



Cold Spring Harbor Symposia on Quantitative Biology

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Cold Spring Harb Symp Quant Biol 1988 53: 629-636

Access the most recent version at doi:[10.1101/SQB.1988.053.01.072](https://doi.org/10.1101/SQB.1988.053.01.072)

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Diverse Biological Functions of Small GTP-binding Proteins in Yeast

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Saccharomyces cerevisiae, the yeast used to make bread, beer, and wine, has been the object of intensive scientific study for more than a century. Modern scientific investigation began with Pasteur's realization that fermentation was the means by which yeast cells acquired energy in a useful form. Yeast soon became a standard system for biochemists and cell physiologists and has remained so until the present day. Genetics was introduced by Winge with the result that yeast became the object of intense study by those interested in the basic mechanisms of meiosis and recombination. Most recently, molecular biologists, always attracted by the possibility of combining genetics with biochemistry, developed this organism to a very high state of experimental tractability (for a review of the properties that make yeast a good model organism today, see Botstein and Fink 1988). Molecular genetic methods have become particularly useful for the study of the molecular aspects of cell biology, which began with the isolation of the cell-division-cycle mutants by Hartwell and colleagues (for a historical review, see Pringle and Hartwell 1981).

Despite the obvious morphological differences between yeast and animal cells, molecular methods have revealed a striking degree of conservation at the protein level between yeast and all other eukaryotes (Botstein and Fink 1988). This conservation has been noted for molecules located throughout the cell, from the nucleus (histones), to the cytoskeleton (actin and tubulin), to the plasma membrane (receptors). The significance of this conservation for the mechanistically inclined biologist lies in the possibility of studying a conserved phenomenon in an organism in which experimental manipulation is easy. In many cases, the existence of a homolog in yeast allows, through genetics, the investigation of *in vivo* function of a protein that otherwise would have to be studied by biochemical means alone.

The major point of this paper is the fact that this conservation of protein sequence (and presumably function) between yeast and animal cells applies to the superfamily of GTP-binding proteins. Others have described in this volume the conservation of the *RAS*

proto-oncogene-related proteins (see also Defeo-Jones et al. 1983; Powers et al. 1984) and the membrane-receptor-associate, signal-transducing, "true" G proteins (Dietzel and Kurjan 1987; Miyajima et al. 1987). We describe here the identification and beginning of characterization of the cellular functions of several small (~20 kD) GTP-binding proteins. The *YPT1* gene product is associated with the intracellular (endoplasmic reticulum [ER] or Golgi) secretion apparatus; it has a close mammalian homolog that appears in the Golgi (Haubruck et al. 1987; Touchot et al. 1987; Segev et al. 1988). The *CIN4* gene product is implicated in the proper mitotic segregation of chromosomes. The yeast *ARF* genes encode a close homolog of the bovine ADP-ribosylation factor, a protein of as yet unknown intracellular function that is required for purified cholera toxin to ADP-ribosylate mammalian G proteins.

A GTP-binding Protein Involved in Membrane Growth and Protein Secretion

The *YPT1* gene was first discovered because it lies between the actin and β -tubulin genes on the yeast genome. Its relationship to the *RAS* family was made clear by its nucleotide sequence (Gallwitz et al. 1983). Genetic arguments quickly established that *YPT1* is functionally quite distinct from the complementary *RAS1* and *RAS2* genes in yeast (Kataoka et al. 1984; Tatchell et al. 1984; Segev and Botstein 1987). Disruptions of the *YPT1* gene are lethal (Schmitt et al. 1986; Segev and Botstein 1987).

We constructed a cold-sensitive point mutation, *ypt1-1*, and have used it extensively to try to understand the normal role of the *YPT1* gene in yeast (Segev and Botstein 1987; Segev et al. 1988). The mutant cells have many phenotypes, including especially cytoskeletal aberrations and lethality, both during vegetative growth and after growth arrest caused by starvation (Segev and Botstein 1987). The large number of phenotypes made it important to try to discern which are primary, reflecting the actual process(es) for which normal *YPT1* function is essential, and which are secondary, reflecting the failure of the cell to carry out the primary process(es) properly. Although the *ypt1-1* mutation does not show a straightforward cell-division-cycle phenotype (i.e., the cells do not arrest at a single

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point in the cell cycle; for review, see Pringle and Hartwell 1981), a large number (~70%) arrest during the first cycle after shift to the nonpermissive temperature at the point in the cell cycle at which bud growth is maximal (Segev and Botstein 1987). There is a strong coupling between secretion and bud growth in *S. cerevisiae*, so attention was naturally turned to the secretion process as a candidate for the primary defect in vegetative growth caused by the mutation in the YPT1 gene.

More recently, we have obtained considerable evidence supporting the idea that primary function of the YPT1 gene may indeed be related to bud growth and protein secretion (Segev et al. 1988). A central piece of that evidence includes the appearance of obviously aberrant membrane structures soon after shift of the *ypt1-1* mutant to its nonpermissive temperature (14°C). Figure 1 shows electron micrographs from an experiment in which *ypt1-1* and control cells were grown at 30°C and were shifted to 14°C for less than one generation time. Figure 1C shows aberrant membranes most easily interpreted as accumulated, abnormal ER, whereas Figure 1D shows accumulated membranous vesicles similar to the "Berkeley bodies" found by Novick et al. (1980) in mutants defective in Golgi functions. Secretion of invertase from the mutant was found to be partially defective. Significantly, glycosylation of invertase is incomplete, consistent with the idea that the mutant is defective either in transfer of material from the ER to Golgi or within the Golgi.

Affinity-purified anti-YPT1 antibodies were prepared and used for immunolocalization studies, both in yeast and in animal cells (Segev et al. 1988). The results in yeast were consistent with the idea that the YPT1 gene product is localized in the ER or Golgi. A particularly suggestive finding was that very small (i.e., young) buds stain very strongly with the affinity-purified sera; unfortunately, it is not known whether such small buds have excess Golgi or Golgi-like membranes in them. The results in animal cells are strongly suggestive of the localization in Golgi of a protein closely analogous to the yeast YPT1 protein. Figure 2 shows mouse fibroblasts (L cells) triply stained with the affinity-purified anti-yeast YPT1, with wheat-germ agglutinin, and with DAPI, a nuclear stain. The almost similar but not identical staining by the anti-YPT1 and wheat-germ agglutinin is fully supportive of the Golgi localization, as wheat-germ agglutinin stains primarily, but not exclusively and not completely, the Golgi apparatus (Tartakoff and Vassalli 1983).

There is considerable biochemical evidence for close analogs of the YPT1 protein in a variety of eukaryotes. Genes having as much as 71% identity have been obtained from rats (Touchot et al. 1987) and mice (Haubruck et al. 1987). These genes encode 23-kD proteins that conserve all the sequence elements of GTP-binding proteins. We have observed cross-reacting species in extracts of fission yeast (*Schizosaccharomyces pombe* and *Xenopus laevis*). We have also detected a

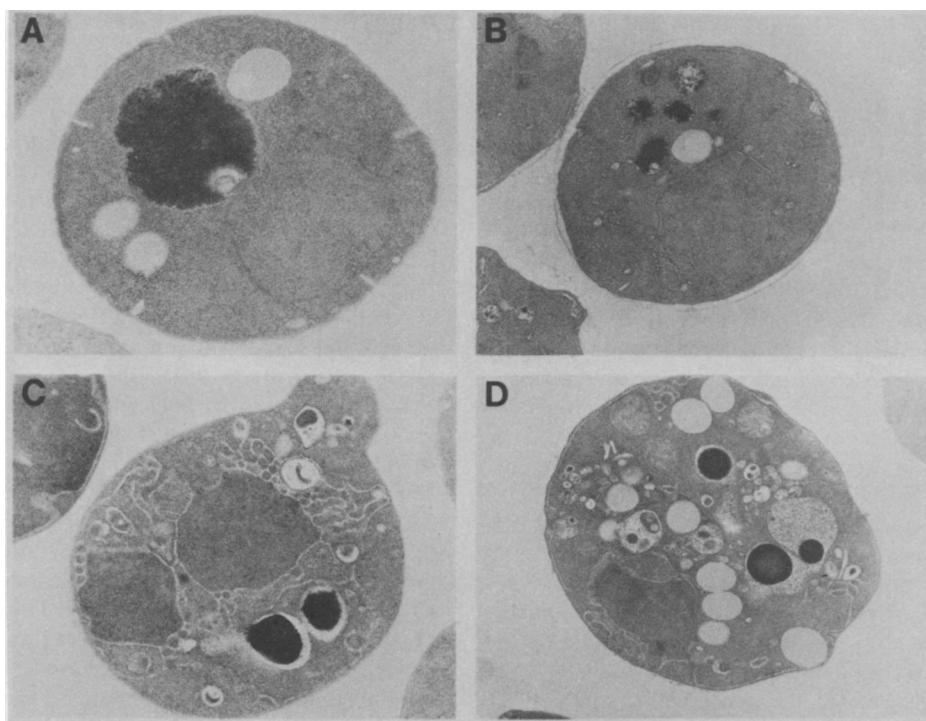


Figure 1. Thin-section electron microscopy of wild-type and *ypt1-1* mutant cells. Cells were grown at 30°C and then shifted to 14°C for various periods of incubation, after which cells were processed for electron microscopy and photographed as described by Segev et al. (1988). (A) Wild type at 14°C for 12 hr; (B) *ypt1-1* at 30°C (before the shift); (C, D) *ypt1-1* at 14°C for 8 hr. Magnifications: (A, B, C) 10,000 \times ; (D) 7,500 \times . (Reprinted, with permission, from Segev et al. 1988.)

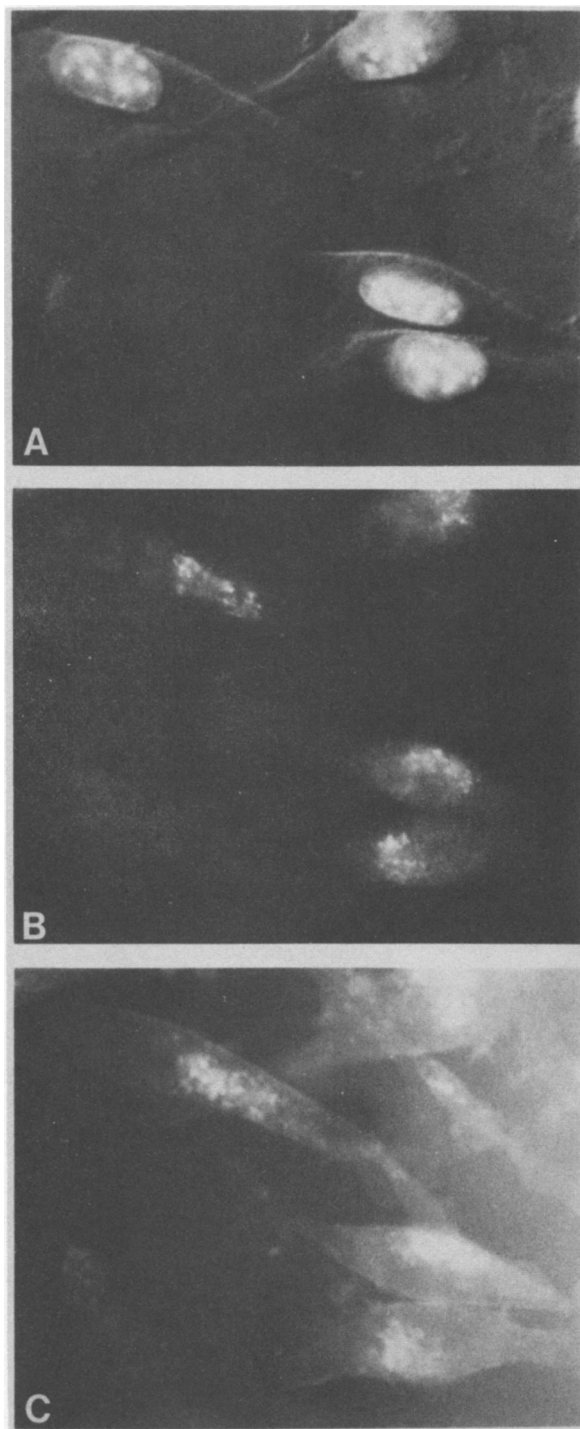


Figure 2. Localization of YPT1-related protein in mouse fibroblast cells. Mouse L cells were fixed and stained for fluorescence microscopy as described by Segev et al. (1988). (A) Nuclear staining with DAPI; (B) staining with affinity-purified anti-YPT1 antibodies at 1:200 dilution; (C) staining with wheat-germ agglutinin. (Reprinted, with permission, from Segev et al. 1988.)

mouse analog with antibodies that fractionates with crude Golgi fractions (N. Segev and J. Rothman, unpubl.). It is particularly noticeable in all of these experi-

ments that the antibodies and nucleic acid probes tend to detect the analogous proteins between even the most widely divergent species more readily than they detect other members of the small GTP-binding family, such as the p21 RAS proteins, within the same species.

The *YPT1* gene product is thus implicated in an early intracellular step in secretion or membrane growth. From its membership in a superfamily of GTP-binding proteins involved in signal transduction through membranes, we are led to propose a fundamentally regulatory role for this protein. From its conservation throughout eukaryotes in apparently the same role, we are led to think that this role is very basic to eukaryotic cell function. Our proposal (Segev et al. 1988) is that the YPT1 protein is involved in intracellular vesicular traffic as part of a system of "labels" that signals the vesicle's origin, destination, and/or contents.

There is a strong precedent for the involvement of a member of a YPT1 homolog in a late step of secretion. Salminen and Novick (1987) found that the *SEC4* gene, known to be involved in the fusion of secretory vesicles to the plasma membrane, encodes a GTP-binding protein very similar in primary sequence to that of *YPT1* (47.5% homology). More recently, Novick and colleagues (Goud et al. 1988; Novick et al., this volume) have determined that the *SEC4* product is located primarily attached to the cytoplasmic face of the membranes of the secretory vesicles, consistent with the view that the role of *SEC4* (and by extension *YPT1*) acts as a regulator of vesicle traffic by somehow labeling the vesicles to which it is bound.

Bourne (Bourne 1988; Bourne et al., this volume) proposes an attractive and mechanistically more specific form of the vesicle-labeling idea. He supposes that the small GTP-binding proteins guarantee the vectorial nature of the vesicular traffic by hydrolyzing their GTP and departing the membrane as GDP proteins only when the vesicle has found and "docked" with proteins attached to the target membrane just prior to fusion. This idea is consistent with what is currently known about *YPT1* and *SEC4* as well.

ARF: A Biochemically Well-defined Small GTP-binding Protein of Uncertain Function In Vivo

ARF was originally identified as the protein cofactor required for the efficient ADP-ribosylation of the stimulatory regulatory subunit (G_s) of adenylate cyclase by cholera toxin (Schleifer et al. 1982). This covalent modification results in the irreversible activation of the adenylate cyclase, and it is the primary cause of all the clinical symptoms associated with cholera. Using a quantitative assay based on the toxin-dependent ADP ribosylation of G_s , ARF activity was detected in a great variety of eukaryotic species, including man, mouse, rat, slime mold, and yeast. Although present in every tissue examined, ARF is most abundant in neural tissue where it may comprise as much as 1–2% of the cellular protein (Kahn and Gilman 1986).

With the purification of bovine ARF (Kahn and

Gilman 1984), it became possible to study the ADP-ribosylation reaction using fully defined components. In addition to the three proteins required (ARF, cholera toxin, and G_s), the reaction uses two nucleotides: NAD (ADP-ribosyl donor) and GTP. It was subsequently possible to demonstrate that ARF binds guanine nucleotides stoichiometrically and with high affinity ($K_D = 10$ – 100 nM) and that it is only the GTP- (or GTP γ S)-bound forms of ARF that are active in the ADP-ribosylation reaction (Kahn and Gilman 1986). Kinetic data suggest that the binding of GTP to ARF during the in vitro reaction promotes the association of ARF and G_s (Kahn and Gilman 1984). The GTP/ARF/ G_s complex is the apparent substrate for cholera toxin. ARF is catalytic in the reaction in that each molecule of ARF can catalyze several rounds of ADP ribosylation and that GTP bound to ARF is not hydrolyzed in the process.

Guanine nucleotide binding to purified ARF requires a hydrophobic environment (optimally, phospholipids and detergent), magnesium ions, and high ionic strength (Kahn and Gilman 1986). Inside the cell, such conditions might most closely be approximated at a membrane-cytosol interface. In fact, ARF was first discovered and purified from membrane sources and subsequently was found to be predominantly a cytosolic protein (Kahn et al. 1988). Yet, the soluble ARF also requires lipid, detergent, and high salt to allow nucleotide exchange.

Two recent observations indicate that a membrane is likely to be the site of action of ARF. First is the demonstration that purified bovine brain ARF contains myristic acid covalently attached to the amino-terminal glycine residue (Kahn et al. 1988). Second is the recent demonstration (J. Holden and R. Kahn, in prep.) that activation of ARF (i.e., binding of a guanine nucleotide triphosphate) promotes its association with purified plasma membranes. Inactive nucleotides (e.g., GDP) fail to promote this membrane binding. The membrane association is not saturable and thus probably involves binding directly to a membrane lipid. A large increase (110%) in intrinsic tryptophan fluorescence of ARF had previously been shown to accom-

pany the binding of active ligands (Kahn and Gilman 1986). Thus, a reasonable hypothesis is that the change in conformation of the protein induced by the binding of the activating nucleotide results in the exposure of a hydrophobic domain (possibly the myristylated amino terminus) capable of strongly associating with the membrane bilayer.

ARF is thus a well-defined biochemical entity with respect to its role in the ADP-ribosylation of G_s by cholera toxin. The presence of ARF in such diverse species as cow and yeast and the conservation of ARF sequences (see below) suggest strongly that ARF has another, more fundamental role in cell physiology. Biochemical studies have already demonstrated a functional relationship between ARF and the adenylate cyclase complex, a role for guanine nucleotides, a reversible membrane association, and identified a membrane as the probable site of ARF action. Yet, to understand the putative regulatory function in vivo, we must still identify the physiological activator(s) as well as the effector(s) of ARF action. Unfortunately, there are no straightforward biochemical means of identifying these.

The finding of ARF activity in yeast suggested the possibility of a genetic approach to the problem. The gene encoding ARF was cloned from a bovine cDNA library using partial protein sequence information (Sewell and Kahn 1988). There appears to be only one gene and one message for ARF in cows. In contrast, we obtained two highly conserved genes, called *ARF1* and *ARF2*, from yeast. Each of the genes (yeast and bovine) encodes a protein 181 residues in length (Fig. 3). The degree of conservation between bovine and either yeast gene is 74% identity, with many conservative substitutions. The two yeast genes are more than 96% identical, with only seven total substitutions, all conservative. The conservation of sequence between mammalian and yeast proteins is thus again very great: Like *YPT1*, it is over 70%, or quite comparable to the tubulins (for more comparisons, see Botstein and Fink 1988).

Disruption of the *ARF1* gene in haploid yeast carrying an intact *ARF2* gene results in slow growth at 30°C,

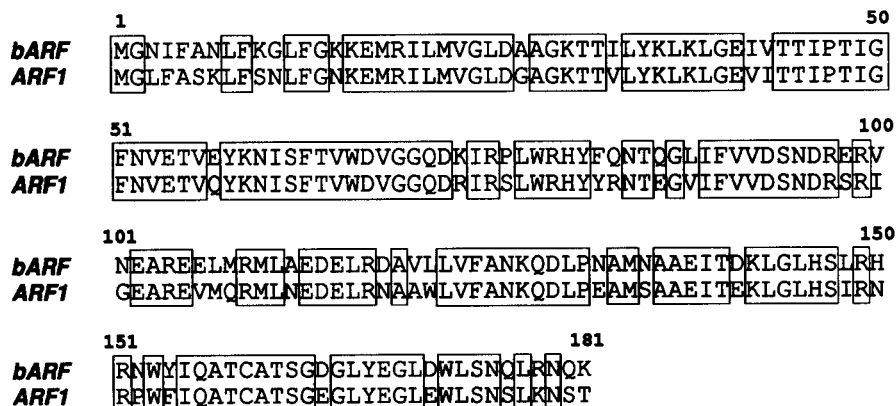


Figure 3. Comparison of predicted amino acid sequences of bovine and yeast ARF. Identities are boxed.

cold-sensitivity (i.e., no growth at 14°C), and hypersensitivity to potassium fluoride. Disruption of *ARF2* in haploids carrying an intact *ARF1* has no discernible phenotype. Disruption of both *ARF1* and *ARF2* is lethal. Thus, it appears that one functional *ARF* gene is absolutely required for cell growth. The reason for the differential requirement for two genes that, by sequence, differ by only seven codons is likely to be different levels of expression, as was found for the two nearly identical α -tubulin-encoding genes *TUB1* and *TUB3* (Schatz et al. 1986). That *ARF2* might be fully able to replace *ARF1* is strongly suggested by the ability of an *ARF1-ARF2* fusion protein (20% *ARF1* at the amino terminus) at the *ARF1* locus to support normal growth at all temperatures. The only difference between the fusion and *ARF2* is a substitution of phenylalanine for tyrosine at position 4 in the coding sequence. Interestingly, the fusion was obtained as a revertant of an *ARF1* disruption, presumably by a gene conversion from the nearby *ARF2* locus (M.A. Hoyt et al., unpubl.). The finding that *ARF* is essential to growth of yeast supports strongly the idea that there is a fundamental role for the *ARF* function in normal cell physiology. Production of point mutations to study the mutant phenotype as a means for understanding this normal role (as was done for *YPT1*, see above) is in progress.

A GTP-binding Protein Involved in Mitotic Chromosome Segregation

Mutations affecting microtubule function were obtained by two independent methods: (1) by collecting mutants hypersensitive to the antimicrotubule drug benomyl and (2) by isolating mutants that lose a marked supernumerary chromosome at a significantly higher frequency than wild type. Complementation and recombination analysis of the mutations obtained with the two different schemes revealed that the same six genes were involved. Three of these are the tubulin genes, *TUB1*, *TUB2*, and *TUB3*; the other three are not linked to any of the tubulin loci and therefore

appear to represent new genes that we have called *CIN1*, *CIN2*, and *CIN4* (chromosome instability). Mutations in any of these three genes result in a similar phenotype: supersensitivity to benomyl, increased frequency of chromosome loss, and weak cold sensitivity.

We cloned the three *CIN* loci from a yeast DNA plasmid library by complementation of the recessive benomyl supersensitivity. For all three genes, deletion mutations are viable and are similar in phenotype to the point mutations. We used the deletion mutations to construct double and triple mutants with the result that even a triple mutant is viable and has a phenotype no more severe than the most severe of the single mutants. This is genetic evidence that the *CIN* genes act in concert as part of the same structure or pathway. Further genetic experiments suggest that these genes play a direct role in microtubule function. Null mutations in any of the *CIN* loci are lethal in combination with a cold-sensitive mutation in the major α -tubulin gene, *TUB1*, or a null mutation in the minor α -tubulin gene, *TUB3*. In addition, a mutation in *CIN1* or *CIN4* is able to suppress the benomyl-dependent phenotype of a specific allele of the β -tubulin gene, *TUB2*.

DNA sequence analysis of the *CIN4* gene revealed that it encodes a protein of approximately 22 kD that has strong homology with a number of GTP-binding proteins. This homology includes the three domains implicated in GTP-binding (Dever et al. 1987), appropriately spaced, but also extends beyond these regions. The strongest homology is with the *ARF* protein. *CIN4* and the yeast gene *ARF1* encode proteins that are 29% identical, and 59% similar, if conservative amino acid substitutions are taken into account (Fig. 4). Although the function of *ARF1* in yeast is unknown, it is clear from analysis of various mutant combinations that *ARF1* and *CIN4* are not functional homologs of each other; doubly heterozygous diploids are phenotypically wild type, haploid double mutants are viable and have the phenotypes of each of the single mutants, unaltered, and the cloned genes on plasmids do not suppress each other's mutant phenotypes.

The most interesting aspect of the *ARF* and *CIN4*

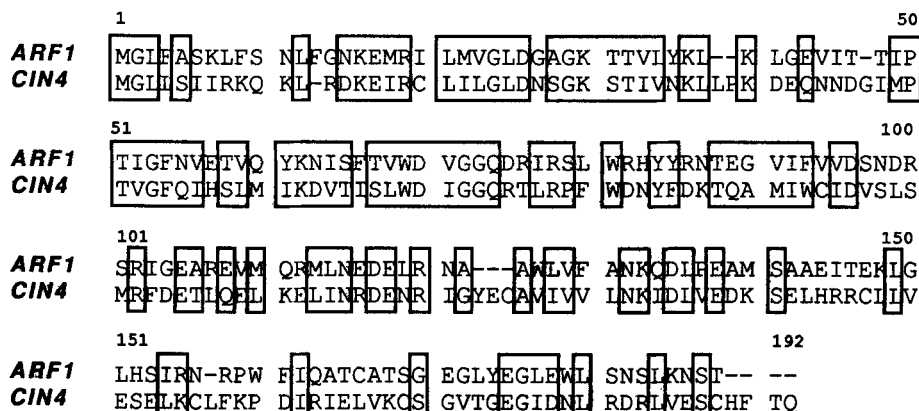


Figure 4. Comparison of predicted amino acid sequences of the yeast *CIN4* and *ARF1* proteins. Similarities (i.e., identities and conservative amino acid substitutions) are boxed.

proteins is that they are clearly related to both the α subunits of G proteins and to the RAS family of GTP-binding proteins (Fig. 5), although the sequence homology with the α subunits is stronger. Indeed, these two proteins are the only known ~ 21 -kD proteins that have the DVGGQ sequence element (DIGGQ in *CIN4*) present in all of the α subunits as part of their GTP-binding consensus sequence. All members of the RAS-related family have the sequence DTAGQ at that position. It remains to be seen whether the sequence homology with the α subunits of G proteins reflects a functional homology; i.e., Do these small GTP-binding proteins act in transduction of a signal across a membrane or do they play some other role? As *CIN4* has been implicated in microtubule function, it is interesting to speculate on possible roles of a GTP-binding protein in the microtubule cytoskeleton. It is worth noting that tubulin itself is a GTP-binding protein (David-Pfeuty et al. 1977), although not of the canonical elongation factor-Tu type, and that the binding and hydrolysis of GTP by tubulin have been proposed to be important in regulating the observed dynamic properties of microtubules (Kirschner and Mitchison 1986). Thus, it is possible that *CIN4* is involved in the interaction of tubulin with GTP. Another possibility is that *CIN4* is involved in the transduction of a signal across the nuclear membrane (the nuclear membrane does not break down during mitosis in yeast). A third possibility is that *CIN4* acts in the regulation of an ion, such as Ca^{++} , that regulates microtubule function.

A closer examination of the phenotype of single, double, and triple mutants reveals some interesting information about the interaction of the three *CIN* genes. Although mutations in the *CIN1* and *CIN2*

genes result in sensitivity to $1 \mu\text{g/ml}$ of benomyl, mutations in *CIN4* usually do not make cells quite as sensitive. Indeed, there are two distinct classes of *cin4* mutations: Certain mutations isolated by ethyl methanesulfonate (EMS) mutagenesis of intact cells (presumably point mutations) result in sensitivity to $1 \mu\text{g/ml}$ benomyl (*cin4-4* in Table 1), whereas null mutations constructed in vitro and certain other EMS-induced mutations result in sensitivity to $2.5 \mu\text{g/ml}$ benomyl and grow well on $1 \mu\text{g/ml}$ (Table 1). One possible cause for this difference is that the more sensitive alleles may be missense mutations that affect function of the *CIN4* gene product in such a way that it disrupts the action of the other gene products involved in this pathway.

Because some *cin4* mutations are less sensitive to benomyl than *cin1* and *cin2* mutations, tests of epistasis can be performed by constructing double and triple mutants. The results of this experiment are presented in Table 1. Briefly, *cin1* mutations are epistatic to mutations in *CIN2* and *CIN4*, and *cin4* null mutations are epistatic to mutations in *CIN2*. This relationship leads us to postulate that the genes do indeed act in the same pathway or structure and that they act in the order defined by the epistasis test: *CIN2* \rightarrow *CIN4* \rightarrow *CIN1* \rightarrow effect. This order assumes that the gene products are regulatory in nature. On the assumption that the *CIN* gene products assemble into a structure, the order will be reversed, *CIN1* \rightarrow *CIN4* \rightarrow *CIN2* \rightarrow effect.

We have thus defined a set of three genes, one of which encodes a GTP-binding protein, that play a role in microtubule function. It will be of great interest to determine the sequence of the *CIN1* and *CIN2* genes, as well as their function, since little is known about the

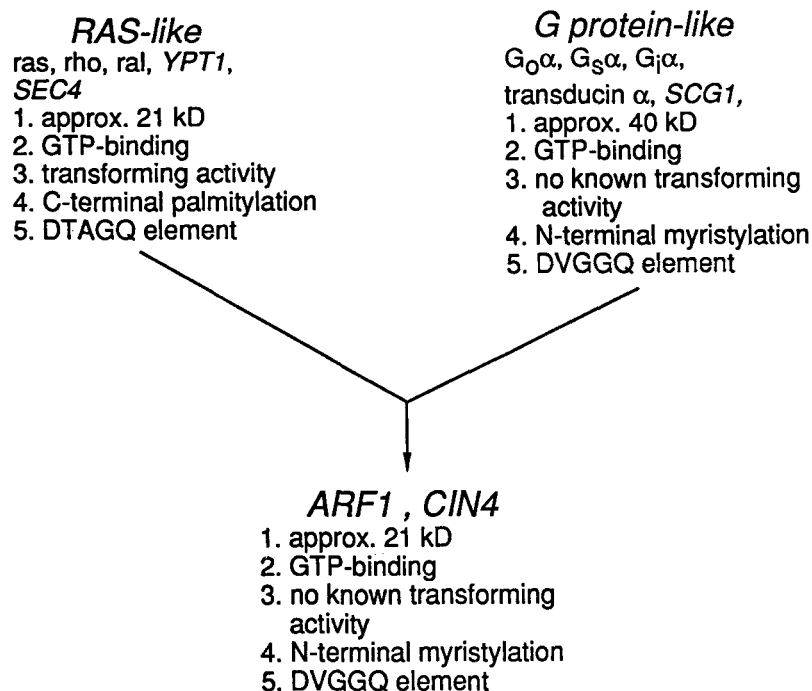


Figure 5. Relationships among members of the GTP-binding protein superfamily.

Table 1. Phenotypic Analysis of Single and Multiple *cin*-null Mutants

Genotype	Benomyl ($\mu\text{m/ml}$)			
	0	1	2.5	5
Single mutants				
Wild type	+	+	+	+
<i>cin1::HIS3</i>	+	-	-	-
<i>cin2::LEU2</i>	+	-	-	-
<i>cin4::URA3</i>	+	+	+/-	-
<i>cin4-4</i>	+	+/-	-	-
Multiple mutants				
<i>cin1 cin2</i>	+	-	-	-
<i>cin1 cin4::URA3</i>	+	-	-	-
<i>cin2 cin4::URA3</i>	+	+	+/-	-
<i>cin1 cin2 cin4::URA3</i>	+	-	-	-

proteins that interact in vivo with small GTP-binding proteins such as RAS, YPT1, ARF, and CIN4.

CONCLUSIONS

The role of the small GTP-binding proteins in cell physiology is still far from clear. However, several points emerge from our studies of these proteins and their in vivo functions in yeast. First, these in vivo roles are vital: *YPT1* and the *ARF* genes encode essential proteins, as does the *SEC4* gene (Salminen and Novick 1987). Second, the genes themselves are very highly conserved in evolution, as strongly, in fact, as the universal cytoskeletal elements like tubulin. It is very striking that the *YPT1* genes of yeast and animals are more similar to each other than *YPT1* is to the *ARF* gene(s) of the same species; likewise, the *ARF* genes of yeast and animals are more similar to each other than *ARF* is to the *RAS* gene(s) of the same species. Subfamilies are clearly emerging within the superfamily. Third, at least in the *YPT1*, *SEC4*, and *CIN4* cases, the current understanding of the mutant phenotypes is consistent with the idea that the small GTP-binding proteins have a fundamentally regulatory role of moving macromolecular structures or organelles inside cells; e.g., the signaling that a vesicle has found its target (*YPT1*, *SEC4*) or the microtubules have been successfully separated such that each chromosome is properly segregated from its homolog during mitosis (*CIN4*). Whether this is a matter of authentic signaling involving transduction of information through membranes or a kind of guarantee of unidirectionality, as proposed by Bourne (1988), is as yet not clear.

Finally, our review of the status of the investigation of the phenotypes of mutations in the yeast versions of each of these proteins stands as a general example of how the genetic system in yeast is helping to unravel the secrets of genes no matter how they were found in the first place: through their DNA sequence (e.g., *YPT1*), their biochemistry (e.g., *ARF1* and *ARF2*), or by direct probe of their function (e.g., *CIN4*). In each case, the ability to produce null and point mutations with facility and to study them whether or not genes are essential

for growth allows the acquisition of information about their role in the cell that is difficult or impossible to acquire any other way.

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