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Research Article

Enzymatic Coupling of Cholesterol Intermediates to a Mating Pheromone Precursor and to the Ras Protein

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The post-translational processing of the yeast a-mating pheromone precursor, Ras proteins, nuclear lamins, and some subunits of trimeric G proteins requires a set of complex modifications at their carboxyl termini. This processing includes three steps: prenylation of a cysteine residue, proteolytic processing, and carboxymethylation. In the yeast *Saccharomyces cerevisiae*, the product of the *DPR1-RAM1* gene participates in this type of processing. Through the use of an in vitro assay with peptide substrates modeled after a presumptive a-mating pheromone precursor, it was discovered that mutations in *DPR1-RAM1* cause a defect in the prenylation reaction. It was further shown that *DPR1-RAM1* encodes an essential and limiting component of a protein prenyltransferase. These studies also implied a fixed order of the three processing steps shared by prenylated proteins: prenylation, proteolysis, then carboxymethylation. Because the yeast protein prenyltransferase could also prenylate human H-ras p21 precursor, the human DPR1-RAM1 analogue may be a useful target for anticancer chemotherapy.

ISOPRENOIDS ARE A CLASS OF STRUCTURALLY RELATED LIPOphilic molecules that perform a wide variety of essential cellular functions. Isoprenoid lipids include such functionally diverse molecules as cholesterol, ubiquinone, dolichols, and chlorophyll, yet all isoprenoids are derived from a common precursor, mevalonic acid. Polyisoprenoid molecules are attached post-translationally to a small class of eukaryotic proteins, which includes nuclear lamins (*1*), trimeric G proteins (*2*), lipopeptide pheromones (*3*), and the Ras family of oncoproteins (*4–6*). The biological activities of several of these proteins require association with the inner surface of the plasma membrane, and this membrane localization is dependent on the post-translational attachment of the polyisoprenoid lipid residue to the COOH-terminus of the protein. This type of protein modification is referred to as protein prenylation. In the case of the *Saccharomyces cerevisiae* **a** mating pheromone (**a**-factor), prenylation is necessary for secretion and biological activity (*6*). Similarly, oncogenic Ras proteins require prenylation for both membrane association and transforming activity (*4–6*). Protein prenylation is a stable and irreversible protein modification that plays a critical role in directing the modified protein to the plasma membrane (*5*).

The mechanism of protein prenylation has recently become of interest because of both the wide range of proteins that undergo this modification and the ability of inhibitors of prenylation to suppress some phenotypes of oncogenic Ras proteins (*7*). Information on protein prenylation comes primarily from studies of the processing of yeast and human Ras proteins, nuclear lamins, and yeast **a**-factor. Analysis of the structure of the modified COOH-termini of Ras protein and **a**-factor revealed at least three chemical modifications that occur post-translationally. These include (i) attachment of an isoprene moiety to a cysteine residue near the COOH-terminus through a thioether linkage, (ii) proteolytic removal of the three amino acids distal to that cysteine, and (iii) formation of a methyl ester at the new COOH-terminus (*3–5, 8, 9*). Secreted **a**-factor and nuclear lamin B contain a farnesyl (C_{15}-lipid) group (*1, 3*), whereas the precise identity of the isoprene group attached to Ras proteins has not been fully resolved (*4, 5*). In some but not all Ras proteins, the COOH-terminus is further modified by the addition of a

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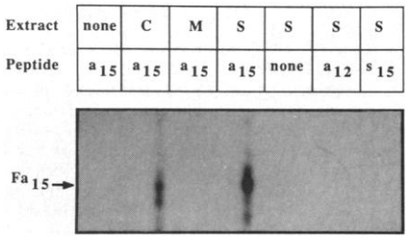


Fig. 1. Protein prenyltransferase activity in vitro. Prenylation reaction mixtures contained a-factor peptide, [^{14}C]IPP, and yeast protein extract as described (*31*), and reaction products were evaluated by TLC. The extract was prepared from *S. cerevisiae* strain JRY1591 (*MATα ade2-101 met his3Δ200 lys2-801 ura3-52*). Crude extracts (C) were prepared as described (*32*) and separated into membrane (M) and soluble (S) fractions by centrifugation for 30 minutes at 100,000*g*. The peptide a_{15} (sequence: YIIKGVFWDPACVIA) has the sequence of mature a-factor with the three amino acid COOH-terminal extension found in the primary translation product. The peptide s_{15} (YIIKGVFWDPASVIA) contains a substitution of serine for cysteine at the presumptive site of prenylation, and the peptide a_{12} (YIIKGVFWDPAC) has the sequence of mature a-factor. Abbreviations for 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. Peptide sequences read from the NH_2-terminus to the COOH-terminus. Fa_{15} indicates the mobility of a farnesylated 15–amino acid standard detected by ninhydrin staining.

palmitoyl moiety through a thioester linkage at a neighboring cysteine subsequent to the first three processing steps (*4*). The exact order of the first three steps is unclear, but in the cases of both Ras protein and a-factor, the prenylation step is essential for membrane targeting and secretion, respectively, and for biological activity (*4–6*).

Some information on prenyltransferases is available from studies of farnesyl diphosphate (FPP) biosynthesis (*10*), ubiquinone biosynthesis (*11*), rubber biosynthesis (*12*), and prenylation of tRNA's (*13*). The FPP synthetase, a prenyltransferase, functions as a homodimer adding isopentenyl diphosphate (IPP) subunits in trans configuration to dimethylallyl diphosphate (DMAPP). In contrast, the synthesis of ubiquinone requires the action of hexaprenyl diphosphate synthetase (HDS), which is composed of two components that must assemble to form an active enzyme (*11*). Rubber biosynthesis requires the concerted action of two proteins: one protein binds to the rubber particle and serves to dock the second protein, FPP synthetase, the stereochemical specificity of which is reversed, leading to the synthesis of long isoprene polymers in the cis configuration. In this example, one polypeptide acts as a regulatory subunit, present in equal stoichiometry with the number of growing rubber molecules, that controls the enzymatic activity of the catalytic subunit (*12*). Taken together, however, these examples do not provide any obvious clue as to the expected structure or subunit composition of the putative protein prenyltransferase.

One candidate for a gene that might encode a component or a regulator of a protein prenyltransferase is the *S. cerevisiae* gene known variously as *DPR1*, *RAM1*, *STE16*, and *SCG2*. Mutants defective in this gene were identified independently on the basis of several different phenotypes (*14*). We refer to the gene as *DPR1-RAM1* and to the protein encoded by this gene as DPR1-RAM1. Loss of *DPR1-RAM1* function blocks deleterious phenotypes caused by the activated *RAS2* gene in yeast. The majority of the Ras2 protein in *dpr1-ram1* mutant extracts is present in the cytoplasm, rather than in the membrane fraction. Also, *dpr1-ram1* mutants of the a mating type are mating-defective because they fail to secrete a-factor, a farnesylated dodecapeptide required for mating. Furthermore, *dpr1-ram1* mutants are defective in the processing of the *STE18*-encoded G protein γ subunit, which is apparently prenylated (*2*). The *DPR1-RAM1* gene has been cloned by functional complementation of the *dpr1-ram1* mutant phenotype, and the predicted sequence of the gene product did not show any homology to previously identified proteins of known function (*15*).

A protein prenyltransferase activity in yeast cells. To identify a yeast protein prenyltransferase, we developed an in vitro assay for this enzymatic activity in yeast extracts. The substrate used for this assay was a 15–amino acid peptide that is a presumptive precursor of mature a-factor lipopeptide (*3*). Prenylation, proteolysis, and carboxymethylation of this peptide would result in a product identical to mature a-factor. The prenyl donor was [^{14}C]IPP, which is a precursor of all isoprene compounds. The peptide and [^{14}C]IPP were incubated together in a yeast extract, and the products were then separated by thin-layer chromatography (TLC) and detected by autoradiography. A synthetic peptide consisting of a farnesylated 15–amino acid peptide was used as a standard for the expected mobility of prenylated product.

In reactions with a crude yeast extract, a product was formed that comigrated with the farnesylated peptide standard. Synthesis of this product was dependent on the presence of the yeast extract, the peptide substrate, and labeled IPP (Fig. 1). Fractionation of the crude cell extract revealed that the protein prenyltransferase activity was present exclusively in the soluble, cytoplasmic fraction. This fraction also contained the IPP isomerase and FPP synthetase enzymatic activities, which together catalyze the synthesis of FPP and other isoprenoid derivatives from IPP (*16*) (Fig. 2). To characterize further the products of the in vitro reaction, we extracted the labeled peptides with chloroform and methanol, and analyzed them by high-performance liquid chromatography (HPLC) (Fig. 3). The HPLC conditions were chosen to separate farnesylated and nonfarnesylated 12– and 15–amino acid peptides, as well as with a *trans,trans*-geranylgeranylated 15–amino acid peptide. At least 8 minutes separated the retention times of any two of these species. The major labeled peptide from the extract eluted with the same retention time as the farnesylated 15–amino acid peptide

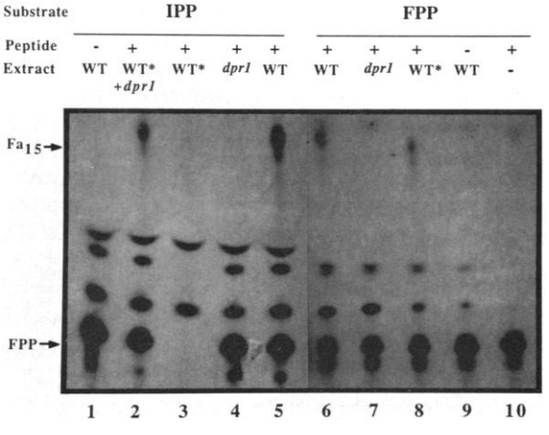
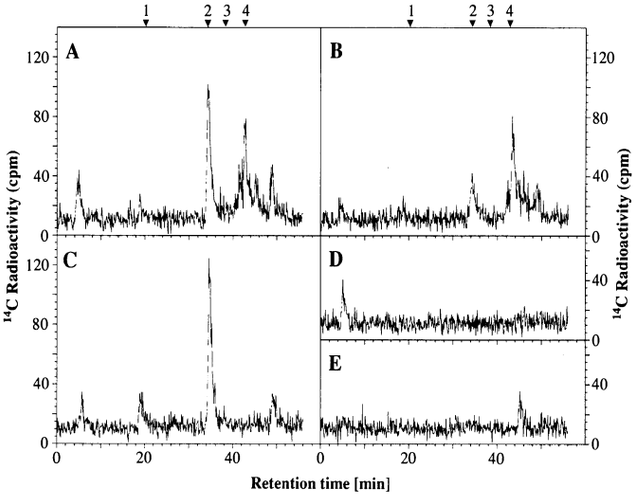


Fig. 2. In vitro labeling of peptide with IPP and FPP, and effect of IPP isomerase inhibitor on protein prenylation. In vitro reactions were performed as described (*31*) with [^{14}C]IPP or [^{3}H]FPP (25 μM, 37 mCi/mmol). Products were separated by TLC in solvent X (*n*-propanol:ammonia:water, 6:3:1). Reaction mixtures contained wild-type (WT) soluble extract prepared from JRY1591 (1 mg/ml), *dpr1* soluble extract from JRY2595 (*MATα ade2-101 met his3Δ200 lys2-801 ura3-52 dpr1::HIS3*) (*33*) (1.6 mg/ml), or WT soluble extract treated with 200 μ*M* 3,4-epoxy-1-butenyl diphosphate (EBPP) (*16*) for 20 minutes at room temperature (WT*). Reactions with FPP as the substrate were performed for 60 minutes. Of the reactions with IPP as the substrate, reactions 1, 3, and 5 were for 20 minutes; reaction 4 was for 30 minutes; and reaction 2 was incubated 10 minutes with the *dpr1::HIS3* extract and an additional 20 minutes after the addition of WT* extract. The products that migrated between the Fa_{15} standard and FPP standards were unidentified isoprenoid derivatives of IPP that were likely to include isopentenyl monophosphate, geranyl monophosphate, and farnesyl monophosphate (see also Fig. 5A).

Fig. 3. HPLC separation of the labeled compounds formed by incubation of extract from a DPR1-RAM1–overproducing strain (JRY2588) with [^{14}C]IPP and a-factor precursor peptide. Incubation conditions were as described (Fig. 1). Samples were extracted twice with chloroform. The chloroform and water phases were removed and the remaining protein interphase was extracted twice with methanol. The methanol extract was dried at reduced pressure, dissolved in acetonitrile:water:trifluoroacetic acid (TFA) (60:40:0.1), and applied to a Vydac C_{18} HPLC column (25 by 0.46 cm). Material was eluted with a linear gradient of 36 to 60 percent acetonitrile in water containing 0.1 percent TFA for 40 minutes, followed by isocratic elution with the 60:40:0.1 mixture. (**A** and **B**) Radioactivity traces of two independent incubations of yeast extract with [^{14}C]IPP and a-factor precursor peptide as substrates. (**C**) Same conditions as in (A) and (B), but no a-factor peptide added. (**D**) Same conditions as in (A) and (B), but no yeast extract added. (**E**) Same conditions as in (A) and (B), but yeast extract from *dpr1::HIS3* mutant used. Arrowheads indicate the retention times of authentic standards: 1, a_{15}; 2, farnesyl monophosphate; 3, Fa_{12}; and 4, Fa_{15}.



standard. Thus, a significant amount of the prenylated product of the in vitro reaction was, like secreted a-factor, modified by a farnesyl moiety. Because the major species of prenylated peptide was apparently not subjected to proteolysis, prenylation appears to occur before proteolysis in vitro.

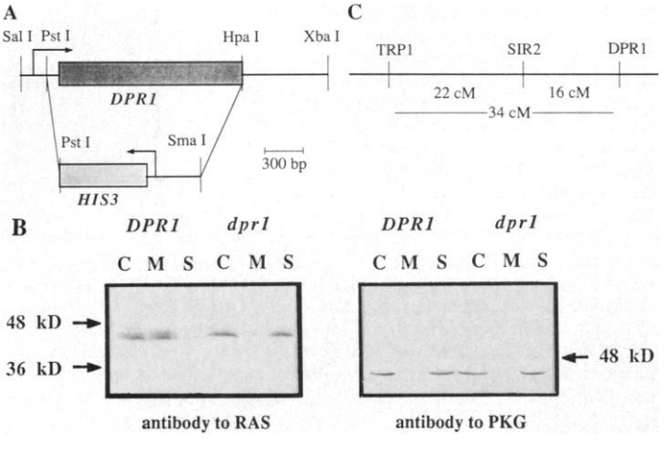
FPP could also act as a prenyl donor in the protein prenylation reaction. The use of [^{3}H]FPP in place of [^{14}C]IPP resulted in the production of a labeled peptide that comigrated with the farnesylated peptide standard (Fig. 2). The formation of this labeled peptide required the presence of both the peptide substrate and the yeast extract. Because FPP could be incorporated into the peptide product and the prenylated product resulting from reaction with IPP appeared to be farnesylated, FPP itself was probably the substrate for the prenylation reaction. However, because commercially available [^{14}C]IPP, when converted to FPP in the reaction, was of higher specific activity and therefore easier to detect than commercially available [^{3}H]FPP, [^{14}C]IPP was used as the lipid substrate in most of our experiments.

The specificity of the protein prenyltransferase activity was consistent with available information on protein prenylation in vivo (*1, 3, 4*). The protein prenyltransferase reaction required the cysteine residue near the COOH-terminus—replacement of this cysteine with a serine prevented the peptide from acting as a substrate in the reaction (Fig. 1). A 12–amino acid peptide lacking the last three amino acids of the a-factor precursor was also not a substrate for prenylation. This result, along with the HPLC analysis and results showing that carboxymethylation requires a farnesylated and proteolytically processed substrate (*17*), suggested that the order of the processing steps was prenylation, proteolysis, and methylation. By scanning the TLC plates it was possible to quantify the ^{14}C-labeled reaction product and to show that the assay was linear with respect to time and amount of extract (*18*).

A role for DPR1-RAM1 in determining the localization of Ras2 in vivo. In order to address the role of DPR1-RAM1 in protein prenylation, we constructed a yeast strain that did not express *DPR1-RAM1*. The deletion allele *dpr1::HIS3* (Fig. 4A), in which the entire *DPR1-RAM1* coding sequence (*15*) was removed and replaced by *HIS3*, with all flanking sequences being left intact, was substituted for one of the two wild-type alleles in a diploid yeast strain by one-step gene replacement (*19*). This diploid, containing both a mutant and a wild-type allele of *DPR1-RAM1*, was slightly defective for growth at high temperature. The diploid was sporulated, and meiotic progeny were isolated to determine the phenotype of haploid segregants lacking functional DPR1-RAM1. Because membrane-associated Ras is essential for cell division, we expected cells completely lacking DPR1-RAM1 to be inviable (*20*). However, *dpr1::HIS3* segregants were viable, but were temperature-sensitive for growth (*21*). Like strains containing a *dpr1* point mutation, *dpr1::HIS3 MAT*a strains were unable to mate.

Because *dpr1* mutations affect the localization of Ras2, we investigated the effect of the complete absence of DPR1-RAM1 on the cellular localization of Ras2. Protein extracts were prepared from isogenic *DPR1* and *dpr1::HIS3* strains carrying *RAS2* on a multicopy plasmid to facilitate detection of Ras2. Soluble and membrane fractions were prepared from the extracts and analyzed by immunoblotting with a monoclonal antibody, Y13-259, to Ras proteins (*22*). An antibody to the soluble enzyme phosphoglyceratekinase

Fig. 4. (**A**) Construction of the *DPR1-RAM1* deletion allele. A replacement of the entire *DPR1-RAM1* coding sequence with *HIS3* and subsequent disruption of the chromosomal *DPR1-RAM1* locus was performed as described (*33*). (**B**) Effect of *DPR1-RAM1* on localization of Ras2 in vivo. The subcellular localization of Ras2 and PGK was evaluated by immunoblotting in isogenic *DPR1-RAM1* and *dpr1::HIS3* strains carrying *RAS2* on a multicopy plasmid as described (*6*). The detection of PGK in the fractionated extracts served as a control to determine the amount of soluble protein contaminating the membrane fractions. C, M, and S refer to the crude extract, membrane fraction, and soluble fraction, respectively, as in Fig. 1. (**C**) Map position of *DPR1*. Genetic distances [in centiMorgans (cM)] were determined by a three-factor cross with mutations in the *DPR1*, *TRP1*, and *SIR2* genes. A total of 34 tetrads were analyzed. The tetrad ratios from which these map distances were derived were as follows: *SIR2-DPR1*, 19 parental ditypes (PD)::9 tetratypes (TT)::0 nonparental ditypes (NPD); *SIR2-TRP1*, 18 PD::14 TT::0 NPD; *TRP1-DPR1*, 9 PD::19 TT::0 NPD. The pattern of crossing over in the cross was consistent with the gene order indicated. All three genes are located on the left arm of yeast chromosome IV.



(PGK) was also used to confirm adequate cell fractionation (*23*). The Ras2 protein in wild-type yeast cells was found primarily in the membrane fraction (Fig. 4B). As expected, most of the Ras2 in the *dpr1::HIS3* strain was found in the cytoplasm, although a small but detectable amount was present in the membrane fraction (Fig. 4B). This result suggested that *DPR1-RAM1* function may not be essential for Ras protein localization at lower temperatures (23°C). Thus, whereas the DPR1-RAM1 protein facilitated membrane association of Ras2 protein, a small fraction of the Ras2 protein became membrane-associated in the complete absence of DPR1-RAM1 protein. This result may explain why the *DPR1-RAM1* deletion is only conditionally lethal. Evidence discussed below suggests the existence of other related enzymes that may be responsible for this low level of membrane association.

In the course of these studies, we determined the map position of *DPR1-RAM1* by a three-factor genetic cross. *DPR1-RAM1* maps approximately 30 map units from the centromere on the left arm of chromosome IV (Fig. 4C).

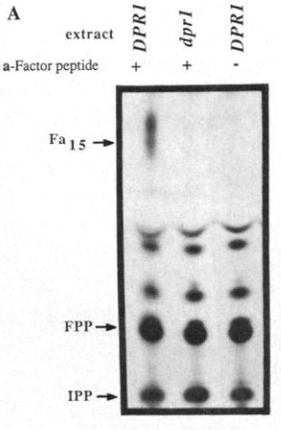
Protein prenyltransferase deficiency of *dpr1-ram1* mutants. The in vitro assay was used to determine whether DPR1-RAM1 was necessary for protein prenyltransferase activity. Protein extracts were prepared from a *dpr1::HIS3* strain, and protein prenyltransferase assays were conducted with the unmodified a-factor precursor as the substrate. These mutant extracts did not contain any detectable protein prenyltransferase activity (Fig. 5A). This deficiency was not due to an inability to convert IPP to FPP, because the amount of FPP generated by the *dpr1::HIS3* extract was equivalent to that generated by the wild-type extract. Extracts prepared from five other *dpr1-ram1* mutants were similarly assayed, and all displayed a defect in protein prenyltransferase activity.

To investigate whether DPR1-RAM1 was limiting for protein prenyltransferase activity in vitro, we prepared protein extracts from both wild-type cells and cells containing *DPR1-RAM1* on a multicopy plasmid. The specific activity of the protein prenyltransferase in the DPR1-RAM1 overexpressing strain was approximately four times higher than that in the wild type (Fig. 5B). The reactions were linear with respect to both time and amount of extract added, and the conversion of IPP to FPP was no greater in the DPR1-RAM1 overexpressor than in the wild-type strain. Thus, DPR1-RAM1 appeared to be both essential and limiting for protein prenyltransferase activity in vitro.

The *dpr1::HIS3* extract was used to explore the specificity of yeast protein prenyltransferase for its lipid substrate. These experiments made use of an irreversible inhibitor of IPP isomerase, 3,4-epoxy-1-butenyl diphosphate (EBPP) (*16*). The IPP isomerase is required for the conversion of the relatively unreactive IPP to the more reactive allylic isomer DMAPP. This conversion is also a necessary step for the production of longer allylic isoprenes such as geranyl diphosphate and FPP. A wild-type extract treated with EBPP was rendered almost completely inactive with respect to the prenylation of the a-factor precursor when IPP was used as the labeled substrate (Fig. 2) and was also unable to produce FPP. However, when FPP was used as the labeled substrate, EBPP treatment did not detectably inhibit peptide prenylation (Fig. 2).

In order to determine whether EBPP, an IPP analog, was directly inhibiting the addition of IPP to the peptide, we performed an in vitro complementation test by mixing the *dpr1::HIS3* strain extract and the EBPP-treated wild-type extract. As noted, the *dpr1::HIS3* cell extract was defective in prenylation of the peptide, but was able to synthesize FPP. When the extracts were mixed, efficient production of prenylated peptide product was observed in the presence of IPP. Thus, with the *dpr1::HIS3* extract generating allylic substrates, the EBPP-treated wild-type extract showed protein prenyltransferase activity (Fig. 2). Therefore, it appeared that IPP itself was not efficiently utilized by the protein prenyltransferase, and that an allylic polyisoprenoid, most likely FPP, was the preferred substrate for protein prenylation.

DPR1-RAM1, a component of a protein prenyltransferase. Because DPR1-RAM1 was essential for protein prenyltransferase activity in vitro, we investigated whether this protein was, in fact, a component of the protein prenyltransferase. An alternative possibility was that DPR1-RAM1 acts indirectly, such as by regulating the synthesis of the protein prenyltransferase. To distinguish between these possibilities, *DPR1-RAM1* was cloned into a bacterial expres-



B

Lane	Extract type	Protein (μg)	Reaction time (min)	Product (pmol)	Specific activity (pmol/min·mg)
1	WT	13.5	7.5	10.3	102
2	WT	13.5	22.5	37.4	123
3	WT	27.0	7.5	23.3	115
4	WT	27.0	22.5	59.9	99
					WT avg. = 110
5	pDPR1	13.5	7.5	38.4	379
6	pDPR1	13.5	22.5	140.4	462
7	pDPR1	27.0	7.5	71.3	352
8	pDPR1	27.0	22.5	212.2	349
					pDPR1 avg. = 386

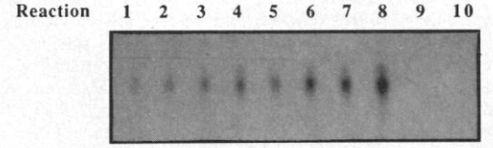


Fig. 5. (**A**) The DPR1-RAM1 protein is required for protein prenyltransferase activity. Protein prenyltransferase assays were performed as described (Fig. 1) except that solvent X was used to separate the reaction products on TLC plates. The *DPR1* soluble extract was prepared from JRY1591 and used at a final concentration of 1 mg/ml. The *dpr1* soluble extract was prepared from JRY2595 and used at a concentration of 1.6 mg/ml. Based on quantitation of product with a beta scanner, the specific activity of the wild-type extract is at least tenfold greater than that of the *dpr1* extract; based on autoradiography, which is more sensitive, the specific activity in the mutant extracts was found to be significantly lower than the limit of detection by beta scanning. (**B**) Comparison of calculated specific activities of isogenic wild-type (WT) and DPR1-RAM1–overproducing (pDPR1) strains. Soluble extracts prepared from wild-type (JRY2587: *MAT***a** *ade2-101 met his3Δ200 lys2-801 ura3-52* carrying the plasmid pSEY8) or DPR1-RAM1–overproducing [JRY2588:*MAT***a** *ade2-101 met his3Δ200 lys2-801 ura3-52* carrying pJR856, a multicopy plasmid consisting of a Sal I–Xba I fragment containing *DPR1* cloned into the polylinker of pSEY8 (*34*)] strains were assayed at two different concentrations and at two different time points as indicated. The prenylated product was detected and quantified with a beta scanner as the number of picomoles of IPP incorporated. An autoradiogram derived from this experiment is shown below. Reactions 9 and 10 were control experiments with either a wild-type or DPR1-RAM1–overproducing extract, respectively, without the peptide substrate. These control reactions contained 27.0 μg of yeast protein and were conducted for 30 minutes. Average (avg.) specific activity values for the two strains are shown.

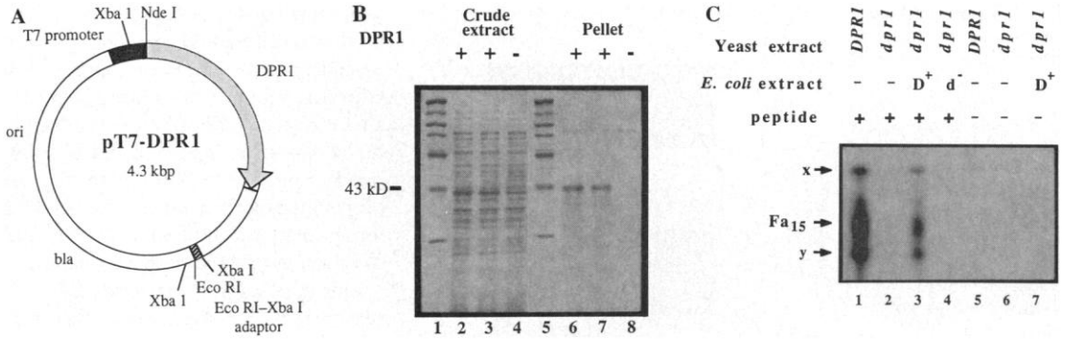


Fig. 6. (**A**) Construction of *E. coli* expression plasmid pT7-DPR1. The *DPR1-RAM1* coding sequence was placed downstream of a phage T7 promoter to allow high level production of DPR1-RAM1 in *E. coli*, as described (*35*); *ori* and *bla* refer to the Col E1 origin of replication and β-lactamose structural gene, respectively. (**B**) Purification of DPR1 after production in *E. coli*. Lanes 1 and 5, molecular mass standards (Bio-Rad): myosin (200 kD), β-galactosidase (116.3 kD), rabbit muscle phosphorylase b (97.4 kD), bovine serum albumin (66.2 kD), hen egg ovalbumin (42.7 kD), carbonic anhydrase (31 kD). The band below myosin is a degradation product of unknown molecular mass. Lanes 2 and 3, crude extracts from *E. coli* strain BL21(DE3) (*36*) transformed with pT7-DPR1; lane 4, crude extract from BL21(DE3) transformed with pT7-7; lanes 6 and 7, inclusion body pellet from BL21(DE3) transformed with pT7-DPR1; lane 8, "inclusion body pellet" fraction from BL21(DE3) transformed with pT7-7. Samples were prepared as described (*37*) and electrophoretically separated on a 7.5 to 15 percent SDS-polyacrylamide gel. (**C**) Renatured DPR1-RAM1 from *E. coli* restored protein prenyltransferase activity to yeast extract lacking DPR1-RAM1. Protein prenyltransferase assays and TLC were performed as described (Fig. 1). Reaction mixtures (8 μl) loaded onto lanes 1 to 4 contained the a-factor 15–amino acid peptide, whereas reaction mixtures loaded onto lanes 5 to 8 lacked the peptide. Soluble fractions were prepared from wild-type (JRY1591) and *dpr1::HIS3* (JRY2595) strains. (D^+) indicates the addition of DPR1-RAM1 from *E. coli* strain BL21(DE3) (*36*) transformed with pT7-DPR1; (d^-) indicates the addition of a renatured protein fraction from *E. coli* strain BL21(DE3) transformed with pT7-7. The TLC plate was developed in solvent B, sprayed with En^{3}Hance (Dupont, Biotechnology Systems), and exposed to film for 3 days. x and y indicate labeled peptide products of unknown identity. Renatured protein was prepared from an inclusion body pellet fraction of *E. coli* expressing DPR1-RAM1 and from an equivalent fraction from an *E. coli* strain that was not producing DPR1-RAM1. Isolation of inclusion body pellets, solubilization in 8 M urea, and renaturation of DPR1-RAM1 was as described (*37*).

sion plasmid so that DPR1-RAM1 could be made in *Escherichia coli* (Fig. 6A). The DPR1-RAM1 produced in *E. coli* had an apparent molecular mass of 42 kD, a value that correlates well with the electrophoretic mobility of in vitro–translated DPR1-RAM1 (*15*) (Fig. 6B). Cell fractionation experiments showed that this protein was localized primarily in inclusion bodies (Fig. 6B). The inclusion body fraction was therefore isolated, solubilized in 8 M urea, and renatured by dialysis.

Addition of renatured DPR1-RAM1 to a *dpr1::HIS3* cell extract resulted in significant protein prenyltransferase activity toward the peptide substrate (Fig. 6C). Adding renatured inclusion body material from *E. coli* that did not express DPR1-RAM1 did not result in measurable activity. The reconstitution of protein prenyltransferase activity in vitro by bacterially produced DPR1-RAM1 indicated a direct role for this protein in the protein prenyltransferase reaction and ruled out the possibility that DPR1-RAM1 regulated the synthesis of the protein prenyltransferase enzyme.

Further evidence that DPR1-RAM1 participated directly in the prenylation reaction was provided by the phenotype of an unusual *dpr1-ram1* point mutation. Several *dpr1-ram1* mutants were isolated in a genetic screen and assayed for protein prenyltransferase activity in vitro under a variety of buffer and pH conditions. One allele, *dpr1-101*, altered the physical properties of the protein prenyltransferase. Extracts from wild-type cells had substantial enzymatic activity in phosphate buffers between pH 7.0 and pH 7.5, but activity was greatly diminished in tris-HCl (pH 7.5) and was even less in tris-HCl (pH 8.2) (Fig. 7). In contrast, extracts made from a *dpr1-101* strain had very little activity under the optimal conditions for the wild-type extract and had substantially more activity under the buffer conditions in which wild-type extracts had little activity. Because both extracts were prepared in tris-HCl buffer, the sensitivity of the mutant protein to phosphate in the reaction buffer indicated that the *dpr1-101* mutation caused a specific alteration in a component that actually functions during the protein prenyltransferase reaction. The simplest interpretation is that *DPR1-RAM1* encodes at least part of the enzyme catalyzing the protein prenylation reaction.

We have been unable to detect enzymatic activity of DPR1-RAM1 purified from *E. coli* in the absence of added yeast extract, with either IPP or FPP as a substrate. Thus, at least one other yeast factor in addition to DPR1-RAM1 may be necessary for protein prenyltransferase activity. This other factor may represent an additional subunit in a multimeric enzyme complex—possibly analogous to HDS of *Micrococcus luteus* (*11*), the active form of which comprises two components. Evidence suggests that the active form of a mammalian protein prenyltransferase may be a heterodimer (*24*).

Requirement for DPR1-RAM1 in prenylation of human H-Ras. Genetic evidence suggests that the DPR1-RAM1 protein prenyltransferase modifies both a-factor and Ras2 in yeast cells. Because human Ras can functionally substitute for Ras2 in yeast, human Ras precursor protein should also be a substrate for modification by DPR1-RAM1. To test this hypothesis, we produced human H-Ras (p21) in *E. coli* and purified the fraction of the protein that was soluble. The soluble protein is still in the precursor form, but when injected into a *Xenopus* oocyte it can be modified in an isoprene-dependent process to activate germinal vesicle breakdown (*6, 25*). Assays were therefore carried out with purified human H-Ras precursor as the substrate. The products of the reaction were separated by electrophoresis, and labeled products were visualized by autoradiography.

Human H-Ras was prenylated in a *DPR1-RAM1*–dependent manner. An in vitro reaction with a wild-type yeast extract and [^{14}C]IPP labeled a 21-kD protein, and this labeling was dependent on addition of both H-Ras and the yeast extract (Fig. 8). Moreover, a *dpr1::HIS3* mutant extract was defective in the prenylation of human Ras in vitro. However, long autoradiographic exposures of gels containing the products of reactions performed with the *dpr1::HIS3* extract at high protein concentrations revealed a small amount of labeled Ras. This result was consistent with the small amount of membrane-associated Ras2 observed in *dpr1::HIS3* mutants and may be attributable to the presence of other protein prenyltransferases in yeast extracts. The defect in in vitro prenylation of Ras precursor observed in the *dpr1::HIS3* mutant extract suggested strongly that the DPR1-RAM1 protein prenyltransferase catalyzes the prenylation of both a-factor and Ras in yeast cells. By inference, a human DPR1-RAM1 homolog may provide the same modification to Ras proteins in human cells.

In independent experiments, others have shown that yeast Ras2 is

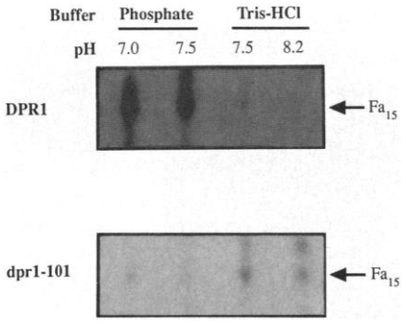


Fig. 7. The *dpr1-101* mutation causes a specific alteration in the protein prenyltransferase enzyme. Reactions were performed as described (Fig. 1), except that KCl was omitted from the reaction mixture. The indicated buffer was present at a concentration of 50 mM. The *DPR1-RAM1* reactions were performed with a soluble extract from JRY1591 (final concentration of 1 mg/ml) for 45 minutes. The *dpr1-101* reactions, containing a soluble extract from YWS101 (*MATa ade2-101 met his3Δ200 lys2-801 ura3-52 RAS2^{val19} dpr1-101*) (final concentration of 1.8 mg/ml), were performed for 75 minutes.

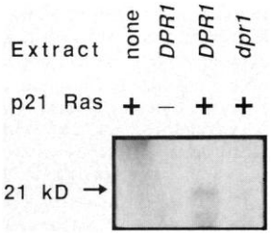


Fig. 8. Prenylation of human Ras precursor in vitro. Protein prenyltransferase assays were conducted as described (Fig. 1), except that purified human H-Ras precursor (100 μg/ml) (*38*) was used in the reactions in place of the a-factor peptide. After a 60-minute incubation at 37°C, 25 μl of reaction mixtures were boiled for 4 minutes in 8 percent glycerol, 1.7 percent SDS, and 75 mM DTT. Products were separated by SDS–polyacrylamide gel electrophoresis and detected by autoradiography. *DPR1-RAM1* (*DPR1*) soluble extract prepared from JRY1591 was present at a final concentration of 1 mg/ml, whereas the *dpr1-ram1* (*dpr1*) soluble extract prepared from JRY2595 was present at a concentration of 1.6 mg/ml.

prenylated in yeast extracts in vitro in a *DPR1-RAM1*–dependent manner. These experiments support the conclusions presented here and extend them by the demonstration that yeast Ras2, like human H-ras, is a prenylated protein (*26*).

Insights into a-factor processing. Because the major prenylated product of our 15–amino acid substrate was apparently not subjected to proteolysis, and the 12–amino acid peptide was not a substrate for prenylation, a-factor prenylation must precede proteolysis. Furthermore, because carboxymethylation of a-factor requires a substrate that has undergone prenylation and proteolysis (*17*), the inferred order of processing is prenylation, proteolysis, and carboxymethylation. It is likely that mutations in any gene encoding a component required for a-factor processing will block the mating ability of cells of the a mating type and have little or no effect on the mating of α cells. Only three such a-specific sterile genes are known: *DPR1-RAM1*, *STE6*, and *STE14*. The *STE6* gene encodes a membrane protein that is required for secretion of a-factor and is the yeast homolog of the human *MDR* locus (*27*). The *STE14* gene encodes or regulates the carboxymethylase (*17*), and *DPR1-RAM1* encodes a component of the prenyltransferase. There are no candidate genes for either the protease that removes the pro sequence of a-factor or the protease that removes the COOH-terminal three amino acids. Similarly, there are no candidates for the missing component or components of the prenyltransferase. Therefore, it is likely that three or more genes required for a-factor processing remain to be discovered, although it is possible that more than one function may be encoded by the same gene.

Protein prenyltransferases as targets for chemotherapy. Ras oncoproteins have been implicated in the pathogenesis of many types of cancer. For example, *ras* oncogenes have been found in 50 percent of all colorectal tumors, 50 percent of lung adenocarcinomas, and 90 percent of pancreatic tumors (*28*). Because prenylation and plasma membrane association are critical for the oncogenic properties of Ras oncoproteins, agents that block this modification in human cells would have therapeutic potential for the treatment of several common types of cancer. In addition, mutations in genes encoding subunits of trimeric G proteins, termed *gsp* mutations, are also oncogenic (*29*). Because the membrane association and biological activity of at least one and possibly all trimeric G proteins also requires prenylation (*2*), *gsp*-related cancers might also be susceptible to this type of treatment. Thus, the potential of a drug that could inhibit prenylation of oncoproteins could extend beyond those types of cancer associated with *ras* oncogenes.

Our data suggest that yeast *DPR1-RAM1* encodes a component of a protein prenyltransferase. Two additional yeast genes have been identified that share sequence homology to *DPR1-RAM1* (*30*). Because our *dpr1-ram1* deletion mutant still contained a small amount of membrane-associated Ras2, and because H-Ras was still labeled by IPP to a small extent in *dpr1-ram1* mutant extracts, at least one of these homologous genes may also encode a component of a structurally related protein prenyltransferase with weak, but measurable, activity toward Ras. The existence of numerous Ras-related proteins with similar but not identical modifiable COOH-termini raises the possibility that some prenyltransferase isozymes may preferentially modify certain prenylated proteins and be specific for various chain lengths in the isoprene substrates. Heterogeneity in the lipid moiety attached by these different isozymes may be important in targeting these different classes of proteins to specific cellular compartments. Sharing of subunits between different protein prenyltransferases, as suggested by the enzymology of rubber biosynthesis, could further contribute to the diversity and potential functional specialization of these modifications.

Because the DPR1-RAM1 protein prenyltransferase is not essential for viability of yeast cells, it is possible that specific inhibitors of individual protein prenyltransferase isozymes of humans would be relatively nontoxic. Furthermore, yeast strains expressing mammalian *DPR1-RAM1* homologs could be used in an in vivo drug screen similar to that described previously (*2*).

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31. The prenylation reaction mixture contained 350 μM **a**-factor peptide, 145 μM [^{14}C]IPP (55 mCi/mmol), 4 mM $MgCl_2$, 50 mM potassium phosphate (pH 6.5), 50 mM KCl, 0.2 mM $ZnSO_4$, 1 mM dithiothreitol (DTT), and yeast protein extract (for crude extract, 2.2 mg/ml). Reactions were performed at 37°C for 30 minutes. Samples (8 μl) of reaction mixture were spotted onto silica gel 60, and products were separated by TLC in solvent B (pyridine:isoamyl alcohol:water:ethanol:acetic acid, 70:70:20:20:5) and detected by autoradiography. The amino acid compositions of the peptides were verified and the structure of the farnesylated 15–amino acid peptide was confirmed by mass spectrometry (*21*). The structure of the *trans-trans* geranylgeranylated peptide was confirmed by nuclear magnetic resonance (*21*).
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33. A Sal I–Xba I fragment containing *DPR1* and its flanking sequences was cloned into the polylinker of the vector plasmid pBluescript KS+. This plasmid was digested with Pst I and Hpa I, and the fragment containing *DPR1* was replaced with a Pst I–Sma I fragment containing the selectable marker gene *HIS3*. This *HIS3* fragment was excised from a plasmid consisting of a Bam HI fragment containing *HIS3* cloned into the polylinker of the pUC19 vector plasmid. The *DPR1-RAM1* deletion plasmid was digested with Sal I and Xba I, and used to transform the yeast strain JRY121, to give strain JRY2582. The structure of the *dpr1-ram1* deletion locus was determined in the diploid and in two haploid segregants of each genotype by DNA blot hybridization (*21*). Strain JRY2595 is a *MATα dpr1::HIS3* haploid segregant of the diploid strain JRY2582.
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35. An Nde I site (CATATG) was created at the ATG initiation codon of *DPR1* by site-directed mutagenesis [F. Ausabel *et al.*, in *Current Protocols in Molecular Biology* (Wiley, New York, 1987), vol. 1, sect. 8.1]. The oligonucleotide (5′-CTGTCGC-ATATGCTTTGATTG-3′) was hybridized to single-stranded template isolated from *E. coli* strain TG1 carrying plasmid pJR857, which is pBluescript KS+ with a 2.4-kb Sal I–Xba I *DPR1* fragment. The resulting plasmid, pJR877, had a single base change from G to A at nucleotide position −2 from the ATG. To construct plasmid pT7-DPR1, we cloned the 1.9-kb Nde I–Xba I fragment of pJR877 together with a synthetic Xba I–Stu I–Eco RI adaptor between the Nde I and Eco RI sites in the T7 promoter expression vector pT7-7. The sequence of the Nde I junction was confirmed by subcloning an Xba I–Apa I fragment of pT7-DPR1 into pRS313 [R. Silorski and P. Hieter, *Genetics* **122**, 19 (1989)] and dideoxy sequencing with Sequenase (U.S. Biochemical).
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37. *Escherichia coli* strain BL21(DE3) transformed with pT7-DPR1 or pT7-7 was grown at 37°C until the absorbance at 600 nm (A_{600}) was 0.5 to 1.0 in media containing 10 g of Tryptone, 5 g of NaCl, 1 g of NH_4Cl, 3 g of KH_2PO_4, 6 g of Na_2HPO_4, 4 g of glucose, and 1 ml of 1 M $MgSO_4$ per liter. Isopropyl-β-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 m*M* to induce expression of the chromosomal copy of the T7 RNA polymerase gene, which is under control of the *lac* promoter in BL21(DE3) (*36*). The induced T7 RNA polymerase transcribes *DPR1-RAM1*, which is under control of the T7 φ10 promoter. After 4 hours growth in the presence of IPTG, cells were harvested, washed once in 50 mM tris (pH 8.0) and 25 percent (w/v) sucrose, and resuspended at 20 mg of cell pellet (wet weight) per milliliter in 50 mM tris (pH 8.0), 25 percent (w/v) sucrose, and 0.01 percent NP-40. Crude extract samples were prepared for electrophoresis by adding one-third volume of 4× sample buffer [4 percent (w/v) SDS, 0.6 M β-mercaptoethanol, 0.24 M tris (pH 6.8), 8 mM EDTA, 32 percent (w/v) glycerol, and 0.001 percent bromophenol blue] and boiling for 4 minutes. Inclusion body pellet fractions for electrophoresis and protein prenyltransferase assays were prepared as follows: Crude extracts were treated with lysozyme (0.1 mg/ml for 30 minutes at 0°C), and then $MgSO_4$, NP-40, and deoxyribonuclease I (DNase I) were added to final concentrations of 5 mM, 1 percent, and 230 units/ml, respectively. After a 20-minute incubation at 0°C, inclusion bodies were separated by centrifugation at 12,000*g* for 10 minutes, washed twice with 50 mM tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.1 percent NP-40, and resuspended at one-third the original volume of crude extract in 8M urea, 50 mM tris (pH 7.5), 5 mM EDTA, 3 mM $MgCl_2$, and 5 mM DTT. For electrophoresis, samples of inclusion body pellets were boiled for 4 minutes in a final concentration of 1× sample buffer. For renaturation of DPR1-RAM1 for protein prenyltransferase assays, solubilized pellets were incubated at room temperature for 1 hour, and dialyzed against two changes of 20 mM tris (pH 7.5), 1 mM EDTA, 0.6 mM $MgCl_2$, 50 mM NaCl, and 0.5 mM DTT at room temperature for 8 hours and then overnight against the same buffer at 4°C. Renatured protein samples were concentrated approximately tenfold in collodion bags (Scheicher & Schuell). Insoluble protein was removed by centrifugation.
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