GENETICS OF CELL PROLIFERATION

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Our laboratory continues to study signal transduction, with the main focus on RAS-dependent signal transduction. In the yeast *Saccharomyces cerevisiae*, RAS proteins regulate adenylyl cyclase. The mechanism by which they do this is unknown, and this is not their only function. Since activation of adenylyl cyclase is not the function of mammalian RAS, the role of RAS in *Schizosaccharomyces pombe* has become a main effort within the group. The command of both *S. pombe* and *S. cerevisiae* systems gives us some clearer perspectives on the evolution of signal transduction pathways and provides us with very powerful tools for the identification and examination of mammalian genes encoding components of signaling pathways.

Signal Transduction in *S. cerevisiae*

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We have been studying the cellular components of *S. cerevisiae* that regulate RAS. Among these are two closely related proteins, IRA1 and IRA2 (Tanaka et al., *Mol. Cell. Biol. 9:* 757 [1989]; Tanaka et al., *Mol. Cell. Biol. 10:* 4303 [1990]). Individually, each has been shown to down-regulate RAS function. These proteins are structurally and functionally related to mammalian GAP, the RAS GTPase-stimulating protein (Tanaka et al., *Mol. Cell. Biol. 9:* 757 [1989]; Tanaka et al., *Mol. Cell. Biol. 10:* 4303 [1990]; Bal-

lester et al., *Cell 59:* 681 [1989]). Although GAP has also been shown to be capable of down-regulating RAS in both mammalian and yeast systems (Ballester et al., *Cell 59:* 681 [1989]; Zhang et al., *Nature 346:* 754 (1990]), there is some experimental evidence supporting the idea that GAP may also be an effector of RAS (Yatani et al., *Cell 61:* 769 [1990]). To test this hypothesis for IRA, both *IRA* genes were disrupted in yeast. The resulting phenotype was that expected for cells with an activated *RAS* allele: heat shock and starvation sensitivity. This phenotype was suppressible by overexpression of the genes encoding cAMP phosphodiesterases. These results suggest that the *IRA* genes do not encode effectors that link RAS to adenylyl cyclase. We cannot eliminate this hypothesis completely, however, since a third homolog of these GAP-like genes may yet be discovered in the genome of yeast, and one can imagine that each of the three might encode redundant RAS effectors. Speculations such as this, which invoke the unseen and undiscovered, will be more readily examined in the future when the complete genomic sequences of organisms favored for genetics will be known. Moreover, we have not eliminated the possibility that the *IRA* genes are required for the alternate function of RAS.

Strains lacking *IRA2* have the typical phenotype of cells with activated RAS, and we have isolated genes that when present on multicopy vectors can suppress this phenotype. Along with the expected genes, including *IRA2* itself, the *PDE* genes, encoding the cAMP phosphodiesterases, and *JUN1*, we found five other genes. These five fail to suppress the mutationally activated $RAS2^{val19}$ allele and hence probably act through the regulation of wild-type *RAS*. One of these genes encodes a carboxy-terminal truncated form of the CDC25 protein that is a RAS-nucleotide exchange catalyst (Broek et al., *Cell 48:* 789 [1987]; Powers et al., *Mol. Cell. Biol. 9:* 390 [1989]). This last result is consistent with the idea that the truncated form of CDC25 interferes with proper CDC25 function, since disruption of the genomic *CDC25* locus is known to suppress the phenotype of an *IRA* disruption. Thus, either the truncated form of CDC25 titrates a factor required for activation of wild-type CDC25 or it forms an ineffective complex with the wild-type CDC25 protein. The other four genes fall into two groups. The two genes of group I also suppress the heat-shock sensitivity of $ira1^{-}$ $ira2^{-}$ $cdc25^{-}$ strains. This result points to the existence of another mode for the regulation of RAS that is not dependent on the *IRA* genes or *CDC25*.

It is not known how RAS proteins stimulate adenylyl cyclase. Last year, we described CAP, an adenylyl-cyclase-associated protein that appears to be required for RAS to be fully capable of activating adenylyl cyclase (Field et al., *Cell 61:* 319 [1990]). Much of our effort to understand RAS/adenylyl cyclase interactions has focused on CAP, and this work is described in a separate section below.

Some time ago, Drs. Kataoka and Powers isolated a number of suppressor mutations of the $RAS2^{val19}$ phenotype (heat shock and starvation sensitivity). Among these were *supH*, or *RAM1*, required for the processing of RAS protein (Powers et al., *Cell 47:* 413 [1986]), and *supC*, encoding CAP, as discussed below (Field et al., *Cell 61:* 319 [1990]). We have recently isolated the gene corresponding to *supF*. It shows no clear homology with proteins in the databases. Complete disruption of *supF* does not completely block the phenotype of $RAS2^{val19}$ and has no effect on the phenotype of cells overexpressing the *TPK* genes, which encode the cAMP-dependent protein kinases (Toda et al., *Cell 50:* 277 [1987]). We hypothesize that *supF* encodes either a protein that facilitates RAS function (e.g., by affecting processing or localization) or a component of an alternate pathway for RAS action. These hypotheses are under study.

Signal Transduction in *S. pombe*

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We have continued and expanded an intensive study of the RAS and adenylyl cyclase signaling pathways in *S. pombe* with the hope that the *S. pombe* systems may bear greater resemblance to mammalian cells and with the expectation that knowledge of the two highly diverged yeasts will lead to a greater understanding of each. Figure 1 diagrams some of the hypothetical wiring of the *S. pombe* systems inferred from our work and the work of other investigators. The figure also embodies some speculative ideas about homologies in these systems and those of *S. cerevisiae* and mammals.

S. pombe contains an adenylyl cyclase, encoded by the *cyr1* gene, that has considerable homology with the *S. cerevisiae* enzyme (Young et al., *Proc. Natl. Acad. Sci. 86:* 7989 [1989]). In *S. pombe*, cAMP levels modulate sexual responses (Beach et

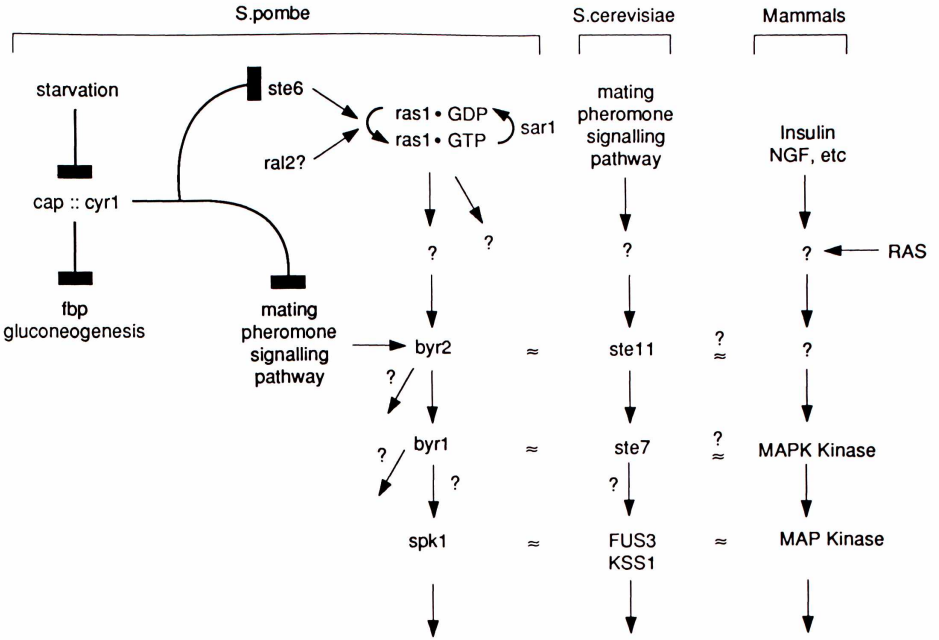

FIGURE 1 Diagram summarizing relationships among signal transduction pathways of the yeasts *S. pombe* and *S. cerevisiae* and mammalian cells. (≈) Homologies between proteins; (→) positive (activating) interactions; (*closed rectangles*) negative (inactivating) interactions; (?) proposed interactions or homologies.

al., *Curr. Genet. 10:* 297 [1985]; Kawamukai et al., *Cell Regul. 2:* 155 [1991]). Cells that overexpress cyr1 are relatively sterile, and cells that lack cyr1 conjugate prematurely. The *S. pombe* cyclase is not regulated by ras1 (Fukui et al., *Cell 44:* 329 [1986]; Nadin-Davis et al., *EMBO J. 5:* 2963 [1986]), the *S. pombe* RAS homolog, but we have inferred that cyr1 is subject to regulatory constraints similar to those of *S. cerevisiae* CYR1 (Kawamukai et al., *Cell Regul. 2:* 155 [1991]). First of all, the *S. pombe* cyr1 contains a leucine-rich repeat, a region homologous to the leucine-rich repeat of the *S. cerevisiae* CYR1 (Young et al., *Proc. Natl. Acad. Sci. 86:* 7989 [1989]). Overexpression of this region in *S. pombe* interferes with cyr1, as does overexpression of the comparable region in *S. cerevisiae* (Field et al., *Science 247:* 464 [1990]). Moreover, the *S. pombe* cyclase binds a protein, cap1, that is homologous to *S. cerevisiae* CAP. Disruption of *S. pombe cap1* causes an apparent failure of *S. pombe* cyr1 to be regulated properly. We do not know what factors activate *S. pombe* cyr1, but it is a question of some importance, since data presented in a subsequent section lead us to speculate that mammalian cells have a cyr1 homolog.

S. pombe contains a gene, *sar1*, that encodes a protein that is homologous to the yeast IRA proteins and the mammalian *GAP* and *NF1* gene products (Wang et al., *Cell Regul. 2:* 453 [1991]). *sar1* was selected as a multicopy suppressor of the activated $ras1^{val17}$ allele. Like its counterparts in *S. cerevisiae,* sar1 appears to down-regulate ras1, and there is no evidence whatsoever that it functions as a ras1 effector. Again, we cannot exclude the possibility that *S. pombe* contains a functional homolog of sar1, but cells with disruptions of sar1 are still ras1-sensitive. sar1 function differs from that of IRA1, IRA2, and GAP in that it is capable of inhibiting mutationally activated ras1. In this respect, sar1 resembles NF1, the product of the von Recklinghausen's neurofibromatosis locus, which can inhibit mutationally activated Ha-ras (Ballester et al., *Cell 63:* 851 [1990]).

S. pombe cells that lack *ras1* are sterile: Haploid cells fail to conjugate and diploid cells cannot undergo sporulation (Fukui et al., *Cell 44:* 329 [1986]; Nadin-Davis et al., *EMBO J. 5:* 2963 [1986]). We have screened for genes that on high copy are capable

of suppressing these defects. One such gene, previously identified by other investigators, is *byr1* (Nadin-Davis and Nasim, *EMBO J. 7:* 985 [1988]). *byr1* encodes a protein kinase and is capable of inducing sporulation in *ras1*$^-$/*ras1*$^-$ diploid. We identified a second gene, *byr2*, that also encodes a protein kinase and also can induce sporulation in *ras1*$^-$/*ras1*$^-$ diploids (Wang et al., *Mol. Cell. Biol. 11:* 3554 [1991]). Genetic interactions suggest that the byr2 product lies upstream of the byr1 product. We have found that both *byr1* and *byr2* can weakly suppress the sterility of *ras1*$^-$ haploids.

byr1 is most homologous to the *S. cerevisiae* gene *STE7*, and *byr2* is most homologous to the *S. cerevisiae* gene *STE11*. Both STE7 and STE11 are kinases that function on the mating pheromone pathway in *S. cerevisiae* (Teague et al., *Proc. Natl. Acad. Sci. 83:* 7371 [1986]; Rhodes et al., *Genes Dev. 4:* 1862 [1990]). Hence, the mating pathways of these two diverged yeasts are at least partially conserved. We can speculate further on these relationships. In both *S. pombe* and *S. cerevisiae*, there are other kinases required for sexual function: in *S. pombe*, spk1 (Toda et al., *Genes Dev. 5:* 60 [1991]), and in *S. cerevisiae,* FUS3 and KSS1 (Elion et al., *Cell 60:* 649 [1990]; Courchesne et al., *Cell 58:* 1107 [1989]). These kinases are related to the MAP/ERK protein kinases that have been identified in mammalian cells as response elements in extracellular signaling mediated through receptor protein tyrosine kinases (Cantley et al., *Cell 64:* 281 [1991]; Cobb et al., *Curr. Opin. Cell Biol. 3:* 1025 [1991]). In mammalian cells, the phosphorylation and activation of the MAP/ERK kinases is RAS-dependent (Thomas et al., *Cell 68:* 1031 [1992]). If we postulate that the KSS1, and FUS3, and the spk1 kinases are regulated by the STE7 and byr1 kinases, respectively, we can imagine drawing a set of homologies between the yeast and mammalian signal transduction pathways as shown in Figure 1. The implication of this speculation, if correct, is that the RAS effector pathways in mammals and *S. pombe* may be homologous.

We have isolated another gene, *byr3*, that can induce sporulation in *ras1*$^-$/*ras1*$^-$ diploid yeast. This gene encodes a protein with seven zinc-finger-binding domains and is homologous to a mammalian protein that was isolated based on its DNA-binding activity (Rajavashisth et al., *Science 245:* 630 [1989]). In collaboration with Dr. Rajavashisth (University of California, Los Angeles), we are examining the conservation of function between these two proteins.

Mammalian Genes That Interact with Yeast Signaling Pathways

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Yeast may be used to select mammalian cDNAs encoding products that interact with yeast signaling pathways (Lee and Nurse, *Nature 327:* 31 [1987]; Colicelli et al., *Proc. Natl. Acad. Sci. 86:* 3599 [1989]; Colicelli et al., *Proc. Natl. Acad. Sci. 88:* 2913 [1991]). We have employed *S. cerevisiae* strains containing RAS2^{val19} to select mammalian cDNAs that suppress the RAS2^{val19} phenotype. Three cDNA genes were so isolated: *JC99*, *JC265*, and *JC310* (Colicelli et al., *Proc. Natl. Acad. Sci. 88:* 2913 [1991]). All three also suppress the phenotype of *ira*$^-$ strains but not the phenotype of cells that overexpress either adenylyl cyclase or the cAMP-dependent protein kinase catalytic subunits. Sequence determinations indicate that *JC99* and *JC265* are in the same gene family. Otherwise, the three genes show no significant relationship to other genes in the databases.

In an attempt to understand how these mammalian genes function, we have expressed them in *S. pombe* and in mammalian cells. Preliminary studies indicate that expression of JC310 in NIH-3T3 cells blocks the mitogenic and morphological responses of these cells to phorbol esters, but not the activation of protein kinase C itself. These results are compatible with the hypothesis that JC310 blocks aspects of RAS function in mammalian cells, for it is known that RAS function is required for the morphologic and mitogenic effects of protein kinase C. This points to a possible evolutionary conservation in the function of JC310. However, expression of JC310 appears to have no evident phenotypic effects in either wild-type or mutant strains of *S. pombe.*

Expression of JC99, on the other hand, has profound effects in *S. pombe.* Expression has minimal effects on wild-type and *ras1*$^-$ cells, slightly promoting sporulation in *ras1*$^-$/*ras1*$^-$ diploids and haploid sporulation in *ras1*$^-$ or wild-type cells; but expression strongly suppresses the sexual dysfunction in cells that have activated the cAMP-signaling pathways. Cells that overexpress adenylyl cyclase or that lack the regulatory subunit of the cAMP-dependent protein kinase are virtually sterile. Moreover, they are elongated and heat-shock-sensitive. Expression of JC99 reverses these phenotypes, yet does not suppress the phenotypes of altered glucose metabolism

associated with activation of the cAMP pathway. Hence, in *S. pombe*, JC99 acts downstream from the cAMP pathway, where it interacts with gene products involved in sexual function. We do not yet understand how to reconcile these results with the effects of JC99 in *S. cerevisiae*, where it does alter phenotypes due to activation of the cAMP pathway but is unable to effect strains with activating mutations in adenylyl cyclase or the cAMP-dependent protein kinases. If expression of JC99 has effects in mammalian cells, these are not yet clear to us.

We have also selected several mammalian cDNAs on the basis of their ability to complement loss of ras1 function in *S. pombe*. These genes are still under study.

CAP, and Adenylyl-cyclase-associated Protein

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The year before last we reported the cloning of *S. cerevisiae CAP* (Field et al., *Cell 61:* 319 [1990]). This gene encodes a product required for full responsiveness to activated RAS. CAP is bifunctional (Gerst et al., *Mol. Cell Biol. 11:* 1248 [1991]). The amino-terminal domain binds adenylyl cyclase and is required for cellular RAS responsiveness. The carboxy-terminal domain is required for a variety of other cellular functions. Cells that lack the carboxyl terminus of CAP are temperature-sensitive, have disrupted actin cables, are enlarged and round, bud randomly from the cell surface, and show a variety of nutritional abnormalities, including valine toxicity, failure to grow in rich medium, and exquisite sensitivity to starvation (Gerst et al., *Mol. Cell. Biol. 11:* 1248 [1991]; Vojtek et al., *Cell 66:* 497 [1991]). The amino and carboxyl termini of CAP appear to act independently and are connected by a proline-rich middle portion that to date appears to be phenotypically silent.

To understand the carboxy-terminal function of CAP, we have sought genes that when overexpressed could suppress the phenotypes that result from its loss. One such gene is profilin, a low-molecular-weight protein that binds actin monomers and also binds phosphoinositides (Vojtek et al., *Cell 66:* 497 [1991]). Overexpression of profilin corrects the temperature-sensitive growth defects and the cytoskeletal, morphological, and nutritional abnormalities of cells lacking the carboxyl terminus of CAP, but not the RAS responsive abnormalities of cells lacking the amino terminus of CAP. Moreover, cells lacking profilin display growth and morphological abnormalities that closely resemble the phenotype of cells lacking the carboxyl terminus of CAP. These results point to an intimate relationship between the function of CAP and the function of profilin, a relationship that is strengthened by the discovery of a mammalian CAP (see below).

Another multicopy suppressor of the loss of the carboxy-terminal function of CAP is a gene we have called *SNC1*. *SNC1* encodes a small protein that has striking homology with the VAMP/synaptobrevin family of vertebrate synaptic-vesicle-associated proteins. These are low-molecular-weight proteins with very hydrophobic carboxy-terminal domains that are thought to function in neurotransmitter release. The presence of VAMP homologs in yeast suggests a more general function, perhaps in membrane vesicle fusion. We do not understand how the function of SNC1 relates to the function of CAP.

Curiously, SNC1 expression suppresses the loss of the carboxyl terminus of CAP only in cells with an activated RAS pathway; activation of the cAMP pathway is not sufficient. A similar result was obtained in the suppression of loss of carboxy-terminal CAP by human profilin: Effective suppression was observed only in RAS-activated strains. These results suggest that RAS, acting through its alternate pathway, in some way effects the functions of CAP, profilin, and SNC1.

The *S. cerevisiae CAP* gene has an *S. pombe* homolog (Kawamukai et al., *Mol. Biol. Cell 3:* 167 [1992]). The existence of this protein was first inferred from the presence of a cross-reacting antigen in preparations of adenylyl cyclase purified from *S. pombe*. The gene was independently cloned in two ways: by immunological screening of *S. pombe* cDNA expression libraries in *Escherichia coli* and by complementation screening of a *cap*$^-$ *S. cerevisiae* strain with cDNAs from *S. pombe*. *S. pombe* and *S. cerevisiae* have homologous carboxy-terminal function. Disruption of the carboxy-terminal domain of *S. pombe* cap1 causes temperature sensitivity and morphological abnormalities. Disruption of the amino-terminal domain causes loss of proper regulation of adenylyl cyclase. Thus, it is likely that in both yeasts, amino-terminal CAP function is required for the

proper regulation of adenylyl cyclase, whereas carboxy-terminal function is somehow related to cell morphology and growth.

Recently, Makoto Kawamukai, upon his return to Japan, cloned a human homolog of CAP. Even more recently, the isolation and purification of an actin-binding protein from pig platelets and the sequence of tryptic peptides were published (Gieselmann and Mann, *FEBS Lett. 298:* 149 [1992]). This sequence showed near identity to the human CAP. Since the amino-terminal domain of the mammalian CAP has homology with those of yeast, we infer that we may find a form of mammalian adenylyl cyclase that is homologous to the yeast adenylyl cyclases. Since the mammalian CAP is associated with actin, and can function in yeast, we infer that the mammalian CAP also regulates the cellular cytoskeleton and that yeast CAP, like yeast profilin, may regulate actin function.

Mammalian cAMP-specific Phosphodiesterases

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Selection for mammalian cDNAs that could suppress the phenotypes of *S. cerevisiae* strains with the $RAS2^{val19}$ mutation led to the cloning of mammalian cDNAs encoding phosphodiesterases. Several of these showed homology with the *Drosophila melanogaster dunce* gene. We have now explored the structure of this gene family in humans and rats. By utilizing polymerase chain reaction (PCR) primers and genomic DNA as a template, we have identified a total of four genes in this family, both from rats and humans. Functional cDNA sequences of three of these four genes have been cloned from humans, although we do not know whether the 5′ extremities of the coding regions have been reached. These genes encode high-affinity cAMP-specific phosphodiesterases which are inhibitable by rolipram. Using yeast lacking endogenous cAMP phosphodiesterases, which also have a heat-shock-sensitive phenotype, we have cloned a human cDNA encoding a novel form of cAMP phosphodiesterase. This gene, given the provisional name TM22, encodes a high-affinity cAMP-specific phosphodiesterase that is not inhibitable by either rolipram or milrinone. It therefore encodes a previously undetected form of cAMP phosphodiesterase.

Genomic Difference Cloning

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Sensitive methods for the detection of sequence differences between like genomes are potentially of great utility in understanding the genetic basis of disease. Such methods have potential application to the discovery of novel pathogens and the definition of genetic lesions in tumor cells. We previously published a method that allows the enrichment of difference sequences between two genomes by factors of several hundred (Wieland et al., *Proc. Natl. Acad. 87:* 2720 [1990]). We have modified this approach in two fundamental ways. First, we simplify the genomes to be compared by taking an "isomorphic representation" of each. This entails making an equal sampling of both genomes and examining sequence differences between these simplified DNA populations. Second, after rounds of subtractive hybridization, we further enrich difference sequences by melting, annealing, and selecting for double-stranded DNA. For sequences enriched by a factor of *N*, this step can, theoretically, give another *N*-fold enrichment. Using this approach, and model systems in which we have added λ DNA or *E. coli* DNA at single-copy levels to human DNA, subtracted against the same DNA without the added sequences, we have enriched the difference sequences by as much as 100,000-fold.

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