport activities, intact sybiosomes were treated with alkaline phosphatase. This treatment resulted in a decrease in malate transport and nodulin 26 phosphorylation both of which were reversible by the addition of calcium and ATP. These data establish the site of phosphorylation of an endogenous substrate of a CDPK and suggest a possible functional correlation between nodulin 26 phosphorylation state and the malate transport activity of symbiosome membranes.

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The amino acid transporter, TEA, has a distinct expression pattern from REC-1 and is alternately spliced at the 5' region of the transcript. K.D. Finley, D. Kakuda, and C.L. MacLeod, Cancer Genetics Program, Department of Medicine, University of California, San Diego, La Jolla, CA 92093

The TEA gene belongs to a growing family of mammalian amino acid transporters. From amino acid sequence analysis (Protein Science, submitted) and preliminary electrophysiology data, the TEA protein is likely to function as a facilitated transporter of basic amino acids in a manner similar to the closely related gene, REC-1. The tissue expression patterns of mRNA transcripts from the two genes are quite distinct. While there are several tissues which co-express both genes, some tissues express TEA or REC-1 predominantly or exclusively. Liver and skeletal muscle tissues express TEA most abundantly, in sharp contrast to REC-1 mRNA, which is absent or present in trace amounts. Since liver and skeletal muscle play a central role in amino acid metabolism, the expression patterns of TEA and REC-1 in those tissues suggest the genes may function to regulate amino acid homeostasis. Among 28 independent T-lymphoma cDNA clones sequenced, evidence for four different spliced isoforms was obtained. Alternate splicing occurs in the 5' untranslated region of the TEA transcript. The genomic sequence of the 3' splice junction site has been identified. Detailed restriction mapping of genomic DNA from a T-lymphoma cell line was compared with DNA from normal mouse liver. The results indicate that no major alteration of the 5' region of the TEA gene is detectable in the transformed cell line. Additional genomic mapping of the Tea gene is underway to clarify the exon structure and experiments are also underway to confirm the amino acid transport function of the TEA protein.

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Developmental Expression of ATP-dependent Bile Acid Transport in Rat Liver canalicular membranes. M.Ananthanarayanan G.Michaud and F.J.Suchy, Department of Pediatrics, Yale University School of Medicine, New Haven, CT. 06510

Canalicular secretion of bile acids in rat liver is mediated, in large part,through a potential-driven carrier-mediated transport mechanism. Earlier studies from this laboratory (Am.J.Physiol.260:G743,1991) have shown that potential-sensitive taurocholate(TC) transport is not present at 7 days of age but is first detected at 14 days postnatally suggesting immaturity of this carrier at this stage of development.In view of the recently described ATP-dependent TC transport in adult cLPM it was of interest to examine whether this second system was functional at 7 days postnatally and determine its role in bile acid secretion. Membrane vesicles from 7 day-old and adult livers were prepared using a nitrogen cavitation method.Marker enzyme enrichments were similar in both preparations (40-50-fold in alk.phos).cLPM from 7-day old rats displayed a clearly demonstrable 5-7-fold higher (^{3}H)TC uptake in the presence of ATP with an overshoot(1.3-fold over equilibrium).Uptake was inhibited by conjugated bile acids (TC=67%,TCDC=62%,TUDCA=58%),and vanadate(50% at 300μM) but was not inhibited by daunomycin (similar to adult membranes).Other nucleotide analogs such as ADP,AMP,AMP-PNP and GTP did not exhibit significant stimulation of uptake compared to ATP.Kinetic analysis revealed that affinities for TC were similar in both membranes(K_m=47μM and 35μM at 7-days and in adult respectively)but $V_{max}$for the transporter at 7-days was only 66% of adults (206 vs 308 pmoles/mg prot/20 sec)indicating reduced carrier density.It is concluded that ATP-dependent TC transport may play a major role in bile acid secretion during the neonatal period when the potential-sensitive transporter is absent.

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Interaction of *Pseudomonas* Exotoxin A with Model Membrane Vesicles. D.M. Rasper & A.R. Merrill, Guelph-Waterloo Centre for Graduate Studies in Chemistry, Dept. of Chem. & Biochem., University of Guelph, Guelph, ON N1G 2W1.

The lipid requirement for binding of wild-type Exotoxin A (ETA) was evaluated by using a fluorescence quenching technique. The binding of toxin to model membrane vesicles prepared by an extrusion method (0.1μm diameter, large, unilamellar vesicles (LUV's); POPC/POPS [60:40, mol:mol]) was pH-dependent with maximal binding observed at pH 4.0. The binding curves demonstrated the presence of a set of high affinity binding sites which were pH-dependent but unaffected by changes in the ionic strength of the incubation mixture. These high affinity binding sites are most likely due to hydrophobic interactions. There was another set(s) of binding sites which is electrostatic in nature and influenced by both pH and ionic strength. Pore formation by ETA, as measured by calcein release from LUV's, was pH-dependent with optimal release occurring at pH 4.0 and approaching zero at pH 5.0 and higher. Calcein release was very sensitive to changes in the ionic strength of the assay buffer with maximal release occurring at 100 mM NaCl. At higher ionic strength ($\geq$200 mM NaCl) pore formation by ETA was dramatically reduced. [Supported by the Medical Research Council of Canada, A.R.M.].

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Nascent Secretory Proteins Pass Through a Tunnel in the Ribosome and into a Sealed Aqueous Compartment at the ER Membrane. K. S. Crowley and A. E. Johnson. Dept. of Chemistry and Biochemistry, University of Oklahoma, Norman, OK 73019.

Fluorescent-labeled secretory proteins were used to investigate the environment of the nascent chain within the ribosome and at the ribosome-membrane junction during the translocation process at the ER membrane. Truncated mRNAs of different lengths were translated in a wheat germ system in the presence or absence of canine signal recognition particle, microsomal membranes, and either N^{ϵ}-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminohexanoyl)-Lys-tRNA or N^{ϵ}-(7-diethylaminocoumarin-3-carbonyl)-Lys-tRNA. This yielded intermediates in the secretion procress in which NBD- or coumarin-labeled lysines were positioned at different locations along the nascent chain pathway through the ribosome and into the ER membrane. Both NBD and coumarin emission (λ_{max}) is sensitive to the polarity of the dye's environment, and the λ_{max} of dyes in nascent chains bound to either free or membrane-bound ribosomes were 550 nm for NBD and 470 nm for coumarin, consistent with their being located in an aqueous environment (the values are 530 and 440 nm, respectively, in a nonpolar solvent). The accessibility of NBD dyes to aqueous solvent was examined using iodide ions as quenching agents. Although all dyes in free ribosomal complexes were accessible to iodide ions, no quenching was observed with membrane-bound ribosome•nascent chain complexes. Thus, dyes within the ribosome and those in the initial stages of translocation are no longer accessible to iodide ions. The nascent chain therefore cannot be passing along a deep groove on the surface of the ribosome. Instead, the nascent chain must pass through an aqueous tunnel in the ribosome that is sealed off from the cytoplasm when the ribosome binds to the membrane. Since the λ_{max} is the same for probes in free and membrane-bound ribosomal complexes, the nascent chains are in an aqueous environment at the early stages of translocation.

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Bidirectional Movement of a Nascent Chain Across Microsomal Membranes Reveals Requirements for Vectorial Translocation of Proteins.
C. E. Ooi and J. Weiss, Dept. of Microbiology, New York University School of Medicine, New York, NY 10016.

The translocation of polypeptides across the endoplasmic reticulum is a vectorial process that occurs probably through a protein channel, by an as yet undetermined mechanism. We demonstrate that a nascent chain may exhibit bidirectional movement across microsomal membranes. A 221-residue N-terminal fragment of the human bactericidal/permeability-increasing protein was translated *in vitro* in the presence of microsomes. The nascent chain was initially translocated into the microsomal lumen as judged by signal cleavage and N-linked glycosylation, but subsequently found on the exterior of microsomes as assessed by protease sensitivity and non-sedimentation with microsomes. Retrograde movement of this translated/translocated product was observed only when the polypeptide was generated from a truncated transcript; addition of a stop codon after codon 221 conferred vectorial movement. Retrograde movement could also be prevented by glycosylation of the nascent polypeptide, as well as by the inclusion of 32 additional amino acid residues that may promote folding of the translocated chain. We propose that the protein translocation channel is a passive pore that does not create a directional bias in polypeptide movement. Rather, we suggest that vectorial translocation is driven by nascent chain elongation, and sustained by post-translocation events such as glycosylation or polypeptide folding that prevent retrograde movement. Since retrograde movement is prevented by the presence of a stop codon, we propose that the stop codon in all natural transcripts provides a signal for the closing of the translocation channel.

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A Direct Association of Ribophorin I and Translocating Preproteins in the Endoplasmic Reticulum of *Xenopus* Oocytes. Y. Sun and D.I. Meyer, Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, CA 90024 (Spon. by X. Liu)

To begin to understand the functions of ribophorin I and II *in vivo*, full length cDNAs encoding ribophorins of *Xenopus laevis* were isolated. By comparing the translation, translocation, and glycosylation of a reporter protein (prepro-α-factor) in the presence of normal or mutant forms of ribophorins I and II, the function of ribophorins was studied. Yeast prepro-α-factor, which we can express with or without its glycosylation sites, was used as a reporter protein. Both forms were synthesized, translocated, and glycosylated in *Xenopus* oocytes. The expression of glycosylated or nonglycosylated prepro-α-factor in oocytes, where various forms of ribophorins had been expressed to endogenous levels, resulted in the translocation and association of pro-α-factor with ribophorin I. An affinity purified antibody against the cytosolic domain of ribophorin I consistently immunoprecipitated ribophorin I together with the translocated pro-α–factor. Pro-α-factor was also found to associate specifically with the ribophorin I luminal domain alone. In contrast, no association was observed with intact or truncated forms of ribophorin II. Moreover, the association was independent of whether or not the prepro-α-factor contained sites for asparagine-linked glycosylation. This is significant as recent studies have shown an association between the ribophorins and oligosaccharyltransferase. We conclude that the large luminal domain of ribophorin I may act as a chaperone within the lumen of the ER, preserving accessibility to potential glycosylation sites, although it does not seem to participate in the glycosylation process *per se*.

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Protein Translocation in the Endoplasmic Reticulum: Ultraviolet Light Induces the Non-Covalent Association of Nascent Peptides with Translocon Proteins. L. Anderson and J.B. Denny, Division of Life Sciences, The University of Texas at San Antonio, San Antonio, TX 78249.

We have photolyzed cell-free translation systems synthesizing beta-lactamase with ultraviolet light. In the presence of rough microsomes (RM), incomplete chains of beta-lactamase became enriched relative to the full-length molecule in pellet fractions obtained following photolysis and alkaline carbonate-extraction. In addition, high molecular weight aggregates were present at the tops of SDS-polyacrylamide gels and occurred only when translocation-competent microsomal membranes were used in translation mixtures. The incomplete chains and high molecular weight aggregates were not obtained on gels when RM were inactivated by reaction with N-ethylmaleimide. The peptides did not bind to Con A-Sepharose, indicating that they had not become covalently crosslinked to membrane glycoproteins. Both photolysis and alkaline carbonate-extraction were required to produce the aggregates. Beta-lactamase peptides sedimented to the bottom of sucrose gradients in slightly increased amounts following photolysis and did not appear as high molecular weight bands on SDS-polyacrylamide gels. The high molecular weight aggregates therefore represent denatured protein complexes rather than crosslinked products, and contain nascent beta-lactamase peptides and microsomal translocon proteins.

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Mutational Analysis of the Invertase Signal Cleavage Site of Yeast *Saccharomyces Cerevisiae*. G. Hawkins and D. Botstein, Dept. of Genetics, Stanford University School of Medicine, Stanford, California, CA 94305.

Our goal is to understand the mechanism of signal peptide cleavage by studying the secreted protein invertase. We are taking two approaches. First, in order to define the amino acid sequence requirements for cleavage, we have mutagenized the signal cleavage site of invertase by replacing 10 base pairs encoding the site with random sequence (technique described by Palzkill and Botstein, 1992. *Proteins* 14: 29-44). This random replacement mutagenesis procedure has yielded a library of invertase mutants which differ at their signal cleavage sites. Analyzing the mutants for cleavage by the following criteria: 1) Growth on sucrose, the substrate for invertase; 2) Invertase assays; 3) Western blots to detect the extent of cleavage, will help us elucidate the sequence requirements for signal peptide processing. Second, we have screened a high copy number yeast library to isolate extragenic suppressors of an invertase mutant *suc2-s1* which is defective in signal peptide cleavage. We are currently analyzing four candidate genes by restriction mapping and DNA sequence to determine what these genes are and how they may act as part of the machinery needed for signal cleavage.

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Role of Charged Amino Acids Within the Segments Flanking the Hydrophobic Core of a Signal/Anchor Sequence in Determining the Transmembrane Disposition of a Protein. S. Monier, D. Sabatini, and M. Adesnik. Dept. of Cell Biology, N.Y.U. School of Medicine, New York, NY 10016.

Cytochrome P450IIB1 and sucrase-Isomaltase (SI) are membrane proteins that contain non-cleaved signal/anchor sequences (S/A) which are located near their N-termini and traverse the membrane with opposite orientations (type I: N_{exo}/C_{cyto} and type II: N_{cyto}/C_{exo}, respectively). The segments that flank the hydrophobic core of the S/A in these proteins differ in their content of charged amino acids. The N-flanking segment of P450 is negatively charged (-1) and that of SI is positive (+2), whereas the C-flanking segment of P450 is highly positive (+5) and that of SI is negative (-1). Analysis of the transmembrane disposition of mutant and chimeric proteins synthesized *in vitro* in the presence of microsomes revealed that: i) absence of a positive charge in the N-flanking region of the S/A of P450 is a critical determinant of its type I disposition, ii) conversion of the net charge of the N-flanking segment of the S/A of SI from +2 to -1, as in normal P450, conferred to the protein the P450 disposition, despite the prominent charge differences at the C-flanking segments, iii) the SI transmembrane disposition was not affected by converting the net charge of the C-flanking region from -1 to +4. We conclude that the net charge of the N-flanking region alone, irrespective of that of the C-flanking one and of the net charge differences between the two, is sufficient to establish the transmembrane disposition of both P450 and SI. We also found that the 36 amino acid N-flanking region (net charge of -1) of the first S/A of opsin is a powerful inducer of the N_{exo}/C_{cyto} disposition and can, by itself, when linked to the N-terminus of SI, partially reverse (>50%) the type II-inducing activity of the SI N-flanking region, despite the overall positive charge of the composite N-flanking segment and of the distance from the membrane (>15aa) of the negatively charged amino acids contributed by Opsin.

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The Role of Proline 345 in Diphtheria Toxin Membrane Translocation. V.G. Johnson*, P.J. Nicholls, W.H. Habig*, and R.J. Youle. *Laboratory of Bacterial Toxins, CBER, FDA and Surgical Neurology Branch, NINDS, NIH, Bethesda, MD 20892. (Spon. by B.S. Packard)

Diphtheria toxin can translocate across endosomal membranes in response to low pH. Buried hydrophobic domains become exposed in response to acidic conditions and are thought to participate in the membrane translocation process. Proline residues are found in high representation and in conserved locations in these hydrophobic domains and, because of their ability to undergo a cis-trans isomerization, have been proposed to play a role in the acid-induced conformational change that is a prerequisite for membrane translocation. The role of the proline at position 345 in membrane translocation was investigated. This residue was selected for mutagenesis because of its conserved and strategic location between two α-helices that compose much of the third hydrophobic domain. Pro was mutagenized to Glu and to Gly using a two-step recombinant PCR procedure and the mutant proteins were expressed *in vitro*. Glu, an α-helix former and Gly, an α-helix breaker, were selected for mutagenesis to distinguish between a structural role for Pro as an α-helix breaker and alternative roles, perhaps involving cis-trans isomerization-related conformational changes. Replacing Pro at position 345 with Glu or Gly resulted in a 99% reduction in toxicity to Vero cells. Since these mutations did not alter the binding or enzymatic activity of the toxin, the reduction in toxicity appears to be due to decreased translocation ability suggesting that the Pro at position 345 plays a specific role in toxin membrane translocation.

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FURTHER CHARACTERIZATION OF TRANSLOCATION OF DIPHTHERIA TOXIN FRAGMENT A FROM ENDOSOMAL MEMBRANES IN A CELL-FREE SYSTEM. C.L. Chang and T. Chang, Division of Gastroenterology, University of Rochester School of Medicine & Dentistry, Rochester, NY 14642.

We have further characterized a cell-free translocation system for diphtheria toxin A chain (DTA) from endosomal membranes of rat hepatocytes preloaded with DTA-asialoorosomucoid conjugate (DTA-ASOR). Endosomal-rich membranes were isolated from rat hepatocytes preincubated at 37° C for 2 h with 20 nM DTA-ASOR by centrifuging the cell membranes in a Percoll density gradient. The membranes were incubated in 0.25 M sucrose/1.5 mM $MgCl_2$ buffered at pH 7.1 in the absence or presence of various agents either alone or in various combinations at 4° or 37° for 30 min. The amount of DTA released into the medium relative to the total DTA content was then determined. A small basal release of DTA at 37° was increased dose-dependently by addition of cytosol. The effect of cytosol was enhanced by 0.1 mM $CaCl_2$ and further enhanced by an ATP regenerating system (ATP-RS). ATP-RS alone was ineffective but decreased the total DTA content that was enhanced by 50 mM KCl or KCl + cytosol and prevented by cytosol + $CaCl_2$. GTPγS dose-dependently increased DTA release but inhibited the dose-dependent effect of cytosol. Dithiothreitol (0.01 - 0.1 mM) dose-dependently enhanced DTA release that was independent of ATP and cytosol. These results indicated that translocation of DTA occurred from an endosomal vesicle in a process requiring a sulfhydryl agent, cytosol and Ca^{2+} and that distribution of DTA-ASOR in this membrane was modulated by ATP, a GTP-binding protein(s) and an ATP/K^+-dependent degradative system. Supported by NIH Grant #GM33754.

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The Role of ATP in Mitochondrial Protein Import. C.N. Wachter and B.S. Glick, Department of Biochemistry, Biocenter, Univ. of Basel, CH-4056 Basel.(Spon. by G. Schatz)

The import of precursor proteins into all mitochondrial compartments requires ATP. However, until recently it was not clear where and how the energy of ATP hydrolysis is utilized. Using an in vitro import system with isolated yeast mitochondria, we could independently manipulate the ATP levels outside and inside the mitochondrial inner membrane. With this novel system we characterized the ATP requirements for import of authentic as well as artifical precursor proteins.

We found that the import of most, but not all precursor proteins requires ATP outside the inner membrane. Moreover all proteins targeted to the mitochondrial matrix need ATP in the matrix to be translocated completely across the inner membrane. In contrast, the ADP/ATP translocator and cytochrome c_1, two inner membrane proteins, require ATP only outside the inner membrane for import and correct insertion into that membrane. These results have implications for the role of chaperone proteins in import and for mechanisms of intramitochondrial protein sorting.

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Characterization and cloning of novel components of the protein translocation system in the mitochondrial inner membrane of the yeast *Saccharomyces cerivisiae*

M. Horst, L. Bolliger, U. Manning-Krieg, T. Jascur, N. Kronidou and P. Scherer Biocenter, University of Basel, Klingelbergstrasse. 70, CH-4056 Basel, Switzerland (Spon. by N. Kronidou)

The mitochondrial genome encodes only a small fraction of the total mitochondrial proteins. To reach the mitochondrial matrix, nuclear-encoded precursor proteins have to cross both mitochondrial membranes. Some of the proteins involved in translocation across the outer membrane have been identified but so far none of the components involved in the translocation across the mitochondrial inner membrane have been characterized. In order to identify inner membrane proteins involved in protein import, we prepared rabbit antisera against inner membrane vesicles. Some of these sera inhibited protein import into these vesicles, but not into intact mitochondria. In order to identify the antigens responsible for these inhibition, we solubilized inner membrane vesicles and fractionated their proteins by anion exchange chromatography. The proteins present in each fraction were coupled to cyanogen bromide-activated Sepharose beads and these affinity beads were then used to affinity-purify antibodies from the inhibitory antiserum. The affinity purified antibodies were tested for their ability to inhibit the import of precursor proteins into inner membrane vesicles. The polypeptides recognized by the inhibiting antibodies were identified by Western blot analysis. So far we have identified two proteins that appear to be components of the mitochondrial inner membrane translocation machinery. These proteins have molecular weights of 40,000 and 45,000. Fab fragments of polyclonal anti 40 kDa IgG's and affinity-purified antibodies against the 45 kDa protein inhibit import into inner membrane vesicles. Interestingly the 45 kDa protein is also found in a crosslink with an import intermediate spanning the inner membrane and therefore seems to be in close proximity to a partially translocated mitochondrial precursor. Oligonucleotides which were generated according to the amino acid sequence obtained by a tryptic digest of the 40 kDa protein and the polyclonal antibodies against the 40 kDa protein were used for screening λgt11 libraries. Five cDNA and two genomic clones were isolated and partially sequenced. So far no significant homology with any known protein was found.

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Mitochondrial Import Receptor Interacts with Signal Sequences Specifying Protein Import Into Mitochondria. H. Murakami, D. Pain, and G. Blobel. Laboratory of Cell Biology, Howard Hughes Medical Institute, The Rockefeller University, New York, NY 10021

Using an antiidiotipic antibody approach, we have previously identified a polytopic integral membrane protein of 32KD (p32) from yeast *S. cerevisiae* mitochondria as a candidate for a signal sequence binding subunit of a putative protein conducting channel for protein import into mitochondria (Pain, D., Murakami, H. and Blobel, G. (1990) Nature (London) 347, 444-449). We have obtained additional data indicating that p32 interacts specifically with signal sequences specifying import into mitochondria. First, p32 and mitochondrial precursor proteins, expressed in *E. coli* or in a cell free translation system, form a complex that could be immunoprecipitated with antibodies against p32 or a mitochondrial precursor protein. p32 did not form complexes with a pre-secretory protein or a precursor for import into chloroplasts. Second, a biotinylated chimeric protein containing a mitochondrial signal sequence linked to a non-mitochondrial protein interacts specifically with p32 when incubated with total mitochondrial membrane proteins that had been separated by SDS-PAGE and transferred to nitrocellulose filter.

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REDUCTION OF CYTOCHROME C BY FRAGMENTS OF HEAT SHOCK PROTEIN 70 SIGNAL PEPTIDE. K.M. Fogarty II, P.T. Nhamburo and C.O. Simpkins, Department of Surgery, University of Maryland, 22 S. Greene Street, Baltimore, Maryland 21201: (Spon. by Daniel Darlington)

We previously demonstrated that hydrophobic di- and tripeptides specifically reduce cytochrome C (CC). Most potent of these peptides was YYF, Km = 4.0mM, V_{max} = 2.2 x 10^{-2} A_{550}/min. The hydrophilic tripeptide, glutathione (ECG) was more potent Km = 22uM and V_{max} = .23 A_{550}/min. The enhanced potency was due to the cysteine residue (Cys). Signal peptides (SP) of heat shock protein 70 (H) are hydrophobic and contain Cys. Using absorption spectroscopy and an O_2 electrode, we studied the ability of well-conserved fragments of SP to reduce CC. SCV (S1) and YSCVGVF (S2) both reduced CC. For S1 Km = 5.8mM and V_{max} = 1.23 A_{550}/min. For S2 Km = 38uM and V_{max} = .14 A_{550}/min. Cytochrome oxidase (CCO) reversed the spectroscopic changes caused by S1 and S2. Moreover, O_2 consumption of 1.28nM/min was observed by reacting S2 with a mixture of CC and CCO. These data suggest that signal peptide of H transfer electrons to cytochromes.

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Transforming Growth Factor-beta1 Regulates Fibrin Gel Contraction and Gel Lysis by Human Dermal Fibroblasts. T.-L. Tuan, D. Sun, and M.E. Nimni, Department of Surgery, University of Southern California School of Medicine, and Laboratory of Connective Tissue Research, Childrens Hospital Los Angeles, Box 54700, Los Angeles, California 90054-0700

Fibrin is a provisional matrix for cell migration and proliferation during wound repair and it is remodeled into collagenous granulation tissue by fibroblasts in the wound. Interactions between fibroblasts and fibrin were studied in a 3-dimensional fibrin gel culture system (Tuan and Grinnell, 1989, J. Cell. Physiol., 140:577). Presently the study has been extended to investigate the effect of transforming growth factor-beta1 (TGF-beta1) on fibroblast contraction of fibrin gels. Fibroblasts caused contraction and lysis of fibrin gels under serum free condition. When TGF-beta1 was added in the medium, it showed a dose-dependent effect on both the contraction and lysis of fibrin gels by fibroblasts. Fibrinolysis was studied using ^{125}I-fibrin and it was found that TGF-beta1 at 5 ng/ml and above could partially inhibit fibrinolysis. Using fibrin zymography and reverse zymography, it was found that TGF-beta1 at 5 ng/ml had reduced fibroblast production of plasminogen activators meanwhile increased the production of plasminogen activator inhibitor. Fibrin gel contraction study was performed by adding Aprotinin (250 U/ml) in the system to prevent lysis. TGF-beta1 showed a dose response (1 to 5 ng/ml) in enhancing gel contraction by fibroblasts. Cells pretreated by cycloheximide (0.1mM) were unable to contract fibrin gels indicating a cell-secreted product is involved in the contraction. These results suggest that TGF-beta1 could regulate fibroblast remodeling of extracellular matrix through cellular products affecting matrix contraction and proteolytic degradation. (This study is supported by NIH Grant AR40409)

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Role of cytokines on the modulation of melanoma cell motility and integrin expression. Dekker S.K., Vink J.*, Pavel S.*, Bruijn J.A.*, Mihm M.C. Jr., Byers H.R. Dermatopathology Division, Dept. of Pathology, Harvard Medical School, Massachusetts General Hospital, Boston, MA 02114. *Dept. of Dermatology and Pathology, University of Leiden, The Netherlands.

The influence of tumor necrosis factor α (TNFα) and interleukin 1α (IL-1α) on the cell migration of a recurrent primary and two metastatic human melanoma cell lines were investigated using two motility assays. In the metastatic cell lines, MM-AN and MM-RU, TNFα and IL-1α induced an increase in number of migrated cells in a micropore transmembrane invasion assay and an increase in mean migration rate in a quantitative video image analysis assay. In contrast, the recurrent primary melanoma cell line RPM-EP showed no significant increase in migration with these cytokines. The expression of the α5 integrin subunit in response to these cytokines in these melanoma cell lines on different (FN) coating concentrations was examined using flow cytometry to determine if the increase of migration was related to an upregulation of α5 integrin subunit expression.

The increase of melanoma cell migration on FN in response to TNFα or IL-1α did not correlate with an upregulation of the expression of the α5 integrin subunit. These data indicate that the cytokine induced increase in melanoma cell migration on FN is mediated by a mechanism other than an upregulation of α5β1 integrin expression.

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Extracellular Matrix of Endothelial Cells Stimulated with TNF$_\alpha$ Induces Expression of 96 kD Gelatinolytic Activity. C.A. Partridge and J.J. Jeffrey. Dept. of Biochemistry and Molecular Biology, Albany Medical College, Albany, NY 12208.

Tumor necrosis factor$_\alpha$ (TNF$_\alpha$) and phorbol myristate acetate (PMA) induce morphological changes and expression of a 96 kD gelatinolytic metalloproteinase in bovine pulmonary microvessel endothelial (BPMVE) cells. BPMVE cells were exposed to 10^{-7}M PMA for 24 hours, and were then incubated in fresh medium which was changed daily. The cells continued expressing 96 kD activity for up to 5 days after exposure, suggesting that a cell-mediated change continued the original stimulus. To examine the role of extracellular matrix in this effect, BPMVE cells were incubated 24 hours with 10^4 U/ml TNF$_\alpha$ or 10^{-7}M PMA, and were then stripped from their extracellular matrices, which were seeded with fresh BPMVE cells. Cells seeded on matrices from induced cells expressed 96 kD gelatinolytic activity and had altered morphology. Addition of TNF$_\alpha$ to stripped matrix from untreated cells did not induce these changes in seeded BPMVE cells. In contrast, bovine pulmonary artery smooth muscle cells did not express 96 kD activity when stimulated with TNF$_\alpha$ or PMA, and produced matrices that did not induce this enzyme in fresh BPMVE. These results indicate that TNF$_\alpha$ and PMA stimulate endothelial cell-mediated alterations of extracellular matrix that induce 96 kD gelatinolytic activity.

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Regulation of Metalloproteinase Gene Expression Following Mechanical Injury *In Vitro*. T. W. James§, R. Wagner§, R. M. Zwolak§, and C. E. Brinckerhoff¶. Depts. of Surgery (§) and Medicine and Biochemistry (¶), Dartmouth Medical School, Hanover, NH 03755 (Sponsored by E. Bresnick).

We describe a novel system for studying the production of matrix metalloproteinases I and III (collagenase, COL, and stromelysin, STM) by a vascular smooth muscle cell line in response to mechanical injury. After growing to a high level of confluence, Rb-1 cells (*In Vitro Cell. Dev. Biol.* 25:892) are disrupted by the passage of a plastic comb; this clears a series of circumferential paths around the plate, each bordered by two "ridges" of displaced cells. Over the next 24 hours cells migrate into the cleared areas. Northern blot analysis demonstrates the accumulation of mRNAs for COL and STM during this process, though they are undetectable prior to injury. Cotreatment with all-*trans*-retinoic acid (RA) markedly decreases the level of mRNAs induced by injury, while dexamethasone (DEX) causes a less pronounced reduction. The addition of exogenous angiotensin II (A_{II}) simultaneously with injury leads to a dose-dependent increase in mRNA levels up to approximately 50% over the high levels induced by injury alone, while in the absence of injury A_{II} has no effect. To localize the cells in which gene induction takes place, *in situ* hybridization studies for STM mRNA and immunohistochemical staining for COL protein were performed on plates of injured cells. The highest levels of STM mRNA and COL protein were in cells in the "ridge" left by the injury; lower levels were seen in some cells migrating into the cleared region. To determine if regulation occurs at the level of transcription, superconfluent cells were transiently transfected with luciferase constructs containing 1.8 kb of the collagenase 5'-flanking region. Injury caused at least a threefold increase in luciferase activity over levels in uninjured cells (Northern analysis showed that calcium-phosphate precipitation itself caused a rise in steady-state COL mRNA levels). Nuclear run-on transcription assays showed a substantial increase in transcription of COL and STM genes following mechanical injury. We conclude that (1) mechanical injury is associated with localized induction of mRNAs for the metalloproteinases I and III, (2) this response is augmented by A_{II} and antagonized by RA, and (3) the regulatory mechanisms responsible for this response include increased gene transcription. A role is suggested for mechanical forces in initiating the changes in gene expression in vascular smooth muscle cells following arterial injury *in vivo*.

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Structural and Functional Characterization of Heparan Sulfate Proteoglycan (HSPG) from the EHS Tumor: The Role of Growth Factors in the Bioactivity of HSPG. R. Kapoor and B.J. Del Buono, Collaborative Biomedical Products, Becton Dickinson and Company, Bedford, MA 01730.

Since HSPG has been implicated in modulating a variety of cellular responses to stimuli, we have examined the role of HSPG and growth factors on cellular physiology. HSPG was extracted from the EHS tumor under dissociative conditions, and purified by ion-exchange and molecular sieve chromatography. The purified proteoglycan was susceptible to heparinase digestion, and chemical analysis indicated 28% protein by weight and a 1:1 ratio of hexosamine (predominately glucosamine) to uronic acid. SDS-PAGE revealed a single band barely penetrating a 4%-20% gel. In bioactivity studies, HSPG stimulated DNA synthesis twofold in HepG2 hepatocellular carcinoma cells, with an ED_{50} = 4 ng/ml. At this concentration, stimulation of DNA synthesis was maximal after 18 hours of treatment with HSPG. Preincubation of HSPG with hepatocyte growth factor abolished this stimulation. Similar results were observed in fetal bovine heart endothelial cells with HSPG ± bFGF. Our results suggest that the proliferation, biosynthetic activity, and spreading of mammalian cells may be modulated by HSPG, in combination with growth factors in the extracellular matrix, or directly by induction of cellular protein kinases.

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Insulin-like Growth Factor I (IGF-I) Stimulates Fibronectin Gene Expression In Cultured Rat Thoracic Aortic Smooth Muscle Cells. **T. Tamaroglio, J. Zhang, and C.S. Lo. Department of Physiology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814.**

Type I diabetics have a greater risk of accelerated atherosclerosis. These vascular lesions are characterized by highly proliferative vascular smooth muscle cells and increased deposition of extracellular matrix proteins. A matrix protein produced by these cells is fibronectin. Because fibronectin can bind directly to cells and other matrix proteins, it may play a role in cell proliferation and matrix deposition. IGF-I exhibits mitogenic effects on vascular smooth muscle cells. To determine if this mitogen affects matrix deposition, the effect of IGF-I on fibronectin mRNA levels was studied. Using Northern blot analysis, fibronectin mRNA levels appear to be dose dependent. Four hours after IGF-I addition, the mRNA levels gradually increased at doses of 1.25 and 2.5 ng/ml reaching a peak at 5ng/ml. Time response experiments show that fibronectin mRNA levels progressively increased and peaked at 4 hours after an IGF-I treatment of 30 ng/ml. These studies suggest that IGF-I may regulate fibronectin gene expression. The interaction between growth factors and matrix proteins may be responsible for the induction of diabetes associated vascular lesions.

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Expression of Alternatively Spliced Fibronectin mRNAs during Fetal Wound Healing. D.J.Whitby, L.F. Brown, M.T. Longaker, and L. Van De Water, Departments of Pathology, Beth Israel Hospital and Harvard Medical School, Boston, MA, 02215; Department of Surgery, UCSF, San Francisco, CA.

Scarless, fetal wound healing differs from adult wound healing in the rapid re-epithelialization of the wound defect and in the enhanced organization of collagen fibrils within the wound bed. The provisional matrix deposited in wounds provides a scaffold for cell migration essential to both processes. The structure of this matrix may thus determine the rate of re-epithelialization and the extent of subsequent collagen organization and scar formation in the wound. Fibronectins (FNs) are a family of glycoproteins present in tissue matrices and in blood plasma. FNs arise by transcription of a single gene to produce mRNAs which can be alternatively spliced in three regions, termed EIIIA , EIIIB and V. Plasma FN (pFN), that lacks the EIIIA and EIIIB domains, is a major constituent of the blood clot and the early provisional wound matrix. During adult wound healing pFN is supplemented by locally synthesized, alternatively spliced FN mRNAs which include the EIIIA and EIIIB domains. In this study the pattern of FN mRNA expression in fetal and adult mouse lip wounds was examined by in situ hybridization. Upper lip incisional wounds were created in fetal (16 d gestation) and adult mice and harvested from 1 to 72 hrs post wounding. The overall level of expression of FN mRNAs in normal, unwounded fetal skin was higher than in the adult tissues. Moreover, these FN mRNAs included forms containing the EIIIA and EIIIB domains. By 6 hrs following fetal wounding, the level of FN mRNAs increased above those found in unwounded fetal skin and persisted until approximately 48 hrs post wounding, a time after which FN mRNA expression within the fetal wound declined. The early expression of alternatively spliced FNs may underlie differences in the rate of wound closure and in the organization of collagen in healing fetal wounds.

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A Cytoplasmic Protein Binding to a Conserved UA Sequence from the 3' Untranslated Region of Fibronectin mRNA May Mediate Ductus Arteriosus Smooth Muscle Cell Fibronectin Synthesis, Feature associated with Intimal Proliferation. B. Zhou, N. Boudreau and M. Rabinovitch, Department of Pathology, University of Toronto, Toronto, Canada.

We previously described increased fibronectin (FN) synthesis in ductus arteriosus (DA) compared to aorta (Ao) smooth muscle cells (SMC), associated with a migratory phenotype in DA SMC related to intimal cushion formation. However, despite the greater amounts of FN protein synthesized by DA SMC, steady-state FN mRNA levels in DA SMC are less than those in Ao. Therefore, the regulation of increased DA SMC FN synthesis appears to be post-transcriptional. Kruys *et al.* (Science 245:852-855, 1989) demonstrated translational blockade imposed by a cytokine-derived UA-rich sequence (UUAUUUAU) present in the 3' UTR. A similar UA consensus sequence is found in FN mRNA and may also function to modulate its translational efficiency. In the present study, we identified a FN mRNA binding protein on gel mobility shift assays using DA and Ao S-100 cytoplasmic extracts, and an 18 mer transcribed from the 3' UTR of FN mRNA and containing the UA consensus sequence. There was increased binding with DA compared to Ao S-100 extracts and we confirmed dose dependency of binding. The formation of the binding complex was RNase T1-resistant and could be competed by cold probe. Binding reactions as short as 5 min yielded the same amount of complex as 1 hour incubations. Complex formation was abolished by prior incubation of the extracts with proteinase K for 30 min. We further established the molecular size of the complex as 57 kD by SDS-PAGE after UV cross-linking. The more efficient translation of DA SMC FN mRNA may be due to increased concentration of a cytoplasmic protein which binds the UUAUUUAU consensus sequence and prevents its repressor function.

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Transfected BHK cells and transgenic mice expressing specific splice variants of tenascin. I. Aukhil and H.P. Erickson, Dept of Periodontics, UNC Dental School and Duke University.

We have assembled full-length cDNAs for human tenascin using clones generously provided by L. Zardi and splicing the segments at unique or rare restriction sites. We have constructed cDNAs for the largest and smallest splice variants, as well as one truncated after the EGF domains. These cDNAs were put into the pNUT vector (generously provided by R. Palmiter) which has a metallothionine promoter and methotrexate selection. BHK cells were transfected with each of the constructs and methotrexate resistant clones were tested for secretion of tenascin. Most clones secreted 3-8 μg/ml tenascin into the conditioned medium after 3 days confluence. The protein was purified from conditioned medium and characterized by gel electrophoresis and electron microscopy. All three constructs were fully assembled into hexabrachions, designated HxB.L, HxB.S and HxB.egf for the large and small splice variants and the truncated EGF segment.

Transgenic mice were prepared with the HxB.L construct, and six founders, identified by PCR of tail DNA were obtained. Tissue homogenates of three lines of transgenic mice were analyzed by Western blotting. Adult brain, liver and serum all showed expression of the large human tenascin. The mice appear to be phenotypically normal, although four of the founders have died of a variety of causes. We are currently breeding the mice for higher copy number and attempting to induce higher expression of the transgene by feeding zinc.

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Altered Laminin Expression in Sjögren's Syndrome. N. Fox and C. McArthur, Department of Oral Biology, University of Missouri-Kansas City School of Dentistry, Pathology Department, Truman Medical Center, Kansas City, Missouri

Recently we have been able to demonstrate the association of laminin or a laminin-like substance with the pathology of Sjögren's Syndrome (SS) (in press). Laminin, a basement membrane protein, is known to promote the attachment and migration of cells and to play a role in differentiation and tumor metastasis, all of which are important in SS. To investigate whether this finding is specific for Sjogren's Syndrome, we used an anti-laminin monoclonal antibody in the immunoperoxidase assay on salivary gland biopsy tissue from 18 SS patients, 20 patients with oral inflammatory lesions and 20 controls. We were able to reaffirm our previous findings as well as demonstrate positive staining for laminin in the chronically inflamed tissues. In the latter, however, the staining was diffuse rather than strictly periductal as in SS. Again, there was no staining of normal tissues. Further, in SS, increased staining for laminin occurred prior to lymphocytic infiltration and glandular destruction. These data lend further support to the notion that laminin is produced and released around the salivary ducts of such patients with SS. They also demonstrate that although this phenomenon may not be specific for Sjögren's Syndrome, it does indicate damage or inflammation and, as such, may lead to a better understanding of this disease.

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Decorin Synthesis in Lectin-Resistant CHO Cells. P.Y. Johnson*, S. McDonnell**, L.M. Matrisian**, and D.A. Blake*, *Department of Biochemistry, Meharry Medical College, Nashville, TN 37208, **Department of Cell Biology, Vanderbilt University, Nashville, TN 37235.

Decorin is a small connective tissue dermatan sulfate proteoglycan which is abundant in loose connective tissue. A full length cDNA which encodes the proteoglycan core protein of decorin was inserted into a mammalian transfection vector and subsequently used to induce decorin synthesis in Chinese hamster ovary (CHO) cells, a cell line which does not normally synthesize decorin. Two strains of CHO cells were transfected, the strain CHO-K1T4 which synthesizes normally glycosylated glycoproteins, and the abrin-resistant strain CHO-D41B which synthesizes glycoproteins containing reduced amounts of cell surface galactose. Both the CHO cell strains transfected with the decorin-expressing plasmid synthesized decorin mRNA and showed an enhanced incorporation of radiolabeled sulfate into dermatan sulfate proteoglycans, as compared to cells transfected with the selection vector alone. The enhanced incorporation of radiolabeled sulfate in the galactosylation deficient cells suggests that the defect does not inhibit synthesis of the oligosaccharide linkage region of glycosaminoglycans and that the galactosylation of proteoglycans may occur in a different compartment than that required for glycoproteins. Decorin-producing CHO cells grew more slowly in culture than did cells transfected with the selection vector alone, and exhibited a lower saturation density at confluence. Supported by 28167-LS-SAH from the U.S. Army Research Office (DAB) and by HD25580 from the NIH (LMM). PYJ holds a graduate fellowship from the DOE (DE-FG05-89ER75530).

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Growth Regulated Expression of p52(PAI-1) in Normal Rat Kidney (NRK) Cells. M. P. Ryan and P. J. Higgins, Department of Microbiology, Immunology and Molecular Genetics, Albany Medical College, Albany, N.Y. 12208

Expression and substrate (matrix) deposition of p52(PAI-1) in NRK cells is associated with changes in cell morphology. Since cell shape influences proliferative activity, expression of p52(PAI-1) in various growth states was examined. Actively proliferating subconfluent NRK cultures contained highest levels of matrix p52(PAI-1) compared to newly confluent and post-confluent cultures. Transition from a quiescent to proliferative state (by addition of media containing 20% serum), was associated with an increased accumulation of matrix p52(PAI-1) (6 and 26 fold over quiescent cultures after 1 and 5 hr., respectively). Induction was actinomycin D sensitive, suggesting a requirement for RNA synthesis. Changes in protein content reflected an increase of p52(PAI-1) cytoplasmic mRNA abundance which peaked at 2 hr (125-fold increase) and subsequently declined (110-fold increase) at 5 hr. post-stimulation. This was in contrast to actin mRNA (which continued to increase) and GAPD mRNA (which remained relatively constant) content. Rapid cytoskeletal reorganization of the actin microfilament network (evident by an increase in transcytoplasmic filaments) and formation of vinculin-containing adhesion plaques proceeded p52(PAI-1) induction. Cytoskeletal reorganization and p52(PAI-1) induction occurred prior to progression of cells through the cell cycle. p52(PAI-1) expression in NRK cells, thus, is dependent on the growth state of the cells and is associated with changes in the cytoarchitecture.

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Evidence for Altered Stromal Epithelial Interactions in Mammary Gland of Rats treated with Chemopreventative Agents Difluoromethylornithine (DFMO) and Retinyl Acetate (RA). P.J. Schedin, R. Strange, M. Singh, M.R. Kaeck, and H.J. Thompson, AMC Cancer Research Center, 1600 Pierce St., Denver, CO 80214.

Extracellular matrix can modulate mammary gland (m.g.) architecture and differentiation. Because these parameters may affect the carcinogenic process, we evaluated the effects of short-term DFMO and RA treatment on maintenance of the stromal-epithelial relationship during hormonally induced m.g. differentiation. Mature female Sprague Dawley rats were fed either a lab chow diet or that diet supplemented with 1% DFMO, 1mM RA or 1% DFMO plus RA for 21 days. From day 22-28 of the feeding period, animals received a s.c. injection of 5 µg 17-ß estradiol and 1.5 mg progesterone. On day 28, animals were injected (i.p.) with 50 mg/kg bromodeoxyuridine (BrDu) and euthanized two hours later. All treatments reduced gland complexity. The number of ducts and size of alveolar units decreased without an apparent effect on cell proliferation as measured by BrDu incorporation. Histological analysis revealed m.g. connective tissue hyperplasia in which large tracts of epithelium and adipose tissue were replaced with fibrous tissue. Focal regression of ductal epithelium with dark condensed epithelial cells, reminiscent of changes in cell morphology associated with apoptotic cell death were observed. Northern analysis revealed an induction of tissue transglutaminase, a crosslinking enzyme expressed during apoptotic cell death, in the combined agent treatment group where these phenotypic changes were most pronounced. These data suggest that fibrous hyperplasia associated with focal gland regression may account in part for the chemoprotective effects of DFMO and RA.

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Effects of Biaxial Deformation on Bladder Smooth Muscle Cells and Urothelium. E.J. Macarak, L. Baskin and P.S. Howard. Connective Tissue Research Institute, University of Pennsylvania, and Children's Hospital of Philadelphia, Philadelphia, PA 19104.

Bovine bladder smooth muscle and urothelial cells were isolated and characterized in vitro. Smooth muscle cells grow as overlapping and woven spindle-shaped cells which ultimately form nodule-like structures reminiscent of the "hills and valleys" morphology observed in cultures of vascular smooth muscle. These cells grow as a non-contact inhibited monolayer in vitro with a doubling time of approximately 6 days. Smooth muscle cells derived from the bladder express the α-actin antigen which is a specific marker protein for smooth muscle cells. Bladder epithelial cells or urothelial cells conversely do grow as a contact-inhibited monolayer and express a cytokeratin specific for urothelium and thus can be easily distinguished from smooth muscle. When both these cell types were subjected to moderate levels of biaxial strain (4.9%), alterations in the expression of type I and III collagen were noted. When these fibrillar matrix components were quantitated by ELISA, type I collagen decreased from 0.783$\pm$.236 ug/well (control) to 0.225 $\pm$.065 ug/well (p=0.006) in cultures being subjected to biaxial deformation. Conversely, type III collagen increased from 0.463$\pm$0.042ug/well (control) to 1.060ug $\pm$ 0.171 ug /well in cultures subjected to biaxial deformation. In urothelial cultures subjected to similar levels of deformation, both type I and type III collagen were significantly increased. These data demonstrate a differential response of bladder tissues to mechanical forces. Supported by NIH Grant HL34005.

Integrin Receptors for Extracellular Matrix (746-749)

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The Organization of Integrin $\alpha_v\beta_5$ Upon Cell Adhesion is Dynamic. I. Stuiver and J.W.Smith. Committee on Vascular Biology, The Scripps Research Institute, La Jolla, CA 92037

There are two integrin receptors known to bind the extracellular matrix protein vitronectin (VN). Integrin $\alpha_v\beta_3$ binds to a broad spectrum of RGD-containing ligands whereas $\alpha_v\beta_5$ binds solely to VN. Using a panel of monoclonal antibodies specific for $\alpha_v\beta_5$, we demonstrate that in MG63 osteosarcoma cells adherent to VN, both integrins are eventually found in focal contacts. However, during initial adhesion to VN, $\alpha_v\beta_3$ is immediately found in focal contacts whereas $\alpha_v\beta_5$ appears to co-localize along actin filaments. After 22 hrs on VN, $\alpha_v\beta_3$ is not detected and $\alpha_v\beta_5$ is found in focal contacts. These results suggest distinct life cycles of $\alpha_v\beta_3$ and $\alpha_v\beta_5$. It has also been reported that MG-63 cells express an integrin that is phosphorylated upon treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA)(E. Freed et al., 1989. EMBO 8:2955-2965) . This receptor was initially termed $\alpha_v\beta_x$. With our panel of anti-$\alpha_v\beta_5$ Mabs, we have begun to examine the relationship of $\alpha_v\beta_3$ and $\alpha_v\beta_5$. FACS analysis of MG-63 cells treated with TPA reveals an apparent down regulation or masking of β_5 epitopes with little change in cell surface α_v. This suggests that β_x may be β_5 and that phosphorylation could have functional significance.

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Regulation of a6, b1, and b4 Integrin Subunits in Response to ECM Proteins. L.Grushkin-Lerner and V. Trinkaus-Randall. Department of Biochemistry and Ophthalmology, Boston University, School of Medicine, Boston, MA 02118.

We have been studying the regulation of a6, b1, and b4 integrin subunits in corneal epithelium in response to either laminin (LM) or fibronectin (FN). Using nonisotope labeled probes, *in situ* hybridization was conducted to determine the presence and distribution of a6 and b4 mRNAs using CLSM. At 3 hours mRNA in cells on FN stained more intensely for a6 mRNA than in cells on LM. However, by 24 hours the intensity of staining for a6 mRNA was equivalent. The intensity of detection for the b4 mRNA was the same at 3 and 24 hours on either surface. To show colocalization of the mRNA with the endoplasmic reticulum (ER) in the perinuclear region, ER was detected with $DiOC_6$ and the golgi was localized with NBD-ceramide. Both were in the perinuclear region. To determine regulation of the a6 and b4 mRNA, RNA was isolated from cells plated on FN. Northern blot analysis indicates an increase in both a6 and b4 mRNA during a 24 hour period. We also studied the regulation of protein synthesis in cells plated on either LM or FN. Metabolically labeled cells were harvested at 3 hour periods over 24 hours. The regulation of a6 and b4 synthesis indicated an increase in subunit synthesis during 24 hour after plating. To detect a change of a6, b1, and b4 subunits on the cell surface, FACS analysis was conducted. The number of cells with a6 on the surface increased from 60% to 90% by 24 hours LM. Cells with b4 increased from 7% to 41% by 24 hours. Cells with b1 (12%) did not increase between the time of plating and 24 hours. Supported by NIHEY06000

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Histochemical Localization of Integrins and Phosphotyrosine Containing Proteins in Normal and Migrating Epidermal Cells of the Adult Newt. D. J. Donaldson, J. T. Mahan, and Hui Yang. Dept. of Anatomy and Neurobiology, U. of Tennessee, The Health Science Center, Memphis, TN 38163.

Epidermal cell migration was initiated in newt limbs by removing a rectangle of skin. Six hours later, when formation of a wound epithelium was well underway, samples were taken from the injured and the contralateral uninjured limb and sectioned on a cryostat. Given sections were stained with one of the following: a polyclonal ab against the β1 subunit of the chick fibronectin receptor (a generous gift from K. Yamada), a monoclonal ab against the β4 integrin subunit (Telios), and a monoclonal ab against phosphotyrosine (UBI). In intact skin, both anti-integrins primarily stained basal cells, where staining was limited to and equally distributed around the cell periphery. In migrating epidermis, there was an increase in staining at the cell-wound matrix interface, often without a reduction of staining in the rest of the cell. In contrast to the pattern with anti-integrins, anti-phosphotyrosine stained the periphery of all but the most superficial cells in uninjured epidermis. In basal cells, the stain seemed weakest at the cell junction with the basement membrane. In migrating cells, increased staining at the cell-wound matrix interface was accompanied by a reduction in staining on the rest of the cell periphery. The anti-integrin results suggest that β1 and β4-containing integrins are involved in the interaction of migrating newt epidermal cells with the wound matrix during wound closure. The results with anti-phosphotyrosine could reflect involvement of phosphotyrosine containing proteins in the transduction of signals generated by integrin-matrix interaction. (Supported by NIH grant AR27940.)

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Integrin localization and expression in the wounded and unwounded stratified squamous epithelium of the cornea. M.A. Stepp,* V. Rajendran,† and I.K. Gipson,†‡ *George Washington University Medical Center, Dept. of Biochemistry, Washington, DC 20037, †Schepens Eye Research Institute and ‡Harvard Medical School, Dept. of Ophthalmology, Boston, MA 02114

The integrins are a family of integral membrane glycoproteins which, as αβ heterodimers, serve as receptors for a variety of extracellular matrix molecules. We have characterized the integrin subunits present in the corneal epithelium and the adhesion properties of individual epithelial cells. The integrin subunits identified in the corneal epithelium include β_1, β_4, β_5, α_2, α_3, α_5, α_6, and α_v. Most of these integrins (β_1, β_5, α_2, α_3, and α_v) are found at sites of apparent cell:cell contact, but some (α_5, α_6, and β_4) localize specifically to the region at the basal aspect of the basal cells. The integrin $\alpha_6\beta_4$ heterodimer plays a unique role in the stratified squamous epithelium since it is a known hemidesmosome component. To study integrin expression in corneal epithelial wound healing, we used organ-cultured rodent corneas that had central epithelial debridement wounds and intact basement membranes to determine integrin production at 3, 6, 9, 12, 18, and 24 hr after wounding. There was no change in the amounts of any of the integrins analyzed, including β_1, β_4, α_3, α_5, and α_6, as compared with the amounts in stationary epithelium. Very little change occurred in the localization of integrins in the migrating epithelial sheets. The cells at the very tip of the leading edge have β_1 integrins on the basal aspect of their basal cell membranes, but the basal and suprabasal cells behind the leading edge localize these integrins in a pattern similar to that of stationary cells. Adhesion assays were also conducted using epithelial cells isolated from stationary and migrating epithelia. No differences were observed in the ability of stationary and migrating cells to adhere to fibronectin and laminin. Thus, integrin production in the stratified squamous epithelium of the cornea is not dramatically altered during epithelial cell migration over debridement wounds.

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Extracellular Matrix (ECM) Regulates Expression of Integrins in Rat Alveolar Epithelial Cells In Vitro

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We previously showed that ECM has profound influence on the function and morphology of alveolar epithelial cells in culture. To study the mechanisms by which the ECM effects are mediated, we examined the regulation of integrin subunits mRNA expression using Northern blot analysis and in situ hybridization. An immortalized rat alveolar type cell-derived cell line (LM5) and primary cultures of activated rat type II cells (type IIB) isolated from silica-injured lungs were cultured on plastic vessels or dishes coated with purified ECM components, or reconstituted murine basement membrane (BM) for 48 to 72 hours. LM5 cells cultured on plastic, type I collagen, and fibronectin, expressed moderate levels of β_1, α_3, α_6, and α_v mRNA. When cultured on type IV collagen or laminin, the cells showed a marked up regulation of α_v and α_3, but no increase in α_6 mRNA. β_1 mRNA was decreased in cells cultured on type IV collagen but increased in cells cultured on laminin. Cells cultured on the reconstituted BM showed increased expression β_1, α_3, and α_v, but not α_6. Primary cultures of rat type IIB cells on plastic showed elevated levels of β_1, α_3, and α_v. mRNA changes were confirmed by in situ hybridization using cDNA probes for the integrin subunit mRNAs. We conclude that purified ECM components can regulate patterns of integrin subunit mRNA expression in alveolar epithelial cells.

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Integrin receptor expression during early development of *Xenopus laevis*.

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The integrins are a family of transmembrane receptors that participate in cell-cell and cell-extracellular matrix (ECM) adhesion. Although many integrin α and β subunits have been identified, very little is known about their individual patterns of expression during embryogenesis. We have approached this problem using degenerate primer PCR methods in order to identify integrins expressed throughout early *Xenopus* development. Our recent efforts have resulted in the isolation of partial cDNAs encoding *Xenopus* α_2, α_3, α_4, α_5, α_6, and α_{1b}. The mRNAs encoding these subunits were quantified at different developmental stages using RNAse protection analyses. Integrin α_2 and α_6 are first transcribed during gastrulation whereas α_{1b} transcripts appear at neurulation. Integrin α_3, α_4, and α_5 mRNAs are first detected in eggs and early cleavage stage embryos and are, therefore, maternally encoded. These data, taken together with previously established expression patterns for the β_1 and β_3 subunits, suggest that multiple integrin receptors are likely to be functioning prior to neurulation. Moreover, because of the lack of evidence for a suitable candidate α subunit to pair with maternally encoded β_3, this current list of integrins is likely to be incomplete for early *Xenopus* embryos. Previously we observed that animal cap cells, derived from mid-blastula embryos, acquire the ability to spread on fibronectin when treated with activin A, a potent mesoderm inducing factor (Smith et al., 1990. Development 108:229). We now report the expression of these integrin subunits in response to mesoderm induction. No increase in integrin β_1, β_3, α_3, and α_5 mRNAs is detected following induction with activin A. Conversely, α_6, α_2, and α_4 mRNA levels appear to be influenced by this mesoderm inducing factor. This work is supported by USPHS grant HD26402 and by the Pew Charitable Trusts.

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Targeted Mutagenesis of β1 Integrins in Embryonal Carcinoma and Embryonic Stem Cells.

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Normal embryonic development requires extensive and continuous remodeling of cell-cell and cell-matrix interactions. The integrins, which comprise a large family of cell surface adhesion receptors, mediate a variety of such interactions and have been implicated in a number of crucial events during embryogenesis such as cell differentiation, the establishment of the basic body plan and in specialized cell migrations. We have chosen to directly assess the role of one family of integrins, the β1 family, in early mammalian development by targeting mutations to the β1 integrin gene in both F9 embryonal carcinoma (EC) cells and in embryonic stem (ES) cells via homologous recombination. To date, we have constructed a β1 integrin targeting vector containing 6kb of the β1 integrin gene, a promotorless neomycin cassette inserted into the first coding exon of the β1 integrin, and an HSV-tk gene. This vector has been introduced into F9 EC cells and colonies selected in neomycin (G418) and gancyclovir (FIAU). Four successfully targeted F9 EC clones were identified (by Southern blotting) out of 80 G418/FIAU resistant colonies. These β1+/β1- clones appear identical to the parent F9 cell line in terms of morphological appearance and in their ability to differentiate to both parietal and visceral endoderm. A second targeting vector containing a hygromycin resistance marker has been constructed to knock out the other β1 integrin allele in these clones to generate an F9 cell line that is completely lacking β1 integrin. The effect of this deficiency on morphological, adhesive and differentiative properties will be examined. In addition, the β1 integrin targeting vector has been introduced into embryonic stem cells for eventual analysis of the role of β1 integrins on development *in vivo*. Resistant clones are currently being screened to identify homologous recombinants.

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Expression and Distribution of the α6β4 Integrin in Myelinating Schwann Cells.

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Formation of the myelin sheath by Schwann cells in the peripheral nervous system depends on the presence of a basal lamina. The molecular mechanisms by which the basal lamina promotes the ensheathment and myelination of axons by Schwann cells are not known, but may involve a functional linkage between the extracellular matrix and the cytoskeleton. Since integrins are known to transduce signals between the extracellular matrix and the cytoskeleton in other cells, we have initiated studies on the role of these proteins in myelinating Schwann cells. To this end, we have examined the expression of the α6β4 integrin, a basal lamina receptor, in cultures of myelinating and non-myelinating Schwann cells. Rat primary Schwann cells grown in the absence of neurons were found to express very low levels of the β4 integrin subunit as determined by immunofluorescence and immunoblotting. However, when Schwann cells were co-cultured with dorsal root ganglia neurons under conditions which promote basal lamina formation and myelination, the expression of this protein in the Schwann cells was significantly upregulated. Substantial levels of the β4 integrin subunit were detected in nascent myelin sheaths that were formed within 3 days of growth under these conditions. After 7 days of myelination, the protein was present throughout the internodes and was enriched in some Schmidt-Lanterman clefts. Expression of α6β4 integrin at the onset of myelination suggests that it may have an important role in the early stages of axonal ensheathment by Schwann cells. This work was supported by a Postdoctoral Fellowship from the National Multiple Sclerosis Society to S.E.and NIH grant NS26001 to J.L.S.. F.G.G. is a Scholar of the L.P. Markey Charitable Trust.

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Platelets express a unique β_1 integrin subunit.

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Platelet adhesion to the subendothelial matrix plays a critical role in the pathophysiology of thrombosis, atherogenesis and inflammation. The integrins expressed on the platelet surface, members of both the β_3 and β_1 subfamilies, mediate adhesion to the extracellular matrix. We have observed that the platelet β_1 subunit is immunochemically different from the classical β_1 subunit expressed in other cells. Polyclonal antibodies directed against three overlapping regions of the cytoplasmic domain of the β_1 subunit failed to react with the platelet integrin β_1 in immunoprecipitation as well as in immunoblotting. In contrast, two monoclonal antibodies against β_1 did react with the platelet subunit. At the mRNA level, the classical β_1 subunit was the predominant form suggesting that a posttranslational modification may be responsible for the unique characteristic of the platelet β_1 subunit. The same immunochemical difference was observed using purified platelet $\alpha_2\beta_1$ and endothelial cell $\alpha_2\beta_1$; the platelet β_1 subunit was not recognized by one of the anti cytoplasmic domain sera, while the endothelial cell subunit was recognized. Since we had previously shown differences in specificity between platelet $\alpha_2\beta_1$ and endothelial cell $\alpha_2\beta_1$, our data suggest that the structural difference in the cytoplasmic domain of the β_1 subunit may modulate the integrin affinity for the ligand. Moreover, the variant cytoplasmic domain may explain the lack of platelet spreading on certain ligands that interact with the β_1 integrins.

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Integrin Subunit Localization in Rat Vascular Smooth Muscle Cells.

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Vascular smooth muscle cells are surrounded by and attached to an extensive extracellular matrix. Integrins are a family of heterodimer transmembrane receptors for extracellular matrix present in many different cell types. Previous studies of integrins in vascular smooth muscle have focused on the elastic aorta. In this study, we have characterized the presence and distribution of several α and β integrin subunits in smaller, more muscular rat arteries. Immunofluorescent staining of tissue was performed on tail and mesenteric artery cryosections. Immunostaining for integrin β_1 and β_3 subunits was present in smooth muscle cells of the vessel media. These cells also stained for α_5 and α_v subunits, while α_3 and α_4 subunits were not detected. Thus, smooth muscle cells in tissue express α_5, α_v, β_1, and β_3 integrin subunits. We then compared this to the integrin subunit immunostaining in isolated vascular smooth muscle cells cultured on various substrates. Smooth muscle cells cultured from the superior artery were grown on coverslips coated with fibronectin, laminin, or type IV collagen and immunostained for integrin subunits. Abundant integrin subunit β_1 staining was present on all substrates. Less integrin subunit β_3 staining was present on all substrates. Integrin subunit α_5 staining was seen on fibronectin, to a lesser extent on laminin, and little on type IV collagen. Integrin subunit α_v was seen on fibronectin and type IV collagen substrates. No α_3 or α_4 subunit staining was detected in cell culture. In conclusion, integrin subunit expression by vascular smooth muscle cells in culture, while similar to that in vascular tissue, can vary with the growth substrate.

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Alterations of Integrins in Transformed MDCK Cells Lacking Apical Polarity. K.S. Matlin[1,2], A. Zuk[1,2], G. Zinkl[1,2], D.M. Kendall[2], and C.A. Schoenenberger[2,3] 1)Renal Unit, Massachusetts General Hospital, Boston, MA; 2) Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, MA; and 3)Maurice Müller Institute, Basel, Switzerland.

We have previously shown that MDCK cells transformed with the K-*ras* oncogene lose apical polarity while retaining the ability to form tight junctions and restrict basolateral proteins to regions of cell-cell contact (J. Cell Biol.*112*::873-889). Because the transformed cells also show defects in cell adhesion, we have begun to examine the integrin family of adhesion receptors to determine if their alteration can be correlated with the transformed phenotype. Normal MDCK cells express a complex assortment of integrins including at least three α subunits associated with β1, one associated with β3, and one associated with β4. The α subunits which form complexes with β1 have been tentatively identified as α2, α3, and α6 based on SDS-gel mobilities with and without reduction and reactivity with specific antibodies. The α6 subunit also associates with β4. The β1 subunit is synthesized in excess of the α subunits such that endoH-sensitive β1 can be continuously detected following pulse-labeling and chase incubations. All of the β1 integrins appear to be targeted directly to the basolateral domain immediately after synthesis and can be localized there by immunofluorescence. In transformed MDCK cells, the relative amount of the β1 subunit declines, and complexes of β1 and presumptive α6 are no longer detectable. Because α6β4 is still expressed in transformants, the possibility exists that the decline in β1 leads to a switch of the α6 subunit from β1 to β4 containing heterodimers resulting in effective loss of the laminin receptor contributed by the α6β1 integrin.

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Changes in the Pattern of β1 Integrins during Malignant Conversion of Mouse Epidermal Keratinocytes. A. Cano, M. Quintanilla and M. Gómez. Instituto de Investigaciones Biomédicas. CSIC. Dept. Bioquímica. UAM. Madrid. Spain.

The expression of β1 integrins has been analysed in several epidermal keratinocyte cell lines representative of different stages of mouse skin tumor progression, ranging from immortalized non-tumorigenic to highly malignant cells. Qualitative changes in the pattern of β1 integrins expressed by the various cell lines have been detected. All the cell lines express similar levels of α3β1, being the predominant β1 integrin in non-tumorigenic cells. The fibronectin receptor, α5β1, is expressed in highly malignant spindle carcinoma cells, but it has not been detected in the rest of cell lines showing epithelial or epithelioid morphology and diferent tumorigenic properties. None of the cells lines exhibit detectable levels of α6β1, in agreement with the low level of adhesion to laminin observed in all of them. The α6 subunit is associated with β4 in all epithelial cells. Immunodepletion of α3β1 shows that all tumorigenic cells express an additional β1 integrin associated to an α subunit (αt) showing the same electrophoretic mobility as α3 on non-reducing PAGE-SDS gels. The expression of αtβ1 is associated with a noticeable increase in adhesion to collagen exhibited by those tumorigenic cells. These data, together with the isoelectric behaviour of mouse β1 integrins, suggest that αt subunit could be the mouse homolog the the human α2 subunit. The characterization of the αt subunit is currently under progress.

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Altered α3β1 and α6β4 Integrin Expression in Invasive and Metastatic Human Prostate Carcinoma Cells. S. Dedhar[1], R. Savlnier[3], R. Nagle[2], and C.M.Overall[3], Universities of Toronto[1], Arizona[2] & British Columbia[3].

Highly invasive human prostate carcinoma cells were selected for by allowing the parental PC-3 cells to invade through reconstituted basement membrane. The invasive subpopulations (I-PC3(2) and (3)) were found to be ~15-fold more invasive *in vitro* than the parental cells, had a rounded morphology and proliferated more rapidly. When injected either SC or IP into immunocompromised SCID mice, the I-PC3 cells formed primary tumours and were highly invasive and metastatic. The I-PC3 attached equally as well as PC-3 cells to fibronectin, laminin, type IV collagen, and vitronectin, but failed to spread on any of these substrates. Whereas the parental cells synthesized substantial amounts of fibronectin, the I-PC3 cell variants did not. Although both PC-3 and I-PC3 cells expressed equivalent levels of cell surface αvβ3, α2β1 and α5β1 integrins, the expression of the α3β1 integrin was markedly reduced on the invasive I-PC3 cells. Finally, whereas the PC-3 cells express α5β1, the invasive I-PC3 cells were found to express the α6β4 heterodimer exclusively. Since the α6β4 integrin is analogous to the A9 tumor antigen which is associated with aggressive human squamous cell carcinomas, α6β4 may also participate in the aggressive behaviour of these prostate carcinoma cells allowing these cells to become more invasive and lead to an increased propensity for metastasis.

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Thrombin Modulation of Expression of Matrix Proteins and Receptors in Human Pulmonary Artery Endothelial Cells. Y.H. Kang, S.E. Quella, R. Williams, and C.H. Lee, Naval Medical Research Institute, Bethesda, Md 20889-5055.

Thrombin stimulates production of IL-1 and secretion of CD62 (GMP-140) in endothelial cells, which is known to be involved in leukocyte adherence. Extracellular matrix proteins such as fibronectin (FN) and laminin (LN) are also known to be implicated in cell adhesion and migration. The present report elucidates the effects of thrombin on the expression of FN, LN, and fibronectin receptor (VLA-5) in human pulmonary artery endothelial cells (HPAEC) in an attempt to gain better understanding of the role of thrombin in inflammatory responses. HPAEC cultures were exposed to various doses of human thrombin for 24 hr and evaluated for expression of FN, LN and VLA-5 by radioimmunoassay, immunofluorescence, and immunoelectron microscopy. Additionally, the effect of thrombin on the growth of the endothelial cell was also studied by ^{3}H-thymidine incorporation. The dose of 0.005 NIH units/ml thrombin showed 5.5 and 7% increases in FN and LN, respectively, while higher doses suppressed production of the proteins. Similarly, thrombin concentrations higher than 0.005 unit/ml also exhibited dose-related inhibition on VLA-5 expression on the endothelial cells. A 42% decrease of VLA-5 was observed in cells treated with 1 unit/ml thrombin. The dose of 0.01 unit/ml thrombin showed a 61% increase in thymidine incorporation. However, dose-related inhibition was observed at doses higher than 0.01 unit/ml. Both FN and LN were localized in the rough endoplasmic reticulum, Golgi apparatus, and secretory granules. FN was also localized on the extracellular fibrils. VLA-5 receptors appeared in fine patches on the cell surfaces and on the extracellular fibrils. The results indicate that a low dose of thrombin may enhance cell adhesion and migration since it stimulates FN and LN production.

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Modulation of Integrin Receptors by Tumor Necrosis Factor in Rat Alveolar Epithelial Cells

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Tumor necrosis factor-α (TNFα) produced by a variety of cell types including alveolar macrophages (AM) is a potent cytokine mediator in a wide spectrum of physiologic and immune functions. Very little information is available regarding the influence of TNFα on alveolar epithelial cells and their response to it in inflammatory states. We hypothesized that TNFα may play an important role in alveolar epithelial cell differentiation by its modulation of integrin receptors. The LM5, a rat alveolar type II cell-derived cell line, was used to overcome AM contamination commonly observed with primary cultures of type II cells. The cells were cultured on plastic dishes in DMEM + 5% FBS to near confluence, then incubated with 0-100 ng/ml TNFα in serum-free medium for 0-16 hours. Steady state mRNA levels for β_1, α_3, α_6, and α_v integrin subunits were analyzed by Northern blotting and confirmed by in situ hybridization. TNFα increased β_1 and α_v subunit mRNA levels in a concentration-dependent manner within 2 hours, whereas, α_3 mRNA levels were decreased and α_6 mRNA levels remained unchanged. We conclude that TNFα modulates alveolar epithelial cell integrin patterns, and that alteration of these adhesion receptors may provide a mechanism for TNFα regulation of epithelial cell interaction with its basement membrane during inflammatory states and lung repair.

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Differential Regulation of Integrin Receptors by Transforming Growth Factor β (TGFβ) in Rat Alveolar Epithelial Cells

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The TGFβ family of genes has been shown to play a role in cell growth and differentiation in many cell types. Little is known about the role of TGFβ on modulation of integrin receptors in lung alveolar epithelial cells. Integrins are heterodimers consisting of α and β subunits which are involved in cell-ECM and cell-cell interactions during cell growth and differentiation. We postulated that TGFβ may play an important role in alveolar epithelial cell differentiation through its regulation of integrins. An immortalized rat alveolar type II cell-derived cell line (LM5) was cultured on plastic dishes in DMEM + 5% FBS to near confluency, then incubated with $TGF\beta_1$ (0-100 pg/ml) in serum-free medium for 0-16 hours. We examined the expression and localization of integrin mRNAs by Northern blot analysis and in situ hybridization. Northern blot analysis demonstrated an increase in β_1, α_v, and α_1 mRNA levels in dose-dependent manner (10 pg/ml). We further investigated the effect of TGFβ on steady state levels of mRNA for β_1, α_v, α_3, α_1, and α_6. Steady state kinetics demonstrated that there was an increase in β_1 and α_v mRNA expression levels but no significant change in induction of α_6 mRNA at 12 hour post-treatment. TGFβ inhibited α_3 mRNA kinetics. We conclude that TGFβ may regulate alveolar epithelial cell differentiation in part through its modulation of integrin gene expression.

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Homotypic Binding of $\alpha 3\beta_1$ in Cell-Cell Adhesion. P. Sriramarao, and K.R. Gehlsen. California Institute for Biological Research, La Jolla, CA 92037.

Several integrin receptors have been found to localize in cell-cell contacts, in addition to their associations in cell-matrix adhesion structures. Our presented data suggests that $\alpha 3\beta_1$ interacts homotypically in cell-cell junctions which contain this receptor. We and others have presented immunofluorescence staining of cells demonstrating localization of $\alpha 3\beta_1$ in cell-cell contacts. To expand on these observations, we purified $\alpha 3\beta_1$ by affinity chromatography and used this receptor as a substrate in cell adhesion assays. In such experiments, $\alpha 3\beta_1$ supported the adhesion of certain cells and this attachment could be inhibited using monoclonal antibodies to both β_1 and $\alpha 3$ integrin subunits. An affinity matrix containing purified $\alpha 3\beta_1$ coupled to CNBr activated-Sepharose, specifically bound only $\alpha 3\beta_1$ from detergent extracts of cell surface proteins. The above interactions were cation-dependent and were augmented in the presence of Mn^{++}. Finally, using a BIACore affinity instrument (Pharmacia), $\alpha 3\beta_1$ was found to only interact homotypically and not with other purified receptors including; $\alpha 5\beta_1$ and $\alpha_v\beta 3$. It is our conclusion from the above data that $\alpha 3\beta_1$ binds in a homotypic manner when found in cell-cell adhesion structures.

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Drosophila PS2(m8) Integrin Mediates RGD-Dependent Cell-Matrix Interactions. T.A.Bunch, M.Zavortink, and D.L.Brower, Department of Molecular and Cellular Biology and Department of Biochemistry, University of Arizona, Tucson, AZ 85721. (Sponsored by S.Ward)

We have examined the functional role of the Drosophila PS2(m8) integrin in mediating cell-matrix interactions. A Drosophila cultured cell line was transfected with genes that express both subunits, β_{PS} and $\alpha_{PS2(m8)}$, of the PS2(m8) integrin. We demonstrate that the PS2(m8) integrin is expressed on the surface of the cells and can mediate cell spreading on purified vertebrate vitronectin, a bacterially produced fusion protein containing the amino-terminal half of mouse vitronectin, and to a lesser extent on fibronectin. Additionally, PS2(m8) integrin can mediate cell spreading on an RGD peptide. The spreading on matrix components or RGD peptide can be inhibited by soluble RGD peptide and is dependent on Mg^{2+} or Mn^{2+}. Mn^{2+} functions better than Mg^{2+} in promoting PS2(m8) integrin-mediated cell spreading while Ca^{2+} appears to have an inhibitory effect. Finally, immunolocalization studies suggest that the PS2 integrin is associated with actin bundles, reminiscent of focal contact sites observed in vertebrate cells.

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Reorganization of Cell-Cell Contacts following Heterologous Expression of the Integrin $\alpha v\beta 6$ in Mouse NIH3T3 Cells. Ric I. Cone and Dean Sheppard. Lung Biology Center, Department of Medicine, University of California, San Francisco, CA 94143.

The integrin $\alpha v\beta 6$ is present in some but not all epithelial cells, and mediates adhesion to fibronectin. Heterologous expression of $\alpha v\beta 6$ was demonstrated on the cell surface of two lines of mouse NIH3T3 cells stably transfected with a neomycin-resistant vector containing human $\beta 6$ subunit cDNA under regulation of a dexamethasone (DEX)-inducible MMTV/LTR promoter. The cell shape of $\beta 6$-transfectants that expressed $\alpha v\beta 6$ was more rectangular compared with the typical fibroblastic appearance of wild-type NIH3T3 cells or NIH3T3 cells stably transfected with vector only. In the absense of DEX, one transfectant line expressed low amounts of $\alpha v\beta 6$ and exhibited a subtle difference in shape, whereas following 24 hours induction, this line expressed substantial amounts of $\alpha v\beta 6$ and a robust shape difference. The other transfectant line expressed very low amounts of $\alpha v\beta 6$ following DEX treatment, and exhibited no detectable difference in shape until 48-72 hours following induction with DEX. Shape differences were evident when cells were plated on several different extracellular matrix proteins, and were not blocked by the addition of GRGDSP. $\beta 6$-transfected NIH3T3 cells plated individually demonstrate the shape difference, and upon confluence the cells formed sheets with (1) increased formation of cell-cell contacts; (2) enhanced organization of tight junctions based on immunolocalization of ZO-1; and (3) enhanced ability to organize fibronectin matrix at cell-cell borders. These results suggest that $\alpha v\beta 6$ plays a role in morphologic changes associated with mesenchyme to epithelial transformation.

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Secretion of functional truncated $\alpha_v\beta_6$ integrin by transfected Chinese hamster ovary cells. A.B. Weinacker and D. Sheppard, Lung Biology Center, Department of Medicine, Cardiovascular Research Institute, University of California, San Francisco, San Francisco, CA 94143.

Integrins are a family of cell surface glycoproteins that mediate adhesion between cells and between cells and extracellular matrix. Each integrin is composed of an alpha and a beta subunit and consists of a cytoplasmic, transmembrane and extracellular domain. We recently identified the integrin, $\alpha_v\beta_6$ on several epithelial cell lines, and characterized this integrin as a fibronectin-binding protein. To determine whether the cytoplasmic or transmembrane domains of either subunit contribute to subunit association or ligand binding, we engineered a secreted form of the molecule by truncating each subunit immediately 5' of the transmembrane domain. Plasmids containing truncated cDNAs encoding each subunit were used to doubly transfect Chinese hamster ovary cells, and successfully transfected cells were selected by inclusion of a neomycin resistance gene on the β_6 vector. The presence of secreted $\alpha_v\beta_6$ heterodimer was demonstrated by immunoprecipitation of ^{35}S labelled protein in media conditioned by transfected cells using an antibody to α_v. Fibronectin affinity chromatography showed that the truncated $\alpha_v\beta_6$ retained its ability to bind to fibronectin, and like the intact heterodimer, was eluted by EDTA. These data suggest that for $\alpha_v\beta_6$ neither subunit association nor ligand binding require the presence of the cytoplasmic or transmembrane domains.

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K562 cells overexpressing α_v show increased adhesion to vitronectin. L. Armstrong, L. Ross*, and J.L. Molony#. Schering-Plough Research Institute, Tumor Biology Department, 60 Orange St., Bloomfield, NJ 07003 and *Glaxo Research Institute, Cell Biology Department, 5 Moore Drive, RTP, NC 27709, #Tanabe Research Labs, 11045 Roselle, San Diego, CA 92121

A novel integrin heterodimer comprised of α_v and β_1 subunits has been described as a vitronectin receptor specific for vitronectin (Vn) [Bodary and McLean, 1990] and as a fibronectin receptor which binds fibronectin (Fn) and the peptide GRGDSPR [Bogel et. al, 1990]. In order to study the specificity attributed to the integrin receptors by the α subunit, the cDNA for α_v was inserted into the mammalian shuttle vector pRc/CMV and transfected into K562 cells. Transient expression of α_v in cells was analyzed at 48h by immunofluorescence microscopy, FACS, and cell-substrate binding assays. Transfected cells showed increased adherence to Vn and Fn substrates. Localizations showed that α_v relocated from a polar position on normal cells to being expressed over the entire surface of transfected cells. FACS analysis of cells labeled with α_v monoclonals and $\alpha_v\beta_3$ polyclonals indicated that transfected cells had increased fluorescence due to increased expression of α_v. However, there was no difference in the expression of $\alpha_v\beta_3$. These data imply that transfected α_v complexed with the β_1 subunit, and that $\alpha_v\beta_1$ induced adhesion to Vn and Fn.

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Functional role of VLA-integrins for T cell locomotion in 3D collagen gels. P. Friedl[1], P.B. Noble[2], E.D. Shields[2], and K.S. Zänker[1]. [1] Naturwissenschaftliche Fakultät, Universität Witten/ Herdecke, Germany, [2] Faculty of Dentistry, McGill University, Montreal, PQ, Canada.

The class of β_1-integrins (CD49/CD29, VLA) play a major role in the interaction of a wide range of cell types with extracellular matrix molecules (ECM). We investigated the effects of monoclonal antibodies (mAb) binding to VLA-2 (α_2, clone Gi9), VLA-4 (α_4, HP2/1), VLA-6 (α_6, GoH3), and LFA-1 (CD11a, 25.3.1) molecules of human $CD4^+$ and $CD8^+$ lymphocytes on the spontaneous and Il-8 induced migration in a 3D collagen gel. The paths of randomly selected cells were digitized and quantitatively analysed.

The spontaneous locomotion of $CD4^+$ and $CD8^+$ cells was significantly inhibited by anti VLA-2 mAb ($P<0.0001$), blocking the major collagen receptor on lymphocytes. The initially normal spontaneous migration was strongly constrained within 30 to 90 min ($CD8^+ > CD4^+$). This inhibition could temporarely be overcome by subsequent stimulation with Il-8, suggesting that the VLA-2 receptor is not directly required for locomotion. In contrast, anti VLA-4 mAb, interfering with a lymphocyte homing molecule, led to a significant increase of spontaneously migrating $CD4^+$ and $CD8^+$ cells from 50 ± 12% to max. 68 ± 8%, whith was not further enhanced by Il-8. Anti VLA-6 and LFA-1 did not affect the spontaneous migration, however, both mAb significantly reduced the Il-8 triggered activation by 60 to 100% ($P>0.001$).

The data suggest that VLA-2, VLA-4, VLA-6, and LFA-1 may act as recognition molecules modulating lymphocyte locomotion in a 3D collagen enviroment.

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Identification and Characterization of a 70kD Band that Co-purifies from Adult Chicken Muscle Tissue on a β_1 Integrin Affinity Column. Z. Bao and A. F. Horwitz, Dept. of Biochemistry, Cell and Structural Biology, University of Illinois, Urbana, IL 61801

Immunoaffinity purification of β_1 integrins from adult chicken muscle show a major band migrating at 70kD. This band was isolated from SDS gels and used to raise monoclonal antibodies. Several monoclonal antibodies were obtained which fall into two classes; which are exemplified by the G9B5 and H1B4 MAbs. The H1B4 MAb immunoblots a major band at 70kD and a minor band at 120kD. It is expressed primarily on skeletal and cardiac muscle and vasculature. Preliminary characterization of this band suggests that it is the α_7 subunit of integrin (full length=120kD). The prominent presence of the 70kD band suggests that its proteolytic cleavage may therefore be important in muscle. While the G9B5 antibody does not blot, it immunopurifies a complex of a subclass of integrins including β_1, α_6 and α_7 subunits and an uncharacterized band. The G9B5 antigen is expressed more widely than H1B4; it is found on all the muscle tissues, peripheral nerve, and vasculature. On skeletal muscle the G9B5 MAb localizes in the myotendinous junction. The localization of the G9B5 antigen in the myotendinous junction suggests that it may play an important role in the muscle cell-matrix interactions.

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Identification of a Synthetic Peptide Derived from the Carboxy Terminal Globular Domain of Laminin that Mediates Keratinocyte α2 Integrin Subunit Binding. M.S. Wilke, J. Vespa, E.A. Wayner, L.T. Furcht, and A.P.N. Skubitz, Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455

Human keratinocyte adhesion to laminin is mediated through the globular domains of laminin. Several keratinocyte binding sequences from these domains have been defined using synthetic peptides (*J. Invest. Dermatol.* 97:141-146, 1991). The present study focused on determining if the cell binding sequences from the globular domains of laminin may represent integrin binding sites. In initial studies, it was found that keratinocyte adhesion to laminin was inhibited up to 80% in the presence of monoclonal antibodies (mAbs) against the β1 integrin subunit, but not significantly inhibited in the presence of mAbs against α2 or α3 integrin subunits. However, when mAbs against the α2 and α3 integrin subunits were added concomitantly, cell adhesion to laminin was inhibited up to 80%. These results indicate that keratinocytes may use either α2β1 and/or α3β1 integrins to adhere to laminin. Cell adhesion to functionally active peptides from the globular domains of laminin were then monitored in the presence of the anti-integrin mAbs. Cell binding to peptide GD-3 was inhibited by a mAb against the α2 integrin subunit, whereas mAbs against the β1 and α3 integrin subunits had little effect. These findings indicate that peptide GD-3 may represent an α2 integrin subunit binding site of laminin. [Supported by NIH grants CA08843 (MSW); Graduate School Grant-in-Aid of Research (APNS); and Jr. Faculty Development Fund (APNS)].

Nucleolar, Nuclear Matrix, and Other Nuclear Proteins (770-773)

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Purification of the Nuclear Matrix MAR-Binding Activity, and the Synergistic Enhancement of this Activity with the Addition of Lamins. T. Spann and J. Newport, Department of Biology, University of California, San Diego, La Jolla, CA 92093-0322.

The nuclear chromatin of eukaryotic organisms is organized into loops. These loops are thought to be attached to an underlying insoluble matrix through interactions between the nuclear matrix and DNA sequences called MARs, matrix attachment regions. Supporting this are the findings that MAR sequences specifically bind nuclear matrices *in vitro*. We fractionated the nuclear matrix in an attempt to determine which proteins were responsible for MAR-binding activity. To measure the MAR-binding activity of matrix fractions, we adapted the nitrocellulose filter binding assay. A high salt, pH 9 extraction temporarily solubilized both the MAR-binding activity and nuclear lamins. However, both nuclear lamins and MAR-binding activity were insoluble under the conditions in which MAR-binding activity was measured. To separate MAR-binding activity from the nuclear lamins, the solubilized matrices were dialyzed into 6M urea and fractionated with ion exchange chromatography. MAR-binding activity was eluted in a lamin depleted fraction, and a 50 kd protein emerged as the probable MAR-binding factor. The MAR-binding activity of the lamin-depleted fraction was compared to the activities of the matrices and the solubilized extract, and we found that the MAR-binding activities of all three samples were saturable and resistant to competition from non-specific DNA. The addition of nuclear lamins or bacterially expressed lamin A enhanced the MAR-binding activity of the lamin-depleted fraction. Our data suggested that the addition of lamins enhanced the activity of the lamin-depleted fraction through a synergistic interaction that created new MAR-binding sites that were not present in either the lamin sample or the lamin depleted fraction before their combination.

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Novel Nuclear Matrix Elements Interact with Two Classes of Sequence-Specific DNA Binding Nuclear Matrix Proteins. J.P. Bidwell, A. van Wijnen, J.L Stein, J.B. Lian and G.S. Stein. Department of Cell Biology, University of Massachusetts Medical Center, Worcester, MA 01655.

The nuclear matrix (NM) may promote gene expression by providing a 3-dimensional architecture that mediates gene localization and concentration of transcription factors. This premise requires the presence of DNA binding proteins in the NM capable of sequence-specific interactions with promoter regulatory elements of distinct target genes. Here we report on the characterization of such NM proteins, NMP-1 and NMP-2, from rat osteosarcoma (ROS 17/2.8) cells that indeed exhibit sequence-specificity to the rat osteocalcin (OC) promoter. NMP-2 (38kD) is NM-specific. NMP-1 is cell growth-dependent, but unlike NMP-2 has a broader subcellular distribution within the nucleus. Binding sequences were characterized at single-nucleotide resolution. Two nuclear matrix elements (NME), each containing both an NMP-1 and NMP-2 binding motif were identified with different organizations. The distal NME, -603/-597, contains overlapping NMP-1 and NMP-2 sites and is dA/dT-rich. In the proximal NME, -452/-435, these sites are non-overlapping and contiguous with regulatory sequences that modulate Vitamin D responsive transcriptional activity of this gene. Our data show that NMP-1 and NMP-2 represent two classes of NM proteins with defined recognition-site specificities. The two rat OC promoter domains (~30bp) containing binding sites for these factors define novel DNA sequences, NMEs, that are different from typical dA/dT-rich nuclear matrix attachment regions (MARs) commonly 200-300bp in length. The short dA/dT-rich NME of the OC promoter resembles a truncated MAR. Although MARs are thought to permanently anchor chromatin to the nuclear matrix, as well as to define transcriptional boundary domains, the NMEs described here may transiently tether gene regulatory regions to this structure, locally concentrating transcription factors. The possibility arises that these NMEs may provide transient spatial cues resulting in developmental and tissue-specific transcriptional competence.

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In Vivo Dynamics and Distribution of Topoisomerase II in *Drosophila* Chromosomes. J.R. Swedlow*, J.W. Sedat# and D.A. Agard#. *Graduate Group in Biophysics, #Department of Biochemistry and Biophysics and Howard Hughes Medical Institute, University of California, San Francisco. San Francisco, CA 94143-0448.

Topoisomerase II is required for full condensation of chromosomes and for proper segregation of sister chromatids at mitosis. To understand the role of the enzyme in condensation and segregation, we have studied the *in vivo* localization and dynamics of topoisomerase II in *Drosophila* syncitial blastoderm embryos. Purified *Drosophila* topoisomerase II was labeled with tetramethylrhodamine. The labeled, catalytically active enzyme was injected into nuclear cycle 9-10 embryos. Time-lapse three-dimensional movies of these embryos were then recorded with a CCD-based, wide field optical sectioning fluorescence microscope. Rhodamine-topoisomerase II (rhod-topo II) is distributed throughout the interphase nucleus, but is also concentrated in at least one, but no more than two sites on the periphery of each nucleus. These sites disappear as nuclei enter prophase and chromosomes condense. The sites may be specific chromosomal loci where topoisomerase II concentrates. During mitosis, rhod-topo II was not localized to an axial chromosome core in these *in vivo* experiments, but instead was distributed throughout the chromosome. Approximately 60% of the rhod-topo II present in the late interphase nucleus leaves and diffuses into the cytoplasm during prophase and prometaphase. During the transition from metaphase to anaphase, the concentration of chromosomal rhod-topo II decreases by approximately 10%. The prophase and anaphase transitions occur immediately after the completion of chromosome condensation and segregation and suggest that separate populations of enzyme are responsible for these activities. Rather than behaving like a purely structural protein, the *in vivo* localization of the enzyme is observed to be highly regulated and correlates with its role in chromosome condensation and segregation.

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The association of RCC1, a regulator of chromosome condensation, with purified chromatin templates and with chromatin in *Xenopus* egg extracts. M. Dasso, Laboratory of Molecular Embryology, NICHD, National Institutes of Health, Bldg. 6, Rm. B1A13, Bethesda, MD 20892. (Spon. by A. Wolffe)

Temperature-sensitive mutants in the RCC1 gene of BHK cells fail to maintain a correct temporal order of the cell cycle, and will prematurely condense their chromosomes and enter mitosis at the restrictive temperature without having completed S phase. In various organisms, the RCC1 protein has been implicated in number nuclear functions in addition to coupling of replication and mitosis. These processes include: RNA splicing, undergoing the G1/S transition, response to a-factor (in *S. cerevisiae*) and nucleotide exchange for *Ran*, a small *ras*-like protein. Studies in *Xenopus* extracts have demonstrated that RCC1 is required neither for chromatin decondensation nor nuclear formation at a gross level, but that it is absolutely required for the replication of added sperm chromatin DNA. These studies have also suggested that RCC1 is not participating enzymatically in replication, but that it may be part of some structure which is a prerequisite for the formation or maintenance of the replication machinery. A structural role for RCC1 may also be indicated by the highly plieotropic nature of RCC1 mutants and by RCC1's high abundance in mammalian, *Drosophila* and *Xenopus* chromatin. (*Xenopus* RCC1 protein is present at a concentration of approximately one monomer per 210 bp of DNA in *in vitro* reconstituted nuclei.) Two experimental approaches have been taken in order to understand how RCC1 interacts with chromatin, what effect this interaction has on RCC1's function and whether RCC1 plays a structural role in chromatin. First, the association of *E. coli*-expressed RCC1 protein with chromatin templates reconstituted *in vitro* from purified DNA and histone components has been examined. Second, chromatin assembled with or without RCC1 using the *Xenopus* egg extract system has been examined in the cell cycle.

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A Yeast Gene Implicated in mRNA Export From Nucleus to Cytoplasm. T. Kadowaki and A. Tartakoff. Pathology Department, Case Western Reserve University School of Medicine, 2085 Adelbert Road, Cleveland, OH 44106. (Spon. by M.D. Snider.)

We have developed enrichment and screening procedures to obtain temperature and cold sensitive mutants of Saccharomyces cerevisiae which are defective in mRNA transport (mtr mutants) from nucleus to cytoplasm [PNAS, 89, 2312]. The first of these, mtr1-1, is characterized by a striking reversible accumulation of poly A+ RNA within the nucleus when the cells are incubated at 37°C. The lesion appears to be in the machinery of transport, rather than the structure of the average poly A+ transcript - both 5' methylation and poly A tail length are as expected.

We have cloned the MTR1 gene by complementation and shown that it is identical to the prp20 and srm1 genes, which are implicated in RNA processing and the mating type response, respectively (Genes & Dev. 3, 1206; Mol. Cell Biol., 9, 2682). The corresponding protein has seven copies of an approximate 60 amino acid long repeat which is rich in glycine. Five different glycine point mutations (mtr1-1, mtr1-2, mtr1-3, prp20-1, srm1-1) are in this repeating structure. All but srm1-1 cause striking intranuclear accumulation of poly A+ RNA when the cells are incubated at 37°C.

By manipulation of a strain in which the MTR1 gene is under galactose control, we have shown that MTR1p is essential for mitotic growth and that depletion of MTR1p leads to striking accumulation of poly A+ RNA in the nucleus.

There is no evidence that MTR1p cycles in and out of the nucleus: an epitope-tagged version of the protein remains in the nucleus even when transcription is interrupted.

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Characterization of Nuclear Centrophilin/NuMA. C. Zeng, D. He and B. R. Brinkley, Department of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030 (Spon. by G. S. May.)

Centrophilin was localized in the mitotic spindle pole and interphase nucleus by mAB 2D3 raised against a kinetochore-enriched chromosome preparation. Earlier studies in our laboratory indicated that centrophilin may play a role in nucleating spindle MT growth in mitosis (Tousson et al, J. Cell Biol. 112:427, 1991). Screening with 2D3, a 1.8 kb cDNA clone was isolated from a lambda gt11 expression library of HT29 cells and a new centrophilin antibody 8.22 was generated against the fusion protein from the clone of centrophilin cDNA fragment. 2D3 and 8.22 were used to investigate the nuclear distribution and function of centrophilin. Although 2D3 and 8.22 resolved the same spindle pole staining in mitosis, 8.22 antibody revealed specific foci in the interphase nucleus instead of the diffuse nuclear staining of 2D3. Such localized staining suggests that centrophilin may exist in two or more forms in the interphase. After removing chromatin with DNase I digestion and salt extractions, most of centrophilin remained insoluble as shown by immunofluorescence and immunoblotting. Immunoelectron microscopy with 2D3 on resinless sections demonstrated that centrophilin is a component of core filaments of the nuclear matrix. We have also performed the double labeled immunofluorescence with 8.22 and αSm, an autoantibody recognizing splicing factors. The 8.22 antigens co-localized with the spliceosomes, suggesting that some forms of centrophilin may be involved in the pre-mRNA process. Centrophilin, also known as NuMA (Compton et al., J. Cell Biol. 116:1395, 1992; Yang et al., J. Cell Biol. 116:1303, 1992), may have a function in the nucleoskeleton as well as the mitotic apparatus.

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Plant Nuclear Scaffold Attachment Regions: Functional Analysis Using Transgenes. L.C. Childs, G.E. Hall, Jr., G.C. Allen, S.L. Spiker, and W.F. Thompson, Departments of Genetics and Botany, North Carolina State University, Raleigh, NC 27695.

Variations in the level of gene expression independent of gene copy number are often ascribed to position effects. These effects are believed to be caused by the transgene's integration into chromatin domains which differ in their availability for transcription. These domains are often defined by scaffold attachment regions (SARs) flanking the gene of interest. Recent data in animal systems indicate that when transgenes including flanking SARs are integrated, position effects are greatly reduced and the resulting levels of gene expression depend on copy number. We are studying SARs in plants in several ways. First, we are examining native SARs near integrated transgenes. We have several tobacco suspension lines which vary dramatically in their levels of expression of a reporter gene and also vary in their distance to nearby SARs. Second, we are also examining SARs near native tobacco genes. Our SARs have the canonical sequences and characteristics identified in SARs from other kingdoms. Third, we made constructs using a yeast SAR which binds in vitro to isolated plant scaffold to study the effects of flanking transgenes with SARs. Using direct DNA transfer to introduce these reporter gene constructs, we have shown that the presence of a yeast SAR increases dramatically the average level of expression over analogous non-SAR constructs.

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Nucleolus-like Particles Can Be Formed *In Vitro*. G.M. Trimbur and C.J. Walsh, Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260.

When 0.4 M NaCl extracts of nucleoli from the amebo-flagellate *Naegleria gruberi* are dialyzed into buffer without NaCl, a subset of the nucleolar components reassociate to form 1 - 3 μm spherical particles (Trimbur & Walsh. 1990. J. Cell Biol. 111:116a). If the particles are prepared from 15,000 x g supernatants they are found to be composed of two layers when examined in the electron microscope. The outer layer is made up of 15 nm granular particles which closely resemble the granular component (GC) of intact nucleoli. The center of the particles is composed of a fine granular material that resembles the dense-fibrillar component (DFC) of nucleoli. Overall the ultrastructure of these nucleolus-like particles (NLPs) resembles a variety of *in vivo* structures including compact nucleoli, nucleolus-like bodies, extrachromosomal nucleoli of oocytes, and nucleoli of early embryos. Treatment of the extracts with RNase before dialysis produces smaller particles that lack the 15 nm granular component, suggesting that this represents RNP particles. If the extracts are centrifuged at 436,000 x g for 60 min before dialysis, they still form particles but the particles are smaller and lack the granular outer component, consistent with the removal of RNP particles. NLPs prepared from 15,000 x g supernatants contain approximately 40% of the nucleolar DNA, RNA, and protein. The protein composition of these NLPs is complex but they are enriched in a specific subset of nucleolar proteins including BN 46/51 (Trimbur & Walsh. 1992. J. Cell Sci., in press). NLPs produced from the high speed supernatant contain no DNA and only about 14% of the nucleolar RNA and protein. In this case the protein composition is significantly simplified. In addition to BN 46/51, the NLPs are composed of 6 major polypeptides as well as a number of minor ones. Supported, in part, by a grant from NSF.

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Expression and Localization of Two Forms of Nucleolar Protein B23 in Rodent Tissues. D. Wang, H. Umekawa and M.O.J. Olson, Department of Biochemistry, The University of Mississippi Medical Center, Jackson MS 39216

Protein B23 is a major RNA-associated nucleolar phosphoprotein and putative ribosome assembly factor. Electrophoretic, cDNA and genomic analyses indicated the presence of two forms of the protein designated B23.1 and B23.2. These are polypeptides of 292 and 257 amino acids, respectively, which differ only at their C-terminal ends and arise from the same gene via alternative splicing at the mRNA level. We have studied the expression of these two B23 isoforms in rat tissues at the mRNA level using northern blot and RNA-PCR analyses and at the protein level by western blotting. In all tissues examined the relative amount of B23.1 mRNA was much higher (3-6 fold) than B23.2 mRNA. For B23.1 the level of mRNA and protein expression was highest in Novikoff hepatoma cells, followed in order by testis, liver and kidney. This suggests that B23.1 expression is correlated with the rate of proliferation of the tissue and/or the rate of ribosome biogenesis. On the other hand, the amount of B23.2 mRNA did not vary significantly among the latter three tissues. The B23.2 protein was present only in trace amounts in those tissues. In the hepatoma cells protein B23.1 was predominantly found in the nucleoli and B23.2 in the nucleoplasm. This suggests that the C-terminal end of B23.1 is important in determining the location of the protein within the cell nucleus. Supported by NIH grant GM28349.

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DISTRIBUTION OF SILVER PROTEINS AND B23 DURING THE MICRONUCLEATION OF METAPHASE CELLS. I.Muharjamova and O.Zatsepina, Institute of Physical and Chemical Biology, Moscow State University, Moscow 119899, Russia

The action of a diluted culter medium on living pig kidney cells (PK cell lines) causes the formation of the nuclear envelope around individual metaphase chromosomes and thus an appearance of numerous (about 30 per cell) micronuclei. We studied a consequence of the nucleolus organizing regions (NORs) reactivation and the fate of the main nucleolar proteins (i.e Ag-proteins and B23) in micronuclei using Ag-staining and indirect immunofluorescence with antiB23 antibodies (presented as a gift by. Dr. R.Ochs). It was shown that the appearance of Ag-proteins and B23 within micronuclei does not correlate with the presence of NORs and can be registrated in all micronuclei simultaniously. Staining of micronuclei with B23 antibodies coincides with the reconstitution of the nucleoli and always follows B23 detection within the cell cytoplasm. It has been concluded that the nuclear targets for Ag-proteins and protein B23 are not connected with the presence of the ribosomal DNA. We did not find the latent NORs in PK cells, as always the total number of nucleoli per micronucleated cell did not exceed the number of Ag-NORs in metaphase cells.

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Expression of Nuclear Coiled Bodies in Hepatocytes of Estrogen-Treated Roosters. R.L. Ochs, K. Brasch*, L.E.C. Andrade, S. Takano, E.K.L. Chan, and E.M. Tan, Autoimmune Disease Center, The Scripps Research Institute, La Jolla, CA 92037 and *Dept. Biology, CSU-San Bernardino, San Bernardino, CA.

As a model for cellular growth and stimulation without accompanying proliferation, we have examined the expression of nuclear coiled bodies in hepatocytes of estrogen-treated roosters. Four week-old roosters were injected with a single intramuscular dose of estradiol at 20 mg/kg body weight and then sacrificed at time points of 8 hr, 48 hr, and 4 weeks postinjection. For immunofluorescence, livers were excised and isolated nuclei were cytocentrifuged onto polylysine-coated microscope slides, fixed with cold methanol/acetone, and labeled with antibodies to the nucleolar protein fibrillarin and to the coiled body-specific protein, p80-coilin. In control animals (no estradiol) or in animals 8 hr postinjection, each hepatocyte nucleus contained an average of 1.0 coiled bodies which appeared randomly distributed in the nucleoplasm. At 48 hr postinjection, there were an average of 3.3 coiled bodies/nucleus and many of these appeared to be in contact with the nucleolus. Pairs of coiled bodies were also observed. By 4 weeks postinjection an average of 1.5 coiled bodies/nucleus were detected, with no apparent relationship to the nucleolus observed. From these data, we hypothesize that in response to estrogen stimulation coiled bodies arise from the nucleolar surface by "budding", replicate by dividing, and then appear randomly distributed in the nucleoplasm. By conventional electron microscopy of intact livers, two different types of round nuclear bodies were observed, sometimes in close proximity to each other. One type of nuclear body was a classical coiled body 0.35 μm in diameter and the other type of body was 0.25 μm in diameter and composed of cortical fibers surrounding a hollow interior. Future studies will involve immunoelectron microscopy to identify which of these nuclear bodies contains p80-coilin.

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Further Characterization of DNA Polymerase Activities from the Ancient Eukaryote Trypanosoma brucei. P. R. Strauss, Department of Biology, Northeastern University, Boston MA 02115

We have previously reported the presence of DNA polymerase β (8-pol) activity in extracts from the ancient eukaryote T. brucei. In this report we extend our purification scheme to include not only pol-β, but also the DNA polymerase α (pol-α) family. T. brucei extracts were resolved as described (Strauss, 1991 J. Cell Biol. 115, 87a) except that activity was obtained from the phosphocellulose flow-through at 50 mM KPO_4, pH 6.8, and retained by single-stranded DNA-sepharose, eluting with increasing concentrations of KCl. The active fraction (D2P1S2) was further resolved by means of glycerol gradients to reveal two activities, one with MWapp of 40 kDa and the other, which was clearly not homogeneous, with MWapp of 113-142 kDa. When D2P1S2 was fractionation further using Filtron microseparation devices(Filtron Tech. Corp., Northborough MA), ~200% of initial activity was recovered with >400 fold purification. Again two distinct activities were recovered. Subfraction A, which presumably corresponded to the 113-142 kDa material on glycerol gradients, had the characteristics of DNA pol-α and family, large proteins and protein assemblages that are sensitive to N-ethylmaleimide and aphidicolin, insensitive to 20 μM ethidium bromide and prefer Mg^{2+} as the divalent cation. Specific activity was 159 units/ mg protein (1 unit is 1 nmole dNTP incorporated into TCA insoluble material/hr); total recovery was 5.5 units/10^9 organisms. Subfraction B, which presumably corresponded to the 40 kDa peak on glycerol gradients, had the characteristics of a vertebrate pol-β, a small protein that is sensitive to dideoxynucleotide triphosphate at a molar ratio of 10:1 but not to aphidicolin, and prefers Mn^{2+} as the divalent cation. Specific activity was 153 units/mg protein; 4.2 units/10^9 organisms was recovered. In conclusion, we have now partially purified both major DNA polymerase classes from T. brucei. Supported by NIH RR07143 and funds from Northeastern University.

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Analysis of Lagging Strand DNA Synthesis in Isolated Nuclei. D.D. Ilsley and R.D. Kuchta, Department of Chemistry and Biochemistry, University of Colorado. Boulder, CO 80309 (Spon. by N. Ahn)

Simian Virus 40 (SV40) origin-dependent DNA replication has been a widely used model for studying eucaryotic DNA replication. Evidence from this system suggests the involvement of two or more polymerases. However, a disadvantage of the SV40 system is its dependence on the viral large T antigen. We have developed a system using isolated cell nuclei from HeLa and L1210 cells to investigate replication. Isolated nuclei contain all necessary components to replicate chromosomal DNA except dNTPs. Nuclei were obtained from cells synchronized in S phase. Compounds which discriminate among the various polymerases were utilized to characterize the role of the polymerases. Aphidicolin decreased the length and amount of products synthesized, leading to the accumulation of short fragments less than 60 bases long. In contrast, butylphenyl-dGTP (BuPdGTP), a selective pol α inhibitor, caused an overall decrease in the amount of products synthesized but no change in their length. Pulse-chase experiments showed BuPdGTP did not inhibit elongation of these short fragments, indicating that an initiation event was being inhibited. These data suggest that pol α likely elongates RNA primers into short DNA primers (30-60 bases), which ar further elongated into Okazaki fragments by another polymerase. This data is consistent with the SV40 model for DNA replication.

Chromatin and Chromosomes (783-784)

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Interactions of Histone Methylation and Ubiqination with Acetylated Chromatin. M.B. Eason*, L. Chang*, C.D. Allis+, and A.T. Annunziato* *Department of Biology, Boston College, Chestnut Hill, MA 02167 and +Department of Biology, Syracuse University, Syracuse, NY 13244-1220

Various relationships among three different histone modifications (acetylation, methylation, and ubiquination) were examined. To study histone methylation, HeLa cells were labeled with [*methyl*-^{3}H]methionine in the presence of cycloheximide (to label post-synthetic methylation only), plus or minus sodium butyrate. In the absence of butyrate, methyl label was largely confined to the unacetylated and monoacetylated forms of H3 and H4. When deacetylation was blocked with sodium butyrate, most methylated H4 remained associated with un- and monoacetylated forms, with only a slight shift toward more highly acetylated subtypes observed. However, following labeling in butyrate H3 methylation shifted predominantly to multiacetylated species, as determined by acid-urea electrophoresis of purified histones. The close association of H3 methylation with acetylation was further confirmed by preferentially immunoprecipitating acetylated *nucleosomes* using antibodies specific for acetylated H4. In contrast, parallel experiments indicated that ubiquinated H2A (uH2A) was not enriched in acetylated chromatin. Thus, while H3 methylation occurs on chromatin regions which are involved in dynamic histone acetylation, [methyl]-H4 and uH2A show significantly less linkage with acetylated chromatin. In light of evidence connecting histone acetylation with gene activity, the data suggest that the methylation of H3 and H4 may serve different functions, and support recent evidence that uH2A is not enriched in transcriptionally active chromatin. (Supported by National Institute of Health Grants GM35837 (to ATA) and HD16259 (to CDA), and by a Boston College Research Grant. L.C. is a Clare Boothe Luce Graduate Fellow.)

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A Complex Pattern of H2A Phosphorylation in the Mouse Testis

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H2A histones are rapidly phosphorylated in plants (Green et al, Plant Physiol. 93:1241, 1990), protozoans (Allis and Gorovsky, Biochemistry 20:3828, 1981; Fusauchi and Iwai, J. Biochem. 95:147, 1984), invertebrates (Green and Poccia, Dev. Biol. 134:413, 1989) and mammals (Green et al, Exp. Cell Res. 195:8, 1991). H2A phosphorylation has not been associated with specific cell cycle events, or obvious changes in chromatin structure and activity, and has therefore been considered "constitutive" (see van Holde, Chromatin, 1989). We show here that H2A phosphorylation in mammalian testes occurs at several sites on different H2A variants. Mouse testis tubule cultures were labeled with $^{32}PO_4$ and histones were analyzed by two systems of two-dimensional polyacrylamide gel electrophoresis, followed by autoradiography of the gels. Of the $^{32}PO_4$ detected in histones, 95% was incorporated by certain modified forms of the H2A variants H2A.1 and H2A.X. Phosphorylation sites were mapped to N- and C-terminal regions of the modified variants by SDS gel electrophoresis and autoradiography of peptides generated by cleavage of *in vivo* labeled peptides with *N*-bromosuccinimide. Incorporation rates differed for N and C-terminal regions from different forms, demonstrating a complex pattern of H2A phosphorylation in the mouse testis. Without resolution of these different events in cell cycle studies, changes in their rates might not be noticed.

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Phosphorylation Weakens DNA Binding by SPKK Motifs

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Sea urchin testis-specific H1 and H2B histones (Sp H1 and Sp H2B) are characterized by reversibly phosphorylated N-terminal regions consisting largely of multiple "SPKK" tetrapeptides, serine-proline dipeptides flanked by two basic amino acids. The SPKK serine residues are not phosphorylated in mature sperm, presumably to create maximal basicity and enhance DNA binding and stabilization of condensed sperm chromatin. During spermatogenesis and again immediately after fertilization, the SPKK serine residues appear as serine phosphate, effectively neutralizing SPKK basicity. To explore the effects of SPKK phosphorylation and dephosphorylation on DNA binding, dephosphorylated N-terminal peptides (NP) and two differentially phosphorylated derivatives (Ph-NPa and Ph-NPb) were obtained from gonadal H1 histones (Sp H1 and its phosphorylated derivative Ph-Sp H1). DNA binding by these peptides was assessed by several criteria, including affinity for DNA-cellulose, ability to precipitate DNA, ability to protect DNA against thermal denaturation and ability to inhibit Hoechst dye binding to DNA. The phosphorylated N-terminal peptides (Ph-NPa and Ph-NPb) eluted from DNA-cellulose in 0.1 M and 0.2 M NaCl, respectively, while their dephosphorylated derivative (NP) eluted in 0.5 M NaCl, demonstrating its higher affinity for DNA. NP precipitated DNA, whereas Ph-NPs did not. Both NP and Ph-NPs protected DNA against thermal denaturation, but NP created a more stabile DNA-protein complex. NP was shown to bind DNA 250 times more tightly than Ph-NPs by its ability to inhibit DNA binding by Hoechst dye. The data support the hypothesis that phosphorylation of SPKK motifs lowers their affinity for DNA.

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A NOVEL Ca^{2+}-CALMODULIN DEPENDENT, CHROMATIN BOUND HISTONE H3/H4 KINASE FROM MOUSE LEUKEMIA CELLS (L1210).

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A Ca^{2+}-Calmodulin (CaM) dependent histone H3/H4 kinase was extracted from mouse leukemia nuclear material with 150 mM NaCl and purified by CaM-Sepharose affinity chromatography. The kinase activity was identified as a component of a complex consisting of 95% protein, 5% DNA and no measurable RNA. An 85 kDa protein, representing the major CaM binding protein, was separated from the rest of the complex after removing the salt by dialysis against water in the presence of DTT. Following centrifugation at 10,000xg, the supernatant contained the purified 85 kDa protein which phosphorylated H3 in a Ca^{2+}-CaM dependent manner. The resulting precipitate also phosphorylated H3 but was independent of exogenous CaM. Neither fraction alone phosphorylated H4. However, upon adding the supernatant to the precipitate in the presence of 140 mM NaCl, the Ca^{2+}-CaM dependent phosphorylation of H3/H4 was fully restored. Since H3 and H4 are the initial histone components involved in nucleosome assembly, their phosphorylation could alter their association with DNA. It is proposed that the 85 kDa protein, when present in the above mentioned complex, phosphorylates both H3 and H4 in a Ca^{2+}-CaM dependent manner thus possibly altering their association with chromatin. Consequently, the 85 kDa protein could play an important role in cell cycle related events including transcription.

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Changes in the Promoter Chromatin Structure Reflect Basal and Vitamin D Enhanced Osteocalcin Gene Transcription.

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Transcription of the osteocalcin (OC) gene, which encodes a bone-specific 6 kD protein, is controlled by the coordinate utilization of modularly organized basal and hormone responsive enhancer elements. Activation of these sequences involves transcription factor binding to the promoter elements. Packaging of DNA as chromatin provides a basis for cooperative interactions between activities of independent regulatory elements that contribute to the level of transcription. Because cleavage by nucleases is indicative of alterations in the chromatin structure, we examined the relationship between DNaseI hypersensitivity of promoter regulatory sequences and the extent to which the OC gene is transcribed. We observed an absence of DNaseI hypersensitivity at principal basal and enhancer sequences in proliferating osteoblasts and in non-osseous cells not expressing OC. In mineralized osteoblasts and in osteosarcoma cells (ROS 17/2.8) actively transcribing the OC gene, DNaseI hypersensitive sites are evident at the basal regulatory CCAAT Box and TATA/GRE elements as well as flanking the vitamin D responsive element (VDRE). Vitamin D enhancement of OC gene transcription is accompanied by progressive changes in nuclease accessibility within these hypersensitive sites. Vitamin D does not affect OC gene transcription or promoter DNaseI hypersensitivity in cells not expressing OC. These results indicate that both gene activation and steroid enhancement of OC transcription involve a functional relationship between changes in chromatin structure and sequence-specific interactions of transactivation factors.

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Human Autoimmune CREST Sera Identify Metaphase Perichromosomal Proteins in *Drosophila melanogaster* and in Human Epithelial Cells.

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Autoimmune CREST sera have been successfully used as probes to clone genes coding for proteins associated with the mitotic apparatus that may have important functions in mitosis. We have detected a metaphase and anaphase perichromosomal staining pattern both in *Drosophila* Kc cells and in human HEp-2 cells using serum from a CREST patient. Western blotting shows that the CREST serum recognizes a group of polypeptides both in total Kc cell extracts and in total HEp-2 cell extracts. We have used this CREST serum to screen a *Drosophila* genomic λgt11 expression library. Fourteen positive clones were obtained which were further grouped in a common set of five. Fusion proteins were induced by the recombinant lysogens. Western blotting of these fusion proteins with the CREST serum confirmed the immunoreactivity. Five overlapping inserts of the main group of clones were sequenced and show homologies with proteins involved in cell proliferation (*myc*, prothymosin). By obtaining antibodies to these fusion proteins, we have been able to produce the initial perichromosomal staining pattern in both cell types and recognize only some of the polypeptides initially stained by the CREST serum. We are currently studying a cDNA clone that may encode the protein(s) immunostained by this CREST serum in an attempt to study their function.

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Cloning of DNA from Detached Centromere/Kinetochore Complexes of Mitotic Cells with Unreplicated Genomes.

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Treatment of some mammalian cell lines with 2 mM hydroxyurea followed by 5 mM caffeine allows them to enter mitosis prematurely without completing DNA replication, giving rise to Mitotic cells with Unreplicated Genomes, or MUGs (Brinkley et. al., 1988, *Nature*, 336: 251-254). Chromosomes in such cells become fragmented and dispersed; however, centromeric fragments remain functional, aligning at the metaphase plate and undergoing segregation in anaphase. These fragments were isolated from CHO cells using a combination of density gradient centrifugation and immunoprecipitation with anti-centromere autoantibodies from the serum of a patient with CREST-scleroderma. DNA was extracted from the resulting preparation and cloned into Lambda ZAPII vector. One of the clones was found to contain a 6 kb insert localizing to the centromeric region of at least one pair of CHO chromosomes by *in situ* hybridization. It has no extensive homology to any known sequences, except for a single copy of an Alu-like repeat. Thus, MUG cells offer a fruitful system for the study of organization of the centromeric DNA.

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Isolation of a DNA-binding domain from the *Euplotes* telomere protein.

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The 51 kD telomere protein from *Euplotes crassus* binds to the extreme terminus of macronuclear telomeres generating a very salt-stable telomeric DNA-protein complex. The protein recognizes both the sequence and the structure of the telomeric DNA. To explore how the telomere protein recognizes and binds telomeric DNA we have examined the DNA-binding specificity of the purified protein using oligonucleotides that mimic natural and mutant versions of *Euplotes* telomeres. The protein binds very specifically to the 3' terminus of single-stranded oligonucleotides with the sequence $(T_4G_4)_{\geq 3}T_4G_2$; even slight modifications to this sequence reduce binding dramatically. Digestion of the telomere protein with trypsin generates an N-terminal protease resistant fragment of ~35 kDa. This 35 kDa peptide appears to comprise the DNA-binding domain of the telomere protein as it retains most of the DNA-binding characteristics of the native 51 kDa protein. For example, the 35 kDa peptide remains bound to telomeric DNA in 2 M KCl. Additionally, the peptide binds well to single-stranded oligonucleotides that have the same sequence as the T_4G_4 strand of native telomeres but binds very poorly to mutant telomeric DNA sequences and double-stranded telomeric DNA. Removal of the C-terminal 15 kDa from the telomere protein does diminish the ability of the protein to bind only to the terminus of a telomeric DNA molecule.

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Euplotes crassus has multiple genes encoding telomere-binding proteins and telomere-binding protein homologs. W. L. Wang, R. Skopp, M. Scofield, and C. Price. Department of Chemistry, University of Nebraska, Lincoln, NE 68588.

We have used genes encoding the α and β subunits of the *Oxytricha nova* telomere protein to identify equivalent genes in *Euplotes crassus*. The *Oxytricha* α subunit probe hybridized to two macronuclear DNA molecules of 1.6 kb while the β subunit probe hybridized to molecules of 1.2 and 1.0 kb. When the two 1.6 kb molecules were cloned and sequenced, one was found to encode the *Euplotes* 51 kDa telomere protein while the other appeared to encode a telomere protein homolog. The gene encoding the telomere protein homolog contained two introns, one of these introns was only 24 bp in length. This is the smallest mRNA intron reported to date. The amino acid sequence identify between the *Euplotes* telomere protein and the α subunit of the *Oxytricha* telomere protein and between either telomere protein and the telomere protein homolog is 35-36%. It is striking that many of the conserved amino acids are identical in all three proteins and that all the extended regions of conservation map within the N-terminal portion that comprises the DNA-binding domain of the *Euplotes* telomere protein. Our findings suggest that some of the conserved amino acids may be situated at the DNA-binding site.

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Using 3-Color Chromosome Painting to Decide Between Chromosome Aberration Models. J.N. Lucas and R.K. Sachs, Biomedical and Environmental Sciences Division Lawrence Livermore National Laboratory, Livermore, CA 94550 (JNL), Department of Physics, University of California, Berkeley, CA 94720 (RKS).

Ionizing radiation produces chromosome aberrations when DNA double strand breaks (DSBs) interact pairwise. For more than 30 years there have been two main, competing theories of such pairwise interactions. The "classical" theory asserts that an unrepaired DSB makes two ends which separate, with each end subsequently able to join any similar (non-telomeric) end. The "exchange" theory asserts that DSB ends remain associated, presumably due to protein constraints, until repair or illegitimate recombination involving a second DSB (i.e. a reciprocal chromosome exchange) occurs. We conducted an experiment to test these models, using 3-color chromosome painting with fluorescent *in situ* hybridization. After *in vitro* irradiation of resting human lymphocytes, we observed cells with "three-color triplets" at metaphase: three derivative chromosomes having permuted colors, as if three broken chromosomes had played musical chairs. On the exchange model, triplets cannot occur. On the classical model, their expected frequency can be calculated. We report data and computer calculations which exclude the exchange model and favor the classical model.

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The Maintenance of the Relative Positions of X Chromosomes During Interphase and their Rearrangement at Mitosis. G. Jeun and M. Locke. Department of Zoology, University of Western Ontario, London, Ontario, Canada, N6A 5B7

The larval epidermis of *Calpodes ethlius* (Lepidoptera, Hesperiidae) is a syncytium of doublets where sibling cells are twins that remain connected by residual midbodies from the last mitosis. Some interphase nuclei of female epidermal cells contain two condensed X chromosomes. Double chromosomes have similar separations in the nuclei of twinned sibling cells, showing that chromosome position is conserved through interphase. Since similar separations do not occur in clones, but differ between pairs of cells in the epithelium, chromosome position is not conserved through mitosis. Although chromosome separation is similar in sibling pairs, their orientation varies, suggesting that the nucleus rotates in the plane of the epidermis without affecting chromosome arrangement. Sibling nuclei divide synchronously but differ in the orientation of their planes of division.

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Non-Random Arrangement of DNA Sequences in Diploid Human Cells. S.C. Henderson and D.L. Spector. Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, NY. 11724-2217.

Fluorescence *in situ* hybridization (FISH) has been used to determine if DNA sequences occupy specific positions within interphase nuclei of human diploid cells. Hybridization conditions were used that preserve the 3-D arrangement of nuclear contents. In fibroblast nuclei, chromosome-specific α-satellite DNA sequences preferentially localize to the nuclear periphery or to the surface of the nucleolus; however, these sequences appear to be randomly arranged with respect to each other. Centromeres from homologous chromosomes are occasionally grouped in pairs, which may reflect temporal rearrangements related to the cell cycle. Human myoblasts were used to determine changes in positioning of DNA sequences during differentiation. In isolated myoblasts, centromeres appear to be randomly arranged. As myoblasts converge, prior to cell fusion, some centromeres align, with the axis of alignment of homologous centromeres parallel to the long axis of each cell. In myotubes, homologous centromeres are aligned parallel to the long axis of the myotube. Similar arrangements were found in frozen sections of human striated muscle; homologous centromeres aligned parallel to the long axis of the muscle fiber. Telomeres appear to be evenly dispersed throughout the nucleoplasm (with many localized to the nuclear periphery) both in cell cultures and in frozen sections of human tissues. FISH with a chromosome 7-specific telomere probe indicated that this telomere is localized to the nuclear periphery. In myotubes, telomere 7 occupies a similar position in many of the sister nuclei. FISH has also been used to examine the position of several other unique DNA sequences. These results suggest that, in a differentiated normal cell system, DNA sequences may occupy non-random domains.

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Functional Interpretation of Interphase Chromosome Domains. T.T. Puck, R. Johnson, and A. Krystosek, Eleanor Roosevelt Institute, Denver, CO 80206.

Previous studies established the existence of DNase I-sensitive (i.e. exposed) chromatin at the nuclear lamina of normal but not in cancerous mammalian cells (PNAS 87, 6560, 1990). Both cell types reveal exposed chromatin at nucleolar sites (Som Cell Molec Gen 17, 489,1991). Digital confocal microscopy with image processing, using whole chromosome painting probes hybridized in situ to interphase nuclei has been carried out. The whole X-chromosome fluorescent probe hybridized to normal human female fibroblasts produces two distinct nuclear domains. At least one abuts the lamina and usually exhibits a diffuse pattern. The second is often but not always in the peripheral region, is brighter and more compact, and is interpreted as representing the condensed Barr body. In normal male fibroblasts the X chromosome probe produces a large diffuse fluorescent region usually in the nuclear periphery and a smaller area often in the interior which presumably represents areas of homology in the Y chromosome. Chromosomes 1, 16 and 21 also reveal significant preference for the nuclear periphery in normal fibroblasts with occasional nucleolar localization. These data are interpretable in terms of differential action of the following parameters: anchoring of chromosomal domains in different nuclear regions; selective condensation; and gene exposure and sequestration and their effects on transcription of different gene classes. Aided by a grant from the Markey Charitable Trust.

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ACTION OF BISBENZIMID HOECHST 33258 ON THE STRUCTURE AND ANAPHASE BEHAVIOUR OF MOUSE CHROMOSOMES.
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Incubation of mouse cells (3T3 fibroblasts) with Hoechst 33258 (H 33258, 40 ug/ml, 1-24 hs) leads to an artificial elongation of centromeric regions of mitotic chromosomes are easily seen under the light microscope level using conventional methods of metaphase spreads. The number of metaphase chromosomes with the elongated centromeres reached about 100 % after 16-24 hs of the treatment. In the control, cells with the elongated centromeres were completely absent. Observation on 29 metaphase living cells after 16-24 hs of incubation with H 33258 showed that the elongated chromosomes were not blocked in metaphase and were able to an anaphase movement towards the cell poles, but in comparison to the normal cells (31 cells were examined) the duration of anaphase was significantly higher (consequently, $6,0\pm0.5$ min and $4,0\pm0.2$ min). It was shown also that the elongated centromeres are stained according to C-technique thus corresponding to the centromeric heterochromatin and are induced by a binding of H 33258 to the chromatin at least in the early G2 period.

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The Effect of Topoisomerase Mutations on Unequal Sister Chromatid Exchange. E.M. Towler, and J.W. Wallis, Department of Biochemistry and Molecular Biology, Saint Louis University, Saint Louis, MO 63104. (Spon by P. Weidman.)

Unequal sister chromatid exchange (USCE) is a recombination event leading to deletion and amplification of DNA sequence. Such chromosomal rearrangements are the molecular basis of many genetic diseases. We have taken advantage of the advanced genetics of the yeast *Saccharomyces cerevisiae* to study the effect of topoisomerase mutations on the rate of USCE in growing cells. Null mutations were used in the case of the nonessential *TOP1* and *TOP3* genes. A temperature-sensitive (ts) mutation was used in the case of the essential *TOP2* gene. All strains carrying the $top2^{ts}$ allele were incubated for one generation at the nonpermissive temperature before assaying for recombination. Each single mutation causes a significant increase in the rate of recombination compared to the wild type control. No synergistic or additive effects were seen in the double mutants tested. The $top1(null)top2^{ts}$ and $top3(null)top2^{ts}$ double mutants rate do not differ significantly from either of the respective single mutations. From this data we conclude that all three topoisomerases are required to prevent elevated USCE.

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A Non-AP1 Phorbol Response Element in the Collagenase Promoter Specifically Binds Fos and Jun *In Vitro*. S.H. Chamberlain, R.M. Hemmer and C.E. Brinckerhoff, Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755-3844

In rabbit synovial fibroblasts the AP1 sequence (ATGAGTCAC) is necessary but not sufficient for induction of collagenase transcription by phorbol esters (PMA) (*Biochem.* 30:4629,1991). To identify additional sequences involved in transcription we transiently transfected fibroblasts with chimeric constructs containing fragments of the rabbit collagenase 5'-flanking DNA linked to the chloramphenicol acetyl transferase gene. We found that both a 5' deletion of the region from -182/-142 and an internal deletion of -182/-141 in a 380bp promoter construct resulted in a 10-fold loss of induction by PMA, thereby implicating a transcriptional role for this region. Electrophoretic mobility shift assays revealed that nuclear proteins derived from PMA-treated fibroblasts bind the -182/-142 region in a specific manner; binding is completely competed by self or only partially competed by the AP1 sequence at 100-fold molar excess levels, implying that proteins binding to the AP1 sequence could also bind to the region from -182/-142. In further experiments the highest degree of specific binding of PMA-induced nuclear proteins localized to the region -182/-162. Competition analysis demonstrated that a 20bp oligoprobe from -182/-162 binds nuclear proteins which also specifically bind to the AP1 sequence. *In vitro* transcribed and translated Fos and Jun proteins exhibited specific binding to both the collagenase AP1 site and a 42bp oligoprobe from -182/-141. DNAse 1 footprinting of the collagenase promoter with purified Jun or Fos/Jun revealed a protected region from -189/-178 which includes an AP1 half-site (ATGA) at -180/-177 in addition to protection at the AP1 sequence located at -77/-69. Our data show that (1) the region from -182/-141 contributes to phorbol induced collagenase gene transcription, (2) Fos and Jun interact *in vitro* with non-AP1 sequences located at -182/-142, and that (3) these proteins may modify transcription by binding to sequences other than AP1.

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A Cytosolic Inhibitor of AP-1-TRE Interaction. Possible Relationships to c-Jun and c-Fos Cytosolic Localization in Normal Human Keratinocytes. P.Briata, F. D' Anna *, A.T. Franzi *, and R. Gherzi, Istituto Nazionale per la Ricerca sul Cancro (IST), Genova, and * Istituto di Anatomia Umana Normale, Università di Genova, Genova, Italy. (Spon. by R.E. Favoni).

The products of c-jun and c-fos proto-oncogenes are nuclear phosphoproteins which associate to form the AP-1 transcription complex. AP-1 constitutes a master switch in the signal transduction cascade that leads to cellular responses as diverse as growth, differentiation, and memory formation. The transcriptional response of many genes to phorbol esters, growth factors, and cAMP has been found to be mediated by the interaction between AP-1 and the TRE motif. Recently, increased levels of c-fos and c-jun expression have been observed in differentiating epithelial cells. We observed, in the upper granular layers of both normal human epidermis and reconstituted human epidermis transplanted onto athimic mice, higher c-Jun (Jun) levels than in the basal layers. On the contrary, when we investigated the AP-1-TRE interaction, by electrophoresis mobility shift assays, in either non differentiated or differentiated normal human keratinocytes (KER), we observed that AP-1 activity is ~8 fold higher in non differentiated than in differentiated KER. In the cytosolic fraction of differentiated KER we found a protein factor (IF) which inhibits AP-1-TRE binding. IF inhibits, in a concentration dependent manner, both mammalian and *Drosophila* AP-1 activity and the TRE binding activity of a recombinant Jun. The activity of the IF seems to be increased by dephosphorylation and reduced by KER treatment with agents rising intracellular cAMP levels. When we examined the localization of Jun and c-Fos (Fos) in KER, we found a preferential cytoplasmic distribution. The nuclear import of latent cytoplasmic Jun and Fos as a consequence of specific stimuli (growth factors, cAMP), under the control of a labile protein factor, has been reported. We hypothesize that the cytoplasmic localization of Jun and Fos, we observed in differentiated KER, could be related to the presence of the cytosolic IF.

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Purification and Characterization of Two Transcription Factors, SP1 and a Novel ATF, that Bind to a Transcriptionally Active Site of a Human Histone H4 Gene. M.T. Bourke, N. Aronin, K.L. Wright, G.S. Stein, and J.L. Stein, Department of Cell Biology, University of Massachusetts Medical Center, Worcester, MA 01655.

Site I (-124 to -90 bp) of the FO108 human histone H4 (FO108) gene promoter is a critical element in generating the maximal level of cell cycle-dependent FO108 gene expression. This transcriptional regulation is dependent upon two trans-acting factors that bind to ATF and SP1 consensus sequences in Site I. Here we report the purification and biochemical characterization of the trans-acting Site I factors, with the use of a one-step DNA affinity chromatographic procedure. Crude HeLa nuclear extracts were incubated with a biotinylated Site I probe-streptavidin-biotin cellulose complex. Complexed proteins were eluted with high salt following extensive low salt washes. Two prominent proteins of approximately 105 kD and 60 kD were identified. Gel mobility shift assays and UV-crosslinking experiments utilizing the eluted factors confirmed that these proteins interacted with the Site I probe in the same manner as the factors in crude nuclear extracts. Western blot analysis revealed that the 105 kD protein interacted with an antiserum that recognizes SP1, and the 60 kD protein was recognized by an anti-serum directed against the leucine zipper region of AP1; this antiserum recognizes ATF-family members. Thus, we have identified proteins that bind to the ATF and SP1 consensus sequences in Site I. The first factor is consistent in size and immunologic presentation with the transcription factor SP1. The second factor appears to be an ATF factor with a molecular mass greater than that of most members of this family. Together with prior observations based on studies with crude nuclear extracts, we conclude that both SP1 and ATF proteins are capable of binding to FO108 Site I, and the purified 60 kD ATF protein may have preferential affinity for the particular ATF region found in Site I. We speculate that this binding specificity is conferred by the regions flanking the ATF consensus sequences.

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bFGF, EGF, and TGF-β Regulation of Upstream Sequences from Three MRP/PLF Genes. M.P.K. Mohideen, C.L. Sexton and M.Nilsen-Hamilton. Program in Molecular ,Cellular and Developmental Biology, Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011.

Mitogen regulated protein or proliferin (MRP/PLF) is a glycoprotein that is secreted by 3T3 cells in tissue culture upon mitogenic stimulation. In vivo, MRP/PLF is secreted into the maternal blood stream by the trophoblastic giant cells of the mouse placenta. Basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and transforming growth factor-beta (TGF-β) regulate MRP/PLF expression in 3T3 cells and might be among the regulators of its expression in vivo. We have analyzed the upstream sequences of three MRP/PLF genes -- plf42, plf149 and mrp/plf3-- for their ability to drive gene expression in the presence and absence of growth factors (bFGF, EGF and TGF-β). Constructs containing 2.0, 1.1, 1.03 and 0.6 kb 5'upstream sequence of mrp/plf3 and 1.1 kb of plf42 and plf149 genes were evaluated using chloramphenicol acetyl transferase (CAT) as the reporter gene. A difference was observed between the regions of upstream sequence that determine the absolute level of CAT expression in the presence or absence of bFGF and the region that determine the ability of bFGF to increase CAT expression. The basal level of CAT expression was lower in cells containing 1.0 kb or less of the upstream sequence than in those containing longer sequences. The level of CAT expression in bFGF stimulated cells was much higher in cells containing the 2.0 kb sequence than in cells containing the shorter upstream sequences. While CAT expression did not increase in response to bFGF in cells containing the 1.1 kb sequences, increased expression was observed with all other constructs. Increased CAT expression was also observed with the 2.0 kb and 1.0 kb sequences upon EGF addition and this increase was inhibited by TGF-β. TGF-β inhibits expression of the endogenous gene. These data suggest, that the 2.0 kb MRP/PLF upstream sequence contains at least two elements by which MRP/PLF expression is regulated -- the response element for bFGF is located within the first -0.6 kb and the region from -1.1 to -2.0 kb contains an element that is necessary for high level of CAT expression in the presence of bFGF.

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TGFß1-inducible Nuclear Factor 1(NF1)-like transcriptional activity is constituitively elevated in Systemic Sclerosis (SSc) fibroblasts. C.W. Hartl, E.A. Smith, E.C. LeRoy and M. Trojanowska, Division of Rheumatology and Immunology, Medical University of South Carolina, Charleston, SC 29425.

Studies with lesional fibroblasts from subjects with SSc demonstrate increased mRNA levels of several extracellular matrix components, including type I collagen; the mechanism of this upregulation at the transcriptional level is not known and forms the basis of this study. Transient transfection experiments with COL1A2-promotor-CAT-deletion-constructs (courtesy of F. Ramirez) indicate that both a TGFß1 responsive element functional in normal human skin fibroblasts (NS) and the sequence involved in intrinsic upregulation of COL1A2 gene expression in SSc fibroblasts are located between -376(Bg12) and -108(Sma1) bp upstream from the start of transcription. A 25bp element (25mer) (bp-318 to bp-294) within this region shares sequence similarity (36% homology) with the consensus sequence for NF1 in adenovirus early promotor. This 25mer (32P-labelled) was used as a probe in gelshift assays performed with nuclear extracts from NS and SSc fibroblasts which show binding to 32P labelled 25mer. SSc extracts bind more intensively than do NS extracts. Binding could be substantially suppressed with competition with non-radiolabelled 25mer (400 fold molar excess). Binding was not affected when the same amount of an unrelated non-radioactive recognition site (GRE) was used as a competitor. Competition with adenoviral NF1 consensus sequence was substantially less effective than was 25mer competition. Binding was increased when cells (NS) were grown in the presence of TGFß1 (1ng/ml) for 24 hrs. Extracts derived from SSc fibroblasts were not inducible by TGFß1, but showed similar binding as extracts from TGFß1 stimulated NS fibroblasts.

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$Fc_\epsilon RI$-mediated Expression of mRNA for c-fos in Rat Basophilic Leukemia Cells Does Not Require Ongoing Aggregation of the Receptor. J. H. Dogar and S.C. Dreskin, Univ. of Colorado Health Sciences Center, Denver, CO.

Cross-linkage of the high affinity receptor for IgE, $Fc_\epsilon RI$, on mast cells leads to release of inflammatory mediators and to late phase responses. It is generally believed that late phase reactions are dependent upon activation of transcription. In order to examine this phenomenon, we have investigated $Fc_\epsilon RI$-mediated enhancement of c-fos mRNA expression in rat basophilic leukemia (RBL) cells. We chose to look at the mRNA for c-fos because c-fos is thought to be important in the general activation of nuclear transcription. RBL cells were sensitized with DNP-specific IgE and incubated at 37°C in the presence of $DNP_{25}BSA$ or BSA alone. Following activation for 0 to 60 minutes, the reaction was terminated by adding ice-cold buffer and placing the tubes in an ice-water bath. Purified RNA was separated on denaturing gels, blotted to nylon membranes and hybridized with a ^{32}P-labelled cDNA probe for c-fos. Increased levels of mRNA for the c-fos proto-oncogene are visible as early as 5-10 minutes after the addition of antigen and increase further in a time dependent fashion over 30 minutes. Unexpectedly, the addition of the hapten, 10^{-4} M DNP-lysine, 5 minutes after the addition of antigen does not alter the amount of message detected at 30 minutes (it is dramatically more than is seen after 5 minutes of stimulation) although this treatment causes immediate cessation of exocytosis. Thus, in contrast to the case with exocytosis and other well-described intracellular events, $Fc_\epsilon RI$-mediated increases in the level of mRNA for c-fos does not require ongoing aggregation of $Fc_\epsilon RI$.

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Over-expression of any fibrinogen chain specifically elevates the expression of the other two chains. S.N.Roy, O.Overton, and C.M.Redman, Lindsley F. Kimball Research Institute, New York Blood Center, NY. 10021.

Hep G2 cells have surplus Aα and γ chains and the synthesis of Bβ may be rate-limiting in fibrinogen production. Earlier studies showed that over-expression of Bβ chains by transfection of Hep G2 cells with Bβ cDNA specifically enhanced the synthesis of the 3 chains. To determine whether over-expression of any of the chains also affects the synthesis of the other 2 chains we developed stable Hep G2 cell lines transfected with individual fibrinogen chain cDNAs. As a control, cells were also transfected with expression vector which did not contain fibrinogen cDNA. In each case transfection with any fibrinogen cDNA increased the synthesis of all 3 chains but did not affect the synthesis of other plasma proteins. Hep G2-Bβ cells produced 3-fold, and Hep G2-Aα and Hep G2-γ cells about 2-fold, more fibrinogen than control cells. Northern blot analyses showed that levels of all 3 fibrinogen mRNAs were increased and were highest in Hep G2-Bβ cells. Nuclear run-on transcription assays demonstrated that increased expression of the chains was due to increased transcriptional activity and was greatest in Hep G2-Bβ cells. These studies show that transcription of the 3 fibrinogen chains is tightly linked and increased synthesis of any chain leads to increased expression of the other 2 chains. The synthesis of 2 cellular proteins, 75kDa and 57 kDa, increased in cells over-producing fibrinogen and was largest in Hep G2-Bβ cells. These 2 proteins may play accessory roles in regulating fibrinogen synthesis.

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The Role of Adenosine 3',5'-Monophosphate in the Regulation of Heme and Apocytochrome Biosynthesis. A.R. PLUMMER and J.R. MATTOON, Biotechnology Center, University of Colorado, 1420 Austin Bluffs Parkway, Colorado Springs, CO 80933-7150 (Spon. C.W. Goff.)

Regulation of mitochondrial respiratory enzyme gene expression has been a very active area of research. Oxygen, heme, carbon source, the heme activator protein (HAP1), and the HAP2/3/4 regulatory complex have been shown to be involved in the regulation of several enzymes in the heme biosynthetic pathway in Saccharomyces cerevisiae. We demonstrate that in cAMP-permeable yeast strains addition of cAMP to glycerol growth medium results in an increase in cytochromes b and c+c1. Transformation of yeast strains with a vector containing the constitutive activator of adenylate cyclase, RAS2val-19, followed by growth in glycerol medium yielded similar results. A cAMP-permeable strain, AM3-4B/U3, was transformed with a CYC1-lacZ fusion plasmid. Cultures were incubated in minimal medium containing glucose or glycerol with or without cAMP supplement. Cells were collected, permeabilized and assayed for β-galactosidase. In the glucose-grown cultures β-galactosidase activity was reduced by 2-fold by cAMP. In glycerol-grown cultures cAMP increased β-galactosidase activity 2 to 3 fold. Intracellular 5-aminolevulinic acid (ALA) assays were conducted on cAMP-permeable strains grown in glycerol medium supplemented with cAMP. Cultures were harvested and intracellular ALA extracted with trichloroacetic acid. In the presence of cAMP, ALA concentrations were increased 3 to 4 fold. These results indicate that cAMP has a positive role in the regulation of heme biosynthesis.

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Analysis of the genetic regulation of human phosphoribosyl amidotransferase (PRAT) expression in response to purine availability. J.W. Barton, and D. Patterson, Eleanor Roosevelt Institute for Cancer Research, Denver, Colorado, 80206.

This laboratory has reported the isolation of a cDNA encoding human phosphoribosyl amidotransferase (PRAT) by correction of a S. cerevisiae ade4 mutation. Due to PRAT's position as the first committed step in the de novo purine biosynthetic pathway it is highly regulated by purine feedback control. We report here an additional level of regulation for the expression of PRAT in response to the availability of exogenously supplied hypoxanthine. Low passage number human fibroblasts, after growth in media supplied with hypoxanthine, were starved for exogenously supplied purines and steady state PRAT mRNA levels were determined at specific time points post-hypoxanthine starvation. PRAT steady state mRNA concentrations increased during hypoxanthine starvation reaching a maximum at approximately 6 hours after hypoxanthine starvation. The mRNA levels then declined to approximately control levels after 24 hours hypoxanthine starvation. This subsequent decrease in steady state PRAT mRNA levels could be eliminated with the addition of 40 µM azaserine. This phenomena implies a possible connection between intermediates produced by the de novo pathway and control of PRAT gene expression. This observation is being explored in greater depth to determine the cause(s) for the increase and subsequent decrease in PRAT steady state mRNA levels and the role of purine biosynthetic intermediates on the control of PRAT mRNA levels. This observation is the first documented example of regulation for any gene involved in the de novo production of Inosine monophosphate.

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Analysis of the Mechanism of Glucocorticoid-Mediated Down Regulation of the Mouse α-Fetoprotein Gene. J.P. Rabek, D.-E. Zhang, C. Torres-Ramos, J. Papaconstantinou, Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch at Galveston, Tx 77555-0643.

Regulation of α-fetoprotein gene expression by the glucocorticoid dexamethasone (Dex) was examined *in vivo* and *in vitro* using primary fetal liver cell cultures. Dex accelerates the developmental down regulation of AFP mRNA pools while not affecting albumin. However, Dex treatment of primary fetal liver cells in culture does not reduce the AFP mRNA pool and may in fact stabilize both AFP and albumin gene expression at a higher level. These results indicate that *in vivo* the effect of Dex may be mediated by another tissue or cell type. The mechanism of the Dex mediated regulation of AFP *in vitro* was examined by DN'ase I footprinting and transient expression assays. Two protein-binding regions of the proximal promoter (III and IV) show significant homology to the GRE consensus sequence. DN'ase I footprinting shows that only region IV can bind purified GR and competition with GRE oligonucleotides indicate that, using adult liver nuclear proteins, no GR is bound in either region. In addition, nuclear proteins from adrenalectomized mice show the same protection as controls. These results indicate that GR does not appear to bind to the AFP proximal promoter in the adult. AFP promoter-CAT expression vectors were used in transient expression assays with primary fetal liver cultures to further examine the effect of Dex on AFP expression. Both proximal and proximal plus distal promoter-CAT constructs were inhibited by 10^{-6}M Dex. However, addition of an AFP enhancer to either of these promoters abolished the Dex mediated inhibition. Similar results were obtained using the human hepatoma line HepG2. We conclude that the *in vitro* effects on transiently expressed AFP directed expression vectors may be a function of vector construction and/or characteristics of the cells used and do not reflect physiologically relevant regulatory mechanisms.