

# Biochemistry of protein prenylation

Patrick J. Casey

Section of Cell Growth, Regulation and Oncogenesis and Department of Biochemistry,  
Duke University Medical Center, Durham, NC 27710

## I. INTRODUCTION

Covalent modification by isoprenoid lipids (prenylation) is now recognized as a mechanism to promote membrane interactions and biological activities of a variety of cellular proteins. Both the 15-carbon farnesyl and 20-carbon geranylgeranyl isoprenoids are involved in these modifications, which occur on carboxyl-terminal cysteine residues of proteins (Fig. 1). Known prenylated proteins include the nuclear protein lamin B and a number of GTP-binding regulatory proteins (G proteins) responsible for controlling a wide spectrum of signal transduction pathways. This review summarizes the development of, and recent studies in, the field of protein prenylation, with particular emphasis on the biochemistry of the processes involved. Several other recent reviews are available for more detailed coverage of aspects which receive limited attention here (1-3).

## II. A BRIEF HISTORY OF THE FIELD

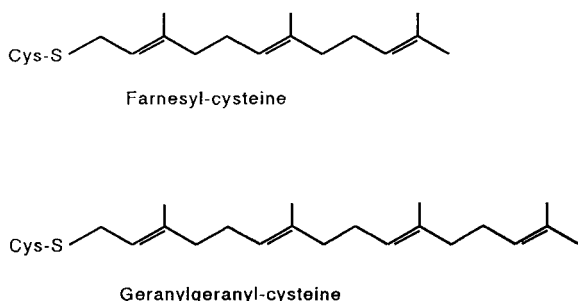
The first evidence that isoprenoids could be covalently attached to proteins came from studies in Japan in the late 1970s on the structures of certain fungal mating factor peptides (4, 5). These investigators found that the 15-carbon isoprenoid, farnesyl, was an integral component of several such mating factors, and defined the linkage to the peptide as being a thioether in which a cysteine sulfhydryl was attached to the C-1 carbon of the isoprenoid (Fig. 1). While the functional significance of this modification was not at all clear, the fact that the modification was a stoichiometric and quite stable one suggested that it was an important component of the active mating factor.

The discovery that mammalian proteins could also be prenylated grew out of studies concerning the effects of compactin, an inhibitor of isoprenoid biosynthesis, on mammalian cell growth and morphology. Compactin acts on HMG-CoA reductase, the enzyme that catalyzes the synthesis of mevalonate (MVA), the precursor to all isoprenoids (Fig. 2). Studies in the late 1970s and early 1980s had shown that treatment of cells with high concentrations of compactin resulted in an arrest of cell growth that could not be reversed by exogenously added cholesterol or other sterols (6, 7); these sterols are the major products of the

isoprenoid pathway. However, small amounts of MVA could reverse the effects of compactin, suggesting that a nonsterol metabolite of MVA (or MVA itself) was important in this recovery from cell growth arrest. When this type of experiment was performed using [ $^3\text{H}$ ]MVA to follow the metabolic fate of the labeled compound, Schmidt, Schneider, and Glomset (8) found that the label was incorporated into a subset of cellular proteins. This observation was confirmed and extended by other groups (9, 10), although some time would pass before the identities of the proteins that were prenylated or the nature of the modifying group were determined.

The first specific mammalian protein identified as being prenylated was the nuclear protein lamin B (11, 12). Parallel studies on a putative lipid modification of the a-factor mating peptide of *Saccharomyces cerevisiae* revealed that this mating factor, like the fungal factors studied a decade earlier, was also prenylated, specifically by a farnesyl isoprenoid (13). A feature common to both lamin B and the a-factor peptide, as well as the fungal mating factors studied a decade earlier, was the presence of a so-called "CAAX-motif" at their carboxyl terminus. This motif, where "C" was a cysteine residue to which the isoprenoid was attached, "A" initially signified an aliphatic amino acid, and "X" denoted an undefined amino acid, was believed at the time to signal addition of a palmitoyl (16-carbon fatty acid) group to proteins containing this lipid (14). The discovery that the CAAX-motif could in fact direct addition of isoprenoids to proteins containing it prompted a re-examination of other proteins containing this motif to determine if they too were prenylated. Chief among these CAAX-containing proteins that were examined were the products of the *ras* family of protooncogenes. The simultaneous discoveries that the *ras* proteins were modified by the 15-carbon farnesyl group, and that this modification was required for the oncogenic forms of these proteins to transform cells (15-17), provided the first functional correlate of this modification and dramatically increased interest in this form of lipid modification.

Abbreviations: MVA, mevalonate; FPP, farnesyl diphosphate; PFT, protein farnesyltransferase; GGPP, geranylgeranyl diphosphate; PGGT, protein geranylgeranyltransferase.



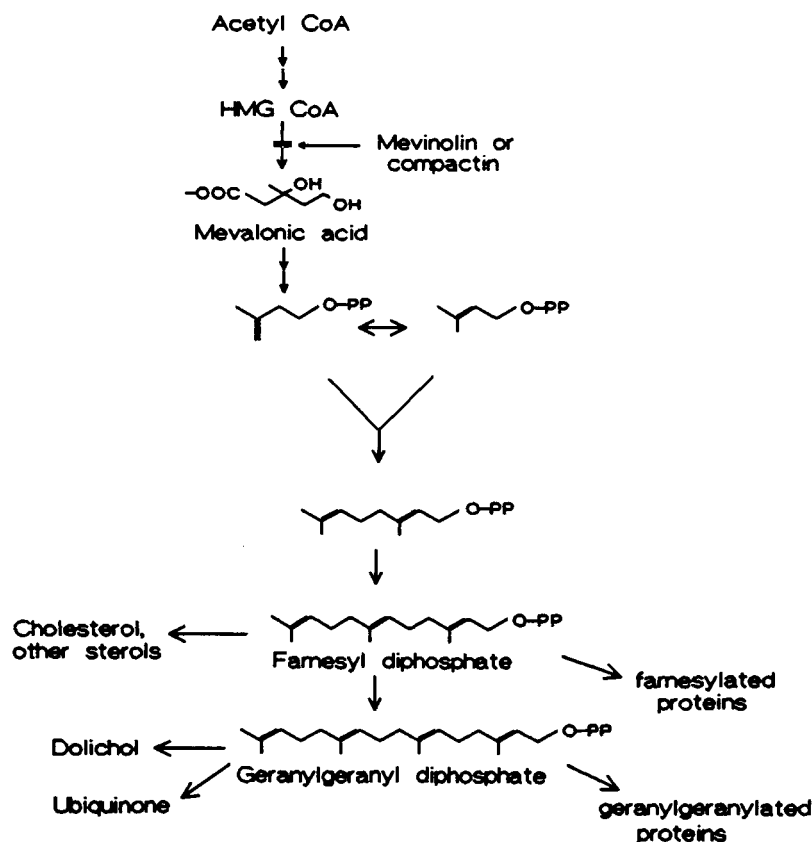
**Fig. 1.** Structures of the farnesyl-cysteine and geranylgeranyl-cysteine. The isoprenoid is attached via a thioether linkage from the C-1 carbon to the thiol of the amino acid.

### III. CLASSES OF PRENYLATED PROTEINS

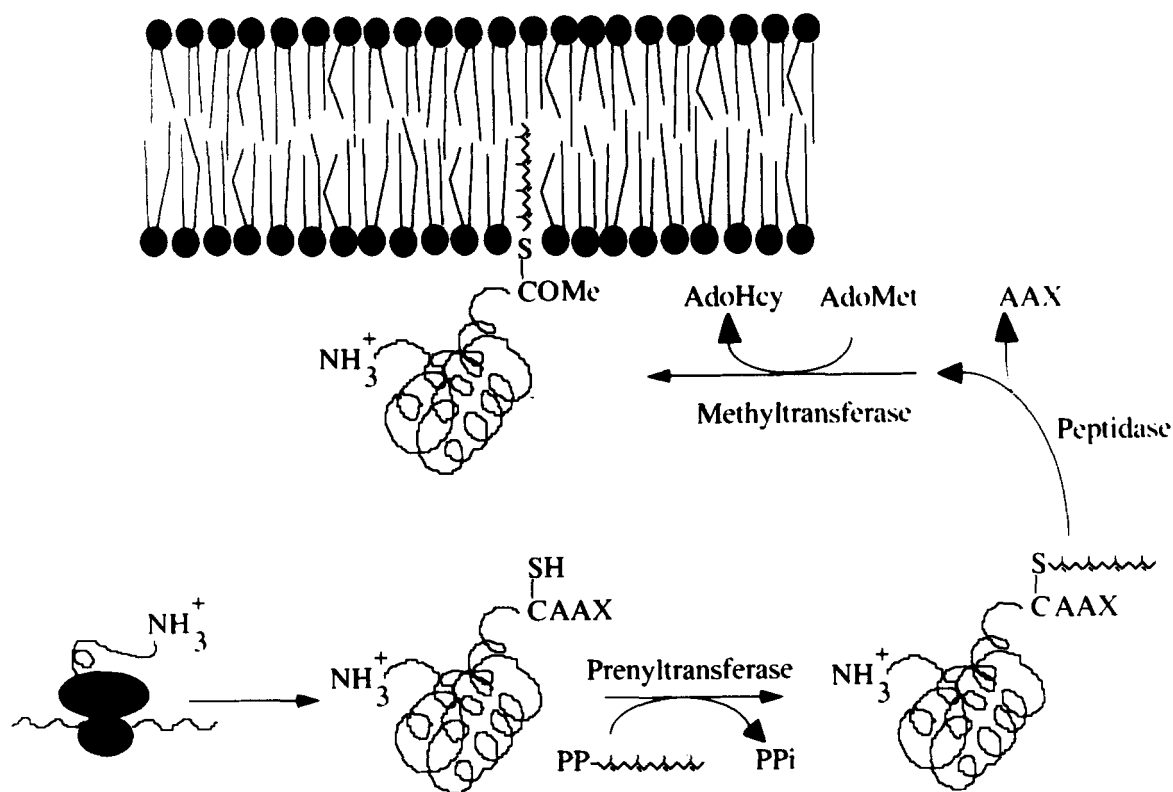
The increasing awareness that the presence of a CAAX-motif at its carboxyl terminus identified a protein as a candidate for prenylation prompted a flurry of activity directed toward determining the prenylation status of such proteins. Searches of the protein sequence data bases initially identified ~40 such candidates; surprisingly, most of these proteins were members of the class of GTP-binding proteins (the so-called "small G proteins") related

to the ras proteins. Furthermore, most of the remaining candidates (e.g., the  $\gamma$  subunits of the heterotrimeric G proteins, cGMP phosphodiesterase) were also known to be involved in cellular signaling events (1, 18). Essentially all of these candidate proteins that have been closely inspected have been shown to be prenylated. To date, only the 15-carbon farnesyl and 20-carbon geranylgeranyl groups have been found to modify proteins, with geranylgeranyl being the predominant isoprenoid attached (19, 20). Of considerable interest to those studying the enzymology of the prenylation processes (see below), the COOH-terminal amino acid ("X") of the CAAX box is now known to specify which isoprenoid is attached to a candidate protein. If this residue is a serine, methionine, or glutamine residue, the protein contains the farnesyl isoprenoid, while a leucine at that position directs geranylgeranyl addition (2, 21).

Prenylation is not the only posttranslational modification performed on proteins containing the CAAX-motif. Specifically, the mature forms of these prenylated proteins lack the three COOH-terminal amino acids (the "AAX"). A cellular peptidase (see Section VI) removes these three amino acids, leaving the prenylated cysteine as the COOH-terminal residue. Additionally, in all cases where



**Fig. 2.** Biosynthesis of isoprenoids in eukaryotic cells. HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) is formed from three molecules of acetyl-CoA. The rate-limiting enzyme in the pathway, HMG-CoA reductase, is the site of action of the pharmacological agent, mevinolin.



**Fig. 3.** Carboxyl-terminal processing of prenylated proteins. The proteins are synthesized as soluble precursors containing a cysteine residue fourth from the COOH-terminus (the so-called "CAAX-motif"). Prenylation is catalyzed by soluble protein prenyltransferases using the appropriate prenyl diphosphate donor. Either the 15-carbon farnesyl or 20-carbon geranylgeranyl group is added, depending on the COOH-terminal residue ("X" of the CAAX-motif). After prenylation, the three COOH-terminal residues are removed by a peptidase and the now-free COOH group of the modified cysteine residue is methylated in a S-adenosylmethionine (AdoMet)-dependent reaction. Recent studies have shown that some proteins that have a natural COOH-terminal cysteine residue, most notably members of the rab/YPT1 family of GTP-binding proteins, are also prenylated although their processing presumably goes by a different pathway. See text for further details. (Graphics by Joyce Higgins)

the prenylated proteins have been closely examined, a substantial fraction have the carboxyl group of this cysteine residue methylated (2, 22). The net result of these three apparently closely linked processing steps is the production of a mature protein with a highly hydrophobic COOH-terminus, a dramatic enhancement of the inherent hydrophobic properties of these proteins (**Fig. 3**).

It has recently become clear that prenylation is not limited to proteins containing the COOH-terminal CAAX-motif, but also occurs on another subset of the GTP-binding proteins termed the rab/YPT1 family (23, 24). Most of these proteins have COOH-terminal sequences ending in Cys-Cys or two cysteines separated by another amino acid (Cys-X-Cys). The rab/YPT1 proteins have been implicated in control of intracellular membrane trafficking pathways, and apparently have the capacity to reversibly associate with membranes (25, 26). Furthermore, in many (if not all) of these proteins, both of the cysteine residues are prenylated with the geranylgeranyl isoprenoid (24). As these modifications occur at the extreme COOH-terminus, no proteolytic processing is required to expose the carboxylate of the prenylated cys-

teine residue for methylation, but this methylation of the carboxyl group has been shown to occur (24).

#### IV. ENZYMOLOGY OF PRENYLATION

The information currently available on the various protein prenyltransferases is summarized in **Table 1**. Efforts to develop *in vitro* systems to examine protein prenylation were initiated immediately after the identification of the farnesyl group as that modifying the ras proteins and lamin B, as the approach then became apparent. Farnesyl diphosphate (FPP) was the logical donor of the isoprenoid since it is the activated form of farnesyl in the isoprenoid biosynthesis pathway (27) (see **Fig. 2**). The protein substrate for the putative enzyme that would transfer the farnesyl was supplied by production of recombinant H-ras protein in bacterial expression systems; purification of the recombinant protein yielded the unmodified form of ras that was recognized by the enzyme responsible for its farnesylation. By following incorporation of [<sup>3</sup>H]FPP into this ras protein, Reiss et al. (28) identified and purified a cyto-



TABLE 1. Properties of protein prenyltransferases

Protein Prenyltransferase	Protein Substrate C-Terminus	Isoprenoid Substrate	Metal Requirements	Subunit Composition (Mammalian)	<i>S. cerevisiae</i> Gene Product	Reference
PFT	-CAAX X = M, S, Q, A	FPP	Zn <sup>2+</sup> , Mg <sup>2+</sup>	48 kDa( $\alpha$ ) 46kDa( $\beta$ )	RAM2( $\alpha$ ) RAM1( $\beta$ )	28-32
PGGT(CAAX)	-CAAX X-L	GGPP	Zn <sup>2+</sup> , Mg <sup>2+</sup>	48 kDa( $\alpha$ ) 43 kDa( $\beta$ )	RAM2( $\alpha$ ) CDC43( $\beta$ )(?)	21, 35-37
PGGT(CC)	-CC (rab proteins)	GGPP	?	?	BET2? Others?	51, 56
PGGT(CXC) [same as PGGT (CC)?]	-CXC	GGPP	?	?	BET2? Others?	55

The PGGT enzymes are separated into three classes, one of which acts on CAAX-containing proteins [PGGT(CAAX)] and two that act on the rab/YPT1 protein family members [PGGT(CC) and PGGT(CXC)], although there may be only one enzyme that can recognize the latter two classes of substrate proteins. Single letter amino acid abbreviations are: C, cysteine; M, methionine; S, serine; Q, glutamine; A, alanine; L, leucine.

solic enzyme from rat brain that catalyzed this reaction. The enzyme was a heterodimer of 48 kDa (designated the  $\alpha$  subunit) and 46 kDa (the  $\beta$  subunit) polypeptides, and exhibited an absolute requirement for divalent metal ions for activity. Activity of this enzyme, designated protein farnesyltransferase (PFT), has now been identified in extracts from a variety of mammalian tissues (29, 30).

An unexpected property of the PFT was its ability to recognize short peptides corresponding to the COOH-terminal sequences of known farnesylated proteins (28). In fact, the enzyme was capable of interacting with a tetrapeptide containing just the four residue CAAX-motif of known farnesylated proteins with essentially the same affinity shown for the intact proteins, suggesting that it is only these four COOH-terminal residues that form the recognition site for attachment of the isoprenoid. This property of the PFT was exploited for its purification, as immobilization of a hexapeptide corresponding to the COOH-terminus of K-ras produced an effective affinity column. The sequence requirements for optimal interaction of these short peptides with the PFT have been extensively examined and have shown that the cysteine is an absolute requirement, with the last amino acid (the "X" residue) also playing an important role (31). While the capacity for a high-affinity interaction of the CAAX peptides with the PFT generally reflects the abilities of these peptides to serve as alternate substrates of the enzyme, variants of these peptides have been identified that serve as true competitive inhibitors of the enzyme without themselves becoming modified (32). Given the level of interest in producing inhibitors of the PFT as potential chemotherapeutic agents in the treatment of ras-associated cancers (33, 34), the high affinity interaction of the short peptides ( $K_i$  values of <200 nM) provides a valuable starting point for the design of specific inhibitors that could serve to block protein farnesylation in vivo.

The discovery that the majority of prenylated proteins were modified not by the farnesyl but by the geranylger-

anyl group suggested that a separate enzyme would be responsible for this modification. Using an approach similar to that described above for the identification of the PFT, an enzymatic activity capable of transferring the geranylgeranyl group from geranylgeranyl diphosphate (GGPP) to candidate proteins and short peptides was identified (21, 35, 36). This enzyme, termed protein geranylgeranyltransferase (PGGT), exhibits properties very similar to those of the PFT described above, including a requirement for divalent metals (see below) and the ability to recognize short peptides encompassing the CAAX-motif of known geranylgeranylated proteins, i.e., those containing a COOH-terminal leucine residue (21, 35). The chief determinant for specific recognition of CAAX-containing proteins by the PGGT is, in fact, this leucine residue, as production of an H-ras protein with a leucine-for-serine switch at the COOH-terminal position converts the protein from a PFT to a PGGT substrate (21). Like the PFT, the PGGT is an apparent heterodimer, with polypeptide components of 48 kDa ( $\alpha$  subunit) and 43 kDa ( $\beta$  subunit) visualized in preparations of the purified enzyme (37). Furthermore, antisera raised against portions of the PFT- $\alpha$  subunit also recognize this polypeptide component of the PGGT (37, 38). Thus, these two transferases apparently share a common, or highly related, subunit. The functional significance of this observation is not yet clear, although it has been proposed that the  $\alpha$  subunit contains the isoprenoid binding site for these enzymes (39). The  $\beta$  subunit of the enzymes apparently contains the binding site for peptide substrates, since radioactively-labeled substrate peptides can be cross-linked to this subunit of the PFT (39). Since the  $\beta$  subunit is the one that distinguishes these two protein prenyltransferases, this property is consistent with the known peptide-binding specificities of these two enzymes.

Analysis of the specificities of interactions of the PFT and PGGT with the metal ions required for activity is being actively investigated. Recent evidence suggests that

both  $Mg^{2+}$  and  $Zn^{2+}$  are required for catalysis by both enzymes (37, 40). By analogy with other enzymes that use diphosphate-containing substrates, it is thought that the  $Mg^{2+}$  contributes to the binding of the diphosphate moiety of the isoprenoid (28, 37). However, the  $Zn^{2+}$  appears to be a component of the enzyme itself, as purification of the enzymes in the presence of a chelating agent or prolonged dialysis against such an agent is required to observe the  $Zn^{2+}$ -dependence (37, 40). The presence of a tightly bound  $Zn^{2+}$  would classify these protein prenyltransferases as Zn-metalloenzymes (41). Given the avidity of  $Zn^{2+}$ -sulfur interactions, it is possible that the  $Zn^{2+}$  atom directly participates in catalysis by assisting in positioning and deprotonation of the incoming thiol group of the protein substrate for isoprenoid transfer.

Experimental analysis of kinetic mechanism of protein prenyltransferases has been mostly limited to studies on the PFT, as this enzyme has been available in highly purified form for study. A recent steady-state approach has shown that substrate binding to the enzyme occurs in random order (42). This analysis is consistent with both the ability of the enzyme to bind to a CAAX-peptide affinity column in the absence of FPP (i.e., the affinity chromatography step used in the purification of the enzyme (28)), and the capacity of the PFT to form a high-affinity complex with FPP in the absence of a protein substrate (39). The important question of the chemical mechanism of the reaction is still unanswered; the two likely possibilities are either a  $S_N1$ -type formation of a farnesyl-cation followed by thiol addition, or direct displacement of the pyrophosphate from FPP by the thiol in a  $S_N2$  reaction. Inhibition studies with farnesyl cation analogs could prove useful in distinguishing between these possible mechanisms (43, 44).

In parallel with the biochemical characterization of these two protein prenyltransferases from mammalian tissues, a genetic approach using the yeast *Saccharomyces cerevisiae* has yielded important information on, and model systems to study the biology of, these modifications. This work grew out of studies on the processing of the yeast a-factor mating peptide. As noted above, this mating factor is modified by the farnesyl group. Initial studies revealed that the product of a yeast gene known as *RAM1* (or *DPR1*) was necessary for the processing not only of the a-factor but also of the yeast ras protein (45, 46). These observations led to the discovery that ras in yeast is also farnesylated, and that the product of the *RAM1/DPR1* gene encoded one of the subunits (the  $\beta$  subunit) of the yeast PFT (17, 47, 48). Additionally, a second gene known as *RAM2* had been implicated in this pathway (49), and it has recently been shown to encode the  $\alpha$  subunit of this organism's PFT (50). The unambiguous identification of these two gene products as the yeast PFT subunits was greatly aided by the cloning of both the  $\alpha$  and  $\beta$  subunits of the mammalian PFT (48, 51, 52); the sequence homol-

ogy between the mammalian cDNAs and their respective yeast genes was sufficient to identify the yeast genes as PFT subunits. This identification has been confirmed by co-expression of the *RAM1/DPR1* and *RAM2* genes in a bacterial system, resulting in the production of enzymatically active PFT (50). This requirement for coexpression of the cDNAs encoding the PFT subunits to observe activity was also seen in the mammalian system developed (48); apparently the subunits expressed alone are not stable.

The identification of the *RAM2* gene product as the  $\alpha$  subunit of the yeast PFT has allowed a genetic test of the hypothesis noted above that this subunit is common to both the PFT and PGGT enzymes. Disruption of the *RAM2* gene in yeast results in a loss of both PFT and PGGT activities in extracts prepared from the mutant strain, an observation which supports this hypothesis (50). Sequence information is not yet available for the  $\beta$  subunit of the mammalian PGGT, although the gene known as *CDC43* (or *CAL1*) is thought to encode this subunit of the *S. cerevisiae* enzyme. Evidence for this designation comes both from sequence analysis of the gene, which shows high homology to both the yeast *RAM2* gene and also to the  $\beta$  subunit of the mammalian PFT, and the observation that disruption of the *CDC43* gene in yeast results in a loss of PGGT activity (51, 53).

Much less is known about the protein prenyltransferase(s) that act on the small GTP-binding proteins containing the Cys-Cys or Cys-X-Cys motifs at their COOH-terminus (e.g., the rab/YPT1 proteins noted above). Soluble enzymatic activities capable of geranylgeranylation of proteins containing both motifs have been identified and partially purified (54, 55). However, it is not yet clear whether there are two separate enzymes or whether a single protein can recognize and modify both types of proteins. The characterized enzyme(s) exclusively use GGPP as the prenyl donor. Unlike the PFT and PGGT enzymes described above that act on proteins containing the CAAX-motif, the rab/YPT1 prenyltransferase(s) do not recognize short peptides encompassing the COOH-terminus of their substrates. Thus, members of this class of protein prenyltransferases apparently contain an extended binding site for the protein substrate, with part of the recognition domain including determinants upstream of the prenylation site.

Yeast genetics has also proven quite useful for analysis of both the enzymology and biology of prenylation of the Cys-Cys- and Cys-X-Cys-containing proteins. In particular, the product of the yeast *BET2* gene has been shown to be involved in the membrane localization of the YPT1 and SEC4 GTP-binding proteins, which are involved in membrane trafficking in this organism (56). Sequence analysis has shown that the deduced product of the *BET2* gene has homology to both the yeast *RAM1* gene product and also the  $\beta$  subunit of the rat PFT (56). Thus, the

*BET2* gene is believed to comprise at least one subunit of the yeast protein prenyltransferase responsible for modification of proteins with the COOH-terminal Cys-Cys motif. The subunit structure of this class of protein prenyltransferases is not known; in particular, the question of whether they also contain the same  $\alpha$  subunit as the PFT and PGGT enzymes that recognize proteins containing the CAAX-motif is important to address. However, this is doubtful since extracts prepared from the yeast *ram2* mutant have essentially unaltered ability to geranylgeranilate the YPT1 protein in vitro when compared with extracts from wild-type yeast (51).

## V. BIOLOGICAL ROLE OF PRENYLATION

Available information on the functional properties imparted to proteins by attachment of isoprenoids and the subsequent COOH-terminal processing steps of proteolysis and methylation is mostly descriptive. The bulk of this information comes from the expression and analysis of mutant proteins that are unable to be prenylated. Mutation of the cysteine residue of the CAAX-motif is the most commonly applied technique; replacement of this residue by a serine abolishes the prenylation, as well as the subsequent processing steps, of the mutant protein (1, 3). Similar results have been obtained by treatment of cells expressing oncogenic ras with the drugs compactin or mevinoxin (17, 57). These compounds block prenylation by inhibiting the enzyme HMG-CoA reductase, the rate-limiting enzyme in isoprenoid biosynthesis (see Section II and Fig. 2), thus eliminating the prenyl diphosphate substrates of the protein prenyltransferases. In most cases examined, the unprenylated proteins produced in these systems associate only poorly, if at all, with cellular membranes, thus confirming the expected contribution of the hydrophobic prenyl group to the membrane targeting of these proteins (2, 3, 58, 59). Additionally, as noted in Section II, the abolition of the transforming capacity of oncogenic ras proteins by blocking their ability to become prenylated has highlighted the importance of prenylation in the biological activity of this family of proteins.

A potentially important property of the nonprocessed ras mutants is that, although they are unable to induce cellular transformation, they can apparently inhibit this activity of processed, membrane-associated, oncogenic ras proteins (60). This implies that some of the activities of the nonprocessed oncogenic mutants are conserved (e.g., binding to regulatory and/or effector molecules of ras proteins). Since the nonprocessed normal ras proteins apparently do not exhibit the inhibitory property of the nonprocessed mutant proteins, specific inhibition of ras function in tumor cells containing an activated *ras* on-

cogene by suppression of PFT activity may be a viable approach to chemotherapy of these tumors (33).

As noted in Section IV, yeast genetics is also proving quite useful for analyzing the role of prenylation in the biological activities of modified proteins. The approaches have included both disruption of the protein prenyltransferase genes, resulting in elimination of the ability of the organism to perform that particular modification, and mutagenesis of the prenylation site of specific prenylated proteins, similar to that described above for studies in mammalian cells. These studies have provided information on the involvement of prenylated proteins in the mating activity of *S. cerevisiae*, both at the level of the activity of the mating factor peptides and in the G protein-dependent signalling pathways involved in this process (61), and also in the secretory pathway of this organism (56, 61). One somewhat surprising result of these studies was that yeast completely lacking the *RAM1* gene product, which comprises a subunit of this organisms' PFT, are still viable even though preventing the farnesylation of the ras protein in this organism (by COOH-terminal truncation or Cys-to-Ser mutation) abolished growth (62). A possible explanation is that, in the *ram1*<sup>-</sup> mutants, enough of the normally farnesylated proteins can become modified by the organisms' PGGT to ensure survival. In support of this hypothesis, the PGGT enzyme can be observed under certain conditions (e.g., high protein substrate) to prenylate proteins normally modified by the PFT (21). Disruption of the *RAM2* gene in yeast, though, abolishes the activities of both the PFT and PGGT (see Section IV), and is lethal (50).

Results from direct biochemical analysis of the functional effects of isoprenoid addition to proteins are still sparse. However, the rapid progress in identifying the cellular machinery involved in the modifications (e.g., isolation of protein prenyltransferases) is allowing development of in vitro methods to expand these studies (see below). Still, some data are currently available in this regard. Competition studies using peptides corresponding to the COOH-terminus of the GTP-binding protein rap1A have indicated that prenylation is required for the interaction of rap1A with a specific protein that stimulates GDP dissociation from this GTP-binding protein (63). A similar approach to the analysis of the influence of prenylation and COOH-methylation on the properties of the small G protein rab3A indicates that the isoprenoid, but not the methyl group, is essential for this protein's interaction with an inhibitory exchange protein (64). Additionally, prenylation of the  $\gamma$  subunit of the retinal GTP-binding protein, transducin, has been reported to be required for efficient signal transduction by this protein, although in this case it was not completely clear that the effects observed were due solely to prenylation-dependent processing of the protein (65).



## VI. FUTURE DIRECTIONS

Many questions remain to be addressed both on the molecular mechanisms of protein prenylation and the functional properties bestowed on proteins by isoprenoid addition (and, where applicable, the subsequent processing steps of proteolysis and methylation). Much of the current effort is centered on analysis of the protein prenyltransferases since these enzymes, in particular the PFT, are logical targets for pharmacological intervention strategies designed to suppress prenyl protein (i.e., oncogenic ras) activity. A key goal here will be to develop subtype-specific inhibitors, particularly cell-permeable forms. Invaluable in the design of such inhibitors will be the acquisition of more detailed mechanistic information on the various protein prenyltransferases (see Section IV). Expression of cDNAs encoding these enzymes should provide systems to produce sufficient amounts of protein for both detailed biochemical analysis as well as the development of model systems for examining more detailed biological questions. Obtaining structural information, particularly on the active sites of these enzymes, will be especially useful in the mechanistic studies. The role of Zn<sup>2+</sup> that apparently occupies a high affinity site in these enzymes will also be important to define. Much insight into this problem will probably come from intensively studied Zn-metalloenzymes such as carboxypeptidase A, where decades of effort have yielded a wealth of information on the role of zinc in enzyme catalysis (66).

The contribution of the isoprenoid to the activities of prenylated proteins should also be a fruitful area of investigation. In particular, the extent to which the attached lipid affects protein-protein as well as protein-membrane interactions needs to be further defined for these modified proteins. The types of competition studies utilizing prenylated peptides described in Section V are proving useful in this regard. Additionally, the ability to produce unmodified proteins by bacterial expression of cDNAs and then modify them *in vitro* using the purified protein prenyltransferases will allow direct examination of the properties conferred on proteins by these modifications.

Another important question that remains to be addressed is the presumed role that prenylation plays in targeting modified proteins to specific cellular membranes. Some prenylated proteins such as ras and the heterotrimeric GTP-binding proteins are specifically localized to the plasma membrane, while others such as the rab/YPT1 proteins are directed to specific intracellular membranes. Since only two isoprenoid modifications, farnesyl and geranylgeranyl, have been found on these proteins, there must be additional determinants inherent in the protein that also contribute to specific membrane localization. At least two possibilities for specific targeting can be envisaged. In the first, specific receptors would be found on target membranes that would recognize not only the iso-

prenoid modification of their specific protein ligand but also some other, more unique, region of the protein. Such a targeting pathway has been proposed to function in the localization of a fatty acylated protein, myristoyl-src, to the plasma membrane of cells (67). The second possibility is that prenylated proteins are recognized by specific soluble "chaperone" proteins, and it is this complex that is directed to a specific membrane. Here again, the target membrane must contain a component that specifically recognizes the complex, although not necessarily the prenyl protein itself. A related problem here is how to achieve reversible association of prenylated proteins with membranes, as the lipid modification is thought to strongly promote interaction of the protein with the membrane once the modified protein gets there. At least in the case of some of the rab/YPT1 proteins, a reversible association with membranes has been detected and is thought to play a crucial role in membrane trafficking pathways (25). To date there is no evidence that prenylation is reversible; pulse-chase studies have indicated that the covalently attached isoprenoids are stable modifications of proteins (16). Thus, some other mechanism needs to be invoked. One possibility is that of phosphorylation near the site of the prenylated cysteine residue, as studies on prenylated small G proteins have shown that this process can result in a decreased association of the protein with both membranes and phospholipid vesicles (68, 69). Additionally, the binding of the small G protein known as smg p25A/rab3A to membranes can be reduced by interaction with an identified inhibitory protein (26), so protein-protein interactions may be important in this process also.

Similar questions concerning the additional modifications of COOH-terminal proteolysis and methylation that occur on many proteins after isoprenoid attachment are also important to address. These modifications probably contribute significantly to the biological activity of prenylated proteins. As prenylation precedes these modifications, they have been more difficult to approach experimentally. However, the ability to produce specific prenylated proteins and peptides is now providing the reagents necessary for more detailed studies of these closely linked modifications. Enzymatic activities for both of these steps have been identified in cell extracts, but neither has been isolated. Two intriguing points have come from these studies. The first is that both processing steps apparently occur in the microsomal membrane fraction (70-73), as opposed to in the cytosolic fraction for prenylation, and the second is that specificity and genetic studies on the methyltransferase suggest there is a single enzyme for modification of both farnesylated and geranylgeranylated proteins (74, 75). If this is the case, and the same holds for the protease, it would suggest that both classes of prenylated proteins traffic through the same membrane compartment in the cell on the way to their final destination. Thus, targeting of fully processed

prenylated proteins may be a problem of vesicular trafficking rather than the trafficking of a single polypeptide or a protein complex. It is possible that the trafficking of this class of lipid-modified proteins is much more like the classical Golgi-endoplasmic reticulum trafficking of membrane proteins than previously thought. Unraveling these pathways and the molecular details of protein prenylation should provide new insight into both protein-membrane association and the regulation of the activities of many crucial proteins involved in cellular signalling pathways. ■

Thanks are due Dan Herschlag and Julia Thissen for critical comments on this manuscript. Work from the author's laboratory was supported by the research grants from the National Science Foundation and the American Cancer Society and an Established Investigator Award from the American Heart Association.

Manuscript received 19 June 1992.

## REFERENCES

- Maltese, W. A. 1990. Posttranslational modification of proteins by isoprenoids in mammalian cells. *FASEB J.* **4**: 3319-3328.
- Glomset, J., M. Gelb, and C. Farnsworth. 1991. The prenylation of proteins. *Curr. Opin. Lipidol.* **2**: 118-124.
- Der, C. J., and A. D. Cox. 1991. Isoprenoid modification and plasma membrane association: Critical factors for ras oncogenicity. *Cancer Cells.* **3**: 331-340.
- Kamiya, Y., A. Sakurai, S. Tamura, and N. Takahashi. 1978. Structure of rhodotorucine A, a novel lipopeptide, inducing mating tube formation in *Rhodospiridium toruloides*. *Biochem. Biophys. Res. Commun.* **83**: 1077-1083.
- Ishibashi, Y., Y. Sakagami, A. Isogai, and A. Suzuki. 1984. Structures of tremogens A-9291-I and A-9291-VII: peptidyl sex hormones of *Tremella brasiliensis*. *Biochemistry.* **23**: 1399-1404.
- Brown, M. S., and J. L. Goldstein. 1980. Multivalent feedback regulation of HMG-CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. *J. Lipid Res.* **21**: 505-517.
- Maltese, W. A., and K. M. Sheridan. 1987. Isoprenylated proteins in cultured cells: subcellular distribution and changes related to altered morphology and growth arrest induced by mevalonate deprivation. *J. Cell. Physiol.* **133**: 471-481.
- Schmidt, R. A., C. J. Schneider, and J. A. Glomset. 1984. Evidence for post-translational incorporation of a product of mevalonic acid into Swiss 3T3 cell proteins. *J. Biol. Chem.* **259**: 10175-10180.
- Faust, J., and M. Krieger. 1987. Expression of specific high capacity mevalonate transport in a Chinese hamster ovary cell variant. *J. Biol. Chem.* **262**: 1996-2004.
- Sinensky, M., and J. Logel. 1985. Defective macromolecule biosynthesis and cell-cycle progression in a mammalian cell starved for mevalonate. *Proc. Natl. Acad. Sci. USA.* **82**: 3257-3261.
- Wolda, S. L., and J. A. Glomset. 1988. Evidence for modification of lamin B by a product of mevalonic acid. *J. Biol. Chem.* **263**: 5997-6000.
- Beck, L. A., T. J. Hosick, and M. Sinensky. 1988. Incorporation of a product of mevalonic acid metabolism into proteins of Chinese hamster ovary cell nuclei. *J. Cell. Biol.* **107**: 1307-1316.
- Anderegg, R. J., R. Betz, S. A. Carr, J. W. Crabb, and W. Duntze. 1988. Structure of *Saccharomyces cerevisiae* mating hormone a-factor. *J. Biol. Chem.* **263**: 18236-18240.
- Treston, A. M., and J. L. Mulshine. 1989. Beyond transcriptional events. *Nature.* **337**: 406.
- Hancock, J. F., A. I. Magee, J. E. Childs, and C. J. Marshall. 1989. All ras proteins are polyisoprenylated but only some are palmitoylated. *Cell.* **57**: 1167-1177.
- Casey, P. J., P. A., Solski, C. J. Der, and J. E. Buss. 1989. p21ras is modified by a farnesyl isoprenoid. *Proc. Natl. Acad. Sci. USA.* **86**: 8323-8327.
- Schafer, W. R., R. Kim, R. Sterne, J. Thorner, S-H. Kim, and J. Rine. 1989. Genetic and pharmacological suppression of oncogenic mutations in RAS genes of yeast and humans. *Science.* **245**: 379-385.
- Stimmel, J. B., R. J. Deschenes, C. Volker, J. Stock, and S. Clarke. 1990. Evidence for an S-farnesylcysteine methyl ester at the carboxyl terminus of the *Saccharomyces cerevisiae* RAS2 protein. *Biochemistry.* **29**: 9651-9659.
- Farnsworth, C. C., M. H. Gelb, and J. A. Glomset. 1990. Identification of geranylgeranyl-modified proteins in HeLa cells. *Science.* **247**: 320-322.
- Rilling, H. C., E. Breunger, W. W. Epstein, and P. F. Crain. 1990. Prenylated proteins: the structure of the isoprenoid group. *Science.* **247**: 318-320.
- Casey, P. J., J. A. Thissen, and J. F. Moomaw. 1991. Enzymatic modification of proteins with a geranylgeranyl isoprenoid. *Proc. Natl. Acad. Sci. USA.* **88**: 8631-8635.
- Gutierrez, L., A. I. Magee, C. J. Marshall, and J. F. Hancock. 1989. Post-translational processing of p21ras is two-step and involves carboxyl-methylation and carboxyl-terminal proteolysis. *EMBO J.* **8**: 1093-1098.
- Kinsella, B. T., and W. A. Maltese. 1991. Rab GTP-binding proteins implicated in vesicular transport are isoprenylated in vitro at cysteines within a novel carboxyl-terminal motif. *J. Biol. Chem.* **266**: 8540-8544.
- Farnsworth, C. C., M. Kawata, Y. Yoshida, Y. Takai, M. H. Gelb, and J. A. Glomset. 1991. C-terminus of the small GTP-binding protein smg p25A contains two geranylgeranylated cysteine residues and a methyl ester. *Proc. Natl. Acad. Sci. USA.* **88**: 6196-6200.
- Fischer von Mollard, G., T. C. Sudhof, and R. Jahn. 1991. A small GTP-binding protein dissociates from synaptic vesicles during exocytosis. *Nature.* **349**: 79-81.
- Araki, S., A. Kikuchi, Y. Hata, M. Iosomura, and Y. Takai. 1990. Regulation of reversible binding of smg p25A, a ras p21-like GTP-binding protein, to synaptic plasma membranes and vesicles by its specific regulatory protein, GDP dissociation inhibitor. *J. Biol. Chem.* **265**: 13007-13015.
- Goldstein, J. L., and M. S. Brown. 1990. Regulation of the mevalonate pathway. *Nature.* **343**: 425-434.
- Reiss, Y., J. L. Goldstein, M. C. Seabra, P. J. Casey, and M. S. Brown. 1990. Inhibition of purified p21ras farnesyl:protein transferase by Cys-AAX tetrapeptides. *Cell.* **62**: 81-88.
- Schaber, M. D., M. B. O'Hara, V. M. Garsky, S. D. Mosser, J. D. Bergstrom, S. L. Moores, M. S. Marshall, P. A. Friedman, R. A. F. Dixon, and J. B. Gibbs. 1990. Polyisoprenylation of ras in vitro by a farnesyl-protein transferase. *J. Biol. Chem.* **265**: 14701-14704.
- Manne, V., D. Roberts, A. Tobin, E. O'Rourke, M. De Virgilio, C. Meyers, N. Ahmed, B. Kurz, M. Resh, H-F.



- Kung, and M. Barbacid. 1990. Identification and preliminary characterization of protein-cysteine farnesyltransferase. *Proc. Natl. Acad. Sci. USA*. **87**: 7541-7545.
31. Reiss, Y., S. J., Stradley, L. M. Gierasch, M. S. Brown, and J. L. Goldstein. 1991. Sequence requirements for peptide recognition by rat brain p21ras farnesyl:protein transferase. *Proc. Natl. Acad. Sci. USA*. **88**: 732-736.
32. Goldstein, J. L., M. S. Brown, S. J. Stradley, Y. Reiss, and L. M. Gierasch. 1991. Nonfarnesylated tetrapeptide inhibitors of protein farnesyltransferase. *J. Biol. Chem.* **266**: 15575-15578.
33. Gibbs, J. B. 1991. Ras C-terminal processing enzymes—new drug targets? *Cell*. **65**: 1-4.
34. Glover, J. 1990. A biochemical connection between ras, yeast, and cholesterol synthesis. *J. Natl. Cancer Inst.* **82**: 1383-1387.
35. Yokoyama, K., G. W., Goodwin, F. Ghomashchi, J. A. Glomset, and M. H. Gelb. 1991. A protein geranylgeranyltransferase from bovine brain: implications for protein prenylation specificity. *Proc. Natl. Acad. Sci. USA*. **88**: 5302-5306.
36. Joly, A., G. Popjak, and P. A. Edwards. 1991. In vitro identification of a soluble protein-geranylgeranyl transferase from rat tissues. *J. Biol. Chem.* **266**: 13495-13498.
37. Moomaw, J. F., and P. J. Casey. 1992. Mammalian protein geranylgeranyltransferase: subunit composition and metal requirements. *J. Biol. Chem.* **267**: 17438-17443.
38. Seabra, M. C., Y. Reiss, P. J. Casey, M. S. Brown, and J. L. Goldstein. 1991. Protein farnesyltransferase and geranylgeranyltransferase share a common alpha subunit. *Cell*. **65**: 429-434.
39. Reiss, Y., M. C. Seabra, S. A. Armstrong, C. A. Slaughter, J. L. Goldstein, and M. S. Brown. 1991. Nonidentical subunits of p21 H-ras farnesyltransferase: peptide binding and farnesyl pyrophosphate carrier functions. *J. Biol. Chem.* **266**: 10672-10677.
40. Reiss, Y., M. S. Brown, and J. L. Goldstein. 1992. Divalent cation and prenyl pyrophosphate specificities of the protein farnesyltransferase from rat brain, a zinc metalloenzyme. *J. Biol. Chem.* **267**: 6403-6408.
41. Spiro, T. G. 1983. Zinc Enzymes. Wiley-Interscience, New York.
42. Pompliano, D. L., E. Rands, M. D. Schaber, S. D. Mosser, N. J. Anthony, and J. B. Gibbs. 1992. Steady-state kinetic mechanism of ras farnesyl:protein transferase. *Biochemistry*. **31**: 3800-3807.
43. Reardon, J. E., and R. H. Abeles. 1986. Mechanism of action of isopentenyl pyrophosphate isomerase: evidence for a carbonium ion intermediate. *Biochemistry*. **25**: 5609-5616.
44. Poulter, C. D., T. L. Capson, T. D. Thompson, and R. S. Bard. 1989. Squalene synthetase. Inhibition by ammonium analogues of carbocationic intermediates in the conversion of presqualene diphosphate to squalene. *J. Am. Chem. Soc.* **111**: 3734-3739.
45. Powers, S., S. Michaelis, D. Broek, S. Santa-Anna, J. Field, I. Herskowitz, and M. Wigler. 1986. RAM, a gene of yeast required for a functional modification of RAS proteins and for production of mating pheromone a-factor. *Cell*. **47**: 413-422.
46. Fujiyama, A., K. Matsumoto, and F. Tamanoi. 1987. A novel yeast mutant defective in the processing of ras proteins: assessment of the effect of the mutation on processing steps. *EMBO J.* **6**: 223-228.
47. Schafer, W. R., C. E. Trueblood, C-C. Yang, M. P. Mayer, S. Rosenberg, C. D. Poulter, S-H. Kim, and J. Rine. 1990. Enzymatic coupling of cholesterol intermediates to a mating pheromone precursor and to the ras protein. *Science*. **249**: 1133-1139.
48. Chen, W., D. A. Andres, J. L. Goldstein, D. W. Russell, and M. S. Brown. 1991. cDNA cloning and expression of the peptide binding beta subunit of rat p21ras farnesyltransferase, the counterpart of yeast RAM1/DPR1. *Cell*. **66**: 327-334.
49. Goodman, L. E., S. R. Judd, C. C. Farnsworth, S. Powers, M. H. Gelb, J. A. Glomset, and F. Tamanoi. 1990. Mutants of *Saccharomyces cerevisiae* defective in the farnesylation of ras proteins. *Proc. Natl. Acad. Sci. USA*. **87**: 9665-9669.
50. He, B., P. Chen, S. Chen, K. L. Vancura, S. Michaelis, and S. Powers. 1991. RAM2, an essential gene in yeast, and RAM1 encode the two polypeptide components of the farnesyltransferase that prenylates a-factor and ras proteins. *Proc. Natl. Acad. Sci. USA*. **88**: 11373-11377.
51. Kohl, N. E., R. E. Diehl, M. D. Schaber, E. Rands, D. D. Soderman, B. He, S. L. Moores, D. L. Pompliano, S. Ferro-Novick, S. Powers, K. A. Thomas, and J. B. Gibbs. 1991. Structural homology among mammalian and *Saccharomyces cerevisiae* isoprenyl-protein transferases. *J. Biol. Chem.* **266**: 18884-18888.
52. Chen, W-J., D. A. Andres, J. L. Goldstein, and M. S. Brown. 1991. Cloning and expression of a cDNA encoding the alpha subunit of rat p21ras protein farnesyltransferase. *Proc. Natl. Acad. Sci. USA*. **88**: 11368-11372.
53. Finegold, A. A., D. I. Johnson, C. C. Farnsworth, M. H. Gelb, S. R. Judd, J. A. Glomset, and F. Tamanoi. 1991. Protein geranylgeranyltransferase of *Saccharomyces cerevisiae* is specific for Cys-Xaa-Xaa-Leu motif proteins and requires the CDC43 gene product but not the DPR1 gene product. *Proc. Natl. Acad. Sci. USA*. **88**: 4448-4452.
54. Moores, S. L., M. D. Schaber, S. D. Mosser, E. Rands, M. B. O'Hara, V. M. Garsky, M. S. Marshall, D. L. Pompliano, and J. B. Gibbs. 1991. Sequence dependence of protein isoprenylation. *J. Biol. Chem.* **266**: 14603-14610.
55. Horiuchi, H., M. Kawata, M. Katayama, Y. Yoshida, T. Musha, S. Ando, and Y. Takai. 1991. A novel prenyltransferase for a small GTP-binding protein having a C-terminal Cys-Ala-Cys structure. *J. Biol. Chem.* **266**: 16981-16984.
56. Rossi, G., Y. Jiang, A. P. Newman, and S. Ferro-Novick. 1991. Dependence of Ypt1 and Sec4 membrane attachment on Bet2. *Nature*. **351**: 158-161.
57. Jackson, J. H., C. G. Cochrane, J. R. Bourne, P. A. Solski, J. E. Buss, and C. J. Der. 1990. Farnesol modification of Kirsten-ras exon 4B protein is essential for transformation. *Proc. Natl. Acad. Sci. USA*. **87**: 3042-3046.
58. Holtz, D., R. A. Tanaka, J. Hartwig, and F. McKeon. 1989. The CaaX motif of lamin A functions in conjunction with the nuclear localization signal to target assembly to the nuclear envelope. *Cell*. **59**: 969-977.
59. Inglese, J., J. F. Glickman, W. Lorenz, M. Caron, and R. J. Lefkowitz. 1992. Isoprenylation of a protein kinase: requirement of farnesylation/alpha-carboxyl methylation for full enzymatic activity of rhodopsin kinase. *J. Biol. Chem.* **267**: 1422-1425.
60. Gibbs, J. B., M. D. Schaber, T. L. Schofield, E. M. Scolnick, and I. S. Sigal. 1989. Xenopus oocyte germinal-vesicle breakdown induced by [Val-12]ras is inhibited by a cytosol-localized ras mutant. *Proc. Natl. Acad. Sci. USA*. **86**: 6630-6634.
61. Schafer, W. R., and J. Rine. 1992. Protein prenylation: genes, enzymes, targets and function. *Annu. Rev. Genet.* **25**: In press.
62. Deschenes, R. J., and J. R. Broach. 1987. Fatty acylation is important but not essential for *Saccharomyces cerevisiae* RAS

- function. *Mol. Cell. Biol.* **7**: 2344-2351.
63. Shirataki, H., K. Kailbuchi, M. Hiroyoshi, M. Isomura, S. Araki, T. Sasaki, and Y. Takai. 1991. Inhibition of the action of the stimulatory GDP/GTP exchange protein for smg p21 by the geranylgeranylated synthetic peptides designed from its C-terminal region. *J. Biol. Chem.* **266**: 20672-20677.
  64. Musha, T., M. Kawata, and Y. Takai. 1992. The geranylgeranyl moiety but not the methyl moiety of the smg-25A/rab3A protein is essential for interactions with membrane and its inhibitory GDP/GTP exchange protein. *J. Biol. Chem.* **267**: 9821-9825.
  65. Fukada, Y., T. Takao, H. Ohguro, T. Yoshizawa, T. Akino, and Y. Shimonishi. 1990. Farnesylated gamma subunit of photoreceptor G protein indispensable for GTP-binding. *Nature.* **346**: 658-660.
  66. Vallee, B. L., A. Galdes, D. S. Auld, and J. F. Riordan. 1983. Carboxypeptidase A. In *Zinc Enzymes*. T. G. Spiro, editor. Wiley-Interscience, New York. 25-76.
  67. Resh, M. D. 1990. Membrane interactions of pp60v-src: a model for myristylated tyrosine protein kinases. *Oncogene.* **5**: 1437-1444.
  68. Hata, Y., K. Kaibuchi, S. Kawamura, M. Hiroyoshi, H. Shirataki, and Y. Takai. 1991. Enhancement of the actions of smg p21 GDP/GTP exchange protein by the protein kinase A-catalyzed phosphorylation of smg p21. *J. Biol. Chem.* **266**: 6571-6577.
  69. Lapetina, E. G., J. C. Lacal, B. R. Reep, and L. Molina-y-Vedia. 1989. A ras-related protein. *Proc. Natl. Acad. Sci. USA.* **86**: 3131-3134.
  70. Stephenson, R. C., and S. Clarke. 1990. Identification of a C-terminal protein carboxyl methyltransferase in rat liver membranes utilizing a synthetic farnesyl cysteine-containing peptide substrate. *J. Biol. Chem.* **265**: 16248-16254.
  71. Tan, E. W., D. Perez-Sala, F. J. Canada, and R. R. Rando. 1991. Identifying the recognition unit for G protein methylation. *J. Biol. Chem.* **266**: 10719-10722.
  72. Ashby, M. N., D. S. King, and J. Rine. 1992. Endoproteolytic processing of a farnesylated peptide in vitro. *Proc. Natl. Acad. Sci. USA.* **89**: 4613-4617.
  73. Hrycyna, C. H., and S. Clarke. 1992. Maturation of isoprenylated proteins in *Saccharomyces cerevisiae*: multiple activities catalyze the cleavage of the three carboxyl-terminal amino acids from farnesylated substrates in vitro. *J. Biol. Chem.* **267**: 10457-10464.
  74. Hrycyna, C. A., S. K. Sapperstein, S. Clarke, and S. Michaelis. 1991. The *Saccharomyces cerevisiae* STE14 gene encodes a methyltransferase that mediates C-terminal methylation of a-factor and ras proteins. *EMBO J.* **10**: 1699-1709.
  75. Volker, C., P. Lane, C. Kwee, M. Johnson, and J. Stock. 1991. A single activity carboxyl methylates both farnesyl and geranylgeranyl cysteine residues. *FEBS Lett.* **295**: 189-194.