MAMMALIAN CELL GENETICS

M. Wigler

C. Yen, M. Hamaguchi, M. Nakamura, D. Esposito, R. Lucito, D. Dong, W. Wei, K. O'Neill, J. Stolarov, H. Tu, K. Chang, Y. Han, L. Rodgers, M. Riggs, J. Brodsky, J. Douglas, J. Troge, B. O'Conner, T. Chen, A. Buglino, A. Solanki, A. Triger

Research in the our lab is divided into three efforts. The first and oldest part is the study of Ras signal transduction pathways. Mutant Ras were the first human oncogenes discovered, and homologs of the Ras genes are found throughout eukaryotic evolution. Our original and major objective was to understand the targets of these small GTPases, but now that we have identified the main Ras target in the budding yeast *Saccharomyces cerevisiae* (Toda et al., *Cell 40:* 27 [1985]), two targets in the fission yeast *Schizosaccharomyces pombe* (Wang et al., *Mol. Cell. Biol. 11:* 3554 [1991]; Van Aelst et al., *Proc. Natl. Acad. Sci. 90:* 6213 [1993]; Chang et al., *Cell 79:* 131 [1994]), and multiple targets in mammalian cells (Van Aelst et al., *Mol. Cell. Biol. 11:* 3554 [1993]; *Cold Spring Harbor Symp. Quant. Biol. 59:* 181 [1995]; White et al., *Cell 80:* 533 [1995]; *J. Biol. Chem. 271:* 16439 [1996]) including the Raf protein kinase, our objective has been to understand how the Ras pathways are organized.

The second part is the application of genomic analysis to the discovery of mutations underlying human pathology, with a special emphasis on cancer. This section is an outgrowth of RDA (representational difference analysis) that was developed here in collaboration with Nikolai Lisitsyn (Lisitsyn et al., *Science 259:* 946 [1993]), now at the University of Pennsylvania School of Medicine. Several novel genetic lesions have been discovered in breast cancer, and one new tumor suppressor gene identified in collaboration with the laboratory of Ramon Parsons at Columbia University College of Physicians & Surgeons (Li et al. 1997).

We have initiated similar efforts to identify genetic lesions in infants with de novo mutations causing a variety of syndromes.

The third part is the exploration of the utility of encoded combinatorial synthesis for the discovery of biologically active molecules. This research is an outgrowth of the development of this methodology in collaboration with Clark Still, at the Department of Chemistry, Columbia University (Ohlmeyer et al., *Proc. Natl. Acad. Sci. 90:* 10922 [1993]). In collaboration with Peter Nestler (CSHL), formerly of the Still lab, we are determining whether we can discover branched peptidic molecules with high affinities for

specific peptides and specific regions of proteins. The longer-term objective is to find low-molecular-weight agonists and antagonists of signal transduction pathways.

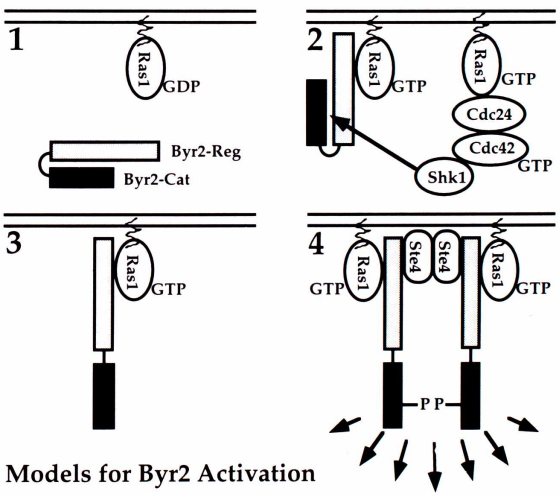
Ras Signaling

K. O'Neill, J. Stolarov, H. Tu, K. Chang

In the budding yeast *S. cerevisiae*, Ras proteins modulate adenylyl cyclase, and Ras is in turn modulated by a guanine nucleotide exchange catalyst, Cdc25 (Broek et al., *Cell 48:* 789 [1987]; Powers et al., *Mol. Cell. Biol. 9:* 390 [1989]), and proteins that accelerate Ras GTP hydrolysis, the Ira proteins (Tanaka et al., *Mol. Cell. Biol. 9:* 757 [1989]). Three clear problems remain: How are the Iras and Cdc25 modulated; what other protein(s) is modulated by Ras; and how does Ras modulate adenylyl cyclase? We are focusing on the latter problem for now. In particular, we have been investigating the role of Cap, an adenylyl-cyclase-associated protein (Field et al., *Cell 61:* 319 [1990]), that is required for the activation of adenylyl cyclase by Ras in vivo but not, apparently, for in vitro reconstituted systems (Field et al., *Cell 61:* 319 [1990]; Fedor-Chaiken et al., *Cell 61:* 329 [1990]; Wang et al., *Mol. Cell. Biol. 12:* 4937 [1997]).

Cap is a bifunctional protein that has been fairly well conserved in evolution (Gerst et al., *Mol. Cell. Biol. 11:* 1248 [1991]; Kawamukai et al., *Mol. Cell. Biol. 3:* 167 [1992]; Gieselmann and Mann, *FEBS Lett. 298:* 149 [1992]). The carboxyl terminus is required for cytoskeletal function and binds actin, whereas the amino terminus, required for adenylyl cyclase function, binds cyclase. We discovered that the amino terminus of the mammalian Cap binds talin, a central cytoskeletal component, and that the amino terminus of yeast Cap also binds a talin homolog, Sla2. We find that these interactions, and the Sla2 protein itself, are required for yeast cells to be responsive to activated Ras. We speculate that these interactions (adenylyl cyclase with Cap, and Cap with Sla2) may be required to facilitate the interaction between adenylyl cyclase and Ras but have not ruled out other mechanisms. (For example, we would not exclude the possibility that Ras, in its activated state, facilitates the interaction of adenylyl cylase with these components.) In any event, these interactions may serve as a mechanism by which the assembly of cytoskeletal components control the assembly of signal transduction components and may serve as a model for the anchorage-dependent control of cell proliferation, a fundamental property of normal mammalian cells that is lost upon their neoplastic transformation.

In the fission yeast *S. pombe*, Ras is required for normal sexual differentiation and cell shape (Fukui and Kaziro, *EMBO J. 4:* 687 [1985]). The target for the latter function is probably Scd1 (Chang et al., *Cell 79:* 131 [1994]) and for the former both Scd1 and the Byr2 protein kinase (Chang et al., *Cell 79:* 131 [1994]; Wang et al., *Mol. Cell. Biol. 11:* 3554 [1991]). Byr2 is a member of the *S. pombe* MAP kinase module, that conserved triad of protein kinases that is a repeating refrain in so many eukaryotic signaling pathways. Byr2 is the functional homolog of the mammalian Raf protein kinase, and its substrate is Byr1, the functional and structural homolog of mammalian MEK. We have recently been focusing on the mechanism of the regulation of Byr2, as an ideal model system for understanding the control of MAP kinase modules in general, and have made rapid progress.



Models for Byr2 Activation

FIGURE 1 We have assembled a model for the activation of Byr2. This model is consistent with our data but is neither complete nor necessarily correct. Byr2 recognizes GTP-bound Ras1, associated with membrane. In this cellular region and through this association, Byr2 encounters other components necessary for its activation, including Shk1, also found in this region through its association with Ras (Chang et al., *Cell 79:* 131 [1994]; Marcus et al., *Proc. Natl. Acad. Sci. 92:* 6180 [1995]). Upon encountering an activated form of Shk1, Byr2 assumes an open configuration. In the presence of Ste4, which is a leucine zipper protein capable of homodimerization, the open configuration of Byr2 dimerizes, leading to its autophosphorylation and activation.

Byr2 has an amino-terminal regulatory domain and a carboxy-terminal catalytic domain. The amino-terminal domain has a binding site for Ras, Ste4 (a leucine zipper protein required for sexual function), and the catalytic domain. These binding sites have now been defined, and mutants in each have been obtained. Cells containing a mutant Byr2 unable to bind Ste4 act as though the Ste4 protein is not present; cells containing a mutant Byr2 unable to bind Ras act as though Ras-deficient; and cells containing a mutant Byr2 with impaired catalytic/regulatory interaction act as though they have a constitutively activated Byr2 kinase. These studies confirm that Ras and Ste4 are required for Byr2 function and suggest that the regulatory domain acts as an inhibitor for the catalytic domain.

Using the two-hybrid system of Fields and Song (*Nature 340:* 245 [1989]), we have been able to demonstrate that mutations which disrupt regulatory/catalytic interaction actually open up the configuration of Byr2 so that it is able to bind its substrate Byr1. Using this as an assay for the open configuration, we have now shown that the Shk1 protein kinase (Marcus et al., *Proc. Natl. Acad. Sci. 92:* 6180 [1995]), the *S. pombe* homolog of *S. cerevisiae* Ste20, also required for sexual differentiation, can induce this confirmational change, presumably by phosphorylating Byr2. Since Ste20 bears the same genetic relationship to the *S. cerevisiae* sexual differentiation MAP kinase module as does Shk1 in *S. pombe*, and these kinases are structurally conserved in evolution, this control mechanism may represent a conserved feature in the regulation of these modules. Figure 1 illustrates one possible model that incorporates our present understandings in a consistent manner.

Genomic Analysis

C. Yen, M. Hamaguchi, M. Nakamura,
D. Esposito, R. Lucito, Y. Han

We have been applying RDA to the analysis of human breast cancer, seeking to find loci that undergo either gene copy amplification or homozygous deletion. This work has been performed in collaboration with Larry Norton at the Memorial Sloan Kettering Cancer Institute, Scott Power's laboratory at the Amplicon Corporation, the laboratory of Ramon Parsons at Columbia University, and Richard McCombie's DNA sequencing group here at the Laboratory.

A number of procedural and technical issues had to be addressed or learned in order to conduct this work properly, involving the procurement of tissue, sorting of cancers by nuclear ploidy, performing RDA on minute amounts of DNA, verification of probe status in the absence of genomic Southern blotting due to limiting material, developing the infrastructure for chromosomal mapping, screening YAC and BAC libraries, establishing banks of cell lines and xenografts, "immortalizing" DNAs through representations, exon trapping, automated sequencing, loss of heterozygosity mapping, informatics for massive data storage and retrieval, and so on. We are still in the midst of the technical developments to accompany and fully exploit the power of RDA and the enormous bank of fixed specimens that have been archived at various medical centers. What we have learned can be applied to other cancer types as well.

The easier part has been to search for amplified loci. Nearly two dozen distinct loci have been identified that become amplified in human breast cancer. In aggregate, about 95% of aneuploid tumors are amplified at at least one of these loci, and many tumors contain multiple amplified loci. Only a few of these loci contain known oncogenes, such as *erb-2*, c-*myc* and cyclin D, so the majority should contain new genes that become activated and contribute to breast cancer. Of particular interest in this regard, we have found by careful analysis that amplification near several loci, such as *erb-2* and c-*myc*, probably constitutes multiple and distinctly separate sites of amplification, suggesting the presence of nearby genes whose amplification may be functionally linked. It is our intention to define the genes that are overexpressed from these loci to help us narrow the field of possible breast cancer oncogene targets.

We have also identified more than a half dozen loci that appear to undergo homozygous loss. Such loci are likely to contain tumor suppressor genes. Among the loci found in this way was the locus for p15 and p16. In principle, because homozygous loss tends to occur over small chromosomal regions, the finding of the suspected tumor suppressor gene is greatly facilitated. In fact, the identification of Brca-2 was facilitated by RDA, although not by us (Schutte et al., *Proc. Natl. Acad. Sci. 92:* 5950 [1995]). In collaboration with Ramon Parsons at Columbia, we determined that the vast majority of loci we have identified by RDA in breast cancer are centers for loss of heterozygosity, consistent with the idea that they contain major new tumor suppressors.

One of the loci we identified and mapped to

chromosome 10q23 was further analyzed by our collaborator Ramon Parsons, who identified a gene, we now call Pten, by exon trapping (Li et al. 1997). Subsequent analysis confirmed that the coding region for this protein is frequently mutated in advanced breast, brain, and prostate cancers. Five considerations make this an especially exciting discovery.

First, the protein has homology with protein phosphatases. Since protein kinases were among the first oncogenes identified, it is highly gratifying to finally find an enzyme that removes phosphate groups from proteins as a tumor suppressor. Second, Pten has homology with cytoskeletal proteins, suggesting it may play a part in cell-cell or cell-matrix interactions. For example, it might play a part in invasiveness, metastasis, or "ancorage-independent" growth. Third, Pten appears to be involved in many cancers, and with high frequency, suggesting it has an important and common role in cancer etiology. Fourth, loss of heterozygosity at the site of Pten appears to correlate with the transition from benign to malignant growths, suggesting it plays a part in an especially critical step in neoplasia. Fifth, work from the Parson's lab indicates that hemizygous loss of Pten appears to be the cause of a rare inherited predisposition to neoplasia called Cowden's disease.

The methodology we have developed for the analysis of mutation in cancers can also be applied to disorders in humans caused by de novo mutation within the germ line. In essence, we can look for genetic alterations present in a child that are not found in either parent. A large number of rare but profound disorders have been recognized by medical geneticists and attributed to de novo mutation, but there may also be cases that underlie frequent but more subtle disorders, such as schizophrenia and autism, that have not generally been considered to arise this way. We are in the process of developing the tools to attack such problems. If we are successful, our methods may provide an alternate and perhaps more effective manner to approach certain human disease than the widely used but cumbersome and costly methods of segregation analysis.

synthesis (Furka et al., *Int. J. Pept. Prot. Res. 37:* 487 [1991]; Lam et al., *Nature 354:* 82 [1991]). Utilizing encoding, investigators can further identify active molecules and rapidly learn their structure. The foundation for this was established in 1993 by our collaboration with Clark Still at Columbia University (Ohlmeyer et al., *Proc. Natl. Acad. Sci. 90:* 10922 [1993]). In collaboration with Peter Nestler here at the Laboratory, we are using this methodology to study the combining power of branched peptidic molecules. In particular, we are searching for branched peptides that are capable of recognizing linear peptides and specific peptide epitopes in proteins. If successful, such approaches should enable us to find affinity reagents for protein molecules to detect, enhance, or diminish their function. We have found some promising molecules that appear to interact with the CAAX motif of Ras and inhibit the farnesylation of Ras. These leads and ideas will be put to more rigorous testing.

PUBLICATIONS

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