GENETICS OF CELL PROLIFERATION

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During the past year, our laboratory has continued its investigations into oncogenes and growth control. The central focus of the laboratory has been on the *RAS* genes, which may play a critical role in a large portion of human cancers. We mainly have been studying the function of the *RAS* genes of the model eukaryotic organism, *Saccharomyces cerevisiae*. As a consequence, we also have been examining growth control in this organism. In addition to these studies, we have been studying two new human oncogenes, the *MAS* and *ROS* oncogenes.

Function of the *RAS* Oncogenes

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Three basic questions about the *RAS* gene products remain unanswered: What controls their activity? What biochemical activities do they control? How does activation of the *RAS*-controlled pathways lead to tumorigenicity? We have explored these questions by using as a model system the *S. cerevisiae RAS*

genes, which encode proteins that regulate yeast adenylate cyclase. This is to date the only *RAS*-coupled effector system known. Although *RAS* proteins probably do not control adenylate cyclase in vertebrates, this yeast system can be used to yield insights into *RAS* function.

Four points of significance have emerged from our yeast *RAS* studies during the past year. First, we discovered that the product of the *CDC25* gene appears to regulate the yeast *RAS1* and *RAS2* proteins, which was the first evidence that *RAS* activity is controlled by other proteins (Broek et al., *Cell 48:* 789 [1987]). *RAS* proteins in cells lacking the *CDC25* gene act as though they are not in an active state: They fail to stimulate adenylate cyclase, although they can be induced to do so by incubation with GTP. Cells containing a $RAS2^{val19}$ gene, a mutant form of *RAS2* that is "activated," are virtually oblivious to the absence of *CDC25*, which suggests that *CDC25* may be involved in maintaining *RAS* proteins in the GTP-bound state. We do not yet know the mechanism of this control, nor whether an analogous mechanism exists in mammals. The normal Ha-*ras* protein when expressed in yeast does not require the *CDC25* gene product to function, but we do have preliminary genetic evidence that the *CDC25* gene product can interact with the mammalian Ha-*ras* protein. A mutant form of *RAS2* can block *CDC25* function, and the analogous Ha-*ras* mutation appears able to do likewise. These results may indicate a direct interaction between the protein products of these genes.

Second, we have developed a novel genetic/biochemical method for purifying the yeast adenylate cyclase complex from cells (Field et al., *Mol. Cell. Biol.* [1988] in press). The method entails the construction and expression of the desired gene product as an epitope fusion protein which is then purified by immunoaffinity chromatography and eluted with a synthetic peptide epitope. This method yields a several hundredfold purification of adenylate cyclase from yeast. The purified adenylate complex is about 600 kD and probably contains two molecules of the 210-kD adenylate cyclase and some number of a 70-kD component. The complex is fully responsive to *RAS* proteins and requires the continued presence of *RAS* proteins to remain active. Analysis of the responsiveness of the purified complex demonstrates that *RAS* proteins have an almost absolute requirement for GTP in order to activate their effector. We are currently investigating the role of the 70-kD component of the complex in *RAS* responsiveness.

Third, we have evidence from genetic experiments that there must be a second *RAS* effector system in yeast (Toda et al., in *Oncogenes and cancer,* Japan Scientific Societies Press, Tokyo, Japan [1987]; Toda et al., *Cell 50:* 277 [1987]). Two observations support this idea. First, although the major effects of *RAS* in yeast are mediated through its effects on adenylate cyclase, deletion of the adenylate cyclase gene is not uniformly lethal in haploid spores, whereas deletion of both *RAS1* and *RAS2* genes is. Second, the slow growth phenotype of adenylate cyclase deletion mutants is fully suppressed by high-copy plasmids expressing a variety of protein kinases, although these same plasmids suppress deletion mutants in *RAS* poorly and in a temperature-sensitive manner. We have demonstrated that the alternate function of *RAS* is also dependent on *CDC25* function. We are currently testing whether human Ha-*ras* can supply this function in yeast and are trying by genetic means to isolate genes encoding components of the alternate *RAS* pathway. These results raise important new questions. Do *RAS* proteins have more than one function in mammalian cells? If so, which are important in malignant transformation?

Fourth, we have discovered "interfering" mutants of the *RAS* genes. These are mutants that either block normal *RAS* function or block "activated" or "oncogenic" *RAS* in a dominant way. We first discovered these mutants during suppressor studies of yeast. A dominant temperature-sensitive *RAS2* mutant was isolated that blocks the function of *CDC25*. A similar mutation in Ha-*ras* also appears to block *CDC25* function, and we are currently testing the ability of this Ha-*ras* mutant to block Ha-*ras* function in mammalian cells. Upon realizing the potential value of such dominant interfering mutants, we deliberately sought mutants of Ha-*ras* in mutagenized pools of Ha-*ras* genes that could block activated $RAS2^{val19}$ function. These were indeed found, and they may be useful tools for exploring *RAS* function, particularly in mammalian cells. Preliminary analysis indicates that these mutants compete for at least one of the *RAS* effectors.

Growth Regulation in *S. cerevisiae*

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Our attempts to understand *RAS* function in yeast have led us to explore in great detail the cAMP-

signaling system and its role in growth regulation. First, we isolated *BCY1*, the gene encoding the regulatory subunit of the cAMP kinases (Toda et al., *Mol. Cell. Biol. 7:* 1371 [1987]). We also isolated three genes, *TPK1*, *TPK2*, and *TPK3*, that encode the catalytic subunits (Toda et al., *Cell 50:* 277 [1987]). Cells lacking *BCY1* display a cluster of phenotypes, including a failure to sporulate, accumulate glycogen, resist starvation, or become heat-shock-resistant. These are the responses of a normal cell to nutrient limitation and can be thought of as the yeast equivalent of entering the G_0 state of the cell cycle. These defective phenotypes appear to be mediated by the unbridled action of the catalytic subunits of the cAMP-dependent protein kinases, since mutant, attenuated *TPK* genes can reverse these phenotypes. These results suggest that the only function of the regulatory subunit is to control the activity of the cAMP-dependent protein kinases. Moreover, cells lacking *BCY1* but containing attenuated *TPK* genes can dynamically regulate responses to nutritional limitation in an apparently normal manner, entering and leaving the G_0 state appropriately (Cameron et al., *Cell* [1988] in press). In such cells, it is even possible to delete the entire ensemble of genes encoding the cAMP-generating and -regulatory machinery (*CDC25*, *RAS*, and *CYR1*) and still retain normal growth regulation. These results support the idea that, although the cAMP signaling system may regulate entry and exit from the G_0 state, there must be cAMP-independent mechanisms for regulating this growth pathway. The "simple" eukaryote, *S. cerevisiae*, may resemble mammalian cells in the complexity of its overlapping signaling pathways.

In the course of our work, we also isolated the cAMP phosphodiesterase genes of yeast. There are two, *PDE1* and *PDE2*, that encode proteins of low and high affinities for cAMP, respectively (Nikawa et al., *Mol. Cell. Biol. 7:* 3629 [1987]). Deletion of these genes only modestly elevates cAMP levels in yeast, a surprising result that suggested the existence of feedback control of cAMP levels in that organism. This idea received dramatic confirmation when we measured cAMP levels in yeast strains lacking *PDE* genes but containing the *RAS2*^{val19} mutation (Nikawa et al., *Genes Dev. 1:* 931 [1987]). Such cells had cAMP levels that were nearly a thousandfold elevated when compared to normal cells. Cells containing *RAS2*^{val19} but possessing *PDE* genes have only modestly elevated cAMP levels. These results indicate that there is feedback control of cAMP levels, that normal *RAS* proteins are part of that feedback pathway, and that the mutant form of *RAS* is not responsive to that feedback. The source of the feedback is almost certainly the activity of the cAMP kinase itself. Cells lacking the regulatory subunit have almost unmeasurable levels of cAMP, whereas cells with attenuated catalytic subunits have dramatically elevated levels of cAMP. Mammalian cells also have feedback systems that modulate cAMP levels, and in this regard, they may resemble yeast cells. However, in neither organism are the pathways of feedback understood, and the extent of the similarity of mechanism cannot presently be assessed.

During our effort to clone the *CDC25* gene by complementation analysis, we isolated a number of yeast genes all possessing the ability to suppress the temperature-sensitive defects of a *CDC25* mutant. Among these were the *TPK* genes and a gene we called *SCH9*. Sequence analysis of *SCH9* revealed that it encodes a protein with a carboxy-terminal domain strikingly similar to that of the catalytic subunits of the cAMP-dependent kinases (Toda et al., *Genes Dev.* [1988] in press). *SCH9* also encodes an amino-terminal domain similar in size to the regulatory domains of protein kinases C and the cGMP-dependent kinases, but without sequence homology. The suppression of *CDC25* by *SCH9* suggested that *SCH9* might have functions that are redundant with the cAMP-dependent protein kinase. Further genetic analysis bears out this conclusion. High-copy plasmids containing the *SCH9* gene can suppress the effects of loss of *RAS*, *CYR1*, and *TPK* genes. Conversely, although loss of the *SCH9* gene is not lethal, it does lead to a slow-growth phenotype, which can, in turn, be suppressed by activating the cAMP pathway. These results indicate that *SCH9* might encode a protein that is a component of a cAMP-independent signaling pathway in yeast controlling growth regulation.

Mammalian Oncogenes

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We have been studying two human oncogenes, *MAS* and *ROS*, which we first isolated using a cotransfer and tumorigenicity assay (Birchmeier et al., *Cold Spring Harbor Symp. Quant. Biol. 51:* 993 [1987]). In both cases, the activation of these genes required a rearrangement occurring during gene transfer. Nevertheless, both genes are of considerable interest. We find that the *ROS* gene is expressed almost

exclusively in tumors of glial origin, but not in normal brain or in tumors of other origins (Birchmeier et al., *Proc. Natl. Acad. Sci. 84:* 9270 [1987]). We are in the midst of cloning and sequencing a full-length copy of *ROS* cDNA and have raised antibodies to its product, which is a membrane spanning tyrosine protein kinase. Sequence analysis indicates a significant homology with the *Drosophila sevenless* gene product, both in the external and internal domains. The gene product is large, perhaps 270,000 daltons, and we estimate the mRNA length to be 9 kb, of which 7 kb may be coding. The full-length product appears to be expressed in many glial tumor cells, with the exception of one glioblastoma cell line in which the *ROS* gene appears to have suffered a rearrangement and now produces a truncated product that has lost its transmembrane domain but still retains tyrosine kinase activity. Our studies suggest a possible role for *ROS* in the etiology of glial tumors, and we wish to test this possibility in an experimental model system. Our findings may provide a useful diagnostic marker for glial tumors.

The *MAS* gene represents a new class of oncogene. Its sequence indicates that it encodes a protein in the same class as the multiple membrane-spanning hormone receptors that activate G proteins (Birchmeier et al., *Cold Spring Harbor Symp. Quant Biol. 51:* 993 [1987]). It is normally expressed at high levels in the cerebral cortex and especially in the hippocampus (D. Young et al., Submitted). Thus, it is likely that *MAS* encodes a neurotransmitter receptor. We do not find it expressed in other tissues or in over 40 different human tumor cell lines we have examined. Activation of the gene during DNA transfer appears to have resulted from rearrangement of the promoter sequences, since merely forcing expression of a normal *MAS* product in NIH-3T3 cells is sufficient to transform these cells. The phenotype produced in NIH-3T3 cells by *MAS* is rather unusual (D. Young et al., Submitted). Such cells are highly tumorigenic and grow to high cell density in culture, but they appear to be morphologically normal and do not grow in agar suspension. *MAS* has an even more limited effect on other rodent cell lines. Our interest in *MAS*, as it relates to oncogenesis, is the biochemical pathway that it activates.

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