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The *ras* Oncogene—an Important Regulatory Element in Lower Eucaryotic Organisms†

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INTRODUCTION

The mechanisms by which normal cells acquire the malignant phenotype constitute a central focus of cancer research. Research efforts have often quantitated overall variations of critical metabolic enzymatic activities and resulting metabolites in cancer cells and then compared the results with those obtained for normal cells. The discovery of oncogenes has led to the identification of the novel proteins (oncoproteins) encoded by these genes. These oncoproteins have been analyzed for their individual biochemical properties and their functions in cell physiology.

Oncogenes can be categorized into at least six general classes based upon the proteins that they encode: growth factors (*sis*), receptors (*neu*, *erbA*, *fms*, *kit*, and *mas*) or truncated receptors (*erbB*), tyrosine kinases (*src*, *abl*, and *fps* as examples), cytoplasmic serine and/or threonine kinases (*mos* and *raf*), guanosine triphosphate (GTP)-binding proteins (*ras*), and nucleus-localized proteins (*myc*, *myb*, *fos*, and *jun* as examples). A description of the various oncogenes can be found in several recent reviews (7, 10, 61). The biochemical properties of oncoproteins suggest that they play a role in signal transduction mechanisms, possibly by relaying extracellular proliferation signals to the nucleus. Proto-oncogenes are the normal nontransforming alleles of oncogenes and encode proteins that are normally found in nontransformed cells. When specific mutations alter the normal biochemical activities of oncoproteins, these proteins can become activated and can cause cellular transformation because the mutations usually result in constitutive biochemical activity. These mutations therefore confer on these oncogenes the dominant ability to transform cells. Although the intrinsic biochemical properties of most oncoproteins have been identified, the exact function of these oncogene products and the pathways which they influence are not known.

To analyze the function of oncoproteins and how they influence the regulation of cell proliferation, lower eucaryotic organisms such as the yeast *Saccharomyces cerevisiae* have been utilized. These cell systems are often easier to manipulate and analyze genetically than mammalian cell systems. Some oncoprotein biochemical activities are present in these lower eucaryotes as normal cellular components. For example, tyrosine kinase activity is detectable in *S. cerevisiae*; however, a close homolog to the *src*-like oncogenes has not been found (24, 130). The *jun* gene product is a transcriptional regulator and shares both sequence and functional homology with the yeast *GCN4* gene product (142, 169). The *ras* gene products were the first oncogene homolog identified in *S. cerevisiae* (29, 34, 117); the structures of yeast *ras*-encoded proteins share amino acid similarity with the mammalian *ras* proteins.

The *ras* genes have attracted a great deal of attention because of their apparently prominent role in malignancy of human cells. Overall, *ras* genes have been identified as activated in 15 to 30% of human tumors of diverse tissue origin (7). In cases involving pancreatic and colorectal cancers, this incidence is as high as 90 and 50%, respectively (3, 168). Recent studies have provided a correlation that *ras* activation may be a causative event in human tumor formation. Activated *ras* genes have been identified during early steps of the cancer process. For example, premalignant colon adenomas can progress to fully malignant colon carcinomas; the 50% frequency rate of activated *ras* genes has been found in both adenomas and carcinomas (168). In myelodisplastic syndrome, analysis of cells from a single patient throughout the disease course indicated that activated *ras* genes were present in lymphocytes cultured during the benign stage as well as in the malignant lymphoma cells analyzed 1 year later (76). In transgenic mice, tumor formation is a function of the transcriptional promoter used to regulate the activated *ras* gene (5, 119, 140). The specific

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† Dedicated to the memory of Irving Sigal.

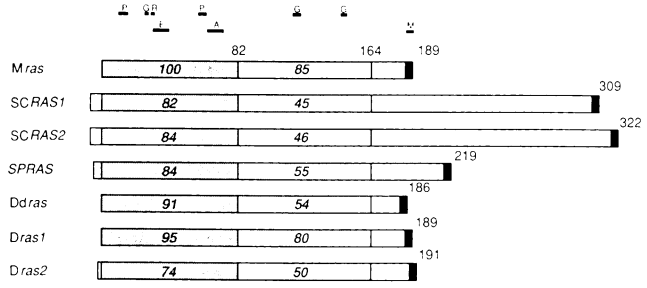

FIG. 1. Schematic representation of the structure of *ras*-encoded proteins. Sources of Ras: M, mammalian; SC, *S. cerevisiae*; SP, *S. pombe*; Dd, *D. discoideum*; D, *D. melanogaster*. Regions critical to guanine nucleotide binding are indicated for the interaction with phosphates (P), the ribose ring (R), and the guanine ring (G). Other abbreviations: E, region critical for effector activity; A, highly conserved epitope for binding the neutralizing antibody Y13-259; M, membrane localization site. Residue numbers are indicated outside the boxes. Numbers inside the boxes represent the degree of amino acid similarity to the M-*ras* proteins (expressed as a percentage).

organ of tumor origin and the time of tumor formation depend on the tissue specificity and developmental sensitivity of individual promoters. The results indicate that the *ras* gene has a major role during early stages of tumor formation.

In this review, we will focus on the *ras* oncogene products in the budding yeast *S. cerevisiae* and in other lower eucaryotic organisms. Earlier reviews on this subject have been presented (7, 137, 146, 150). The evidence provided by structure-function, biochemical, and genetic analyses supports a role for these gene products as important growth-regulatory elements. The *ras* genes make up a family of highly conserved sequences. For mammalian cells, three members have been described: Harvey (Ha), Kirsten (Ki), and N-*ras*. These genes encode homologous 21-kilodalton (kDa) proteins of 189 amino acids (p21). Genes homologous to the mammalian *ras* genes were identified in the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe*, the slime mold *Dictyostelium discoideum*, and *Drosophila melanogaster* (29, 50, 98, 103, 106, 121). Although some divergence is observed at the nucleic acid level, the amino acids encoded by these genes are at least 74% homologous over the first 80 residues. Divergence is present in the C-terminal residues, but the last four residues are again highly conserved. A schematic representation of *ras* proteins from various organisms is shown in Fig. 1. In this review, we will refer to the gene as *ras* and the gene product as Ras, except for *S. cerevisiae*, for which standard nomenclature will be used to describe wild-type and mutant genes (*RAS* and *ras*, respectively). The yeast protein will be referred to as RAS.

PROPERTIES OF *RAS* PROTEINS

The biochemical and structural properties of the *ras* proteins are consistent with a functional role as a GTP-binding regulatory protein (G protein). G proteins regulate diverse biochemical processes such as protein biosynthesis (elongation factor Tu) or the transduction of extracellular signals to intracellular enzymes (for example, G_s, G_i, and G_o) by a GTP-guanosine diphosphate (GDP) cycle (58). When complexed with GTP, these proteins function by stimulating a target protein. This stimulation is turned off upon hydrolysis of the GTP to GDP. GDP-to-GTP nucleotide exchange reinitiates the cycle. All the *ras* proteins bind GTP and GDP, specifically, with approximately the same affinity, of 50 to 100 pM (40, 41); possess a slow GTP-hydrolytic activity (57, 84, 94, 144, 154); and are localized on the inner face of the plasma membrane (136, 174). Regions essential for guanine nucleotide binding are conserved with known G proteins and are located at residues 11 to 16, 116 to 119, and 140 to 145 (25, 33, 39, 139, 171). Membrane localization occurs by palmitoylation of a Cys residue in the conserved four ultimate residues. The membrane localization, which is required for the cell-transforming activity of Ras (175), has led to speculation that Ras might be involved with signal transduction processes.

Biological activation of Ras can occur by two mechanisms, both of which are consistent with the G-protein hypothesis of Ras function. First, amino acid substitutions at residue 12 or 61 located near the phosphates of GTP in the GTP-binding domain can impair GTP-hydrolytic activity (57, 84, 94, 144, 154). These mutations are predicted to inhibit the ability of Ras to turn off its function and result in a constitutively activated Ras-GTP complex. Analysis of the guanine nucleotides bound to *ras*-encoded proteins in yeast and mammalian cells has confirmed that forms of Ras with impaired guanosine triphosphatase (GTPase) activity are bound to more GTP than that observed for the wild-type protein (56, 128a). Second, residues 16, 116, 119, and 144 are essential for nucleotide binding, and substitutions at these positions reduce GTP- and GDP-binding affinity constants from subnanomolar up to micromolar values (25, 39, 139, 171). These mutant *ras* proteins have greater cell-transforming potency than wild-type Ras does (139, 171). An increased off rate of bound GDP observed for these mutants results in rapid nucleotide exchange kinetics. In the cell, the facilitated nucleotide exchange is predicted to result in more GTP complexed to Ras. Guanine nucleotides are present at high micromolar levels and therefore would saturate Ras nucleotide-binding sites. In addition, cells contain approximately 5 to 10 times more GTP than GDP (36, 65). Since *ras* proteins bind GTP and GDP with similar affinities, one would predict, on the basis of the law of mass action, that GTP would be bound to Ras.

Two other regions encompassing residues 30 to 40 and 63 to 73 are highly conserved among the *ras* proteins found in evolutionarily diverse organisms. Residues 63 to 73 bind a monoclonal antibody, Y13-259, that is diagnostic for the identity of *ras* proteins (138). Although mutations in the region from residues 63 to 73 are without effect on Ras biology, antibodies directed at this epitope are neutralizing (54, 99, 138). The amino acids from residues 30 to 40 have been identified as being critical for biological activity (87, 138, 141, 176). Mutation of amino acids in the region from residues 30 to 40 impairs the ability of mammalian Ras to transform cells; however, the proteins bind GTP and localize to the membranes. The region containing residues 30 to 40, sometimes referred to as the effector region, may interact with a target protein or influence the formation of an active conformation. Antibodies directed at the region containing residues 30 to 40 are neutralizing (73).

RAS IN THE YEAST *S. CEREVISIAE*

In *S. cerevisiae*, two genes, *RAS1* and *RAS2*, encode homologous proteins that are characterized by a 180-amino-acid domain highly conserved with mammalian Ras and a unique divergent region of approximately 120 residues (29, 34, 117). The *RAS1* and *RAS2* genes are located on chromosomes XV and XIV, respectively (70, 151). Either of the two *RAS* genes will promote *S. cerevisiae* growth (70, 151). A

disruption of either the *RAS1* or the *RAS2* gene alone has no effect on growth in rich media containing glucose. However, haploid spores containing disruptions of both *RAS* genes fail to germinate and grow. Thus, the *RAS* proteins are essential for yeast cell viability. This defect can be complemented by mammalian *ras* genes, indicating a conservation of biological properties among members of the *ras* gene family (30, 69). In a converse experiment, a modified form of RAS1 containing the activating Leu-68 substitution and the membrane localization region but lacking the unique 120-amino-acid C-terminal region transformed NIH 3T3 mouse fibroblast cells upon transfection (30).

RAS1 and RAS2, which are 36 and 40 kDa, respectively, are larger than the other 21-kDa members of the family of *ras* gene products (48, 112). The most highly conserved residues are in the N-terminal half of the protein and contain the structural features necessary for guanine nucleotide binding, GTPase activity, and effector function. Like the mammalian *ras* proteins, yeast RAS binds GTP and GDP, specifically, with high affinity and hydrolyzes GTP to GDP (147a, 148, 153, 154). Importantly, mutations at position 19 or 68 (mammalian Ras residues 12 and 61) that activate biological activity impair GTPase activity, as is observed with mammalian Ras.

The unique 120-amino-acid domain is divergent between RAS1 and RAS2. At the C-terminal end of the 120-amino-acid divergent region are four residues conserved with all *ras*-encoded proteins that are critical for transport to the plasma membrane. Two steps have been identified in the posttranslational modification of RAS that are necessary for membrane localization. First, a processing step occurs that causes RAS to be more mobile on sodium dodecyl sulfate-polyacrylamide gels (26, 48). This step requires the *DPR1* gene product, also called *RAM* (47, 118, 147). In *dpr1* cells, *RAS* proteins remain as the precursor and accumulate in the cytosolic fraction, with a concurrent reduction in particulate RAS. The cytosolic RAS is not acylated, although small amounts of RAS are found in the membranes in acylated form. Second, acylation with palmitate occurs subsequent to the processing step. The palmitoylation seems to involve a thiol-ester linkage on Cys-319, analogous to Cys-186 of mammalian Ras. This modification apparently directs RAS to the membranes, since [^{3}H]palmitate-labeled RAS is found exclusively in the membrane fraction. The processing and acylation steps observed with RAS in yeast cells appear to be similar to events that occur with Ras in mammalian cells (83, 136). Carboxymethylation of *ras* p21 in mammalian cells prior to acylation has been described recently (27). This modification would make the C terminus more hydrophobic by eliminating the negative charge of the free carboxylate. It remains to be determined whether carboxymethylation of *RAS* proteins in *S. cerevisiae* also occurs.

Membrane localization is a very important but not essential step for RAS action in *S. cerevisiae* (31). At physiological levels of *RAS* expression, the ability of *RAS* protein to associate with membranes is necessary for biological action. Upon deletion of the last four residues or substitution of Cys-319 with Ser, *RAS* proteins cannot be palmitoylated and therefore do not localize to membranes. In the absence of a wild-type *RAS* gene, yeast cells expressing the mutant *RAS* gene at normal levels are nonviable. However, overexpression of these mutant genes restores viability even though no membrane-bound *RAS* protein is detected (31). The results indicate that membrane localization is important for the efficiency of RAS action rather than being an absolute requirement.

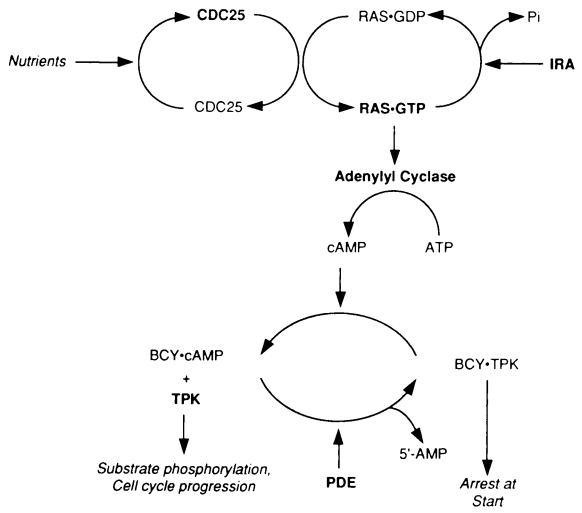

FIG. 2. The cAMP pathway in *S. cerevisiae*. The functions of the indicated gene products are discussed in the text and summarized in Table 1.

The Essential Requirement of cAMP in *S. cerevisiae*

Adenosine 3',5'-cyclic monophosphate (cAMP) is essential for the viability of *S. cerevisiae* cells. This requirement was most clearly demonstrated by using genetic analyses of the cAMP pathway. *S. cerevisiae* cells have an adenylyl cyclase that synthesizes cAMP, both high- and low-Michaelis-constant (K_m) phosphodiesterases that degrade it, and cAMP-dependent protein kinases that transduce the metabolic signal (21–23, 66, 68, 77, 78, 90, 91, 108, 128, 155, 160, 162). A mutation in the adenylyl cyclase gene *CYR1* (*CDC35*) that inhibits cAMP production is lethal unless the cells are supplemented with cAMP in the growth media or the cells contain a suppressor mutation that bypasses the requirement for cAMP. One such suppressor was termed *bcy1* (92). Strains having the *bcy1* mutation expressed a constitutively activated protein kinase that did not require cAMP for maximal activity. The regulation of cAMP metabolism in *S. cerevisiae* involves multiple proteins. A schematic representation of the cAMP pathway is shown in Fig. 2, and a discussion of the various gene products is presented below. A summary of the nomenclature can be found in Table 1.

The adenylyl cyclase of *S. cerevisiae* is localized at the plasma membrane and is regulated by guanine nucleotides. Casperson et al. (23) were the first to show that adenylyl cyclase activity in *S. cerevisiae* was stimulated by GTP and inhibited by GDP in a manner reminiscent of the mammalian enzyme. Furthermore, they provided evidence in temperature lability studies that guanine nucleotide sensitivity was conferred by a protein distinct from catalytic cyclase. Like the situation with mammalian adenylyl cyclase, GTP markedly stimulated basal adenylyl cyclase activity assayed with Mg^{2+} ion but had minimal effect in assays containing Mn^{2+} ion, which measures catalytic activity independent of GTP-binding regulatory proteins. These observations suggested that cAMP metabolism in *S. cerevisiae* might be regulated by a signal transduction mechanism. However, there are no reports to date of receptor-coupled stimulation of cAMP formation in *S. cerevisiae*.

The biosynthesis of cAMP in *S. cerevisiae* has been shown to be an essential requirement for progression of normal cells through the cell division cycle (90–92). Temperature-sensi-

TABLE 1. Genes of the *RAS*-cAMP pathway

Nomenclature (alleles)	Gene product
RAS1, *RAS2*	GTP-binding regulatory proteins
CYR1 (*CDC35*, *SRA4*, *SSR2*, *CRI14*)	Adenylyl cyclase
BCY1 (*SRA1*, *CYR3*)	Regulatory subunit of the cAMP-dependent protein kinases
TPK1, *TPK2*, *TPK3* (*SRA3*)	Catalytic subunits of the cAMP-dependent protein kinases
PDE1	Low-affinity (high-K_m) cAMP phosphodiesterase
PDE2 (*SRA5*)	High-affinity (low-K_m) cAMP phosphodiesterase
CDC25	Enzymatic function unknown; probably involved in regulation of the RAS nucleotide state
IRA1 (*PPD1*)	Enzymatic function unknown; probably involved in regulation of the RAS nucleotide state
DPR1 (*RAM*, *SUPH*, *STE16*)	Gene product required for processing of RAS prior to fatty acid acylation
SRA6	Required for repression of *RAS1* transcription on nonfermentable carbon sources

tive mutations in genes affecting the cAMP pathway (*CDC35* and *CDC25*) result in growth arrest at the G_1 phase of the cell cycle when cells are shifted to the restrictive temperature for growth (19, 62, 68, 145). As has been observed in higher eucaryotic cells, cAMP functions as a second messenger, regulating protein phosphorylation, levels of storage carbohydrates such as glycogen and trehalose, and mitochondrial function (45, 46, 79, 98, 111, 116, 124, 126, 159). In addition, cAMP appears to influence sporulation and sensitivity to heat shock, although the mechanisms for these effects are not clearly defined (17, 161). The effects on both storage carbohydrates and mitochondrial function are regulated by protein phosphorylation-dephosphorylation. The metabolic effects of cAMP in *S. cerevisiae* can be quickly and easily tested by staining for the storage carbohydrates or by growing the cells on media containing fermentable or nonfermentable carbon sources. For example, the intensity of staining with iodine vapors is directly proportional to the amount of glycogen and trehalose in the cell. High levels of cAMP reduce glycogen levels, whereas low levels of cAMP promote glycogen storage.

RAS Regulation of cAMP

The analysis of RAS function in *S. cerevisiae* initially focused on the effect of *RAS* gene disruptions or expression of the activated [Val-19]*RAS2* allele on the above-mentioned phenotypes (44, 70, 152). Disruption of the *RAS2* gene produces three phenotypes: (i) hyperaccumulation of glycogen and trehalose; (ii) failure to grow on nonfermentable carbon sources such as glycerol, ethanol, and acetate; and (iii) sporulation in the absence of nutrient deprivation. In contrast, cells expressing [Val-19]*RAS2* fail to accumulate glycogen and trehalose, do not sporulate efficiently, and lose viability under suboptimal growth conditions (starvation, storage in the cold, or heat shock).

The effects of *ras2* mutations on glycogen and trehalose accumulation led Wigler and co-workers to the discovery that RAS in *S. cerevisiae* regulates cAMP metabolism by stimulating adenylyl cyclase activity (69, 156). [Val-19]*RAS2* phenotypes were similar to those associated with *bcy1*, which bypasses the requirement for a functional adenylyl cyclase. Furthermore, *bcy1* suppressed lethality in *ras1 ras2* mutant strains. The catabolic enzyme trehalase, which is stimulated by cAMP-dependent protein kinase, was maximally activated in extracts of [Val-19]*RAS2*-transformed cells and was insensitive to further incubation with cAMP and cAMP-dependent protein kinase. This result indicated that the transducer of cAMP, the cAMP-dependent protein kinase, and substrate targets such as trehalase were maximally activated. Consistent with these observations, intracellular cAMP levels were fourfold higher in [Val-19]*RAS2* cells and fourfold lower in *ras2* mutant cells. In vitro adenylyl cyclase activity in membranes derived from [Val-19]*RAS2* or *ras2* cells was proportionately high or low, respectively, compared with the wild type (156). A more recent study indicates that *S. cerevisiae* cells lacking both *RAS* genes do not synthesize cAMP in response to glucose induction (93).

More refined analyses provided evidence that *RAS* proteins stimulated *S. cerevisiae* adenylyl cyclase in a GTP-dependent manner. Reconstitution of purified yeast or mammalian Ras with membranes derived from a strain genetically devoid of *RAS* protein restored adenylyl cyclase activity (15, 32, 42). This reconstitution was observed only with RAS-GTP and not with RAS-GDP. Furthermore, RAS-GDP did not compete with RAS-GTP (42). These results provided the first clear evidence that RAS could modulate an effector activity in a manner dependent on the complexed guanine nucleotide. In addition, the data indicated that RAS-GDP either does not interact with the target protein or does so poorly compared with RAS-GTP.

Regulation of cAMP metabolism appears to be a major function of RAS in *S. cerevisiae*. Suppressors of *ras2* mutations (*SRA*) as screened by growth on nonfermentable carbon sources correspond to key enzymes within the cAMP cascade (Table 1) (20). These suppressors include a constitutive adenylyl cyclase (*SRA4*), an impaired low-K_m phosphodiesterase (*sra5*), and altered regulatory (*sra1*) or catalytic (*SRA3*) subunits of the cAMP-dependent protein kinase. With the exception of *sra5*, these suppressors also bypass the requirement of RAS for cell viability. The presence of a second phosphodiesterase with a high K_m for cAMP (*PDE1*) explains the inability of *sra5* to be *RAS* independent. Cells that are devoid of both *RAS* genes and both *PDE* genes are viable because a sufficient steady-state level of cAMP is maintained (107, 108).

The strong positive control of cAMP metabolism by *RAS* proteins in *S. cerevisiae* is subject to negative regulatory mechanisms. Evidence for such a mechanism was most clearly seen in cells devoid of both *PDE1* and *PDE2* (107, 108). Intracellular cAMP levels in *pde1 pde2* cells were only two- to threefold higher than that found in wild-type cells. This feedback requires cAMP-dependent protein kinase activity (107). The *RAS2* protein may be one of the sensitive substrates in vivo (27a, 120, 140a). In vitro, phosphorylation of the *RAS2* gene product by cAMP-dependent protein kinase inhibits stimulation of adenylyl cyclase (120). Interestingly, [Val-19]*RAS2* cells lacking both *PDE* genes have 1,000-fold-higher cAMP levels than wild-type cells do, sug-

gesting that activated *RAS* proteins can overcome the negative feedback controls (107). Adenylyl cyclase may also be a target for feedback mechanisms involving protein phosphorylation. Using a temperature-sensitive *RAS2* allele, De Vendittis et al. isolated a mutant adenylyl cyclase, *CRI4*, that could suppress impaired RAS2 function at the nonpermissive temperature (32). *CRI4* yeast cells are characterized as having an elevated RAS-independent activity as well as an oversensitive RAS-dependent activity. Therefore, the mutation does not uncouple adenylyl cyclase from *RAS* protein. Rather, *CRI4* adenylyl cyclase appears to be resistant to down-regulation mechanisms. The mutation responsible for the *CRI4* phenotype is a Thr-to-Ile change at residue 1651, located in the catalytic domain of adenylyl cyclase (68, 89, 164). The residues immediately surrounding amino acid 1651 share homology with known protein kinase substrates. If phosphorylation were to occur at this residue, it could influence adenylyl cyclase catalytic activity (32). However, this hypothesis has not yet been tested directly. Mutations in the *CAS1* allele also enhance adenylyl cyclase activity in vitro (11). Although *CAS1* is not allelic with *CYR1* or *RAS*, the nature of the *CAS1* gene product is unknown.

RAS1 versus RAS2

The divergence within the unique region of RAS1 and RAS2 led to speculation that these related proteins might have different functions (29, 70, 117). Consistent with these hypotheses were the observations that the presence of the chromosomal *RAS1* gene could not complement the phenotypes associated with *ras2* mutations such as defective growth on nonfermentable carbon sources and low levels of intracellular cAMP (44, 70, 152). Furthermore, no clear phenotype was apparent for the *ras1* null and the activated [Val-19]*RAS1* alleles. The absence of a biological phenotype for *ras1* mutant strains appears to be due to transcriptional and translational controls that distinguish *RAS1* from *RAS2*. The steady-state level of *RAS1* messenger ribonucleic acid (mRNA) and the rate of *RAS1* protein synthesis are reduced as cells approach the mid-logarithmic phase of growth on glucose (12). In contrast, synthesis of the *RAS2* protein is low during early logarithmic growth, whereas *RAS2* mRNA levels are high during all phases of growth (13). When yeast cells are grown in media containing nonfermentable carbon sources, *RAS2* mRNA levels remain constant, with increased synthesis of *RAS2* protein during the early logarithmic phase. The amounts of *RAS1* mRNA and protein are diminished during cultivation on nonfermentable carbon sources (12). Therefore, the inability of *RAS1 ras2* strains to grow on nonfermentable media is a direct result of reduced *RAS1* gene expression, which leaves the yeast cells functionally RAS^-. The extragenic suppressor *sra6* bypasses the growth defect of *RAS1 ras2* strains on nonfermentable carbon sources by permitting elevated levels of *RAS1* mRNA (12). When expressed from the constitutive *ADH1* promoter, *RAS1* can fully suppress *ras2* null mutations (86). Furthermore, when constitutively expressed, the activated [Val-19]*RAS1* and [Leu-68]*RAS1* alleles cause the same characteristic phenotypes as activated alleles of *RAS2* do (86). These results provide evidence that the gene products of *RAS1* and *RAS2* are biologically equivalent, although transcriptional and translational controls determine under what physiological conditions either *RAS* protein will be expressed.

RAS Interaction with Adenylyl Cyclase

A critical question concerning how *RAS* proteins regulate adenylyl cyclase function is whether RAS directly interacts with adenylyl cyclase or acts through an unidentified intermediate(s) to regulate adenylyl cyclase activity. Mutagenesis studies of mammalian and yeast *ras* proteins have identified a region of the protein critical for effector function and biological activity (38, 87, 138, 141, 176). This putative effector domain includes residues 30 to 40 of mammalian Ras and 37 to 47 of yeast RAS. Deletions or single-amino-acid substitutions within this region dramatically impair the transforming activity of mammalian Ras. Likewise, these mutations in yeast or mammalian *ras* proteins impair the ability to complement the *ras2* defect or to stimulate adenylyl cyclase activity. This putative effector region of RAS has been identified by systematic as well as random mutagenesis approaches (38, 87, 138, 141, 176). Interestingly, both approaches also revealed a second critical region. Mutations at yeast RAS2 residues 82 and 84 (corresponding to mammalian Ras residues 75 and 77) or mammalian Ras residue 78 also partially inhibit Ras function in the yeast (38, 138). In the crystal structure of mammalian Ras, residues 75 to 78 lie on the far surface of the protein adjacent to the C-terminal alpha helix (33). Residues 30 to 40 are on the near surface adjacent to the guanine nucleotide-binding domain.

Using [Ser-42]*RAS2*, which only weakly complements defective growth on nonfermentable carbon sources in *ras2* strains, we developed a genetic screen to identify the immediate target protein of RAS in *S. cerevisiae* (87). The goal of this screen was to isolate a dominant gene that would allow [Ser-42]*RAS2* to promote cell growth in media containing a nonfermentable carbon source. One of the suppressors isolated was dominant, *RAS* dependent, and biologically responsive to at least two different mutant *RAS* genes having impaired effector function. This second-site suppressor mutation, *SSR2*, mapped to the adenylyl cyclase locus (87). Cloning of *SSR2* revealed that it was the structural gene for adenylyl cyclase and that it encoded an adenylyl cyclase with a single point mutation at nucleotide 5300. This mutation is predicted to create a single-amino-acid substitution of Asp to Tyr at residue 1547. Biochemical experiments indicated that the *SSR2* adenylyl cyclase required *RAS* protein to produce cAMP and was responsive to both wild-type RAS and mutant RAS having impaired effector activity. A second-site mutation in the gene encoding an actual target protein would be expected to promote such an interaction with biologically inactive *RAS* proteins.

On the basis of structure-function studies of the 2,026-residue adenylyl cyclase protein, the position of the SSR2 mutation at amino acid 1547 is within a domain that is required for interaction with RAS. Gene disruption and biochemical experiments indicate that the adenylyl cyclase protein consists of a catalytic domain, a membrane domain, and regulatory sequences (68, 163, 164). The catalytic domain resides in the carboxy-terminal 400 amino acids (approximately residues 1609 to 2026) and catalyzes the formation of cAMP. The region between residues 734 and 1300 may be important for membrane localization based upon a sequence that consists of tandem amphipathic repeats (68). A domain conferring RAS-sensitive adenylyl cyclase activity has been localized to the 293-amino-acid region between the amphipathic and catalytic domains (68, 164). Expression of the 3′-terminal 2.1-kilobase (kb) region of the *CYR1* gene in both *Escherichia coli* and *S. cerevisiae* gave an adenylyl cyclase activity that was RAS dependent and sensitive to

stable GTP analogs only in the presence of *RAS* protein (164). This analysis of adenylyl cyclase, as well as the identification of the *SSR2* mutation at position 1547, provides evidence that the amino acids within the region 1300 to 1600 are critical for the interaction of adenylyl cyclase with *RAS* proteins.

Physical studies of *S. cerevisiae* adenylyl cyclase have provided some insights into the structural characteristics of the active enzyme complex. Extraction of wild-type yeast membranes with detergent and salt released an adenylyl cyclase complex having a molecular mass of 450 kDa (166) or 594 kDa (64). Analysis of the 594-kDa complex showed that it possessed a catalytic activity in the presence of Mn^{2+} which was insensitive to guanine nucleotide stimulation. The size of this complex is consistent with the presence of one or more adenylyl cyclase molecules (predicted monomer size of 220 kDa) and other associated proteins. The 594-kDa complex behaved identically when isolated from a *ras1 ras2* strain of *S. cerevisiae*, suggesting that *RAS* proteins did not coextract with adenylyl cyclase. Field et al. have also observed that RAS apparently does not form a stable complex with adenylyl cyclase (43). Using an epitope addition method, they purified adenylyl cyclase from *S. cerevisiae* cells with a specific monoclonal antibody (43). This antibody was directed against a polypeptide epitope which had been fused to adenylyl cyclase by using recombinant deoxyribonucleic acid techniques to modify the *CYR1* gene. Additional purification over a glycerol gradient followed by analysis on sodium dodecyl sulfate-polyacrylamide gels identified two protein components (200 and 70 kDa) associated with the fraction containing adenylyl cyclase activity. The 200-kDa polypeptide was identified as adenylyl cyclase; however, the nature of the 70-kDa form has yet to be identified. The copurification of the 70-kDa protein with adenylyl cyclase suggests that it may be complexed with adenylyl cyclase. The purified adenylyl cyclase complex could be activated by purified *RAS* protein bound to GTP, indicating that the RAS-adenylyl cyclase interaction requires few components.

The *CDC25* Gene

The *CDC25* gene product is involved in the regulation of the cAMP pathway in *S. cerevisiae* (19, 88) at a point upstream of both *RAS* and *CYR1* (16, 86, 125). The *CDC25* gene was originally identified as a temperature-sensitive class II start mutant showing altered intracellular levels of cAMP at the restrictive temperature (62). Biochemical and genetic evidence demonstrated that the *CDC25* gene product was part of the RAS-adenylyl cyclase pathway. The temperature-sensitive *cdc25* growth defect could be suppressed by the addition of exogenous cAMP (88), and suppressors of *ras* mutations were also capable of bypassing the requirement for a functional *CDC25* gene product (16, 20, 75). In addition, the activated [Val-19]*RAS2* allele but not normal *RAS2* could bypass the growth arrest of the *cdc25-1* mutation (16, 86, 125). These results suggested that CDC25 functioned in the cAMP metabolic pathway at some point upstream of RAS. Furthermore, it appeared that CDC25 was required for normal RAS action.

The ability of the activated [Val-19]*RAS2* protein with impaired GTPase activity to bypass the requirement for CDC25 suggested that a functional interaction between normal RAS and the *CDC25* gene product might involve the formation or stabilization of the active RAS-GTP complex. Biochemical studies have provided data consistent with the hypothesis that the *CDC25* protein acts as an exchange factor to regenerate RAS-GTP from RAS-GDP (16, 27b, 86, 125). In membranes isolated from *cdc25* strains, basal adenylyl cyclase activity assayed in the presence of Mg^{2+} is barely measurable. Activity was restored upon addition of exogenous GTP analogs such as Gpp(NH)p or GTP[S] (16, 86). The K_{act} (concentration required for half-maximal activation) for Gpp(NH)p stimulation of adenylyl cyclase activity was the same (1 μM) in wild-type and *cdc25* strains, suggesting that CDC25 does not influence the coupling between RAS and adenylyl cyclase (16). These results indicate that RAS in membranes derived from the *cdc25* strain is bound to GDP, whereas RAS in wild-type cells exists complexed to some GTP.

RAS proteins, having facilitated guanine nucleotide exchange kinetics, are also able to bypass the requirement for a functional *CDC25* gene product, a result consistent with the hypothesis that CDC25 function involves formation of the active RAS-GTP complex. [Ile-152]*RAS2* (a mutation at a position analogous to mammalian Ras residue 144) was isolated as a natural suppressor mutation of *cdc25-5* (18). This RAS2 mutation is within the nucleotide-binding site and has been shown to reduce the affinity of mammalian Ras for guanine nucleotides (18, 39). Expression in *S. cerevisiae* of the [Asn-16]Ha-*ras* mutant, which possesses reduced affinity for guanine nucleotides, was found to be lethal to wild-type cells growing at 37°C (139). This phenotype was postulated to result from the nucleotide-free species of Ras forming a dead-end complex with an exchange factor (139). The [Asn-16]Ha-*ras* lethal phenotype was shown to be suppressible by either [Val-19]*RAS2* or inactivation of the *CDC25* gene (86, 139). Conversely, the *cdc25-1* phenotype was itself suppressed by the expression of [Asn-16]Ha-*ras* probably reflecting GTP bound to the mutant *ras* protein (86). Lethality is also observed with [Ala-22]*RAS2* or [Ala-15]Ha ras, and this phenotype can be suppressed by [Val-19]*RAS2* or by overexpression of *CDC25* (118a). If the *CDC25* gene product does in fact function as a GDP-GTP exchange factor, cells with increased CDC25 activity might be expected to be phenotypically similar to cells expressing the [Val-19]*RAS2* gene. At least one such activated allele of *CDC25* has been identified by its ability to induce heat shock sensitivity into wild-type strains (16).

The wild-type *CDC25* gene has been cloned and sequenced and found to potentially encode a protein of 1,587 amino acids and a predicted molecular size of 178 kDa (16, 19). The *CDC25* gene product does not have obvious homology to any proteins in the sequence data bases and lacks any strong hydrophobic or transmembrane domains. A preliminary structure-function study revealed that expression of the 3′ region of *CDC25* encoding the C-terminal 712 residues was sufficient to complement a null *cdc25-5* mutation (19). The N-terminal half of the protein may have a distinct function, such as regulating the catalytic activity of the C-terminal domain.

In a search for genes capable of suppressing the *cdc25-5* mutation, a genomic *S. cerevisiae* plasmid library was screened (E. Boy-Marcotte, F. Damak, J. Camonis, H. Garreau, and M. Jacquet, Gene, in press). One plasmid capable of suppressing the *cdc25-5* growth defect contained a DNA fragment encoding a protein 45% homologous to the CDC25 C-terminal domain. Further sequence analysis of overlapping DNA clones showed that the complementing open reading frame was in fact the 3′ end of a much larger open reading frame. The complete gene encoded a protein of 1,251 amino acids with extensive regions of homology with the predicted *CDC25* gene product. This *CDC25* homolog

TABLE 2. Regulation of yeast RAS function by its C-terminal domain and the *CDC25* gene product[a]

RAS gene	Adenylyl cyclase activity[b]		*cdc25* bypass[c]	% GTP bound[d]
	Wild-type	*cdc25*		
RAS2	9.9	0.9	–	0
RAS2 Δ[e]	12.2	6.8	+	3
[Ala-18 Val-19]*RAS2*	6.4	12.2	++	16
[Ala-18 Val-19]*RAS2* Δ[e]	18.1	31.2	+++	33

[a] The results in this table are summarized from references 56 and 86.

[b] Wild-type and *cdc25-1 S. cerevisiae* cells were transformed with the indicated *RAS* gene, membranes were prepared, and basal adenylyl cyclase activity was assayed in the presence of Mg^{2+} ion (86). Results are expressed as picomoles of cAMP per minute per milligram of protein.

[c] The ability of *RAS* genes to bypass the lethality of a temperature-sensitive *cdc25-1* mutation was scored by the level of growth at 37°C (86).

[d] Yeast cells were labeled with $^{32}P_i$, *RAS* proteins were immunoprecipitated, and the guanine nucleotides bound to RAS were quantitated by thin-layer chromatography (56). Results are expressed as the percentage of GTP bound relative to total GTP plus GDP detected.

[e] Δ, Deletion of RAS2 residues 175 to 300.

was designated *SCD25* (for suppression of *cdc25-5* mutation) (Boy-Marcotte et al., in press). Only expression of the 3′ portion of the *SCD25* gene and not the full-length *SCD25* gene suppressed the lethal phenotype of *cdc25* strains. The complementing fragment of *SCD25* corresponds to the minimum complementing domain of the *CDC25* protein. Apparently *CDC25* and *SCD25* encode proteins with similar activities, but in the case of *SCD25*, the target of this activity remains unknown.

Regulatory Function of the RAS C-Terminal Domain

As previously mentioned, both the *RAS1* and *RAS2* proteins differ from their mammalian homologs by the presence of divergent C-terminal domains of approximately 120 amino acids. A functional role for the unique RAS2 C-terminal domain is evident from the observation that *RAS2* mutant proteins lacking the unique C-terminal domain (residues 175 to 300) but retaining the membrane attachment site (RAS2Δ) can bypass the requirement for CDC25 (86). Both normal and oncogenic mammalian *ras* proteins, which do not have a domain equivalent to the RAS2 C-terminal unique region, can also bypass the requirement for a functional *CDC25* gene product (86). Biochemical experiments are consistent with the observed biology. When membranes derived from wild-type cells expressing full-length *RAS2* protein are used, basal adenylyl cyclase activity is low, but the activity is stimulated two- to fourfold by the addition of Gpp(NH)p. When membranes derived from a strain expressing RAS2Δ are assayed, basal adenylyl cyclase activity is two- to fourfold higher than that observed in wild-type RAS2-containing cells and is not stimulated upon addition of exogenous GTP analogs. Similar results were obtained when wild-type mammalian Ras was expressed in *S. cerevisiae* (26, 86). These results suggest that *RAS* proteins lacking a C-terminal domain (RAS2Δ, mammalian Ras) are stably bound to more GTP than is full-length RAS2. Analysis of the guanine nucleotides bound to RAS2Δ in growing yeast cells demonstrated that this protein was complexed to a small amount of GTP, whereas the full-length *RAS* protein was bound entirely to GDP (56). A *RAS2* mutant protein lacking the C-terminal domain and having impaired GTPase (Val-19) was the most potent for bypassing *cdc25* lethality, and this protein had the largest amount of bound GTP. A summary of the biological and biochemical potencies of various *RAS* proteins is presented in Table 2. The results closely correlate biochemical and biological activity with the amount of GTP complexed to the *RAS* protein in vivo. These results suggest that the RAS C-terminal domain serves a negative regulatory function by promoting the GDP-bound form of RAS either through interaction with another protein component or through its effect on the conformation of the protein.

The *IRA1* Gene

Recently Tanaka et al. identified another component of the RAS-cAMP pathway designated IRA1 (149). The *IRA1* (inhibitory regulator of the RAS-cAMP pathway) gene product seems to be required for maintaining low levels of cAMP in the yeast cells. Strains containing *ira1* mutations contained increased levels of cAMP and displayed phenotypes associated with the *bcy1* and [Val-19]*RAS2* mutations such as sporulation deficiency and sensitivity to nitrogen starvation and heat shock. Genetic analysis of *ira1* mutants suggests that IRA1 acts upstream of RAS2 and CYR1 in the cAMP pathway. Null alleles of *IRA1* were observed to suppress the growth defect of *cdc25-1* but not the lethality of a *ras1 ras2* double mutation or a *CYR1* gene disruption. For a *cdc25 ira1* double null mutation, mutual suppression between the two genes was observed with both the *cdc25* lethality and the *ira1* heat shock phenotype suppressed. Interestingly, the *ira1* phenotype could also be completely suppressed by a *ras2* mutation, but not at all by a *ras1* mutation. On the basis of these results, Tanaka et al. (149) concluded that IRA1 functions upstream of RAS2 but not RAS1 in the cAMP pathway, possibly in a manner antagonistic to CDC25. Another gene, *IRA2*, has been identified as a homolog of *IRA1* and may influence *RAS1* protein function (K. Matsumoto, personal communication).

A clue to the biochemical function of IRA1 comes from analysis of the predicted protein sequence determined from cloned *IRA1* DNA. The *IRA1* gene could potentially encode a large protein, of 2,938 amino acids (149). When the amino acid sequence of IRA1 was compared with the amino acid sequence of bovine brain or human placenta Ras GTPase-activating protein (GAP [158a, 167]), a significant similarity was observed between residues 1683 to 1780 of IRA1 and residues 870 to 975 of GAP. GAP, a cytosolic 125-kDa monomeric polypeptide, binds the GTP complexes of both normal and oncogenic Ras and stimulates 100-fold the intrinsic GTPase activity of normal Ras but not that of oncogenic Ras with impaired GTPase activity (55, 158, 167). Although GAP-like activity has not been detected in extracts of yeast cells (1), mammalian GAP can stimulate the GTPase activity of yeast *RAS* protein (55; J. B. Gibbs, U. S. Vogel, M. D. Schaber, M. S. Marshall, R. E. Diehl, E. M. Scolnick, R. A. F. Dixon, and I. S. Sigal, *in* L. Bosch, B. Kraal, and A. Parmeggiani, ed., *The Guanine-Nucleotide Binding Proteins: Common Structural and Functional Properties*, in press). The C-terminal 40-kDa domain of GAP encompassing residues 702 to 1044 is sufficient to bind Ras and to catalytically stimulate Ras GTPase activity (87a). Interestingly, the region of GAP homologous to IRA1 is within this C-terminal catalytic domain. This finding suggests that IRA1 might bind *RAS* protein and possess GAP-like activity. The genetic studies of *IRA1* support the concept that IRA1 acts to down-regulate RAS2 activity, and hence adenylyl cyclase activity, by stimulating the GTPase activity of *RAS2* protein. Since *ras1* mutations do not suppress the *ira1* phenotype, RAS1 may not be regulated by IRA1, although the intrica-

cies of *RAS1* mRNA expression make this observation difficult to interpret. Should IRA1 act only upon RAS2, it is possible that specificity might be determined by the unique RAS2 C-terminal domain.

Alternative Pathways

Although regulation of cAMP metabolism appears to be a major function of RAS in *S. cerevisiae* cells, there is some evidence, both genetic and biochemical, that RAS may have some other actions as well. The cAMP signal produced by RAS stimulation of adenylyl cyclase is transduced by cAMP-dependent protein kinases. These kinases belong to a family having three members in *S. cerevisiae* that are encoded by the genes *TPK1*, *TPK2* and *TPK3* (155). Disruptions of any two of the three *TPK* genes are not lethal; however, at least one functional gene is required for normal cell growth. When expressed on a multicopy plasmid, any one of the *TPK* genes can bypass the requirement for functional CDC25 and CYR1. Overexpression of *TPK1* also is capable of bypassing the lethality of a *ras1 ras2* double disruption. However, overexpression of *TPK2* or *TPK3* only weakly bypasses this phenotype. Upon disruption of all three *TPK* genes, cells either are nonviable or germinate and grow very slowly. Although these kinases are downstream of RAS function, the small amount of growth possible in the absence of functional TPK is in sharp contrast to the absolute requirement for functional RAS (155). Assuming that no other cAMP-dependent protein kinases are present in *S. cerevisiae*, this result implies that cAMP-mediated action is extremely important but not absolutely essential for cell viability. Toda et al. have interpreted these results as being consistent with other possible actions of RAS in *S. cerevisiae* (155).

In mammalian cells, the formation of inositol trisphosphate stimulates calcium release from microsomal compartments and results in elevated levels of intracellular calcium (8). This increase in calcium levels then activates calmodulin-dependent enzymes. In *S. cerevisiae*, changes in calcium levels can occur during the cell cycle or in response to extracellular signals such as alpha mating factor or glucose (67, 109, 110). The transducer of calcium signals, calmodulin, is present in *S. cerevisiae* and is an essential gene (28). Polyphosphoinositides have been identified in *S. cerevisiae* cells, and glucose can stimulate turnover in this metabolic pathway. There has been a report that *RAS* proteins in *S. cerevisiae* may influence phosphoinositide metabolism and subsequent changes in intracellular calcium (67).

PROTEINS HOMOLOGOUS TO RAS IN OTHER LOWER EUCARYOTES

The presence of *ras* genes in other evolutionarily diverse organisms such as *S. pombe*, *D. discoideum*, and *D. melanogaster* highlights the apparently central importance of Ras in cell physiology (49, 97, 102, 106, 121). Furthermore, analysis of Ras biology in these different cells allows comparisons of different biochemical pathways and different developmental cycles. A common feature that distinguishes these organisms from *S. cerevisiae* is that none have a Ras-sensitive adenylyl cyclase, as is also observed with higher eucaryotes (51, 101, 122). The pathway(s) influenced by Ras in these other lower eucaryotes may be more closely related to mammalian Ras function.

The fission yeast *S. pombe* has a single gene, *ras1*, that is homologous to mammalian *ras* (49, 102). The gene encodes a single 1.2-kb mRNA. The coding sequence for 219 amino acids consists of a 175-amino-acid N-terminal domain containing the guanine nucleotide and effector regions, a 40-residue unique region, and the C-terminal 4 amino acids required for membrane localization. Disruptions of *ras1* block mating function, which appears to involve a diffusible pheromonelike factor secreted by h^- cells (50, 51, 101). The *ras1* disruption in h^- cells does not impair secretion of the factor; however, *ras1* h^+ mutant cells are unable to respond (50). This result suggests that *S. pombe* Ras1 is involved in the signal transduction mechanism for responding to mating pheromone. The mating defect phenotype can be complemented by mammalian Ras. Ras function in *S. pombe* may require protein kinase activity (100).

In *D. discoideum*, a single *ras* gene that encodes a 187-residue protein with 60% overall homology to mammalian Ras has been described (121). The gene encodes two mRNA species, of 0.9 and 1.2 kb, that are differentially expressed. The 1.2-kb mRNA is found in vegetative cells and can be induced by cAMP under appropriate culture conditions. Under nutrient deprivation conditions, *D. discoideum* cells aggregate and form spore and stalk cells. During the initial phases of differentiation in response to starvation, the level of the 1.2-kb mRNA rapidly decreases. Later, both the 0.9- and 1.2-kb mRNAs reaccumulate in prestalk cells. A single translation product of 23 kDa that reacts with anti-Ras antibodies is detected in *D. discoideum* cells, and the level of this protein appears to remain constant during the changes in mRNA levels (113, 121, 123, 172). Expression of Ras in *D. discoideum* cells is apparently essential, because transfection of antisense deoxyribonucleic acid is lethal (123). The essential role of Ras in these cells does not involve regulation of adenylyl cyclase activity or intracellular cAMP levels (122). However, a role of *D. discoideum* Ras in signal transduction is suggested by reduced chemotactic sensitivity to cAMP in cells expressing the activated [Thr-12]*ras* gene (165). Cells expressing [Thr-12]*ras* have elevated steady-state levels of water-soluble inositol phosphates and a diminished intracellular cGMP response when challenged with the chemoattractant cAMP (37, 165). This attenuated response is due to down-regulation of cAMP binding (80). The mechanism of this down-regulation may involve Ras-induced protein kinase C activation, because in vitro, cAMP binding to wild-type membranes can be down-regulated by a mixture of adenosine triphosphate (ATP), Ca^{2+}, and either phorbol ester or GTP (80). This mixture had no effect on the down-regulated cAMP binding to membranes derived from cells expressing the [Thr-12]*ras* protein, implying that the same mechanisms were involved.

Three *ras* genes have been identified in *D. melanogaster* (14, 97, 106, 129). The *D. melanogaster ras* gene products are referred to as Dras. Dras1 is 75% homologous to mammalian Ras, and Dras2/64B and Dras3 share 50% homology with mammalian Ras. Dras3 is more closely related to the mammalian Ras homologs Rap1A, Rap1B, and Rap2 (114, 115). It has been proposed that the *rap* gene products may act as antagonists of Ras (70a, 72a, 114, 115). The RNAs of all three *Dras* genes are expressed with similar tissue distribution as analyzed by in situ hybridization (74, 96, 133). During early development, *Dras* transcripts are uniformly distributed among all tissues. In larvae, the transcripts are found predominantly in dividing cells such as reproductive and neural tissue. In adults, the most abundant localization is in the reproductive tissue and brain, which at this stage are differentiated and nondividing. This high concentration of Ras in neural cells is also observed in mammals and *Aplysia*

TABLE 3. G proteins in *S. cerevisiae*

Gene	Function
RAS1, *RAS2*	Regulation of adenylyl cyclase activity; both *RAS* genes required for viability
RHO1, *RHO2*	Function unknown; *RHO1* is an essential gene
GPA1, *GPA2*	*GPA1* (*SCG1*) is required for the viability of haploid cells as well as response to mating pheromone; *GPA2* (*SCG2*) may affect the cAMP pathway but is not an essential gene
YPT1	Possibly involved in regulation of intracellular Ca^{2+} levels; *YPT1* is an essential gene
SEC4	Required for protein secretion at the post-Golgi stage; *SEC4* is an essential gene

californica (52, 143). Transgenic *D. melanogaster* cells expressing an activated *Dras2*/64B gene have low fertility and develop a doral-to-ventral eye scar during development (9). The low fertility probably reflects expression of Ras in the gonads. As noted by Bishop and Corces (9), the abnormal eye development is particularly interesting, because a mutant allele of *Notch* also causes this abnormality. *Notch* is a *D. melanogaster* homolog of the epidermal growth factor receptor (71, 173). *Sevenless*, a putative membrane-spanning tyrosine kinase, also causes eye abnormalities (6, 60). Bishop and Corces speculate that tyrosine kinases and Ras are part of a signal transduction pathway during *D. melanogaster* eye development (9).

RAS-RELATED PROTEINS

Several genes have been identified in *S. cerevisiae* that encode proteins approximately 20 kDa in size and that share up to 40% amino acid homology with mammalian Ras. The list to date includes *YPT1*, *RHO1* and *RHO2*, and *SEC4* (53, 81, 127). The strongest homology among the proteins encoded by these genes exists in regions required for guanine nucleotide binding. Consistent with this observation, biochemical studies have demonstrated that both YPT1 and RHO bind and hydrolyze GTP (4, 170). SEC4 also binds GTP as determined by a GTP-blotting assay (59). However, it is clear that these proteins exert biological effects that are distinct from RAS; none complement RAS defects in *S. cerevisiae*. Furthermore, *YPT1*, *RHO*, and *SEC4* are essential genes which do not complement each other (53, 82, 127, 134). Genetic studies described below also indicate that these GTP-binding proteins influence very different biochemical events. Summaries of G-protein functions in *S. cerevisiae* are listed in Table 3.

The *YPT1* gene was originally discovered as an open reading frame between the actin and beta-tubulin genes (53). In the absence of a functional *YPT1* gene, cells cease to divide and are unable to sporulate. Morphologically, the cells become multibudded, with abnormally long and apparently disorganized microtubules (132, 134). Actin structure is also altered. Actin and beta-tubulin gene expression are not affected, indicating that the action of YPT1, either directly or indirectly, influences the microtubule organization. Consistent with YPT1 having a function distinct from RAS, the *bcy1* mutation (constitutive cAMP-dependent protein kinase activity) is unable to bypass the requirement for YPT1 (134). The GTP-binding properties of YPT1 apparently are essential to its function, because a mutation in the nucleotide-binding site (Asn-121 to Ile) results in a dominant lethal phenotype (132). YPT1 localizes to membranes after palmitoylation on a C-terminal cysteine residue (95) and may be required for protein secretion and calcium homeostasis (131, 135). A central role of YPT1 in cell physiology is suggested by its conservation in mammalian tissues (63, 157).

The *rho* gene was originally identified in the marine snail *A. californica*, and homologs were subsequently identified in both human and *S. cerevisiae* cells (81, 82). In the yeast *S. cerevisiae*, two genes, *RHO1* and *RHO2*, are present that are 70 and 57% homologous, respectively, to *Aplysia rho*. *RHO1* but not *RHO2* is essential for viability (82). The lethal RHO1$^-$ phenotype cannot be bypassed by either [Val-19]*RAS2* or overexpression of the catalytic subunit of cAMP-dependent protein kinase, indicating that RHO does not act through the adenylyl cyclase regulatory system. An activated allele, [His-68]*RHO1*, inhibits sporulation in a dominant manner (82). This phenotype is similar to that observed with the activated [Val-19]*RAS2* allele, even though the two genes are functionally distinct. Mammalian *rho* protein serves as a substrate for adenosine diphosphate (ADP) ribosylation by the exoenzyme of botulinum toxin, although the physiological significance of this modification is not clear at present (72, 96).

Studies of protein secretion in *S. cerevisiae* have identified a series of genes, *SEC*, necessary for various functions at the endoplasmic reticulum, Golgi apparatus, and post-Golgi vesicle stages. The *SEC4* gene product, required for a post-Golgi event, shares 32 and 48% homology with mammalian RAS and YPT1, respectively (127). Like *YPT1*, *SEC4* encodes a serine at the position analogous to Ras Gly-12. In YPT1, this mutation inhibits intrinsic GTPase activity (170). Upon disruption of the *SEC4* gene, cells cease to divide, indicating that it is an essential locus. In yeast cells having a temperature-sensitive allele of *SEC4*, invertase secretion stopped 100% within 15 min of a shift to the nonpermissive temperature (127). The rapidity of this effect suggests that SEC4 has a direct action on protein secretion late in the process. Consistent with this idea, the wild-type *SEC4* gene can suppress lethal *sec* alleles involved in post-Golgi vesicle events but not those required at the endoplasmic reticulum or Golgi apparatus stages. Although the *SEC4* protein has a terminal cysteine residue and is localized to membranes, it does not require a functional *DPR1-RAM* gene product (59).

In addition to G proteins of the 20-kDa class, *S. cerevisiae* cells have two genes, *GPA1* and *GPA2*, that are predicted to encode G proteins of 40 kDa (35, 103, 104). These proteins share 40 to 60% homology with the mammalian 40-kDa class G proteins such as G_s, G_i, and G_o. *GPA1*, also called *SCG1*, is required for mating-factor response and may be part of a pheromone signal-transducing pathway (35). Null alleles of *GPA1-SCG1* cause a haploid-specific growth arrest, similar to that observed upon treatment of haploid cells with mating pheromone. Genetic studies of *GPA1* suggest that the *GPA1* protein itself does not have effector function, but rather that it releases a signal-transducing subunit upon exposure of the cell to mating pheromone (35). The growth arrest phenotype of *gpa1* mutants can be suppressed by the *sgp2* mutation, which is allelic with *DPR1* (105). Since *SGP2* (*DPR1*) is required for membrane localization of other proteins in *S. cerevisiae*, a component downstream of GPA1 in the mating-factor signal transduction pathway probably must be membrane associated to transmit a growth arrest signal (105). Gene disruptions studies with *GPA2* indicate that it is not an essential gene (104). Although *GPA2* does not suppress lethal mutations in *ras*, *cdc25*, or *cdc35*, expression of *GPA2* at high copy number induces high levels of glucose-induced cAMP accumulation in yeast cells containing a temperature-sensitive *ras2* allele (104).

CONCLUSIONS

The *ras*-encoded proteins clearly have important functions in the normal cellular physiology of lower eucaryotic organisms. In *S. cerevisiae*, RAS is essential for viability, whereas in *S. pombe* and *D. discoideum*, Ras is necessary for appropriate responses to mating factor and chemoattractants, respectively. Analysis of a more complex organism such as *D. melanogaster* reveals a role of Ras in development. In each case, the intrinsic biochemical properties of Ras (GTP binding and hydrolysis) are central to Ras function. Alteration of these properties leads to aberrant physiology or development. Thus, fundamental mechanisms of Ras action appear to be conserved among these lower eucaryotic organisms as well as in mammalian cells.

The greatest level of detail of how Ras exerts its physiological actions has been attained for the yeast *S. cerevisiae*. Experimental results for this system provided the first definitive evidence that *ras*-encoded proteins were biochemically and biologically active only when complexed to GTP. Furthermore, the interaction of RAS with other proteins appears to regulate the interconversion between the GTP and GDP complexes of RAS. The *CDC25* gene product acts as a positive element to promote the formation of the GTP complex, whereas the *IRA* gene product appears to be a negative regulator, possibly stimulating the conversion of RAS-GTP to RAS-GDP. It is interesting that there is a high degree of duplication of proteins in the cAMP pathway of *S. cerevisiae*: two positive regulators (CDC25 and SCD25), two negative regulators (IRA1 and IRA2), two G proteins (RAS1 and RAS2), two phosphodiesterases (PDE1 and PDE2), and at least three protein kinases (TPK1, TPK2, and TPK3). Only adenylyl cyclase (CYR1) and the regulatory subunit of the cAMP-dependent protein kinases (BCY1) are present as the products of single genes. The redundancy may be required for appropriate responsiveness of *S. cerevisiae* cells to a wide variety of nutrient and growth conditions. Although RAS regulation of adenylyl cyclase activity appears to be unique to *S. cerevisiae*, the discovery of this interaction was the first evidence that RAS could couple with another enzyme and modify its activity. If RAS regulates other pathways in *S. cerevisiae* cells, the effector(s) may be more conserved with the target(s) of Ras action in other lower eucaryotes and in mammalian cells.

G-protein regulation of metabolic activities is conserved in evolution, as evidenced by the multiple G proteins found in *S. cerevisiae*. The G proteins in *S. cerevisiae* are required for appropriate responses to extracellular signals and for secretion mechanisms as have been observed for events in mammalian cells. G proteins are also present in procaryotes; the *E. coli era* gene product is a GTP-binding protein that is essential for normal cellular growth (2, 85). Although G proteins regulate many fundamental cellular processes, in mammalian cells only Ras has the ability to transform cells and stimulate tumor formation. Obviously, elucidating the metabolic pathway controlled by Ras is critical for solving the mechanism(s) of Ras-mediated cellular transformation. The studies of the *ras* oncogene in lower eucaryotic organisms have offered insight on the action of Ras and how it integrates with metabolic pathways. The concepts learned by this approach should continue to increase our understanding of the role of Ras in human cancer.

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