Genetic and Pharmacological Suppression of Oncogenic Mutations in *RAS* Genes of Yeast and Humans

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The activity of an oncoprotein and the secretion of a pheromone can be affected by an unusual protein modification. Specifically, posttranslational modification of yeast a-factor and Ras protein requires an intermediate of the cholesterol biosynthetic pathway. This modification is apparently essential for biological activity. Studies of yeast mutants blocked in sterol biosynthesis demonstrated that the membrane association and biological activation of the yeast Ras2 protein require mevalonate, a precursor of sterols and other isoprenes such as farnesyl pyrophosphate. Furthermore, drugs that inhibit mevalonate biosynthesis blocked the in vivo action of oncogenic derivatives of human Ras protein in the *Xenopus* oocyte assay. The same drugs and mutations also prevented the posttranslational processing and secretion of yeast a-factor, a peptide that is farnesylated. Thus, the mevalonate requirement for Ras activation may indicate that attachment of a mevalonate-derived (isoprenoid) moiety to Ras proteins is necessary for membrane association and biological function. These observations establish a connection between the cholesterol biosynthetic pathway and transformation by the *ras* oncogene and offer a novel pharmacological approach to investigating, and possibly controlling, *ras*-mediated malignant transformations.

THE *Ras* FAMILY OF ONCOGENES AND PROTO-ONCOGENES encode guanine nucleotide binding proteins that participate in the control of eukaryotic cell proliferation. Ras proteins associate with the inner surface of the plasma membrane, where they interact with effector molecules to control cell division. Ras proteins are found in two states: a GTP-bound active state and a GDP-bound inactive state. Active Ras protein is converted to an inactive form by an intrinsic guanosine triphosphatase (GTPase) activity that is stimulated by interaction with a GTPase activating protein (GAP). Upon binding GTP, Ras proteins become activated and are capable of stimulating cell proliferation, although the mechanism of growth stimulation is unknown (*1, 2*). The proteins encoded by oncogenic alleles of cellular *ras* genes are often mutated in a manner that decreases their intrinsic GTPase activity (*3*) by a conformational change in the guanine nucleotide binding site of the protein (*4, 5*) or by a nonproductive association with GAP protein (*2*). Hence, the proteins encoded by such alleles remain in the active conformation longer. In addition to regulating cell division in mammalian cells, Ras proteins can induce meiosis in *Xenopus* oocytes (*6*) and cause specific development defects in *Drosophila* (*7*). The yeast *Saccharomyces cerevisiae* contains two *RAS* genes known as *RAS1* and *RAS2*. Mutations in the yeast *RAS2* gene that correspond to oncogenic alleles of the human Ras protein, such as $RAS2^{val\ 19}$, prevent the yeast cell from arresting in the G_1 phase of the cell cycle in response to nutrient limitation (*8, 9*). The Ras proteins have an essential function in yeast since at least one intact *RAS* gene is required for viability. The Ras2 protein is more abundant than the Ras1 protein and has received more attention.

Membrane association is critical for the oncogenic transformation caused by activated Ras proteins (*10*). Ras proteins of both yeast and mammals are posttranslationally modified near the carboxyl terminus by the attachment of a lipophilic moiety. This modification mediates association of Ras protein with the inner surface of the plasma membrane without altering the structure of the protein. Three-dimensional structures of the intact and catalytic domain of human c-H-*ras* proteins show that the region of protein modification is flexible and would not alter the conformation of the catalytic domain (*5, 11*). Mutation of the cysteine codon located four residues from the COOH-terminus of the protein, to which the lipophilic moiety is thought to be attached, blocks membrane association of the protein, prevents neoplastic transformation of cells, and causes accumulation of a slower-migrating unmodified species. Previous work suggested that the lipophilic modification of Ras is palmitylation of the COOH-terminal cysteine because mutants lacking that cysteine failed to become palmitate-labeled (*10*).

The posttranslational processing of yeast Ras proteins is similar to that of a yeast mating pheromone. Haploid yeast of the *MAT***a** mating type secrete a 12–amino acid peptide known as **a**-factor, which arrests cells of the α mating type in the G_1 phase of the cell cycle in preparation for mating. The **a**-factor is synthesized on cytoplasmic ribosomes and secreted independently of the classically defined secretory pathway (*12*). The **a**-factor of yeast, like the mating pheromones of some other fungi, is posttranslationally modified by the attachment of a farnesyl moiety, derived from

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mevalonate, to a COOH-terminal cysteine by a thioether linkage (*13*). It is not known what role farnesylation plays in the biogenesis of active **a**-factor. In contrast, the mating pheromone of *MAT*α yeast, known as α-factor, is processed through the secretory pathway and does not appear to be a lipopeptide (*12*).

Genetic and biochemical evidence indicates that the posttranslational processing of **a**-factor and Ras protein share a number of common features. Mutations in the gene known variously as *DPR1, RAM1, STE16*, or *SGP2* (referred to here collectively as *RAM1-DPR1*) have been independently identified on the basis of two different phenotypes. One allele was identified as an **a**-specific sterile mutation, whereas other alleles were identified as suppressors of the viability defects associated with activated *RAS* mutants. The *RAM1-DPR1* mutants are defective in the posttranslational processing and secretion of **a**-factor, as well as in the processing and membrane localization of Ras2 protein. Thus, the simplest interpretation of these findings is that the *RAM1-DPR1* gene product is required for the posttranslational modification of both **a**-factor and yeast Ras protein (*14*). Furthermore, the COOH-terminal amino acid sequences required for processing of **a**-factor and Ras are strikingly similar. In addition, some of the chemical modifications involved in **a**-factor processing appear to be shared by Ras protein processing. Specifically, the COOH-terminal processing of **a**-factor involves (i) proteolytic removal of the last three amino acids resulting in a COOH-terminal cysteine, (ii) carboxymethylation of that cysteine, and (iii) attachment of a farnesyl group to that cysteine (*13*). Processing of N-*ras*, one of three Ras proteins found in all mammalian cells, involves proteolytic removal of the last three amino acids, leaving a COOH-terminal cysteine, and methylation of the carboxyl group of that cysteine (*15*). The possibility that Ras proteins might also be farnesylated provided the basis for the experiments described below.

Mevalonate biosynthesis is essential for mating ability of *MAT*a cells. The enzymes 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoA synthase) and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) are required for the synthesis of mevalonate, the precursor of isoprenoid molecules such as farnesyl pyrophosphate, cholesterol, dolichol, and ubiquinone. Hence, null mutations in the genes for these enzymes render both yeast strains and mammalian cell lines mevalonate auxotrophs (*16*). Because uptake of mevalonate by yeast cells is inefficient, mevalonate auxotrophs are partially starved for mevalonate even when grown on medium containing mevalonate (*17*). In genetic crosses with strains containing mutations in either *ERG13*, the gene encoding HMG-CoA synthase, or in the two genes encoding the HMG-CoA reductase isozymes (*HMG1* and *HMG2*), we observed that *MAT*α mating type segregants were relatively mating proficient, but the *MAT***a** mating type segregants were mating deficient. The mating deficiency of the *MAT***a** segregants was exacerbated by incubating the cells in the absence of mevalonate (Table 1). Thus, blocks in early steps of the isoprene biosynthetic pathway resulted in **a**-specific sterility.

Mating defects of *MAT***a** cells result either from failure to secrete active **a**-factor or from failure to respond to α-factor (*18*). To characterize the mating defect of the mevalonate auxotrophs, we cultured wild-type *MAT***a** cells and *MAT***a** cells containing a mutant HMG-CoA synthase gene on medium containing mevalonate; individual cells were then micromanipulated near a source of α-factor. Most (43 out of 50 cells tested) of wild-type *MAT***a** cells responded to α-factor within the first cell division by undergoing a characteristic change in morphology. Similarly, many (19 out of 50 cells tested) of the mutant *MAT***a** cells supplemented with mevalonate also responded normally to α-factor. These results suggested that the **a**-specific sterility of mevalonate auxotrophs was due to a defect in **a**-factor production rather than a defect in α-factor response.

We tested this hypothesis directly by replica plating wild-type cells and cells deficient for HMG-CoA synthase or for HMG-CoA reductase onto a lawn of α cells rendered supersensitive to **a**-factor by the presence of the *sst2* mutation (*19*). Wild-type cells produced a halo of growth inhibition indicative of the secretion of active **a**-factor. In contrast, the HMG-CoA synthase mutant was markedly defective in the production of active **a**-factor and the HMG-CoA reductase mutant was completely defective in the production of active **a**-factor (Fig. 1). Thus, mevalonate auxotrophy impaired or blocked the production of active **a**-factor. The ability of the HMG-CoA synthase mutants to secrete some **a**-factor may have been due either to its better growth on mevalonate-containing medium or to residual enzyme activity encoded by the disruption-allele of *ERG13*.

To identify the step of **a**-factor biosynthesis or processing defective in mevalonate auxotrophs, we prepared extracts from ^{35}S-labeled cells and immunoprecipitated with antibody to the 12-residue **a**-factor peptide (*12*). The culture medium was also analyzed for secreted **a**-factor. The immunoprecipitate was evaluated by SDS–polyacrylamide gel electrophoresis (PAGE) and fluorography (Fig. 2). In wild-type cells, most of the mature **a**-factor was recovered from the culture medium (fraction S), whereas in the mevalonate-supplemented HMG-CoA synthase mutant cells some precursor and processed forms could be detected intracellularly (fraction I; Fig. 2). In contrast, in mevalonate-starved HMG-CoA synthase mutant cells no mature-sized **a**-factor could be detected in either the culture medium or in the cell lysate. Moreover, when normalized for ^{35}S incorporated (counts per minute) into protein, the mevalonate-starved mutant cells accumulated 2.5 times more of the **a**-factor precursor than did the isogenic wild-type cells. Compactin is an inhibitor of HMG-CoA reductase activity and hence inhibits mevalonate synthesis (*20*). Prior treatment of *MAT***a** cells with compactin (40 μg/ml) for 1 hour blocked the secretion of **a**-

Table 1. Mevalonate auxotrophy results in *MAT***a**-specific sterility. Strains were grown to A_{600} of approximately 2 in YPD medium (*36*). Mevalonate auxotrophs were supplemented with mevalonate (5 mg/ml). Cultures of the cells to be tested were serially diluted in YPD medium lacking mevalonate and plated onto minimal medium containing uracil and histidine. Either 0, 2, or 4 hours later, the test plates were spread with a freshly grown lawn of 10^8 cells of the mating-type tester. Since only diploids would be able to grow on the minimal medium, the efficiency of mating was determined as the fraction of cells plated that were capable of forming prototrophic colonies. The **a** and α mating-type tester strains, JRY412 (α) and JRY1721 (**a**), were isogenic and had the genotype *can1 his4 trp1 ura3-52 gal*. The genotype of the α *sst2* strain (JRY2176) was *MAT*α *HML*α *HMR*α *sst2 leu2 ade2 ura3*. The genotypes of the other strains are in (*34*).

Strain	Relevant genotype	Time (hours)	Mating efficiency		
			×**a**	×α	×α *sst2*
JRY527	**a** *HMG1 HMG2*	0		0.67	0.25
		2			0.24
		4			0.55
JRY1593	**a** *hmg1 hmg2*	0		4.0×10^{-6}	1.5×10^{-4}
		2			3.1×10^{-4}
		4			4.8×10^{-5}
JRY2138	**a** *erg13* (*35*)	0		9.9×10^{-5}	2.2×10^{-3}
		2			3.1×10^{-3}
		4			3.6×10^{-4}
JRY528	α *HMG1 HMG2*	0	1.27		
JRY1597	α *hmg1 hmg2*	0	3.2×10^{-2}		
JRY2223	α *erg13*	0	6.2×10^{-2}		

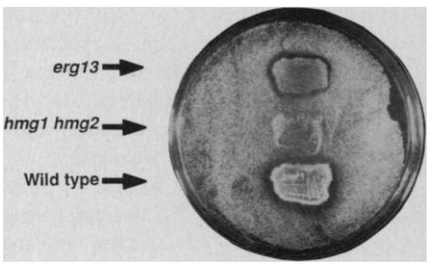

Fig. 1. The production of a-factor was detected by replica-plating a lawn of α *sst2* cells onto a plate containing patches of the strains being tested. The *sst2* mutation renders cells supersensitive to arrest by mating pheromones (*19*). The top strain contains a disruption of the *ERG13* gene (*35*) and hence lacks HMG-CoA synthase activity, the middle strain contains a disruption of both *HMG1* and *HMG2* and hence lacks HMG-CoA reductase activity. The bottom strain is wild type for all genes of the isoprene biosynthetic pathway. The middle and bottom strains are isogenic. The residual halo of growth inhibition produced by the *erg13* mutant may be a result of its ability to assimilate mevalonate from the medium better than the HMG-CoA reductase mutant, or to residual activity encoded by the disruption allele.

factor, an indication that a mevalonate-derived product was required for processing and secretion of a-factor. Since farnesyl pyrophosphate is derived from mevalonate and the secreted form of a-factor is farnesylated, the mevalonate dependence of a-factor processing and secretion most likely reflects a requirement for farnesylation of the precursor for subsequent processing and secretion. Thus, one cause of the a-specific sterility of mevalonate auxotrophs presumably is the impaired ability to farnesylate a-factor.

A biochemical connection between a-factor and Ras proteins of yeast. Mevalonate depletion resulted in a defect in a-factor processing that resembled the processing defect in *RAM1-DPR1* mutants. As noted previously, *RAM1-DPR1* mutants are also defective in the posttranslational processing and membrane localization of Ras2 protein. These mutants accumulate a precursor that has a slower electrophoretic mobility than mature Ras2 protein and is found in the cytoplasmic rather than the membrane fraction of a crude cell extract (*14*). If the COOH-terminal processing of a-factor and Ras were identical, then mevalonate depletion should block the posttranslational processing of Ras2 protein as well. Thus, the electrophoretic mobility and the subcellular localization of Ras2 protein in mevalonate-depleted cells were examined by immunoblotting. A wild-type strain and an isogenic mevalonate auxotroph were transformed with a multicopy plasmid containing the *RAS2* gene as an aid in detection. Crude extracts were prepared from cells either grown in the presence of mevalonate or starved for mevalonate for 12 hours. These extracts were separated into membrane and soluble fractions and analyzed by immunoblotting with a monoclonal antibody (Y13-259) specific for Ras proteins (*21*) (Fig. 3). Mevalonate-supplemented cells (Fig. 3, lanes 1 to 3) contained a single form of Ras2 protein, which was present exclusively in the membrane fraction (Fig 3, lane 2). After 12 hours of mevalonate depletion (Fig. 3, lanes 4 to 6), cells accumulated a second, slower migrating form of Ras2 protein with a mobility characteristic of Ras2 precursor (Fig. 3, lane 6); these experiments indicated that mevalonate or a mevalonate-derived product was required for the processing and membrane association of Ras2. Wild-type cells contained a higher steady-state level of Ras2 protein than did the mevalonate auxotrophs (Fig. 3, lanes 7 and 2). This difference was observed consistently. Efforts to detect modification of Ras2 protein by a mevalonate-derived product directly have so far been unsuccessful, possibly because of poor uptake of mevalonate from the medium, the low specific activity of commercially available radioactively labeled mevalonate, or an indirect requirement for mevalonate in the activation of Ras protein. The detection of the farnesyl moiety on a-factor required direct chemical analysis of the purified peptide, which has not yet been attempted with Ras protein.

Since membrane association is required for both oncogenic transformation by activated Ras proteins in mammalian cells and for the biological activity of activated Ras proteins in yeast (*10*), and since mevalonate depletion blocks membrane association of yeast Ras2 protein, we sought to determine whether mevalonate depletion would suppress the phenotypes of the $RAS2^{val19}$ allele in yeast. Stationary phase yeast cells arrest in the G_1 phase of the cell cycle as unbudded cells. The $RAS2^{val19}$ mutation interferes with normal G_1 arrest of stationary phase cells, particularly in glucose-grown cells. Thus, the $RAS2^{val19}$ allele prevents the accumulation of unbudded, G_1–arrested cells. On low nitrogen medium, these non-G_1–arrested cells become translucent, acquire a deformed morphology, and rapidly lose viability (*22*). Overexpression of wild-type Ras2 protein from the GAL10 promoter causes a similar, but less severe arrest defect. To determine whether mevalonate starvation would suppress the cell cycle defect of activated Ras2 mutants, isogenic wild-type and HMG-CoA reductase deficient strains (*hmg1 hmg2*) were transformed with plasmids containing either the $RAS2^{val19}$ gene expressed from its own promoter, the *RAS2* gene expressed from the GAL10 promoter, or a multicopy plasmid containing *RAS2*. These strains were grown to stationary phase in various media, and the ability of wild-type and mevalonate-starved cells to arrest as unbudded cells and to survive stationary phase was measured (Table 2).

When cells produced normal amounts of Ras2 protein (on glucose medium), mevalonate auxotrophy had no effect on the fraction of stationary phase cells that arrested in G_1 (Table 2, experiment 1). However, when Ras2 was overproduced (on galactose medium), the wild-type strain was unable to arrest in G_1, whereas the mevalonate auxotroph was able to accumulate mostly as unbudded cells. Thus mevalonate auxotrophy restored normal growth control to cells overproducing Ras2 protein. On this high nitrogen medium, mevalonate auxotrophy had perhaps a slight effect on the ability of cells overproducing Ras2 protein to survive stationary phase. On low nitrogen medium mevalonate auxotrophy restored both the capacity to arrest in G_1 and the ability to survive stationary phase to Ras2 overproducing cells (Table 2, experiment 2). The effect of $Ras2^{Val19}$ protein on G_1 arrest and stationary phase

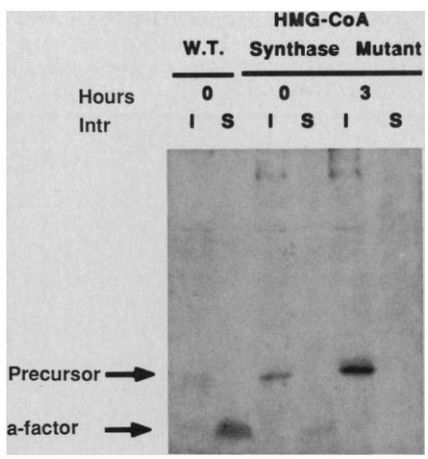

Fig. 2. A diploid (JRY-2139) heterozygous for the *erg13::HIS3* allele of the HMG-CoA synthase structural gene was transformed with a multicopy plasmid containing the *MFa1* gene, the structural gene for a-factor (pAB182). After sporulation and tetrad dissection, *erg13::HIS3* and wild-type sister spores were grown on low-sulfate medium [LSM (*12*)] to mid-log phase in the presence of mevalonate (5 mg/ml) (Sigma). Approximately 2×10^7 cells were harvested, washed, and resuspended in 1 ml of LSM. The *erg13::HIS3* mutants were labeled at 0 time or after a 3-hour incubation in LSM in the absence of mevalonate. Labeling of wild-type and mutant strains was carried out for 1 hour in LSM containing 10 μ*M* ammonium sulfate and 1 mCi of $[^{35}S]H_2SO_4$. Secreted a-factor (S), from the culture medium, and intracellular a-factor (I), from whole cells, were isolated (*12*) and precipitated with antibodies to the peptide portion of mature a-factor. The immunoprecipitates were electrophoretically separated on a 10 to 20 percent gradient SDS-polyacrylamide gel and the presence of a-factor–related material was detected by autoradiography.

viability was compared in a wild-type strain and in a mevalonate auxotroph. In the wild-type strain, expression of Ras2^{Val19} caused most of the cells to be budded or deformed in stationary phase, with a concomitant fivefold decrease in viability. In contrast, in the isogenic mevalonate auxotroph the detrimental effects of Ras2^{Val19} were substantially suppressed. Mevalonate auxotrophy suppressed these phenotypes in stationary phase cells maintained on media containing either glucose or galactose (Table 2, experiments 3 and 4). Glucose exacerbated the loss of stationary phase viability cause by Ras2^{Val19} in low nitrogen medium. Nevertheless, even the low viability caused by these conditions was substantially suppressed by mevalonate limitation (Table 2, experiment 4). These data showed that mevalonate limitation resulted in a significant amelioration of the cell cycle defect caused by activated *RAS2* mutants; this effect is probably due to altered localization and decreased levels of Ras2 protein in the mevalonate auxotroph.

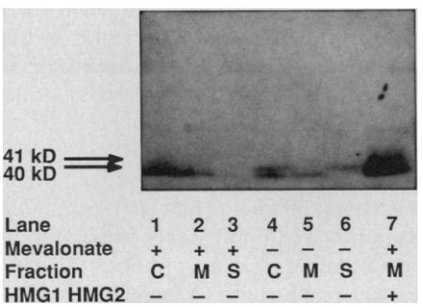

Fig. 3. HMG-CoA reductase mutant cells containing *RAS2* on a multicopy plasmid were grown to mid-log phase in the presence of mevalonate, or starved in mevalonate-free medium for 12 hours. Cell extracts were prepared by glass bead lysis; debris and unbroken cells were removed; membrane and soluble fractions were then separated by centrifugation at 100,000*g* for 30 minutes. Extracts from isogenic mevalonate prototrophic strains containing or lacking the *RAS2* plasmid were also prepared. Cell extracts were separated by SDS-PAGE, transferred to nitrocellulose, immunoblotted with the monoclonal antibody Y13-259 (to Ras), and detected by alkaline phosphate-conjugated antibody to rat immunoglobulin G (IgG) (Jackson Laboratory) visualized with Nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (NBT-CBIP) development. C signifies a crude cell extract, whereas M and S signify membrane and soluble fractions, respectively. The *RAS2* multicopy plasmid, pJR670, consists of an Eco RI to Hind III fragment from YEp13-*RAS2* inserted into the polylinker of pSEY8 (*37*). The lower arrow represents the position of mature Ras2 protein, and the upper arrow represents the position of the precursor. The size markers are extrapolated from protein markers and are consistent with published values. Although equal amounts of protein were present in each lane, an increase of Ras2 protein in the *HMG1 HMG2* strain was routinely observed in each of several experiments.

Recessive mutations in the *BCY1* gene cause many of the same phenotypes as *RAS2^{val19}* including rapid loss of viability in stationary phase (*22*). *BCY1* encodes the regulatory subunit of cyclic AMP (adenosine monophosphate)–dependent protein kinase and mediates most of the effects of yeast Ras2 protein (*22*). Isogenic *bcy1* and *hmg1 hmg2 bcy1* strains were grown to stationary phase on mevalonate-containing medium and incubated for 4 days to determine the number of cells that survived stationary phase. The viable fraction of *bcy1* cells was 0.13 percent, whereas the viable fraction of *hmg1 hmg2 bcy1* cells was <0.008 percent. Similarly, 37 percent of the *bcy1* cells arrested in G_1 and 29 percent of the *hmg1 hmg2 bcy1* cells arrested in G_1. Thus, suppression of the phenotypes of *RAS2^{val19}* by mevalonate limitation was specific to the mechanism by which Ras2 acts since the genetically equivalent phenotype caused by the *bcy1* mutation was not suppressed by mevalonate limitation. Complete starvation for mevalonate resulted in both nonspecific growth arrest and rapid cell death, phenotypes also caused by activated Ras (*22*). Therefore it was not possible to investigate the effect of total mevalonate depletion on activated *RAS2* mutants. Nevertheless, suppression of the phenotypes caused by activated Ras2 protein did occur under conditions of partial mevalonate starvation that still permitted cell growth.

Activation of human Ras protein is mevalonate-dependent. To determine whether the effects of mevalonate starvation on Ras-mediated processes was specific to yeast, or whether the properties of the oncogenic human *ras* protein also might be sensitive to mevalonate limitation, we utilized the properties of the *Xenopus* oocyte. Injection of *Xenopus* oocytes with the human c-H-ras^{Val12} protein induces meiosis as monitored by breakdown of the germinal vesicle; injection with wild-type human Ras protein is far less effective in inducing meiosis. The human c-H-ras^{Val12} protein used

Table 2. Mevalonate limitation suppresses the effects of Ras activation. Each strain was grown to late stationary phase on the indicated medium. Cells were spread on petri plates and the budding index was determined for 100 randomly chosen cells. Percent G_1 refers to the fraction of cells that were unbudded and of normal shape. These cells were removed by micromanipulation into an ordered array and incubated for 2 days at 30°C on YPD (experiments 1 and 3) or complete minimal medium lacking uracil (experiments 2 and 4) supplemented with mevalonate to determine viability. In experiment 4, viability was determined by plating approximately 6.8×10^5 cells onto supplemented plates and incubating for 2 days at 30°C. Glucose and galactose were added to media at a concentration of 2 percent. High nitrogen medium contained Casamino acids (2 percent), ammonium sulfate (0.5 percent), amino acid supplements, and yeast nitrogen base (0.17 percent). Low nitrogen medium contained only yeast nitrogen base. Mevalonate was added to media at a concentration of 38.5 m*M*. JRY125 *MATα HMG1 HMG2 ade2-101 his3Δ200 lys2-801 met ura3-52* pJR646, JRY2314 *MATa HMG1 hmg2::HIS3 ade2-101 his3Δ200 lys-2-801 met ura3-52* pJR672; JRY2315-*hmg1::LYS2*, otherwise identical to JRY2314; JRY2329 *MATa hmg1::LYS2 hmg2::HIS3*, otherwise identical to JRY125. Remaining strains were isogenic to JRY2329 except as indicated and JRY124 was *MATα*. Plasmids with the *RAS2^{val19}* mutant allele were YCp50-based. pJR670 (YEpRAS2) is a multicopy plasmid consisting of an Eco RI–Hind III fragment from YEp13-*RAS2* inserted into the polylinker of the vector pSEY8 (*37*). pJR672 consisted of an Eco RI fragment containing pGAL10 fused to *RAS2* coding sequences and the 2-μm plasmid origin of replication inserted into the Eco RI site of YIp5.

Strain	Relevant genotype	Plasmid	Carbon source	Nitrogen	Cells in G_1 (%)	Viability (%)
		Experiment 1				
JRY2314	*HMG1 HMG2*	p*GAL-RAS2*	Glu	High	80	43
JRY2315	*hmg1 hmg2*	p*GAL-RAS2*	Glu	High	78	37
JRY2314	*HMG1 HMG2*	p*GAL-RAS2*	Gal	High	29	37
JRY2315	*hmg1 hmg2*	p*GAL-RAS2*	Gal	High	80	62
		Experiment 2				
JRY2419	*HMG1 HMG2*	Vector	Gal	Low	92	50
JRY2422	*hmg1 hmg2*	Vector	Gal	Low	86	52
JRY124	*HMG1 HMG2*	*YEpRAS2*	Gal	Low	34	8
JRY2326	*hmg1 hmg2*	*YEpRAS2*	Gal	Low	90	52
		Experiment 3				
JRY1594	*HMG1 HMG2*	None	Gal	Low	84	37
JRY1593	*hmg1 hmg2*	None	Gal	Low	71	43
JRY125	*HMG1 HMG2*	*RAS2^{val19}*	Gal	Low	17	8
JRY2329	*hmg1 hmg2*	*RAS2^{val19}*	Gal	Low	70	38
		Experiment 4				
JRY125	*HMG1 HMG2*	*RAS2^{val19}*	Glu	Low	41	$<1.3 \times 10^{-5}$
JRY2329	*hmg1 hmg2*	*RAS2^{val19}*	Glu	Low	78	2.1×10^{-2}

in our experiments was purified from *Escherichia coli* and hence lacked eukaryotic posttranslational modifications. However, the oocyte is capable of posttranslationally modifying Ras proteins (*6*). Although it was not possible to limit mevalonate synthesis in the oocyte genetically, drugs such as compactin or lovastatin, also known as mevinolin (*20*), which competitively inhibit HMG-CoA reductase, should result in mevalonate starvation and, provided that the oocyte's pool of distal intermediates is low, should also result in the depletion of all isoprenoid compounds.

The ability of compactin to block c-H-ras^{Val12} induced breakdown of the germinal vesicle was tested. In the first experiment, injection of compactin over a 100-fold concentration range did not, by itself, cause germinal vesicle breakdown (Table 3, experiment 1). As would be expected, injection of c-H-ras^{Val12} protein purified from *E. coli* resulted in breakdown of the germinal vesicle at all but the lowest concentration tested (Table 3, experiment 2). In experiment 3 (Table 3), oocytes were first injected with compactin and then with c-H-ras^{Val12} to determine whether depletion of mevalonate would block the action of Ras protein. Even the lowest concentration of compactin produced a complete block in germinal vesicle breakdown even when relatively high levels of the oncogenic Ras protein were present. The addition of mevalonate to oocytes that had been previously injected with compactin and c-H-ras^{Val12} protein restored the ability of the protein to induce germinal vesicle breakdown (Table 3, experiment 4). Therefore, the inhibition of Ras function by compactin was due exclusively to a block in mevalonate synthesis. These results demonstrated that a mevalonate-dependent step is required for the biological properties of human c-H-ras^{Val12}. Furthermore, this process may be mevalonate-limited in the oocyte since injection of mevalonate shortened the time required to observe germinal vesicle breakdown when c-H-ras^{Val12} protein was co-injected (legend, Table 3).

Progesterone treatment causes germinal vesicle breakdown in *Xenopus* oocytes in a manner that does not require activity of Ras protein (*6*). In an experiment to evaluate the specificity of the inhibition of germinal vesicle breakdown by compactin, 11 of 11 progesterone-treated oocytes exhibited germinal vesicle breakdown and 9 of 9 oocytes injected with compactin and then treated with progesterone exhibited germinal vesicle breakdown. Thus, the ability of compactin to block germinal vesicle breakdown was specific to its ability to block Ras protein function.

General importance of mevalonate-based protein modification. The mevalonate-dependent modification of Ras2 protein reported here may be a common and important modification of many proteins. All *ras* gene products contain a cysteine residue positioned four amino acids from the end of the open reading frame (Table 4). This cysteine residue corresponds to Cys^{186} encoded by the human c-H-*ras* gene and to the cysteine known to be farnesylated in yeast **a**-factor. On the basis of the genetic and biochemical evidence linking **a**-factor and yeast Ras protein processing, it is possible that Cys^{186} is farnesylated in human c-H-ras and perhaps in all Ras proteins. This amino acid is required for both membrane localization and fatty acylation of Ras protein and has been presumed to be the site of palmitylation of Ras proteins (*10*). However, there is no direct evidence that the palmityl group is actually attached to this particular cysteine. In fact, Ras proteins contain nearby cysteine residues which may be the site or sites of palmitylation (Table 4). Our data are consistent with the mevalonate-dependence of Ras activation and membrane association being due to the farnesylation of Ras protein at Cys^{186} or at another nearby cysteine. Palmitylation of Ras could in fact be a consequence of, rather than a cause of, membrane association. In this regard it should be noted that the palmitylation of bovine opsin, an integral membrane protein of rod outer segments, apparently occurs by a nonenzymatic mechanism (*23*). However, our data are consistent with other interpretations as well. For example, one of the enzymes used to process Ras proteins may require a mevalonate-dependent modification for its activity. It is critical to determine both whether Ras protein is in fact farnesylated and, if it is, where it is farnesylated and whether the farnesylation is the basis of the mevalonate-dependent activation.

On the basis of sequence similarity and cellular localization, other proteins that may be modified by a mevalonate-derived molecule include the γ subunit of the G_s class of G proteins and the α subunit of the G_i and G_o class. Constitutive activation of the *STE4-STE18*–encoded G protein $\beta\gamma$ subunits that mediate yeast mating factor response results in arrest of cell division (*24, 25*). We have recently found that mevalonate limitation suppresses the division arrest associated with constitutive STE18 protein. The sequence of the COOH-terminus of STE18 as deduced from the gene sequence bears striking similarity to the carboxy sequence of Ras and **a**-factor (Table 4). All of these proteins except DRAS (*Drosophila* RAS) have the sequence Cys-A-A-X where A and X refer to aliphatic and any amino acid, respectively. It is possible that the function of STE18 protein and other G proteins depends on a mevalonate-dependent modification. Since response to both **a**-factor and α-factor occurs through *STE18*, a mevalonate-dependent modification of the *STE18*-encoded γ subunit could explain the slight yet

Table 3. Mevalonate-dependent activation of human c-H-ras^{Val12} in *Xenopus* oocytes. Oocyte injections were performed as described (*6*). For each experiment three to five oocytes were activated by progesterone treatment as a control for the ability of the oocytes from a particular frog to initiate meiosis. In all experiments a total volume of 50 nl was injected. Initiation of meiosis was measured visually by the appearance of germinal vesicle breakdown (GVBD), which occurred at 8 hours after injections except for those experiments labeled with an asterisk, in which the germinal vesicle breakdown occurred at 5 hours after injection. In experiments 3 and 4, oocytes were injected with compactin (25 nl) and then incubated for 1 hour at room temperature to allow depletion of the isoprene pool; they were then injected with human c-H-Ras^{Val12} protein (25 nl) or a mixture of this protein and mevalonate (25 nl). Compactin was diluted from a stock solution (25 mg/ml) in 25 percent ethanol. Human c-H-ras^{Val12} protein was purified from *E. coli* as described (*38*), stored in 50 m*M* Hepes, *p*H 7.4, 1 m*M* EDTA, 1 m*M* DTT, and diluted into 50 m*M* β-glycerophosphate, *p*H 7.5, 3 m*M* $MgCl_2$ before injection.

Source	Compactin (ng)	H-Ras^{Val12} (ng)	Mevalonate (ng)	Oocytes with GVB
Experiment 1	1.25	0	0	0/6
	0.125	0	0	0/6
	0.0625	0	0	0/6
	0.0125	0	0	0/6
Experiment 2	0	56	0	6/6
	0	28	0	6/6
	0	14	0	6/6
	0	7	0	0/6
Experiment 3	12.5	56	0	0/6
	1.25	56	0	0/6
	0.125	56	0	0/6
	12.5	0	0	0/3
	1.25	0	0	0/3
	0.125	0	0	0/3
	0	56	0	5/5
Experiment 4	0.125	28	4	6/6*
	0.125	28	40	6/6*
	0.125	28	400	6/6*
	0	28	0	3/3
	0.125	0	0	0/3
	0	0	4	0/3
	0	0	40	0/3
	0	0	400	0/3

detectable reduction in mating efficiency of mevalonate auxotrophs of the α mating type and would contribute to the sterility of *MATa* mevalonate auxotrophs (Table 1). With regard to the mevalonate dependence of G protein function in general, two observations are particularly relevant. In many cell types metabolically labeled with radioactive mevalonate, the major labeled proteins are in the size range of G protein subunits (*26*). Furthermore, compactin treatment blocks PDGF (platelet-derived growth factor)–mediated stimulation of DNA replication of mammalian cells in tissue culture (*26*). Taken together, these observations suggest that mevalonate-dependent modifications may be required for the transduction of many growth factor signals.

Not all relatives of the Ras family have a cysteine positioned four amino acids from the COOH-terminus of the sequence. The *Saccharomyces cerevisiae YPT1* gene and related genes in yeast and mammals do, however, encode a cysteine as the last amino acid in the coding sequence of the gene, and are membrane associated (Table 4). In the case of YPT1, at least one of the COOH-terminal cysteines is required for membrane association, biological function, and palmitate labeling (*27*). Mutant *YPT1* genes have been constructed that contain a 3′ extension to the open reading frame that add the last three amino acids encoded in the H-*ras* sequence. This modified YPT1 protein is still biologically active and apparently fatty acylated (*27*). Although our data do not bear directly on the YPT1 protein, it is of interest to determine whether the activity and membrane association of YPT-related proteins is mevalonate-dependent.

Other proteins with COOH-terminal sequence similarity to the Ras family include nuclear lamins of both the A and B subtypes (*28*).

Table 4. A sequence comparison of the carboxyl terminal ten amino acids of Ras and Ras-related proteins. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

a-factor				V	F	W	D	P	A	C	(39)
RAS Proteins											
RAS1				E	Y	S	G	G	C	C	(9)
RAS2				S	G	S	G	G	C	C	(9)
DRAS				P	N	C	R	F	K	C	(7)
HRAS				G	C	M	S	C	K	C	(40)
KRAS				K	K	S	K	T	K	C	(41)
RAL				K	R	I	R	E	R	C	(42)
APLYSIA RHO				K	K	K	K	G	G	C	(43)
SC RHO1				E	K	K	K	K	K	C	(43)
SC RHO2				E	P	G	A	N	C	C	(43)
S.POMBE.RAS				E	V	S	T	K	C	C	(44)
G Proteins											
STE18				N	S	N	S	V	C	C	(24)
TRANSDUCIN γ Subunit				K	E	L	K	G	G	C	(45)
RAT Giα1				K	N	N	L	K	D	C	(46)
RAT G0α				A	N	N	L	R	G	C	(46)
YPT1 Family											
SC YPT1	S	L	T	N	T	G	G	G	C	C	(47)
RAB1	P	V	K	Q	S	G	G	G	C	C	(48)
RAB2	G	G	Q	Q	A	G	G	G	C	C	(48)
RAB3	Q	A	P	P	H	Q	D	C	A	C	(48)
RAB4	Q	A	P	S	A	Q	E	C	G	C	(48)
SEC4	S	G	N	S	S	K	S	N	C	C	(49)
Other											
HMG1				K	D	G	S	V	T	C	(31)
Nuclear Lamins											
A Subtype											
Xenopus				Q	U	A	P	Q	N	C	(28)
Human				T	Q	S	P	Q	N	C	(28)
B Subtype											
Xenopus				K	S	G	N	K	N	C	(28)
Drosophila				Q	Q	S	N	E	K	C	(28)

Lamin B appears to be associated with the inner layer of the nuclear envelope. Furthermore, there is direct evidence that the protein is posttranslationally esterified with a methyl group and covalently modified by mevalonate or a mevalonate-derived product (*29, 30*). The sequence similarity with **a**-factor and Ras suggests that these modifications may occur at the carboxyl terminus, raising the possibility that mevalonate-derived products may mediate association of proteins to a variety of cellular membranes. It may also be significant that the carboxyl terminal sequence of HMG-CoA reductase, a protein of the endoplasmic reticulum that catalyzes the rate limiting step in isoprene biosynthesis, also encodes a cysteine residue four amino acids from the end of the open reading frame (*31*) (Table 4).

Pharmacological implications of mevalonate-dependent Ras processing. The demonstration of a requirement for mevalonate-derived molecules for the modification and in vivo activity of Ras proteins suggests practical ways to block Ras activation. Specifically, these results identify a class of drugs that is capable of inhibiting at least some of the effects and properties of an activated human c-H-ras oncoprotein. Moreover, the analogous studies with yeast confirmed genetically that activated Ras can be suppressed by inhibiting isoprene biosynthesis to a degree that still permits cell growth and viability. Activated *ras* genes have been found in 10 to 20 percent of all human tumors. Approximately 50 percent of colorectal tumors and more than 90 percent of pancreatic tumors have activated *ras* genes (*1, 32*). Nevertheless, the role of Ras in the properties of these tumors is unknown. Drugs that inhibit HMG-CoA reductase or enzymes downstream in the cholesterol biosynthetic pathway may prove valuable in determining in what fraction of these tumors the Ras mutation acts in tumorigenesis and also in controlling the tumors. Since drugs that inhibit human HMG-CoA reductase are used to control hypercholesteremia and have relatively few detrimental side effects (*33*), it is conceivable they may be of some value in treating certain tumors involving activated Ras protein. The involvement of G protein–mediated responses of tumor cells to growth factors, and the possibility of a mevalonate dependence for G protein function may extend the pharmacological utility of these and related drugs in controlling cell proliferation.

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35. A clone of the *ERG13* gene, encoding HMG-CoA synthase was provided by K. Jarman and J. Proffitt (Amoco Corp.). A Bam HI fragment containing the yeast *HIS3* gene was inserted into a Bgl II site in the HMG-CoA synthase coding region to create a disruption allele, which was then used to disrupt the chromosomal *ERG13* locus, rendering the cell a mevalonate auxotroph.
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Peptide Binding and Release by Proteins Implicated as Catalysts of Protein Assembly

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Two members of the hsp70 family, termed hsc70 and BiP, have been implicated in promoting protein folding and assembly processes in the cytoplasm and the lumen of the endoplasmic reticulum, respectively. Short hydrophilic (8 to 25 residues) synthetic peptides have now been tested as possible mimics of polypeptide chain substrates to help define an enzymatic basis for these activities. Both BiP and hsc70 have specific peptide binding sites. Peptide binding elicits hydrolysis of adenosine triphosphate, with the subsequent release of bound peptide.

BECAUSE MANY PROTEINS REFOLD AFTER DENATURATION (*1*), it has long been assumed that protein folding and assembly occurs spontaneously in cells. However, recent and diverse studies of protein folding, unfolding, and related processes (such as translocation across membranes) in vivo suggest that in many instances these events may be catalyzed. If so, then these catalysts can determine the nature of folding pathways and their location in cells, defining in essence what folded states consist of and coordinating protein assembly in relation to existing cellular organization. How this works is unknown and has been difficult to determine for lack of a well-defined in vitro system. Here we describe an assay system that may mimic essential steps in this process.

Two distinct families of adenosine triphosphate (ATP)–dependent proteins have been implicated as catalysts, namely members of the groEL family (termed chaperonins) found in bacteria, mitochondria, and chloroplasts (*2, 3*); and members of the hsp70 family, found both in the cytoplasm and in the lumen of the endoplasmic reticulum (ER) (*4–6*). The heat shock protein hsp70 family consists of several members (ranging from 70 to 78 kD) that are induced at elevated temperatures (*4, 5*), and of other members that are constitutively expressed (*6*). These constitutively expressed proteins include hsc70, also known as the uncoating ATPase for clathrin-coated vesicles (*7*) and a binding protein BiP (*8*), also known as

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