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Biochemistry

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

(protein prenylation/C-terminal amino acid/cell polarity/bud positioning)

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ABSTRACT **Protein prenylation occurs by modification of proteins with one of at least two isoprenoids, the farnesyl group and the geranylgeranyl group. Protein farnesyltransferases have been identified, but no such enzyme has been identified for geranylgeranylation. We report the identification of an activity in crude soluble yeast extracts that catalyzes the transfer of a geranylgeranyl moiety from geranylgeranyl pyrophosphate to proteins having the C-terminal sequence Cys-Ile-Ile-Leu or Cys-Val-Leu-Leu but not to a similar protein ending with Cys-Ile-Ile-Ser. This activity is dependent upon the *CDC43/CAL1* gene, which is involved in budding and the control of cell polarity, but does not require the *DPR1/RAM1* gene, which is known to be required for the farnesylation of Ras proteins. These results indicate that the protein geranylgeranyltransferase activity is distinct from the protein farnesyltransferase activity and that its specificity depends in part on the extreme C-terminal leucine in the protein to be prenylated.**

Protein prenylation is a post-translational modification that occurs on many eukaryotic proteins (1, 2). Two types have been discovered thus far: farnesylation and geranylgeranylation. Geranylgeranylation appears to be the more frequent type of modification in mammalian cells (3–5). Farnesylated proteins include Ras proteins (6–8), lamin B (9), the a mating factor of *Saccharomyces cerevisiae* (10), and the γ subunit of transducin (11). Geranylgeranylated proteins include the γ subunit of brain trimeric guanine nucleotide binding (G) proteins (12, 13), Rap1A/Krev-1 (14), Rap1B/smg p21B (15), and bovine brain G25K/CDC42 (16), the mammalian homolog of the yeast protein CDC42Sc (17–19).

An enzyme catalyzing transfer of farnesyl groups from farnesyl pyrophosphate (Fpp) to Ras proteins *in vitro* [protein farnesyltransferase (PFT)] has been reported in mammalian cells (20–22) as well as in yeast (23, 24). The mammalian PFT is a 90-kDa dimeric protein that recognizes the C-terminal "CAAX" motif in Ras proteins (where A represents an aliphatic amino acid and X is the C-terminal amino acid) (20–22, 24). The yeast PFT has been shown to be dependent upon *DPR1/RAM1* (referred to here as *DPR1*) (23, 24) and *RAM2* gene products (23). In contrast, no such enzyme has been found thus far for geranylgeranylation. The predicted amino acid sequence of this region in known geranylgeranylated proteins is Cys-Xaa-Xaa-Leu (where Xaa is an unspecified amino acid) whereas no known farnesylated proteins contain leucine at the extreme C terminus.

CDC42Sc and RSR1 are putative GTP-binding proteins of yeast containing the Cys-Xaa-Xaa-Leu motif (19, 25). CDC42Sc shares homology with Rho proteins whereas RSR1 shares homology with Rap proteins. Mammalian counterparts of these proteins [G25K/CDC42 for CDC42Sc and Rap1A for RSR1 (25)] are known to be modified by a geranylgeranyl moiety (16, 14). Genetic studies of CDC42Sc and RSR1 point to their involvement in the establishment of cell polarity and localization of budding and secretion (19, 25, 26). Two other genes, *CDC43* (26) and *CDC24* (27, 28), are also known to be involved in this physiological process. *CDC24* is identical to *CLS4*, which contains two putative Ca^{2+}-binding domains (29). The *CDC43* gene is identical to the *CAL1* gene (30, 31). Ohya *et al.* (31) noted a significant sequence similarity between the *CAL1* gene and the *DPR1* gene. About 50% similarity was observed between *CAL1* and *DPR1* in a region containing approximately two-thirds of the protein. This led us to test the hypothesis that *CDC43/CAL1* is required for the geranylgeranylation of proteins.

We report here the existence of an activity [which we name protein geranylgeranyltransferase (PGGT)] in *S. cerevisiae* that transfers geranylgeranyl groups from geranylgeranyl pyrophosphate (GGpp) to proteins containing C-terminal Cys-Ile-Ile-Leu (CIIL) or Cys-Val-Leu-Leu (CVLL) sequences but not to a protein containing a C-terminal Cys-Ile-Ile-Ser (CIIS) sequence. It also displays specificity toward the prenyl donor GGpp and thus appears to be distinct from the PFT. We also demonstrate that the *CDC43* gene is required for PGGT activity.

MATERIALS AND METHODS

Materials. [1-^{3}H]Fpp and [1-^{3}H]GGpp (20 Ci/mmol; 1 Ci = 37 GBq) were purchased from NEN. XL1-B bacteria harboring the pGEX-CDC42Hs plasmid (17) were a gift of R. A. Cerione (Cornell University, Ithaca, NY). The pGEX-CDC42Hs plasmid contains the glutathione *S*-transferase (GST) gene fused to a human placental CDC42Hs cDNA. The cDNA encodes a protein ending with the C-terminal CVLL sequence (17).

Yeast Strains and Crude Soluble Extracts. The following *S. cerevisiae* strains were used: KMY5-2A-N (*MAT***a** *dpr1 leu2 his3 ura3*) and KMY2-3A (*MAT***a** *leu2 his3*) (23); CJ198-2B (*MATα cdc43-2 ura3 trp1 gal2*) and TD1 (*MATα ura3 his4 trp1 gal2*) (30); and RS16-4C (*MATα ura3 his3 trp1 ade8 ade2 can1 SUP^{84L}*) (32). Transformation of CJ198-2B yeast cells by the *CDC43*-containing plasmid YEp(43)2 (30) and YEp352 (33) was performed as described (30). Crude soluble extracts

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Abbreviations: PFT, protein farnesyltransferase; PGGT, protein geranylgeranyltransferase; Fpp, farnesyl pyrophosphate; GGpp, geranylgeranyl pyrophosphate; GST, glutathione *S*-transferase.

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of the above yeast strains were prepared as described (23). Briefly, cells were grown in YPD medium or minimal medium (34) lacking uracil (for cells harboring plasmids) at 22°C (KMY5-2A-N and CJ198-2B) or 30°C (KMY2-3A, TD1, and RS16-4C) to late logarithmic phase, collected, and broken by glass beads. The resulting lysate was centrifuged at 100,000 × *g* for 1 hr, yielding ≈5 mg of protein per ml, as measured by the method of Bradford (35).

Construction of Vectors for *Escherichia coli* Expression of GST–CIIS and GST–CIIL Proteins. Oligonucleotides were designed to encode the sense strand of the C-terminal 12 amino acids of yeast RAS2 with serine as the C-terminal amino acid (5′-GATCCGTCCAAATCCGGCTCCGGCG-GCTGCTGCATCATCTCCTGA-3′ for GST–CIIS protein, where underlined sequence represents the C-terminal amino acid) or with leucine as the C-terminal amino acid (5′-GATCCGTCCAAATCCGGCTCCGGCGGCTGCTGCAT-CATCCTGTGA-3′ for GST–CIIL protein). Codon substitutions were made to reflect the codon usage bias between *E. coli* and *S. cerevisiae* (36). Complementary oligonucleotides (5′-AGCTTCAGGAGATGATGCAGCAGCCGCCGGAGC-CGGATTTGGACG-3′, GST–CIIS; 5′-AGCTTCACAG-GATGATGCAGCAGCCGCCGGAGCCGGATTTG-GACG-3′, GST–CIIL) were synthesized and annealed to the sense oligonucleotides, producing fragments with *Bam*HI (5′) and *Hin*dIII (3′) cohesive ends, which were cloned into pGEX-1/RCT (37). The 3′ portion of each of these fusion genes was confirmed by DNA sequencing.

Purification of GST Fusion Proteins. The glutathione affinity-precipitation method was used to purify GST–CIIS, GST–CIIL, and GST–CDC42Hs proteins, essentially as described (38). Briefly, *E. coli* cells harboring GST fusion protein expression plasmids were induced with isopropyl β-D-thiogalactopyranoside and then treated with lysozyme and Lubrol PX to lyse cells. Glutathione-agarose beads were mixed with the lysate and washed, and protein was eluted from the beads with reduced glutathione, yielding ≈2 mg/ml (GST–CIIS, GST–CIIL) or ≈0.7 mg/ml (GST–CDC42Hs), as measured by the method of Bradford (35).

PFT and PGGT Assays and Analysis of Product of PGGT Reaction. The PFT filter-binding assays were performed essentially as described (23), with the exceptions that (*i*) the reactions were performed at 37°C, (*ii*) dithiothreitol was used at 1 mM, and (*iii*) the substrates used were GST–CIIS and GST–CIIL (≈40 μg per 60-μl reaction mixture), and GST–CDC42Hs (≈13 μg per 60-μl reaction mixture). Reaction mixtures (60 μl) were incubated at 37°C, 10 μl was withdrawn at various times and spotted onto Whatman no. 3 paper, and the trichloroacetic acid-insoluble radioactivity was measured. The PGGT assays were performed under the same conditions as the PFT assays, with the exception that 1.0 μM [^{3}H]GGpp was used as substrate. The lipid transferred to the GST–CIIL protein in the PGGT reaction was analyzed as described (23). Briefly, the radioactive band from SDS/PAGE was cut out, and radioactive material was eluted by digestion with trypsin and then treated with Raney nickel/pentane. The pentane-soluble material was analyzed by radiometric gas chromatography before and after hydrogenation, and the migration of the sample was compared to that of authentic standards.

RESULTS

Detection of PGGT Activity. The cDNA-predicted sequences of all known geranylgeranylated proteins have leucine as their C-terminal amino acid. This led us to design a substrate to detect PGGT. The protein GST–CIIS contains GST fused to the C-terminal 12 amino acids of yeast RAS2. We changed the C-terminal serine of GST–CIIS to leucine, resulting in GST–CIIL. Both fusion substrates were purified

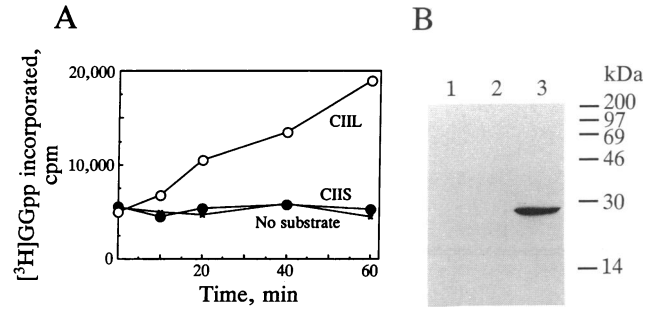

FIG. 1. PGGT uses CIIL but not CIIS as a signal in incorporating [^{3}H]GGpp into protein *in vitro*. (*A*) Time course of incorporation of [^{3}H]GGpp (60-μl reaction mixture). Soluble yeast extracts of KMY2-3A cells (113 μg) were incubated with GST–CIIS (solid circle, 40 μg), GST–CIIL (open circle, 40 μg), or no substrate (×) in the presence of [^{3}H]GGpp for the time indicated and 10 μl was assayed for incorporation of radioactivity into protein by a filter-binding assay. (*B*) SDS/PAGE analysis of products of PGGT reaction. Reaction mixtures of 30 μl were incubated as in *A* for 60 min, and the entire sample was separated on a 12.5% gel. Fluorography was performed on the resulting gel after treatment with Amplify fluorographic enhancer (Amersham). Lanes: 1, no added protein; 2, GST–CIIS; 3, GST–CIIL.

by an affinity purification method using glutathione beads. When [^{3}H]GGpp was incubated with GST–CIIL in the presence of crude soluble extracts of yeast cells (conditions analogous to those used for PFT activity), radioactivity was incorporated into the protein as determined by a filter-binding assay (Fig. 1*A*). In contrast, no geranylgeranyl radioactivity was incorporated into the GST–CIIS protein, and no radioactivity was incorporated if GST–CIIL protein was not added. These results were confirmed by SDS/PAGE analysis of the reaction products. The incorporated radioactivity was associated with a protein of M_r ≈27,000, the molecular weight of GST–CIIL (Fig. 1*B*, lane 3), but no radioactive band was detected with the GST–CIIS protein (Fig. 1*B*, lane 2) or in the absence of added protein (Fig. 1*B*, lane 1).

The PGGT and PFT activities had different substrate requirements (Table 1). The GST–CIIS protein served as a good substrate for the PFT activity, whereas only a relatively small amount of incorporation was observed in a PFT assay using the GST–CIIL protein as a substrate (Table 1). In contrast, the GST–CIIS protein did not serve as a good substrate for PGGT. These results suggest that the C-terminal leucine is important for the recognition of substrates by the PGGT. A small amount of radioactive Fpp was incorporated into GST–CIIL when Fpp was used. This incorporation was not affected by the *dprl* mutation.

Table 1. PGGT and PFT filter-binding assays using GST fusion substrates

Exp.	Substrate	[^{3}H]Geranylgeranyl incorporated, cpm	[^{3}H]Farnesyl incorporated, cpm
I	None	4,487	5,052
	GST–CIIL	18,881	7,966
	GST–CIIS	5,199	18,583
II	None	5,220	6,286
	GST–CDC42Hs	12,569	6,700

PGGT and PFT assays (60 μl) were carried out at 37°C using no added protein or 40 μg of GST–CIIL (GST-SKSGSGGC**CIIL**) or GST–CIIS (GST-SKSGGC**CIIS**) (experiment I) or 13 μg of GST–CDC42Hs (experiment II), mixed with 113 μg of crude soluble extracts of KMY2-3A cells (experiment I) or 120 μg of crude soluble extracts of RS16-4C cells (experiment II). Samples of 10 μl were withdrawn after 60 min (experiment I) or 40 min (experiment II) and assayed for incorporation of radioactivity by a filter-binding method.

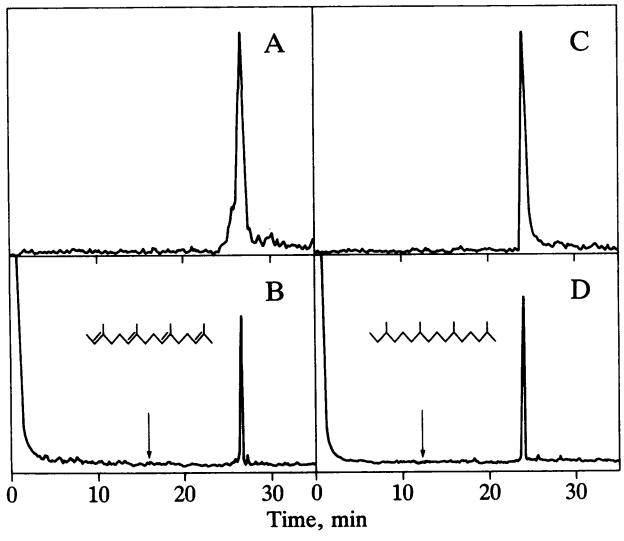

FIG. 2. Identification by gas chromatography of lipid transferred to GST–CIIL. Reaction mixtures (30 μl) were incubated as in Fig. 1 for 60 min, and the entire reaction mixture was subjected to SDS/PAGE. The radioactive band (as in Fig. 1*B*) was cut out and treated with trypsin to elute protein fragments. Soluble material was then lyophilized, solubilized with formic acid/ethanol, 1:4 (vol/vol), and treated with Raney nickel to release lipid. The products were analyzed by gas chromatography with both flame ionization and radiometric detection. (*A*) Nonhydrogenated ^{3}H-labeled sample released from GST–CIIL. (*B*) Authentic all-*trans*-2,6,10,14-tetramethyl-2,6,10,14-hexadecatetraene. Arrow marks the retention time of authentic all-*trans*-2,6,10-trimethyl-2,6,10-dodecatriene. (*C*) ^{3}H-labeled sample released from GST–CIIL that was hydrogenated over platinum. (*D*) Authentic phytane. Arrow marks the retention time of authentic farnesane.

In addition to the GST–CIIL substrate, we have found that the GST–CDC42Hs protein can serve as a substrate for the PGGT activity. This protein is a fusion with the human placenta GTP-binding protein CDC42Hs, which contains the predicted C-terminal sequence CVLL (17). The bovine brain isoform of this protein is known to be geranylgeranylated *in vivo* (16). By using the glutathione affinity method, we purified this M_r ≈45,000 fusion protein and tested it in the *in vitro* reaction, using crude soluble extracts and either [^{3}H]GGpp or [^{3}H]Fpp. As can be seen in Table 1, the GST–CDC42Hs protein was geranylgeranylated *in vitro* but did not appear to be farnesylated.

Identification of the Lipid Attached to the GST–CIIL Protein. The radioactivity incorporated into the GST–CIIL protein was recovered as a geranylgeranyl group. A sample from a PGGT assay was eluted from the gel after SDS/PAGE, treated with Raney nickel to cleave thioether bonds, extracted with pentane, and analyzed by radiometric gas chromatography before and after hydrogenation. Before hydrogenation the sample yielded a single peak of radioactivity (Fig. 2*A*) corresponding to the authentic C_{20} isoprenoid 2,6,10,14-tetramethyl-2,6,10,14-hexadecatetraene (Fig. 2*B*). After hydrogenation the sample yielded a single peak of radioactivity (Fig. 2*C*) corresponding to phytane (Fig. 2*D*). No C_{15} isoprenoid (farnesane) was observed. These results indicated that the geranylgeranyl moiety was transferred without further modification from GGpp to GST–CIIL protein, to give a thioether-linked geranylgeranyl group on the protein.

PGGT Was Not Affected by *dprl* but Was Affected by *cdc43*. The above results clearly demonstrate that yeast cells contain two types of prenyltransferase activities, PFT and PGGT. We previously showed that the *dprl* mutant is defective in PFT activity (23). To test whether PGGT activity is affected by the *dprl* mutation, we prepared extracts from a *dprl* mutant and examined them for PGGT activity. As shown in Fig. 3*A*, enzymatic incorporation of [^{3}H]GGpp into GST–CIIL protein was just as efficient when extracts of *dprl* mutant cells were used as when extracts of a parental strain were used. However, the *dprl* extracts used were deficient in PFT activity relative to wild-type extracts, when tested using [^{3}H]Fpp and GST–CIIS protein (Fig. 3*B*). Thus, PGGT activity is not affected by the *dprl* mutation.

In contrast to *dprl*, we found that cells containing the temperature-sensitive *cdc43-2* mutation (26) had greatly reduced PGGT activity but wild-type levels of PFT activity. The *CDC43* gene, which is involved in bud positioning and the control of cell polarity in yeast, encodes a protein with significant sequence similarity to the protein encoded by the *DPR1* gene. Extracts made from the *cdc43-2* mutant exhibited PGGT activity at 37°C that was reduced by a factor of at least 10 as compared to a parental strain (Fig. 4*A*). In contrast, the *cdc43-2* mutant had wild-type levels of PFT activity (Fig. 4*B*). PGGT activity in *cdc43-2* mutant extracts was greatly reduced even at 22°C. The defect in the PGGT activity was *cdc43*-specific, since introduction of the *CDC43* gene into the *cdc43-2* mutant resulted in the recovery of PGGT activity (Fig. 4*A*). These results indicate that the *dprl* and *cdc43* mutations affect two separate activities and provide genetic evidence that the PGGT is distinct from the PFT.

DISCUSSION

We have shown herein that *S. cerevisiae* cells contain a PGGT activity that transfers a geranylgeranyl moiety from GGpp to protein substrates GST–CIIL and GST–CDC42Hs. GST–CIIL and GST–CDC42Hs end with the CIIL and CVLL sequences, respectively. The PFT plays an analogous role by transferring a farnesyl moiety from Fpp to Ras proteins,

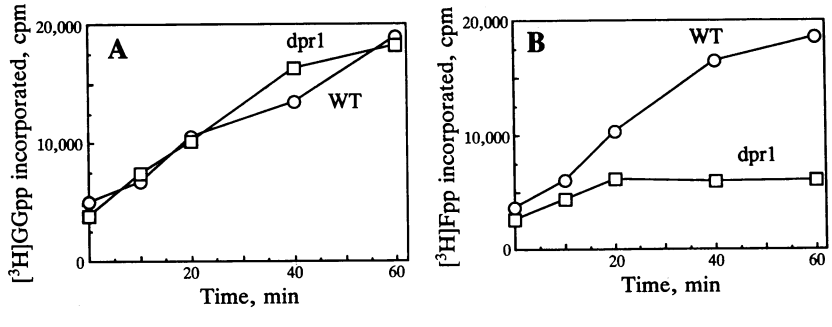

FIG. 3. Effect of *dprl* mutation upon PGGT and PFT activities *in vitro*. Soluble extracts of yeast mutants were assayed for PFT (using GST–CIIS and Fpp) or PGGT (using GST–CIIL and GGpp) by a filter-binding assay. (*A*) PGGT activity of KMY2-3A (circle) or KMY5-2A-N (square) crude soluble extracts. Assays were performed as in Fig. 1 using GST–CIIL and [^{3}H]GGpp. (*B*) PFT activity of strains as in *A*. Assays were performed at 37°C as in Fig. 1 using GST–CIIS and [^{3}H]Fpp.

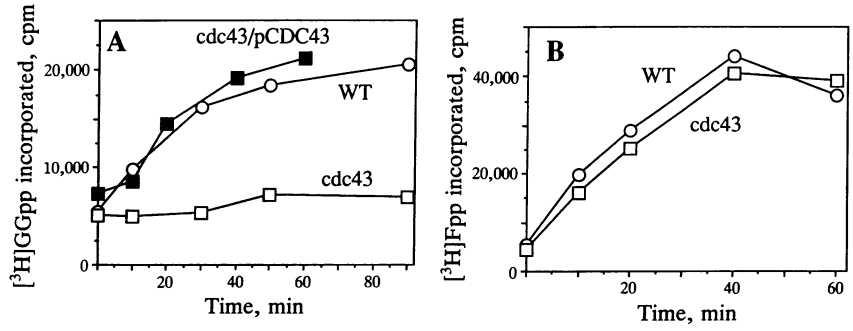

FIG. 4. Effect of *cdc43-2* mutation upon PGGT and PFT activities *in vitro*. Assays were performed at 37°C as in Fig. 3. (*A*) PGGT activity of crude soluble extracts of yeast strains TD1 (open circle), CJ198-2B (open square), or CJ198-2B [YEp(43)2] (solid square). (*B*) PFT activity of crude soluble extracts of yeast strains TD1 (circle) or CJ198-2B (square).

which contain a similar C-terminal sequence but do not end with leucine. Biochemical and genetic evidence strongly suggest that the PGGT activity is distinct from the PFT activity. (*i*) These activities displayed different qualitative and quantitative specificity for protein acceptor and prenyl donor (GGpp and Fpp). A farnesyl group was transferred to the GST–CIIS protein, whereas a geranylgeranyl group was preferentially transferred to the GST–CIIL and GST–CDC42Hs proteins *in vitro* (Table 1). (*ii*) Two yeast mutants, *dprl* and *cdc43*, displayed different defects in the two prenyltransferase activities (Figs. 3 and 4). The *dprl* mutant cells were defective in PFT but retained wild-type levels of PGGT. Conversely, the *cdc43* mutant cells were defective in PGGT but not in PFT. Thus, different genes are required for the two activities. Although the *cdc43* mutant is temperature-sensitive for growth, we have not been able to obtain temperature-sensitive PGGT activity in the *cdc43* mutant extracts. The PGGT activity of the *cdc43* mutant extracts was greatly reduced even at a low temperature. This could be due to instability of the activity in the mutant extracts. Alternatively, this low-level activity might have been enough to support the growth of the *cdc43* mutant at a low temperature.

Geranylgeranylation appears to function on GST fusion substrates containing the 12 C-terminal amino acids of yeast RAS2 with the C-terminal amino acid changed from serine to leucine (GST–CIIL) and on a fusion substrate with an entire protein (GST–CDC42Hs). These results suggest that the determinants for geranylgeranylation reside within the few C-terminal amino acids. In particular, the identity of the extreme C-terminal amino acid plays a critical role in determining whether a protein is farnesylated or geranylgeranylated, since the single amino acid difference between GST–CIIS and GST–CIIL at their C termini results in a reversal of specificity for both prenyltransferase activity (PFT and PGGT) and prenyl donor (Fpp and GGpp, respectively). The importance of the C-terminal sequence in determining the type of modification has also been shown by Buss *et al.* (14). A chimeric Ras protein with the C terminus for Rap1A was shown to be geranylgeranylated, whereas a chimeric Rap1A protein with the C terminus for Ras was farnesylated.

We have shown (23) that the PFT requires the *RAM2* gene in addition to the *DPR1* gene. We tested whether the *RAM2* gene is required for PGGT activity. Preliminary results suggest that PGGT activity is greatly reduced in *ram2* mutant extracts (A.A.F., S.R.J., and F.T., unpublished results). Thus, the *RAM2* gene might be required for both PFT and PGGT activities. This raises the possibility that there are two types of genes involved in prenyltransferases. *DPR1* and *CDC43* might be involved in defining the specificity of a transferase, whereas *RAM2* might play a common role in the two prenyltransferases. More work will be needed to identify which, if any, of these genes encode the prenyltransferase. Nonetheless, it is clear that the genes *DPR1*, *RAM2*, and *CDC43* affect the activity of yeast prenyltransferases.

What proteins are substrates for PGGT in yeast cells? Candidates for such proteins are the yeast proteins CDC42Sc and RSR1, putative GTP-binding proteins that contain the Cys-Xaa-Xaa-Leu motif (19, 25). These proteins are both involved in the same physiological process of controlling cell polarity. In this paper, we have shown that the CDC42Hs protein can be geranylgeranylated *in vitro* by the yeast PGGT (Table 1). Yeast CDC42Sc protein purified from *E. coli* can also be geranylgeranylated *in vitro* (A.A.F., D.I.J., and F.T., unpublished results). Since the *CDC43* gene is required for PGGT activity, the *CDC43* gene appears to be involved in the modification of the CDC42Sc protein. This may explain the similar phenotypes of *cdc43* and *cdc42* mutants, and the fact that a $cdc43^{ts}$ $cdc42^{ts}$ double mutant is inviable at the permissive temperature for the individual mutations (26). Further work is needed to examine whether RSR1 protein is also geranylgeranylated. Many yeast genes have been isolated whose predicted amino acid sequence contains a cysteine at or near the C terminus. For example, RHO1 ends with Cys-Val-Ile-Leu (CVIL) and RHO2 ends with CIIL (39). The CDC43-dependent PGGT assay described herein provides a powerful means to determine potential substrates of the PGGT, which should lead to the elucidation of the C-terminal sequence specificity for yeast PGGT substrates. Future studies using the yeast system should enable us to examine whether alteration of the C-terminal amino acid to leucine results in geranylgeranyl modification *in vivo* and whether this alteration affects the function of proteins such as yeast RAS proteins.

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