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RAS Genes and Growth Control in *Saccharomyces cerevisiae*

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ras genes have been identified in such evolutionarily diverse organisms as mammals, insects, and fungi. Initial interest stemmed from the role of these genes in the etiology of mammalian cancers, but they now appear to play central biological roles in other eucaryotes as well. Because genetic manipulations can be performed with relative ease in the yeast *Saccharomyces cerevisiae*, the most rapid advances in our knowledge of the *RAS* gene function come from studies of this organism. Studies of the *RAS* genes in *S. cerevisiae* have provided important information about the control of cell growth in yeasts, information which may be relevant to the function of the *ras* gene in mammalian cells as well.

ras genes were originally identified as those responsible for the transforming ability of Harvey and Kirsten murine sarcoma viruses (reviewed in reference 12). These helper-dependent retroviruses induce tumors in mice after a short latent period, usually 2 to 3 weeks. The transforming genes present in the viruses code for similar 21,000-dalton proteins, termed p21. Independent experiments have revealed that human tumors contain DNA sequences capable of transforming mouse fibroblast NIH 3T3 cells. Cloning of these sequences has indicated that many are cellular homologs of the viral *ras* genes. Three distinct genes have been identified in this manner: two genes closely related to viral Harvey and Kirsten *ras* (cHa-*ras* and cKi-*ras*) (11, 28, 31) and a third gene with no viral counterpart (N-*ras*) (34). All three genes code for closely related proteins, with less than 20% amino acid divergence among members of the family.

Transforming *ras* genes, whether of viral or cellular origin, differ from their normal cellular homologs by the presence of missense mutations at a limited number of positions. Mutations at amino acid positions 12, 13, 59, 61, and 63 are sufficient to confer a transforming phenotype (13, 32, and references therein). The observation that these missense mutations reduce the intrinsic GTPase activity of p21 (16, 26, 36, 42) strongly suggests that the GTPase activity of this molecule is central to its biological function. Association with the cell plasma membrane via a covalently bound lipid also appears necessary for the *ras* function. Mutant p21 proteins that lack a conserved cysteine residue near the C terminus do not bind lipid, fail to associate with the cell membrane, and fail to transform NIH 3T3 cells (46).

The GTPase specific activity of p21 is similar to that of a number of G proteins including the GTP-binding proteins of signal transduction systems (17, 35) and bacterial elongation factor EF-Tu (18). This similarity between p21 and the G proteins of the signal transduction pathway, the fact that the *ras*-encoded transforming proteins are dominant in heterozygous diploid cells, and the cell surface location of p21 have led to the proposal of a model in which p21 functions as part of some signal transduction pathway, possibly for one or more of the growth hormones. It is clear, however, that p21 is not the G protein of several well-characterized signal transduction systems. The function of p21 in other less-well-characterized systems remains an open question.

Although the *ras* gene is clearly involved in either the induction or development of some cancers, its precise role remains unknown. Most studies of *ras* function have utilized dominant transforming genes. Although it is widely believed that *ras* plays a role in normal cellular function(s), the lack of recessive loss-of-function mutations in the *ras* gene has hindered the identification of these functions. The multigene nature of the *ras* gene also complicates studies. It is not known, for instance, whether each member of the *ras* gene family has a unique biological function, a specific window of developmental expression, or unique tissue distribution. For these reasons, the identification of *ras* genes in *S. cerevisiae* has been met with enthusiasm. The sophisticated genetics of this organism, which include the ability to create null mutations, make it possible to investigate aspects of *ras* function that cannot be readily studied in mammalian systems. The hope is, of course, that this information will also apply to the function of the *ras* gene in mammalian cells.

Two genes have been cloned from *S. cerevisiae* by using the viral *ras* gene as a hybridization probe (9, 29). The complete DNA sequences of these two genes, designated *RAS1* and *RAS2*, reveal open reading frames with significant blocks of homology to *ras* (8, 29). Amino acids 7 to 65 have over 80% homology with Ha-*ras*; the homology in the next 100 amino acids is less but still greater than 40%. All of the amino acid residues corresponding to the positions of the transforming mutations of p21 (positions 12, 13, 59, 61, and 63) are identical to those in the normal nontransforming p21 protein. This is not true of *YP2*, a third yeast gene which shares some homology with *ras* (<40% homology) (15). The yeast *RAS1* and *RAS2* genes code for 34,000- and 35,000-dalton polypeptides, respectively. This greater size relative to the 21,000 daltons of the mammalian p21 is the result of a 7-amino-acid extension at the N terminus and a 100- to 120-amino-acid insertion near the C terminus. The large C-terminal insertions in *RAS1* and *RAS2* are not homologous with each other and occur at a position in *ras* which corresponds to that at which the greatest amount of divergence occurs among members of the mammalian *ras* gene family. The extreme C termini of both yeast *RAS* proteins contain the cysteine residue conserved in all *ras* genes (described above). A mutant *RAS2* gene, containing a serine residue in place of cysteine at amino acid position 319 (four amino acids from the C terminus) is nonfunctional (J. Broach, personal communication).

Monoclonal antibodies against viral *ras* immunoprecipitate yeast *RAS* gene products (38, 41). The yeast *RAS* gene products, like the mammalian *ras* proteins, bind GDP and GTP (38, 41) and hydrolyze GTP (37, 42). Mutations analogous to those that give transforming ability to p21 decrease this GTPase activity (37, 42).

These structural and biochemical similarities provide compelling evidence that the mammalian and yeast *ras* genes are related but by no means prove that they carry out the same function. As described below, the yeast *RAS* genes are essential for cell growth. However, yeast strains lacking both *RAS* genes are viable if they contain either the normal mammalian or viral Ha-*ras* genes (10, 20). Conversely, a

modified yeast *RAS1* gene, containing a valine codon at position 19 and an in-frame deletion removing the nonhomologous region near the C terminus, will transform NIH 3T3 cells (10). Thus, *ras* genes from *S. cerevisiae* and mammals are functionally homologous and could carry out the same function in both organisms.

The first clue as to the function of the *RAS* genes in *S. cerevisiae* came from gene disruption experiments. In a now almost routine experiment, cloned *RAS* genes were made nonfunctional by inserting fragments of DNA coding for selectable markers into the middle of the *RAS* coding sequence. These disrupted genes were reintroduced into *S. cerevisiae* so that the disrupted allele replaced the wild type. These experiments indicate that *RAS1* and *RAS2* are members of an essential gene family. Strains lacking either a functional *RAS1* or *RAS2* are viable, but spores with disruptions in both genes either fail to germinate or growth is arrested after germination but before the formation of the first bud (21, 39). The growth of yeast strains that contain, as their sole *RAS* gene, a gene under the control of an inducible promoter, is arrested when the *RAS* gene is not induced, resulting in populations of unbudded, nongrowing cells (20). The growth of strains with temperature-sensitive *RAS* alleles is also arrested in G1 at the restrictive temperature (J. Broach, personal communication). This terminal phenotype, arrested cell cycle development in G1, characterized by unbudded, nongrowing cells, is shared by a class of temperature-sensitive cell division cycle mutations (*cdc19*, *cdc25*, *cdc33*, and *cdc35*) and by nutritionally arrested, wild-type cells (30). The *RAS* gene therefore appears to be required either for growth or passage through the cell cycle. This evidence alone does not indicate that the *RAS* gene plays a key or pivitol role in cell growth control, because any number of metabolic blocks might cause a similar G1 arrest phenotype. For example, one member of this class of mutations, *cdc19*, is the structural gene for pyruvate kinase (G. Kawasaki, Ph.D. thesis, University of Washington, Seattle, 1979). The *RAS* gene could encode an enzyme, in any number of metabolic pathways, that is no more or no less interesting than pyruvate kinase. However, additional observations suggest that the *RAS* gene does indeed have a regulatory role in controlling cell growth.

Yeast strains that contain a *RAS2* gene with valine substituted for glycine at amino acid position 19 (analogous to the transforming-position-12 mutation in the mammalian *ras* gene) fail to accumulate storage carbohydrates, are unable to sporulate efficiently, and lose viability rapidly when starved (21, 43). All of these traits are dominant to the wild-type *RAS2* gene. In contrast, strains that lack *RAS2* have virtually the opposite phenotype; they sporulate on rich media (normal strains of *S. cerevisiae* require starvation conditions to initiate the meiotic cell cycle) (40, 43) and hyperaccumulate storage carbohydrates (40). Surprisingly, mutations in *RAS1* have neither of these phenotypes. The *RAS1*^{val19} mutation has none of the dominant effects of the *RAS2*^{val19} mutation, and disruptions of *RAS1* which cause a loss of function create no observable phenotypic change when *RAS2* is present (unpublished observations). This difference could be caused by decreased expression of the *RAS1* gene relative to that of the *RAS2* gene or by a qualitative difference between the two genes.

The ability of *RAS2* mutants to sporulate on rich media is shared by the cell division cycle mutants carrying the *cdc25* and *cdc35*/*cyr1* mutations (23, 33). *cyr1* (allelic to *cdc35*) is thought to be the structural gene for adenylate cyclase. In strains that can take up cyclic AMP (cAMP), the lethality of *cyr1* mutations can be rescued by exogenous cAMP (25). The growth of such strains is arrested, and they are unbudded in G1 of the cell cycle in the absence of exogenous cAMP. *cyr1* mutants have very low levels of adenylate cyclase activity, and one allele, *cdc35-10*, has been reported to have temperature-sensitive adenylate cyclase activity (3). Elevated expression of the cloned *CYR1* gene leads to increased levels of adenylate cyclase activity in *S. cerevisiae* (6, 19) and *Escherichia coli* (19, 45). Matsumoto et al. (25) have isolated a mutation, *bcy1*, which bypasses the cAMP requirement of the *cyr1* mutation. The same mutation also bypasses the requirement for the *RAS* gene, allowing a cell without either *RAS* gene to complete the mitotic cell cycle (43). *bcy1* mutants have decreased levels of the regulatory subunit of the cAMP-dependent protein kinase, thereby allowing the protein kinase to function in the absence of cAMP (25). Recent sequence analysis of the cloned *BCY1* gene indicates that it likely is the structural gene for the cAMP-binding regulatory subunit (M. Wigler, personal communication).

Yeast strains with disruptions of the *RAS2* gene are unable to efficiently utilize nonfermentable carbon sources such as ethanol, glycerol, or pyruvate (14, 40). Although the nature of this gluconeogenic or respiratory defect is not fully understood, it has proven to be useful in selecting mutants that suppress the *ras2* defect. In one extensive mutational analysis, five suppressor genes, termed *sra* for "suppressor of *ras*," have been identified (J. Cannon, J. Gibbs, and K. Tatchell, Genetics, in press). One recessive complementation group, *sra1*, is allelic to *bcy1* and thus suppresses the *ras* gene by stimulating the cAMP-dependent protein kinase. *SRA4* is represented by a set of dominant suppressors that are tightly linked to *cyr1*. Adenylate cyclase activity is higher than normal in *SRA4*-containing strains, even in those which lack a functional *ras* gene. Alleles of another recessive suppressor, *sra5*, result in decreased levels of cAMP phosphodiesterase activity.

Mutations in *bcy1* and *SRA4* and dominant mutations at another locus, *SRA3*, allow strains lacking both *ras* genes to grow, thus bypassing the essential requirement for *ras*. All three mutations also suppress the conditional lethality of recessive mutations in adenylate cyclase (*cyr1*/*cdc35*) (unpublished observations). Alleles in the recessive complementation group *sra6* do not bypass deficiencies in both *ras* genes and do not suppress mutations in adenylate cyclase. Preliminary evidence suggests that *sra6* may function by increasing the expression of *RAS1* on media containing only nonfermentable carbon sources since *RAS1* mRNA does not normally accumulate on such media (D. Brevairio, A. Hinnebusch, J. Cannon, K. Tatchell, and R. Dhar, Proc. Natl. Acad. Sci. USA, in press).

Where does the *RAS* gene fit in the adenylate cyclase-cAMP-protein kinase pathway? *S. cerevisiae* contains an adenylate cyclase activity that is stimulated by GTP (7), a property shared by the mammalian adenylate cyclase. This stimulation is dependent on the *RAS* gene; cells without either *RAS* gene have almost undetectable GTP-stimulated adenylate cyclase activity, while strains containing the dominant *RAS2*^{val19} mutation have increased levels of adenylate cyclase activity (43). Mutations in adenylate cyclase activity suppress the dominant phenotypes of *RAS2*^{val19} (M. Wigler, J. Broach, and L. Robinson, personal communication). Broek et al. (4) have reconstituted the system in vitro by adding purified RAS protein to membrane fractions devoid of RAS protein. The adenylate cyclase activity in these experiments was dependent on the presence of exogenously

added RAS protein. It has recently been reported that adenylate cyclase activity, with the biochemical characteristics of the yeast enzyme, can be recovered by expressing both the *CYR1* and *RAS2* genes in *E. coli* (42), but caution should be exerted in overinterpreting this interesting report. It is not clear whether a full-length adenylate cyclase protein was expressed in *E. coli*, and it is known that some *cyr1* deletions that retain the C-terminal portion of the protein have catalytic activity (19). Another report comes to the opposite conclusion, i.e., that the system can not be reconstituted by expressing only *RAS2* and *CYR1* genes in *E. coli* (19).

The simplest interpretation of both the genetic and biochemical data is that the *RAS* gene product directly stimulates adenylate cyclase in a manner analogous to the stimulation of mammalian adenylate cyclase by the G or N protein (17). Such a function would not have been predicted for the yeast *RAS* gene product, based on the evidence from animal systems. Mammalian cell lines often have reduced levels of adenylate cyclase activity upon infection with *ras*-containing viruses (5), and p21 does not appear to activate mammalian adenylate cyclase (1). Furthermore, the injection of *RAS* proteins into *Xenopus* oocytes does stimulate oocyte maturation but without concomitant stimulation of adenylate cyclase (2). The high degree of homology between mammalian and yeast *RAS* genes suggests that the macromolecules that interact with *RAS* proteins, or portions thereof, are also conserved. How can this apparent paradox be resolved? The yeast adenylate cyclase gene *CYR1* codes for a polypeptide that contains 2,026 amino acid residues; however, only a small portion of the C terminus is required for adenylate cyclase activity (19). If *RAS* protein interacts directly with adenylate cyclase, perhaps that portion of the molecule which interacts with *RAS* protein has a homolog in animal cells, such as a domain of another protein. This is not the only model, however. There is still no evidence that RAS protein and adenylate cyclase interact directly. The general assumption has been that *RAS* protein functions as a G protein, but not all of the accumulated data are consistent with such a model. In a bona fide G system, only the GTP adduct of G stimulates adenylate cyclase. However, both the GTP and the GDP-bound forms of *RAS* protein stimulate adenylate cyclase in the in vitro reconstitution system (4). In addition, the reconstituted adenylate cyclase system prepared from *E. coli* has a significantly lower level of GTP stimulation than that prepared from *S. cerevisiae* (45). This suggests that adenylate cyclase activity depends on more than just *CYR1* and *RAS* gene products. The *RAS* gene product could be interacting with an unidentified component which, in turn, could stimulate adenylate cyclase. A more detailed biochemical and genetic analysis of the system should help resolve the apparent paradox.

Taken together, these results implicate cAMP as a key element in regulating cell growth in *S. cerevisiae* (reference 24 reviews these data in detail). The development of cells that lack the ability to synthesize cAMP, i.e., that contain mutations in the *ras* gene or adenylate cyclase, is arrested in G1 of the cell cycle, and such cells remain unbudded and nongrowing. The main function of cAMP appears to be the activation of the cAMP-dependent protein kinase, which it does by binding to the inhibitory subunit (*bcy1*), thereby releasing the active catalytic subunit. Phosphorylation of specific substrates by this kinase leads to cell growth. This system is tuned to the nutritional state of the cell. By some unknown mechanism, depletion of essential nutrients (nitrate, sulfate, phosphate, or carbon) leads to loss of adenylate cyclase activity and consequently to decreased phosphorylation by the cAMP-dependent protein kinase. Although it has not been rigorously shown that starvation directly inhibits adenylate cyclase, it is known that the loss of a nitrate, sulfate, phosphate or carbon source leads to rapid accumulation of storage carbohydrates (22), which are known to be regulated by the cAMP-dependent protein kinase (27, 44, 47). Regulation of entry into the meiotic cell cycle also appears to be regulated by cAMP metabolism. Normally, sporulation is induced only by starvation conditions. However, strains with mutations causing defects in adenylate cyclase or *ras* activity (*ras2*, *cdc35*/*cyr1*) sporulate on rich media. Conversely, strains that contain *RAS2*^{val19}, *bcy1*, or *SRA4* mutations fail to sporulate under any conditions.

It is worth pointing out that the *RAS* gene and adenylate cyclase do not affect the cell cycle per se but primarily affect cell growth. Specific control of the cell cycle is regulated by another set of genes, identified by the *cdc28* class of cell cycle mutations, which cause cell cycle arrest at the restrictive temperature without affecting cell growth (30). This class of cell division cycle mutants is not suppressed by mutations that suppress the *ras* gene and adenylate cyclase mutations, i.e., *bcy1* (unpublished observations). Clearly, the systems of growth control and cell cycle control must be integrated at some point. One would expect that some signal or signals telling the cell that the necessary nutritional requirement had been met would be one of the preconditions required for cells to enter a new cell cycle. Perhaps a phosphorylation event, mediated by the cAMP-dependent protein kinase, is one such signal.

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