Common Modifications of Trimeric G. Proteins and ras Protein: Involvement of Polyisoprenylation

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Science, New Series, Volume 249, Issue 4965 (Jul. 13, 1990), 165-169.

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examined sequences required for change of function (suppression of transformation). Given that amino acids 32–44 are identical in K*rev*-1 and *ras*, that amino acid 38 is required for K*rev*-1 suppression, and that K*rev*-1–specific amino acids between residues 21 and 31 are also essential for suppression (*9*), our results imply that suppression by K*rev*-1 is mediated by a region in K*rev*-1 that is analogous to the region of *ras* previously defined by the deletion mutants as the ras effector region.

Ras protein crystal structure indicates that residues 26–36 form an exterior loop (*12*), which suggests that this region is sterically available for interaction with Ras target molecules. Amino acid residues 26, 27, and 31, which lie within the NH_2-terminal half of the loop, are three of the four divergent amino acids within segment 21–31 that were essential for transformation (Fig. 4). This structure is also consistent with results from experiments with *ras* deletion mutants that implicated these residues in effector function (*7*). Because specific amino acids between residues 45–54 are required for efficient transformation by *ras*, amino acids within this segment may impose subtle alterations on the effector region that are critical for transformation but not for suppression.

Previous studies with a chimera formed between the α subunits of the stimulatory (G_s) and inhibitory (G_i) G proteins of adenylate cyclase, two larger guanine nucleotide binding proteins, suggested that COOH-terminal sequences contribute to their effector function (*13*). Another study showed that a chimeric gene formed between *ras* and the *ras*-related gene R-*ras* was transformation-defective when the NH_2-terminal 111 amino acids were encoded by *ras* and the COOH-terminal amino acids were encoded by R-*ras* (*14*). Although this loss of function suggests that residues located downstream from codon 111 might contribute to Ras effector function (*14, 15*), results obtained herein suggest an alternative explanation. We observed nearly full biological activity (determined by fragment A or a) when the COOH-terminal amino acids were derived from the same gene, but several chimeras whose COOH-terminal amino acids were derived from combinations of the two genes displayed anomalously low activity. Perhaps functional integrity of residues carboxyl to amino acid 61 must be maintained for biological activity, but these residues do not contribute to the specificity of the activity (transformation versus suppression). Indeed, our data indicate that the amino acids carboxyl to residue 61 in the Ras and Krev-1 protein can substitute functionally for each other, although fewer than 50% are identical. Because these COOH-terminal residues in Ras are known to be required for membrane association and guanine nucleotide binding, our results suggest that the corresponding region of the K*rev*-1–encoded protein mediates these same functions. We infer that other gene families encoding small guanine nucleotide binding proteins with similarity to Ras proteins, such as *rho*, *ral*, R-*ras*, and *rab* (*16*), also possess a similar functional organization.

Our results would be expected if K*rev*-1 suppressed *ras*-induced transformation by competing with Ras protein for a target molecule. Whereas our results are most consistent with such a model, they do not exclude the possibility that K*rev*-1 antagonizes *ras* function by transmitting its signal to distinct target molecules that in turn mediate the anti-*ras* activity of K*rev*-1. It will be difficult to distinguish unambiguously between these two possibilities until the target molecules are identified.

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20. We thank S. Murakami and T. Matsuzaki for excellent technical assistance, B. Willumsen for helpful discussions and critical reading of the manuscript, and P. Martin for critical reading of the manuscript and preparation of the figures.

10 January 1990; accepted 10 May 1990

Common Modifications of Trimeric G Proteins and ras Protein: Involvement of Polyisoprenylation

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The heterotrimeric guanine nucleotide–binding regulatory proteins act at the inner surface of the plasma membrane to relay information from cell surface receptors to effectors inside the cell. These G proteins are not integral membrane proteins, yet are membrane associated. The processing and function of the γ subunit of the yeast G protein involved in mating-pheromone signal transduction was found to be affected by the same mutations that block ras processing. The nature of these mutations implied that the γ subunit was polyisoprenylated and that this modification was necessary for membrane association and biological activity. A microbial screen was developed for pharmacological agents that inhibit polyisoprenylation and that have potential application in cancer therapy.

GUANINE NUCLEOTIDE BINDING proteins (G proteins) participate in eukaryotic signal transduction. Common characteristics of these proteins are guanosine triphosphate (GTP) binding and GTPase activity, which are central to the G protein's signal-transducing ability. G proteins are grouped into at least two classes: the hormone-linked trimeric G proteins (*1*) and the single-subunit G protein typified by the ras family (*2*). Trimeric G proteins are membrane-associated, consist of three different subunits (α, β, and γ), and transduce signals from cell surface receptors to downstream effector proteins on or near the inner surface of the plasma membrane. This class includes the G proteins G_s, recently identified as an oncogene (*3*), and G_i [which transduces a signal from adrenergic receptors to stimulate or inhibit cyclic adenosine

monophosphate (cAMP) production by adenylate cyclase (*1*)], and transducin (G_t) [which mediates visual signal amplification by stimulating a cyclic guanosine monophosphate (cGMP) phosphodiesterase in response to absorption of a photon by rhodopsin (*4*)]. The ras protein, a representative of the single-subunit class, is localized to the plasma membrane and is involved in cell division signaling. The nature of the signal that it responds to, however, is presently unknown.

The membrane association of the ras proteins involves a targeting and processing mechanism distinct from that of integral membrane proteins. Ras membrane association involves COOH-terminal post-translational addition of a polyisoprenoid group to a cysteine through a thioether linkage (*5, 6, 7*), removal of three COOH-terminal amino acids and carboxyl methylation (*8*), and addition of palmitic acid (*9*). These modifications result in increased hydrophobicity of the protein, thus promoting membrane association. In yeast, the *DPR1/RAM* gene, which encodes a hydrophilic protein of 431 amino acids (*10*), is required for early steps in the processing of the ras proteins and the mating pheromone a-factor (*11*), and, for the sake of brevity, is referred to simply as *DPR1* hereafter.

In contrast, little is known about the mechanism of membrane association of the trimeric G proteins. We studied the *Saccharomyces cerevisiae* G protein G_m, which is involved in mating-factor signal transduction (*12*). Yeast mating requires peptide pheromones that bind to cell type–specific receptors (*13*). G_m transduces this mating signal from the receptor to an unidentified effector. Three genes that encode the subunits of G_m have been characterized: *GPA1* ($G_{m\alpha}$), *STE4* ($G_{m\beta}$), and *STE18* ($G_{m\gamma}$) (*12, 14*). We now present evidence that $G_{m\gamma}$, the product of the *STE18* gene, is membrane-associated, and that mutations that affect processing of ras proteins also affect processing of the STE18 protein.

To detect the STE18 protein, and to follow its processing, we used an epitope addition method (*15*). A short epitope of 19 amino acids from the influenza virus hemagglutinin protein was added to the NH_2-terminus of STE18 (*16*) (Fig. 1A). The addition of the epitope did not affect the function of STE18; the fusion gene, under control of the *GAL10* promoter, complemented the mating deficiency of *ste18* mutants in a manner indistinguishable from wild type. We analyzed cells that expressed the fusion gene by immunoblotting cellular extracts with a monoclonal antibody to the epitope (Fig. 1B). A protein of 15 kD was detected, in agreement with the predicted molecular mass of 14.7 kD for wild-type STE18. This band was missing in extracts from yeast cells that contained the expression vector alone.

To evaluate the membrane association of STE18 protein, we broke cells overexpressing the fusion protein, separated them into

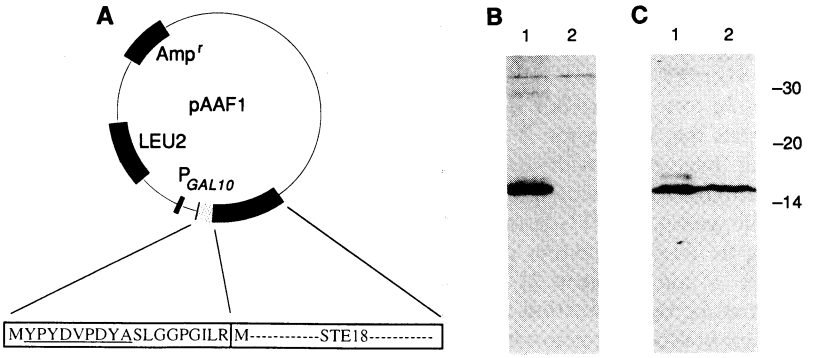

Fig. 1. An epitope-STE18 fusion protein expressed in yeast was associated with a membrane fraction, but a presumptive precursor form was cytoplasmic. (**A**) Construction of the pAAF1 plasmid (*16*) showing the predicted amino acid sequence of the fusion gene. The epitope recognized by the monoclonal antibody is underlined. (**B**) Yeast UC100 (**a** *leu2 trp1 ura3 pep4 prb1*) cells carrying pAAF1 plasmid (lane 1) or YEp51 vector alone (lane 2) were grown in synthetic medium (*28*) containing 5% (w/v) galactose with no leucine or methionine, and crude lysates of cells were obtained by resuspension in RIPA buffer [150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, and 50 mM tris-HCl, pH 8.0] and homogenization by shaking in the presence of glass beads. The proteins of the lysate were then separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% polyacrylamide gel and transferred to nitrocellulose (0.45-μm pore size, Schleicher & Schuell) by electroblotting. The nitrocellulose was incubated with murine ascites fluid containing 12CA5 antibody (*30*). The bound antibody was detected by a biotinylated antibody to mouse immunoglobulin and an avidin–horseradish peroxidase complex (Vectastain ABC kit, Vector Laboratories), which reduces diaminobenzidine in the presence of hydrogen peroxide, producing a brown color. (**C**) Cells expressing STE18 were fractionated into soluble (lane 1) and membrane fractions (lane 2) as previously described (*17*), except ultracentrifugation was performed at 230,000*g*. Immunoblotting as described above. Markers indicate kilodaltons.

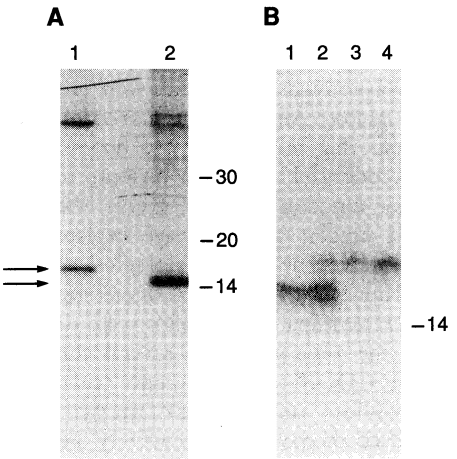

Fig. 2. Mutations in either the *DPR1* gene or in the genes for HMG-CoA reductase resulted in accumulation of a possible precursor form of STE18, as determined by immunoprecipitation. (**A**) Strains TK161R2V (*MAT***a** *leu2 his3 ura3 trp1 ade8 can1 RAS2^{val19}*) (lane 2) and HR12 (*MAT***a** *leu2 his3 ura3 trp1 ade8 can1 RAS2^{val19} dpr1*) (lane 1) carrying pAAF2 plasmid (*16*) were grown at room temperature in synthetic medium (*28*) containing 2% (w/v) glucose, lacking uracil and methionine, to late log phase and labeled with [^{35}S]methionine (17 μCi/ml) (Tran^{35}S-label, ICN, 1155 Ci/mmol) at 30°C for 5 min. Cells were then collected, resuspended in 50 mM potassium phosphate, 150 mM NaCl, pH 7.5, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride and broken with glass beads. STE18 protein was immunoprecipitated from the lysate with the 12CA5 antibody and separated by SDS-PAGE. Arrows indicate the difference in position of the bands in lanes 1 and 2. (**B**) Yeast strain JRY1593 (*MAT***a** *ade2 met his3 lys2 ura3 hmg1::LYS2 hmg2::HIS3*) (lanes 2 to 4) was transformed with pAAF2 (*16*) and grown at 30°C in synthetic medium (*28*) containing 2% (w/v) glucose with no uracil or methionine and supplemented with mevalonic acid lactone (5 mg/ml) and 2% (w/v) casamino acids to mid to late log phase. Cells were then centrifuged and resuspended in the same medium lacking casamino acids, incubated for 1 hour to deplete methionine pools, and a 10-ml sample of culture was labeled with [^{35}S]methionine and immunoprecipitated as in (A), lane 2. Mevalonic acid lactone was then removed from the medium of the remaining culture, which was incubated at 30°C for 1 or 3 hours, and portions were labeled and immunoprecipitated as above. Yeast cells UC100 transformed with pAAF2 were labeled and immunoprecipitated as above for a wild-type control (lane 1). Markers indicate kilodaltons.

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soluble and membrane fractions by ultracentrifugation at 230,000*g* (*17*), and analyzed them by immunoblot. A significant portion of STE18 was found in the membrane fraction (Fig. 1C), which also contained a majority of Sec61p, an integral endoplasmic reticulum membrane protein involved in protein secretion (*18*). Sucrose density gradient centrifugation experiments (*19*) showed that a majority of STE18 sediments at a density near that reported for the plasma membrane (*20*). The form detected in the membrane fraction was 15 kD. In the soluble fraction, in addition to the 15-kD form, a minor, slower migrating form was detected at 15.5 kD (Fig. 1C). This minor form of STE18 appeared to be a precursor protein, since the 15.5-kD form was detected only by a brief labeling with [^{35}S]methionine followed by immunoprecipitation (*19*). The majority of 3-phosphoglycerate kinase was detected by immunoblot analysis in the soluble fraction, and serves as a control for the fractionation procedure. Appearance of a precursor protein in the cytosol and mature-sized proteins in the cytosol and membrane is also characteristic of ras processing (*11*).

We further investigated the similarity of the processing of STE18 and ras proteins by examining STE18 protein expressed in the *dpr1* mutant, in which the processing of ras is blocked; precursor ras proteins accumulate in the *dpr1* mutant (*17*). We transformed a *dpr1* mutant and a parental strain with a plasmid containing the *STE18* fusion gene and immunoprecipitated STE18 from crude lysates (Fig. 2A). The STE18 protein in a *dpr1* mutant migrated as a 15.5-kD protein, slightly slower than the 15-kD STE18 protein detected in the parental strain. Thus, the processing of STE18 appeared to be blocked in the *dpr1* mutant.

The processing of STE18 was also affected by mutations of the genes *HMG1* and *HMG2*, both of which encode 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an enzyme required for isoprenoid biosynthesis (*21*). Mutation of these genes is lethal unless mutant cells are supplemented with mevalonate (the product of the reaction catalyzed by HMG-CoA reductase) and starvation for mevalonate blocks polyisoprenylation of ras proteins (*7*). The *hmg1 hmg2* mutant cells expressing STE18 were grown in the presence of mevalonate and were then subjected to mevalonate starvation for 0, 1, or 3 hours, at which times cellular proteins were labeled with [^{35}S]methionine and extracts were made (Fig. 2B). The STE18 protein immunoprecipitated from extracts of cells grown in the presence of mevalonate migrated mainly as a 15-kD mature protein (lane 2). This protein was identical in mobility to that detected in a wild-type strain (compare lanes 1 and 2). However, when the *hmg1 hmg2* strains were starved for mevalonate, STE18 protein accumulated as the slower migrating form (lanes 3 and 4). The mevalonate starvation does not change the mobility of STE18 protein in a control parental strain with wild-type HMG-CoA reductase genes (*19*). Thus, mevalonate starvation resulted in a block of STE18 processing, indistinguishable from that caused by *dpr1*. These results indicated that STE18 processing was mevalonate-dependent, and suggested that STE18 protein was polyisoprenylated.

The role of G_m is to block the G_1 to S transition of the cell cycle in cells treated with mating pheromones, to permit mating of yeast. This block is apparently mediated by free βγ subunits, which are released from the α subunit upon binding of GTP by the α subunit, in response to hormone bound to the receptor. Disruption of *GPA1* ($G_{m\alpha}$) causes cell cycle arrest, presumably because of the constitutive growth arrest signal of free βγ subunits (*12, 14*). Because the *hmg1 hmg2* double mutant, which blocks processing of yeast RAS2, also suppresses the lethality of *RAS2*^{val19} (*7*), we tested whether the same double mutant, which blocks processing of STE18, would suppress the lethality of the α subunit disruption. The results of such genetic experiments are shown in Table 1, which were fully consistent with suppression of *gpa1* lethality by *hmg1 hmg2*. A similar suppression of *gpa1* lethality was obtained by the use of lovastatin, an inhibitor of HMG-CoA reductase, which decreases the available pool of polyisoprenoids in treated cells. Yeast cells that have a temperature-sensitive allele of *GPA1* do not grow at a nonpermissive tempera-

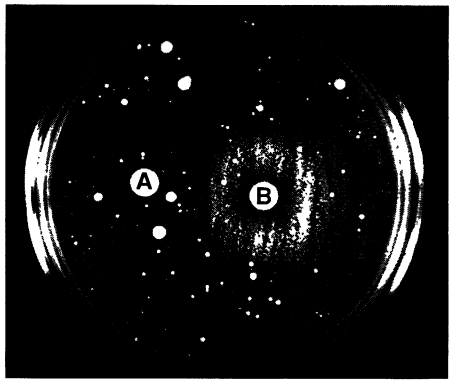

Fig. 3. The drug lovastatin (a HMG-CoA reductase inhibitor) suppressed the lethality of *gpa1* mutation. Yeast strain JRY2583 (*MATa ade2 gpa1-5*^{ts}), which has a temperature-sensitive allele of *GPA1*, was grown at room temperature to stationary phase in yeast extract–peptone dextrose (YEPD) medium (*28*), diluted 1:100, and 150 μl were plated as a lawn on YEPD plates. Two filter disks, soaked in either (**A**) 25% ethanol alone or (**B**) 25% ethanol containing lovastatin (25 mg/ml), were placed on the lawn of cells, and the plate was incubated at 37°C for 4 days. Cells closest to the lovastatin-containing filter disk were inhibited by the high concentration of the drug. At lower drug concentrations, lovastatin blocked the growth arrest of the *gpa1* mutant, presumably by interfering with STE18 processing, and allowed a halo of growth of the lawn. The single colonies scattered about the plate were secondary mutations in genes downstream of *STE18*, since they were sterile. Mutations in genes such as *STE4*, *STE5*, *STE7*, and *STE12* would be expected among these colonies, accounting for the high apparent reversion frequency.

Table 1. Mevalonate auxotrophy resulted in suppression of lethality of *gpa1* mutation. Tetrad analysis was performed on an a/α diploid whose relevant genotype was *GPA1/gpa1::URA3 hmg1::LYS2/HMG1 hmg2::HIS3/hmg2::HIS3* (*27*). Eleven tetrads were dissected and tested for viability on plates containing a limiting amount of mevalonate. Two mutually exclusive hypotheses were tested by this tetrad analysis. Hypothesis 1 represented the expected pattern of viability if mevalonate auxotrophy did not suppress the lethality of *gpa1*. Hypothesis 2 represented the expected pattern if *gpa1* lethality was suppressed by mevalonate auxotrophy. The observed pattern conformed with hypothesis 2. Furthermore, the genotypes of the dead segregants as predicted by tetrad analysis agreed in all cases with those predicted by hypothesis 2. Although mevalonate supplementation would be expected to restore the characteristics of the *hmg1 hmg2* double mutant to those of a wild-type cell, mevalonate uptake by yeast is inefficient (*7*). Thus, cells supplemented with mevalonate remain mevalonate-limited, which allowed us to test the mevalonate dependence of a process in viable, growing cells.

Class of tetrads	Hypothesis 1	Hypothesis 2	Observed	No. of each class
Parental ditypes:				
2 *GPA1 HMG1*	2 live	4 live	4 live	5
2 *gpa1 hmg1*	2 dead			
Nonparental ditypes:				
2 *GPA1 hmg1*	2 live	2 live	2 live	3
2 *gpa1 HMG1*	2 dead	2 dead	2 dead	
Tetratypes:				
1 *GPA1 HMG1*	2 live	3 live	3 live	3
1 *GPA1 hmg1*				
1 *gpa1 hmg1*	2 dead			
1 *gpa1 HMG1*		1 dead	1 dead	

Table 2. Quantitative mating assay of wild-type *STE18* and a mutant of Cys^{107} to Ser show the importance of this COOH-terminal Cys. A tester strain DC17 (*MAT*α *his1*) at 2.8×10^7 cells per milliliter was mated to transformants of strain S11 (*MAT***a** *leu2 ura3 his3 ste18::LacZ*) containing either plasmid M70p2 (*STE18 URA3*), or plasmid C107S ($ste18^{cys107ser}$ *URA3*), both at 1.7×10^7 plasmid-containing cells per milliliter. After 4 hours of mating, the mixtures were vortexed and suitable dilutions were spread on minimal plates (*28*) to detect prototrophs resultng from successful matings. S11 has previously been shown to be completely sterile (*14*). The C107S mutation was constructed in two steps. Initially an oligonucleotide ACTCAAATAGTGTTTagTcgACGCTTATGTAATGA was used to change four nucleotides (lowercase letters), introducing a stop codon and a Sal I site (underlined) in the wild-type *STE18* sequence. This mutation was identified by the presence of the Sal I site, and subsequently modified by an oligonucleotide CTCAAATAGTGTTTgtTCGACGCTTAT, which destroyed the Sal I site and replaced the nonsense codon with a Cys codon, leaving only the C107S change. All mutations were generated by the method of Kunkel (*29*) and confirmed by sequencing. One quantitative experiment was performed and confirmed by several patch (qualitative) mating experiments.

Strain	Relevant genotype	Viable cells	Prototrophic diploids	Mating efficiency
S11 (M70p2)	*STE18*	1.7×10^7	3.7×10^6	0.22
S11 (C107S)	$ste18^{cys107ser}$	1.7×10^7	0	<0.00000006

ture. However, this growth arrest was rescued on a petri dish in the region near filters soaked in lovastatin (25 mg/ml) (Fig. 3). This effect of lovastatin is analogous to its ability to block the function of H-$ras^{val\ 12}$ in frog oocytes (7). Similar suppression of the lethality of a *gpa1* disruption by the *dpr1* mutation has previously been reported by Nakayama *et al.* (*22*). Thus, the same genetic and pharmacological treatments that block the processing of polyisoprenylated ras protein also blocked STE18 processing and rescued the lethality of *gpa1* mutants. These results implied that STE18 protein was dependent on polyisoprenylation for processing and for full biological function. It is likely that the STE18 protein itself is polyisoprenylated, although the possibility that another protein in the mating pathway downstream of GPA1 is modified by polyisoprenylation is not excluded. We have developed conditions to purify STE18 protein from yeast using immunoaffinity purification. Further structural analysis should reveal the exact nature of the modifications.

Because STE18 protein is crucial to the mating process, a block in its processing is expected to lead to decreased mating efficiency. When *hmg1 hmg2* mutants are grown in a limiting amount of mevalonate, they have a reduced mating efficiency (3×10^{-2}) compared to a parental strain (7). The mating defect is more pronounced in the *MAT***a** cells (4×10^{-6}), because **a**-factor processing is also affected by the limiting mevalonate (7). The *dpr1* mutation also resulted in decreased mating efficiency in *MAT*α cells (1×10^{-1} to 5×10^{-3}) (*23*). The mutant *MAT***a** *dpr1* cells are known to be severely mating defective (*11, 17, 24*).

An examination of the STE18 amino acid sequence revealed homology with the ras proteins at the COOH-terminus, where a conserved sequence Cys-A-A-X is found (A is any aliphatic amino acid and X is the last amino acid). In the ras proteins, this sequence is a signal for the post-translational modifications at the COOH-terminus required for membrane association and biological activity (*25*). The conserved cysteine is the site of polyisoprenylation of the ras proteins (*5, 6*) and by analogy was predicted to be important for STE18 function. This prediction was supported, since a mutation of the conserved Cys^{107} to Ser in this sequence resulted in sterility (Table 2). In a quantitative mating assay (Table 2) and in several patch matings, no mating was detected in *ste18* disruption mutant cells expressing a plasmid-borne Ser^{107} mutant of the *STE18* gene. Yeast RAS and STE18 contain another Cys adjacent to the conserved Cys. Mutation of this other Cys^{106} to Ser greatly reduces mating, but does not completely abolish it (*23*). In ras, this other Cys is the site of palmitoylation (*5*).

Our results suggest that the processing and membrane association of STE18 share a set of modifications with the ras proteins that includes polyisoprenylation. Mammalian G protein γ subunits of G_i/G_o and G_t also contain the Cys-A-A-X consensus (*26*). This common sequence suggests that the ras processing mechanism is used by the yeast G protein as well as by heterotrimeric G proteins from a variety of species. Inhibitors of polyisoprenylation have the potential to block the biological effects of oncogenic *ras* mutations (7). Our results show that such inhibitors can also be used to block the biological effects of G proteins. Because G proteins have been implicated in human pituitary tumors (*3*), these results extend the possible applications of such inhibitors. Furthermore, the results presented here provide a microbial-based screen for inhibitors of polyisoprenylation in which specific inhibitors are detected by their ability to promote the growth of *gpa1* mutant. Such a screen should substantially simplify the search for pharmacological agents that selectively interfere with this important class of eukaryotic protein modifications.

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16. Synthetic oligonucleotides HA1 (5′-AATT GTC GAC ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT AGC TTG GGT GGT CCA GG-3′) and HA2 (5′-AATTCC TGG ACC ACC CAA GCT AGC GTA ATC TGG AAC ATC GTA TGG GTA CAT GTC GAC-3′) encoding an epitope of the influenza virus hemagglutinin protein were annealed and inserted into the Eco RI site of pTZ18R (Pharmacia), destroying the original Eco RI site but creating new Eco RI and Sal I sites, resulting in pTZHA. The sequence of the epitope region of pTZHA was confirmed by DNA sequencing more than 30 bp on either side of this region. The *STE18* gene was added to the epitope by cloning an Eco RI–Bam HI fragment of M89p9 containing the *STE18* gene into pTZHA at the 3′ end of the epitope. M89p9 was created by cloning the Pst I–Sst I fragment containing the *STE18* gene (*14*) into pVT100U [T. Vernet, D Dignard, D Y Thomas, *Gene* **52**, 225 (1987)], followed by in vitro oligonucleotide-directed mutagenesis to change a T at −11 to G, creating an Eco RI site at the 5′ end of the gene. The fusion gene was then cloned as a Sal I–Bam HI fragment into either YEp51 cleaved previously with Sal I and Bam HI, yielding pAAF1, or into pVT100U cleaved previously with Xho I and Bam HI, yielding pAAF2, under control of the *GAL10* (pAAF1) or *ADH1* (pAAF2) promoters (Fig. 1). Plasmids pAAF1 and pAAF2 were transformed into yeast strains by the spheroplast method (*28*).
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27. For this experiment, an a/α diploid (JRY121) of the genotype *ade2-101/ade2-101 his3Δ200/his3Δ200 lys2-801/lys2-801 met/met ura3-52/ura3-52 HMG1/hmg1::LYS2 hmg2::HIS3/hmg2::HIS3* was transformed with a linear DNA fragment containing the *gpa1 URA3* disruption allele, selecting for uracil prototrophy. Sporulation and analysis of the asci revealed that *GPA1* was disrupted on only one homolog.
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31. We thank J. Field for advice on the epitope addition method, T. Vernet for advice on site-directed mutagenesis, D Dignard for sequencing, and K. Matsumoto, S.-H. Kim, and D. Thomas for stimulating discussions. Supported by NIH grant CA 41996 (F.T); GM 35827 and a grant from the Lucille P. Markey Charitable Trust (J.R.); a NSF predoctoral fellowship (W.R.S.); and NIH Molecular and Cellular Biology Training grant GM 07183 (A.A.F.). F.T. is an Established Investigator of the American Heart Association. Lovastatin was a gift from A. Alberts (Merck, Sharp & Dohme Research Laboratories). The *gpa1*^{ts} allele was a gift from G. Cole and the *gpa1* disruption was a gift from K. Matsumoto. Antibodies to 3-phosphoglyerate kinase and to Sec61p were gifts from J. Thorner and from C. Stirling and R. Schekman, respectively. 12CA5 antibody was provided by I. Wilson. This is National Research Council of Canada publication number 31158.

9 January 1990; accepted 26 April 1990

Inhibition of GTPase Activating Protein Stimulation of Ras-p21 GTPase by the K*rev*-1 Gene Product

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K*rev*-1 is known to suppress transformation by *ras*. However, the mechanism of the suppression is unclear. The protein product of K*rev*-1, Rap1A-p21, is identical to Ras-p21 proteins in the region where interaction with guanosine triphosphatase (GTPase) activating protein (GAP) is believed to occur. Therefore, the ability of GAP to interact with Rap1A-p21 was tested. Rap1A-p21 was not activated by GAP but bound tightly to GAP and was an effective competitive inhibitor of GAP-mediated Ras-GTPase activity. Binding of GAP to Rap1A-p21 was strictly guanosine triphosphate (GTP)–dependent. The ability of Rap1A-p21 to bind tightly to GAP may account for K*rev*-1 suppression of transformation by *ras*. This may occur by preventing interaction of GAP with Ras-p21 or with other cellular proteins necessary for GAP-mediated Ras GTPase activity.

GTPASE ACTIVATING PROTEIN (GAP) (*1, 2*) has been implicated as an effector of *ras* action by the following observations: (i) antibody to Ras-p21 inhibits *ras* function and prevents GAP interaction (*1*); (ii) GAP interacts with Ras-p21 at the effector binding site (*3–5*); and (iii) Ras-p21 mutants that bind tightly to GAP but cannot localize in the cell membrane inhibit the function of membrane-associated Ras proteins. This inhibition is relieved by addition of GAP (*6*).

The K*rev*-1 gene, also called *rap*1A, suppresses transformation of cells by the K*ras* oncogene (*7–9*). The predicted protein product is 53% identical to the K*ras* gene product, and contains all the sequences typical of a guanine nucleotide binding protein (*10*). Furthermore, the putative effector binding sequence of Ras proteins (a region essential for biological activity of *ras* oncogenes) is conserved in the K*rev*-1 gene product, Rap1A-p21. Thus, we tested the hypothesis that Rap1A-p21 may interact with the same effector as the Ras proteins and may suppress the *ras* transformation function by competing with Ras-p21 for that effector molecule.

We purified from *Escherichia coli* intact, recombinant Rap1A-p21 (60% purity) as well as a COOH-terminal truncated version of the protein that contained amino acids 1 to 168 (>95% pure). Like other small guanine nucleotide–binding proteins, Rap1A-p21 contains 1 mol of tightly bound nucleotide [GTP or guanosine diphosphate (GDP)], which is released slowly in the presence and rapidly in the absence of Mg^{2+} with kinetics similar to those of Ras-p21. The intrinsic GTPase activity (*11*) of Rap1A-p21 (4×10^{-3} min^{-1} at 37°C, Fig. 4, insert) was slow compared to that of Ras-p21 (2.8×10^{-2} min^{-1}) (*12*). Rap1A-p21 GTPase activity was not stimulated by addition of GAP (Fig. 1). Increasing the GAP: Rap1A-p21 molar ratio to 0.16 did not stimulate the GTPase activity. Under the same conditions, the GTP complex of Ras-p21 was hydrolyzed in 3 min.

Because oncogenic mutant forms of Ras protein that are not sensitive to GTPase stimulation by GAP nonetheless bind to GAP (*5*), we tested the ability of Rap1A-p21 to bind GAP. Affinity of binding was

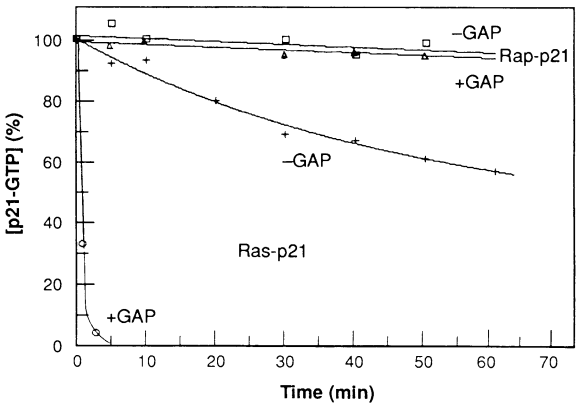

Fig. 1. Activation of the GTPase reaction of Ras-p21 and Rap1A-p21 by GAP. Normal and truncated (1–168) Rap1A-p21 were expressed in *Escherichia coli* with the ptacRas-expression system described for Ras-p21 (*21*). All proteins and the mutant (T61Q) (Fig. 4) were purified with the two-column procedure described (*12, 21*). Purity of Ras-p21, >95%; Rap1A-p21; ~60%; Rap1A (1–166), >95%; Rap1A(T61Q), 60%. Complexes between Ras-p21 or Rap1A-p21 and [γ-^{32}P]GTP were formed by incubating the proteins and radioactive nucleotide with 1 mM EDTA and 64 mM tris-HCl, pH 7.5, 1 mM dithioerythritol (DTE). Excess nucleotide was removed by gel filtration on a G-25 commercial column (NAP-5, Pharmacia LKB). The protein was eluted from GAP reaction buffer: 20 mM Hepes, pH 7.5, 1 mM DTE. The GTPase reaction was started by addition of 2 mM $MgCl_2$ and where indicated, the appropriate amount of recombinant human GAP (80 nM). Human GAP was expressed in sf9 insect cells (*22*) and was purified with a conventional S-Sepharose column and high-performance liquid chromatography (HPLC) on Mono-Q resin. The GTPase activity was measured as described (*2, 3, 5*) by following the decrease in concentration of [γ-^{32}P]GTP bound to p21 by filtration of the reaction mixture through nitrocellulose filters (0.45 μm). The data are plotted as the percentage of total Ras-p21-GTP converted to Ras-p21-GDP with time. The 100% value corresponds to 500 nM Ras-p21 or Rap1A-p21 (28,000 cpm).

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