

MAMMALIAN CELL GENETICS

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Our laboratory has been involved in three distinct activities: studying aspects of signal transduction; developing methods for the discovery of small molecules with desirable chemical properties; and applying a new system for genomic analysis for the discovery of genetic lesions in human cancers. This year has witnessed important progress emerging from four

breakthroughs that were described in last year's Annual Report: the discovery of RAS targets; the definition of a conserved protein kinase signal transduction cascade; the development of a molecular indexing system for complex combinatorial chemical synthesis; and the invention of a powerful method for comparative genomic analysis. We have used heavily the

two-hybrid genetic system developed by Fields and Song (*Nature* 340: 245 [1989]) that allows the detection of complex formation between any two proteins expressed in the yeast *Saccharomyces cerevisiae*.

RAS in Yeasts

R. Ballester, H.-P. Xu, E. Chang, L. Van Aelst, S. Marcus, V. Jung, K. Sen, A. Polverino, M. Barr

RAS homologs in the budding yeast, *S. cerevisiae*, and the evolutionary distant fission yeast, *Schizosaccharomyces pombe*, have highly diverged functions. In the budding yeast, RAS proteins are essential for growth. They regulate adenyl cyclase and probably another as yet unidentified target. In the fission yeast, ras1 is required for sexual differentiation and pheromone-inducible responses as well as an elongated cell shape. As we previously described, one of the target effectors for ras1 in *S. pombe* is the byr2 protein kinase required for mediating pheromone responses. Complex formation between RAS and byr2 was detected using the two-hybrid genetic systems of Fields and Song (Van Aelst et al. 1993). Mutations in the effector loop of RAS abrogate this interaction. The byr2 protein kinase is part of the conserved MAP kinase module, described in last year's Annual Report and in more detail below. Additional regulators of byr2 have been found (see later section).

byr2 does not mediate the effects of ras1 on cell morphology (Wang et al., *Mol. Cell. Biol.* 11: 3554 [1991]), and hence ras1 must have additional mediators. Mutant hunts unearthed two, scd1 and scd2, both required for elongated cell shape and conjugation. scd1 and scd2 are homologs of *S. cerevisiae* CDC24 and BEM1, respectively, each involved in morphogenic events in that organism, as is RSR1, a RAS homolog (Miyamoto et al., *Gene* 54: 125 [1987]; Bender and Pringle, *Proc. Natl. Acad. Sci.* 86: 9976 [1989]; Miyamoto et al., *Biochem. Biophys. Res. Commun.* 181: 604 [1991]; Chenevert et al., *Nature* 356: 77 [1992]). In the budding yeast, the CDC42 protein, a member of the RHO family of small GTPases, is also functionally linked with CDC24 (Bender and Pringle, *Proc. Natl. Acad. Sci.* 86: 9976 [1989]; Zheng et al., *J. Biol. Chem.* 269: 2369 [1994]). Likewise, we found that the *S. pombe* CDC42 homolog, cdc42sp, is functionally linked

with ras1, scd1, and scd2. Genetic studies led to the conclusion that ras1 and scd2 act in concert upon scd1, which interacts with cdc42sp. Biochemical studies indicate that scd1 and scd2 interact directly. Studies with the two-hybrid system confirm this and further indicate that scd1, scd2, cdc42sp, and ras1 can all form a complex: scd2 binds cdc42sp; ras1, in its GTP-bound state, induces scd1 to bind cdc42sp; and scd2 induces scd1 to bind ras1. It seems likely that the morphogenic module we have described in fission yeast will have been conserved in evolution, perhaps even in metazoans.

We have continued our studies of the new interfering mutants of RAS found in *S. pombe*, first mentioned in last year's Annual Report. In collaboration with Dan Broek's group at the University of Southern California, we have confirmed that these mutants (ras1^{Tyr62} and HRAS^{Tyr57}) block nucleotide exchange function by binding virtually irreversibly to exchange factors of the CDC25 type. ras1^{Tyr62} blocks all ras1 function, beyond that dependent on the ste6 exchange factor, which is required for conjugation but not cell shape (Jung et al. 1994). We therefore infer that ras1^{Tyr62} blocks other regulatory molecules involved in morphogenesis. One candidate protein has been identified through two hybrid interactions and is now being characterized genetically.

RAS in Mammalian Cells

L. Van Aelst, M. White, A. Polverino

As reported last year, we detected complex formation between RAS and RAF in the yeast two-hybrid genetic system (Van Aelst et al. 1993). RAF is itself an oncoprotein (Rapp et al., *Proc. Natl. Acad. Sci.* 80: 4128 [1983]), a protein kinase capable of activating MAP kinase through a MAP kinase kinase (Dent et al., *Science* 257: 1404 [1992]; Howe et al., *Cell* 71: 335 [1992]; Kyriakis et al., *Nature* 358: 417 [1992]), and able to bypass dominant negative mutants of RAS to transform mammalian cells (Bonner et al., *Nucleic Acids Res.* 14: 1009 [1986]; Cia et al., *Mol. Cell. Biol.* 10: 5314 [1990]). Genetic evidence for the direct interaction of RAS and RAF comes from mutational studies. We first made a mutagenized library of RAS and then screened for mutations of RAS that could not complex with RAF, yet still formed complexes with byr2. One, HRAS^{Gly37}, was chosen for

further study. We reasoned that if RAS and RAF interact directly, we could find mutants of RAF that restored interaction with HRAS^{Gly37}. Several mutants were found. The compensating mutations in RAF all mapped to the conserved CR2 domain in the RAF regulatory domain. These studies give evidence of direct physical contact between the effector loop of RAS and this domain of RAF.

The mutant HRAS^{Gly37} was activated to an HRAS^{Val12,Gly37} double mutant. The double mutant failed to induce transformed foci in NIH-3T3 cells. However, when the RAFs with mutations in the CR2 domain were cotransfected with HRAS^{Val12,Gly37}, transformed foci were readily observed. The mutant RAFs were unable to induce foci on their own but synergized with the mutant HRAS^{Gly37} greater than tenfold better than did wild-type RAF. These are the first studies to show that interaction between RAS and RAF is necessary for transformation.

Evidence has been presented in previous Annual Reports, and in this one as well, that RAS has multiple target effectors in yeasts. We have long speculated, therefore, that RAS would have multiple target effectors in mammalian cells. To test this idea, a second type of mutant RAS was sought, one that bound RAF but failed to bind byr2. We reasoned that such a mutant might fail to bind other effectors essential for RAS transformation of mammalian cells. Such a mutant, HRAS^{Ser35}, was found. As expected from its failure to interact with byr2 in the two-hybrid assay, HRAS^{Ser35} fails to replace *ras1* function in *S. pombe*. This in itself is rather astonishing since serine is a conservative substitution for threonine, the wild-type residue at position 35. Gratifyingly, HRAS^{Val12,Ser35} fails to transform NIH-3T3 cells but efficiently induces transformed foci when cotransfected with HRAS^{Val12,Gly37}. This result argues very strongly that HRAS transforms cells by interacting with two different target effectors.

Using the two-hybrid system again, we have screened mammalian cDNAs for those encoding proteins capable of interacting with RAS. Among those so found were cDNAs encoding members of the RAF family of protein kinases, a guanine nucleotide exchange protein for RAL (Albright et al., *EMBO J.* 12: 339 [1993]), and a number of other as yet unidentified cDNAs. We should be able to determine which of these candidates are actually required for the transforming potential of RAS by utilizing the type of approach we have already taken for RAF.

Regulation of the Conserved MAP Kinase Module

H.-P. Xu, L. Van Aelst, S. Marcus, A. Polverino, M. Barr

We described in last year's Annual Report the realization that a cascade of protein kinases, which we call the MAP kinase module, was conserved in evolution. The first examples come from the pheromone signaling pathways in yeasts: in *S. cerevisiae*, STE11, STE7, and FUS3; in *S. pombe*, byr2, byr1, and spk1 (Neiman et al. 1993). These protein kinases are now known to have homologs in vertebrates: FUS3 and spk1 are homologous to the mitogen-activated protein kinases (MAPKs or ERKs); STE7 and byr1, to the MAP kinase kinases (MEKs); and STE11 and byr2, to MEK kinases (MEKKs) (Neiman et al. 1993). In addition, this kinase cascade motif is repeated multiple times within other signaling pathways within yeasts (Errede and Leven, *Curr. Opin. Cell Biol.* 5: 254 [1993]), and one has to presume that this module is a "good approximate solution" to some recurring problem of adaptation. Our interest in this cascade began when we and other investigators discovered evidence for the involvement of RAS in its regulation in *S. pombe* and in mammalian cells (see Nieman et al. 1993; Van Aelst et al. 1993). We have been interested in the control and integration of the MAP kinase module, both in *S. pombe* and in *S. cerevisiae* (even though, in the latter, RAS does not appear to be involved).

byr2 and byr1 are required for the basal level expression of the pheromone receptor gene *mam2* (Xu et al. 1994). Interestingly, spk1 is not required for this, either because the kinase cascade branches prior to activation of spk1 or because spk1 has redundant kinase function. The latter is a distinct possibility, given that multiple genes encoding MAP kinase homologs are found in other organisms, but spk1 is essential for many aspects of sexual differentiation. We conclude that it is probable that byr1 has functions other than the activation of spk1.

The *S. pombe* kinase module appears to require both *ras1* and the pheromone sensing apparatus (Neiman et al. 1993). Disruption of *ras1* or *gpa1*, encoding the α subunit of the heterotrimer mediating receptor occupancy, abolishes basal level expression of *mam2* in *h⁹⁰* (homothallic) *S. pombe*. Epistatic studies indicate that *ras1* and *gpa1* act in concert upon *mam2* expression. High-level expression of *gpa1* can induce *mam2* even in the absence of *ras1*;

yet disruption of *ras1* produces a depression of *mam2* expression even in the absence of *gpa1* in heterothallic *S. pombe* strains. From these results, we infer that the MAP kinase module is under independent but cooperative regulation by *ras1* and the upstream mating pheromone response pathway (Xu et al. 1994).

We have identified a new component of the *S. pombe* MAP kinase module: *ste4*. *ste4* was found in a two-hybrid screen of a library of *S. pombe* cDNAs encoding proteins capable of binding to *byr2*. It was previously known that *ste4* was essential for conjugation and encoded a leucine zipper protein (Okazaki et al., *Nucleic Acids Res.* 19: 7043 [1991]). Using the two-hybrid system, we demonstrated that *ste4* indeed forms dimers, binds to the regulatory domain of *byr2*, and causes *byr2* to dimerize. Hence, we propose that *ste4* has a role in activating *byr2* by inducing autophosphorylation through dimerization. We suspect that a *ste4* homolog may exist in *S. cerevisiae*, since we find that STE11 dimerizes in that organism. Although *ras1* does not bind directly to *ste4*, overexpression of the *byr2* regulatory domain induces complex formation between *ras1* and *ste4* detectable with the two-hybrid system. Hence, *ste4* and *ras1* occupy separate sites on the regulatory domain of *byr2*. We are investigating whether this binding is cooperative. If so, this may partly explain the regulatory function of *ras1*.

One of the proteins of *S. cerevisiae* that participates in pheromone signaling is STE5. Genetic studies have placed STE5 upstream of the MAP kinase module (Hasson et al., *Mol. Cell. Biol.* 14: 1054 [1994]). Using the two-hybrid system, we demonstrated that STE5 binds all three of the protein kinases that comprise the MAP kinase module, STE11, STE7, and FUS3. The binding between STE11 and STE7 is not apparent unless STE5 is present. Hence, we have proposed that STE5 is a scaffolding protein that promotes interactions between the members of the cascade. In addition to possibly activating the pathway thereby, STE5 may serve to "insulate" the pathway from cross talk with other MAP kinase modules within the same cell that are dedicated to other signal transduction functions. We feel it likely that scaffolding proteins like STE5 may exist for other MAP kinase cascades given that we cannot detect interactions between MEKK and MEK and between *byr2* and *byr1* with the two-hybrid system.

Finally, we have found that the *S. cerevisiae* STE20 protein kinase (Leberer et al., *EMBO J.* 11:

4815 [1993]) may be a conserved element of the MAP kinase cascade. STE20 functions weakly in *S. pombe* as a suppressor of *ras1*^{null} cells. Moreover, STE20 protein can induce the phosphorylation of MAP kinase in *Xenopus* oocyte cell-free extracts. We therefore sought homologs of STE20 in *S. pombe* and mammalian cells. In collaboration with Dr. M. Cobb's laboratory at the University of Texas Southwestern Medical School, we identified several clear STE20 homologs in both fission yeast and mammalian cells. Their properties are currently under investigation.

Combinatorial Chemistry

R. Swanson

As we began to describe in last year's Annual Report, we have collaborated with Clark Still and Michael Ohlmeyer of the Department of Chemistry at Columbia University to develop a general method for the molecular tagging of solid-phase synthesis beads used in "split" synthesis to generate chemical libraries of great complexity. In split synthesis, solid-phase synthesis beads are used as substrates to build molecules by iterative synthetic steps (Furka et al., *Int. J. Pept. Protein Res.* 37: 487 [1991]; Lam et al., *Nature* 354: 82 [1991]). By consecutive batching and segregating into different reaction vessels, huge libraries of diverse compounds are created, with each bead bearing a single predominant moiety according to its particular reaction history. We have devised a method of using tags to record the reaction history of each bead (Ohlmeyer et al. 1993). After beads containing a desirable moiety are identified, the composition of the moiety is inferred by reading the reaction history of the bead encoded by the tags. The tags are read, after detachment from the bead, by gas chromatography and electron capture. We have utilized this system to tag a combinatorial library of oligopeptides of greater than 10⁵ complexity and demonstrated the correct identification of peptide epitopes reactive with a monoclonal antibody. Many conserved amino acid substitutions were allowed within the epitope, but to our surprise, we found that certain very conservative substitutions reduced the binding of the epitope to the antibody by several orders of magnitude. The tagging method is perfectly general and can be applied to libraries of organic molecules created on beads by virtually any synthetic

route. In this vein, Dr. Still and co-workers have used the method to tag libraries of nonlinear peptidic moieties containing structures that would be difficult to elucidate by other means (Borchardt and Still, *J. Am. Chem. Soc.* 116: 373 [1994]). In collaboration with other investigators, we are testing the application of this method for the discovery of chemical agonists and antagonists of components of signal transduction pathways.

Comparative Genomic Analysis

N. Lisitsyn, H.-P. Xu

We have developed a method for genomic difference analysis that we call representational difference analysis, or RDA, and are applying the method to clone probes that define genetic lesions occurring in cancer cells or are linked to inherited disease loci. This method allows the detection and cloning of specific DNA sequences ("difference" sequences) present in one population of DNA molecules ("tester") that are not equivalently present in a highly related second population of DNA molecules ("driver"). The RDA procedure is composed of two parts. In the first part, we make a reductive sampling of the two DNA populations that are to be compared, thereby deriving two new populations of DNA molecules, called representations. The sequence complexity of the representation is considerably lower than that of the DNA population from which it derived. In the second part, we utilize "sequence subtraction" and "kinetic enrichment" to clone the difference sequences. A detailed description of this powerful method was presented in last year's Annual Report and recently published in *Science* (Lisitsyn et al. 1993). At its core, our method detects small restriction fragments in one DNA population that are not present in another.

The method can be used to find probes linked to inherited disease in animals that can be bred (Lisitsyn et al. 1994). In collaboration with Eric Lander's group at MIT, we designed two protocols that exploit the ability of RDA to detect polymorphic differences between individuals or groups of individuals. The first protocol requires the use of congenic strains, in which a strain carrying an inherited disease is repeatedly back-crossed into a polymorphic strain. The second protocol, which is more general, merely requires the generation of F₂ progeny from the cross

of a disease-carrying strain to one that is disease-free. It is possible to generate thereby polymorphic probes linked to the disease locus for any experimental animal that can be bred. Preexisting genetic maps are not needed. Similar strategies may be applicable to humans.

The method can be applied to define the lesions in cancer cells in either of two ways. Using DNA from normal cells as tester and DNA from cancer cells as driver, we can isolate probes that are homozygously lost in cancers or that detect restriction endonuclease polymorphisms that are heterozygously lost in cancers. To be applied in this way, the cancer and normal must be from the same individual and the cancer DNA (driver) must be nearly free of contaminating normal DNA (tester). As starting material, we have used paired normal and cancer cell lines, derived in the main from renal cell carcinomas or colon cancers. We have also successfully implemented the protocol on cancer biopsies using nuclei fractionated by flow cytometry into aneuploid (cancer) and diploid (normal) fractions. In this manner, we have identified seven loci that undergo homozygous deletion in at least one cancer sample. Two of these loci were observed to undergo very frequent loss. Presumably, these are the sites of genes the loss of which contribute to the initiation or progression of cancer.

When cancer DNA is taken as tester, probes arising from several types of lesions can, in principle, be detected: point mutations, genetic rearrangements, gene amplifications, and pathogens. In practice, we have observed point mutations and amplifications. We can calculate that on the order of 1/5000 point mutations should create a detectable tester fragment. The presence of such fragments may indicate an increased frequency of point mutation in the cancer. The application of RDA to the detection of amplifications may be of substantial value, since amplification is a useful prognostic indicator for the disease. We have not yet documented examples of RDA probes that detect the other types of lesions.

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