Yeast Geranylgeranyltransferase Type-II: Steady State Kinetic Studies of the Recombinant Enzyme†

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ABSTRACT: Rab proteins in mammalian cells, or Ypt1p and Sec4p in yeast, regulate vesicular traffic. Prenylation of these small GTP-binding proteins is required for membrane attachment and subsequent biological activity. Yeast protein geranylgeranyltransferase type-II (PGGTase-II) catalyzes the prenylation of Ypt1p in the presence of an escort protein, Msi4p. The genes encoding the α- (*BET4*) and *β*- (*BET2*) subunits of PGGTase-II were translationally coupled by overlapping the *BET4*−*BET2* stop/start codons and by adding a ribosome-binding site near the 3′-end of *BET4* that fused an -EEF C-terminal α-tubulin epitope to Bet4p. Active recombinant heterodimer was purified by chromatography on DE52 and anti-α-tubulin columns. Recombinant Msi4p with an N-terminal polyhistidine leader was purified on a Ni^{2+}-Sepharose column, followed by gel filtration and ion exchange chromatography. An escort protein, Msi4p, was necessary for geranylgeranylation of Ypt1p by yeast PGGTase-II. Michaelis constants for GGPP and Ypt1p were 1.6 and 1.1 μM, respectively; $V_{max} = 1.7$ nmol min^{-1} mg^{-1} for yeast PGGTase-II. Typical Michaelis−Menten behavior was also seen for the enzyme for varied concentrations of Msi4p, with a maximal catalytic activity seen for a 10-fold excess of escort protein over enzyme. In contrast to previous reports, PGGTase-II requires both Zn^{2+} and Mg^{2+} for maximal activity, although Zn^{2+} becomes inhibitory at concentrations above ∼10 μM. Prenylated Ypt1p obtained after incubation of Ypt1p with PGGTase-II, Msi4p, and geranylgeranyl diphosphate was digested with trypsin. The C-terminal peptide fragment from modified Ypt1p was purified by HPLC and analyzed by electrospray mass spectrometry. The mass of the fragment is consistent with the 12-mer C-terminal amino acid fragment predicted from proteolysis by trypsin with both cysteine residues modified by geranylgeranyl moieties.

Three different protein prenyltransferases modify a variety of eukaryotic proteins with C_{15} farnesyl or C_{20} geranylgeranyl units. The best characterized of the group is protein farnesyltransferase (PFTase).[1] This enzyme transfers a C_{15} isoprenoid unit to the cysteine moiety in a C-terminal CaaX sequence, where the C is Cys, a is normally an aliphatic amino acid, and X is Met, Ser, Gln, Cys, or Ala (Reiss et al., 1995; Omer et al., 1993; Moores et al., 1991; Caplin et al., 1994). Similarly, protein geranylgeranyltransferase type-I (PGGTase-I) adds a geranylgeranyl group to proteins bearing a C-terminal CaaX sequence, where the X is Leu or Phe (Yokoyama et al., 1991; Moores et al., 1991; Caplin et al., 1994). The third, and least understood, protein prenyltransferase is the type-II geranylgeranyltransferase. This enzyme attaches two 20-carbon geranylgeranyl groups to newly synthesized Rab proteins.

PGGTase-II was discovered following the observation that GTP-binding proteins lacking a CaaX box were post-translationally modified by geranylgeranyl residues (Farnsworth et al., 1991). Subsequently, several groups reported that a cytosolic extract from bovine brain catalyzed the geranylgeranylation of proteins bearing C-terminal Cys-Cys and Cys-Ala-Cys moieties (Horiuchi et al., 1991; Moores et al., 1991). Upon chromatography on DEAE-Sephacel, the prenyltransferase activities for PFTase, PGGTase-I, and PGGTase-II in a bovine brain extract were resolved (Moores et al., 1991). Seabra et al. (1992a) found that two components, A and B, were required for geranylgeranylation of Rab proteins with C-terminal Cys-Cys and Cys-X-Cys sequences. They also reported that PGGTase-II was strongly inhibited by Zn^{2+}. In contrast, PFTase and PGGTase-I are Zn^{2+} metalloproteins, and the metal is required for activity.

The catalytic unit in the mammalian PGGTase-II complex, component B, consists of a heterodimer of tightly associated α- and *β*-subunits with molecular masses of 60 and 38 kDa, respectively. The amino acid sequences of the two subunits resemble the respective α- and *β*-subunits of PFTase and PGGTase-I (Armstrong et al., 1993). However, the type-II heterodimer requires a third protein, component A, for full activity. The complete three-protein complex catalyzes geranylgeranylation of both cysteines in Rab1A (-XXCC), Rab3A (-XCXC), and Rab5A (-CCXX) (Farnsworth et al., 1994).

Component A, also called Rab escort protein (REP-1), was isolated from rat brain and found to be similar to a protein associated with choroideremia, an X-linked retinal degeneration disease (Seabra et al., 1992b). The amino acid sequence of Rab escort protein also resembles that of Rab guanine

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[1] Abbreviations: Amp, ampicillin; BME, 2-mercaptoethanol; CaaX, cysteine (C), aliphatic amino acid (a), any amino acid (X); FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; IPTG, isopropyl thio-*β*-D-galactoside; orf, open reading frame; PFTase, protein farnesyltransferase; PGGTase, protein geranylgeranyltransferase; PMSF, phenylmethylsulfonyl fluoride; RBS ribosome-binding site; REP, Rab escort protein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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nucleotide dissociation inhibitor (Rab GDI). GDI forms a complex with prenylated proteins and facilitates their return to the cytosol (Araki et al., 1990). Andres et al. (1993) proposed that component A was a Rab escort protein (REP-1) that binds to Rab proteins and facilitates their recognition by component B, which in turn catalyzes the prenyltransfer reaction. This hypothesis is consistent with the observation that REP-1 binds unprenylated Rab1A and remains bound after the protein is geranylgeranylated (Andres et al., 1993). In the absence of a detergent, the prenyl transfer reaction terminates after one cycle, presumably because all of the component A is bound to newly prenylated substrate. However, the association and dissociation of rab proteins and REP-1 do not require Rab GDI or other cytosolic factors (Alexandrov et al., 1994), and the mechanism of REP-1-mediated membrane association of Rab5A seems to be similar to that mediated by Rab GDI. Thus, REP-1 appears to bind unprenylated protein, escort the substrate to the catalytic component B, and then shuttle the prenylated product to the target membrane, whereupon it dissociates and returns to the cytosol for another round of transport.

A comparison of the amino acid sequences of the α- and β-subunits of rat PGGTase-II suggested that their counterparts in *Saccharomyces cerevisiae* are encoded by the *BET4*[2] and *BET2* genes, respectively (Armstrong et al., 1993). The α-subunit, Bet4p (Jiang et al., 1993), and the β-subunit, Bet2p (Rossi et al., 1991; Kohl et al., 1991), of yeast PGGTase-II were both shown to be necessary for the *in vitro* geranylgeranylation of Ypt1p and Sec4p (Jiang et al., 1993). Jiang et al. also found that an escort protein, Msi4p, was required for the prenylation and membrane attachment of Ypt1p and Sec4p *in vivo* (Jiang & Ferro-Novick, 1994). Msi4p is ∼30% identical to both mammalian REP-1 and Rab GDI and presumably fulfills a similar role in the catalytic cycle of yeast PGGTase-II. The C-terminal sequences for the yeast proteins Ypt1p and Sec4p are GGGCC and KSNCC, respectively. These proteins and a Ypt1p mutant (GGCSC) are geranylgeranylated *in vitro*, although it was not known if the yeast enzyme modifies both C-terminal cysteines. We now report the construction of *Escherichia coli* strains for the overproduction of the three yeast proteins required for geranylgeranyltransferase activity. The yeast enzyme and escort protein were purified to apparent homogeneity for product and kinetic studies.

EXPERIMENTAL PROCEDURES

Materials. Standard molecular biology procedures were carried out as described elsewhere (Sambrook et al., 1989). The protein content of samples was determined by the method of Bradford (1976). Enzymes for molecular biology were obtained from New England Biolabs or Boehringer Mannheim. Plasmid pBlueScript II SK(+) was purchased from Stratagene. DNA sequencing was performed by the Sequencing Services Laboratory (University of Utah). Asp-Phe and geranylgeraniol were purchased from Sigma. [1-^{3}H]-Geranylgeranyl diphosphate (6.7 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. Unlabeled geranylgeranyl diphosphate (GGPP) was synthesized from geranylgeranyl bromide and tris(tetrabutylammonium) pyrophosphate (Davisson et al., 1986). Stock solutions of GGPP were prepared in 25 mM NH_4HCO_3 (pH 8.3), and concentrations of GGPP were determined by phosphate analysis (Reed & Rillings, 1976). Liquid scintillation spectrometry was performed on a Packard TRI-CARB (4530 or 2300TR) liquid scintillation analyzer using CytoScint scintillation cocktail purchased from ICN Laboratories. Dimethyl formamide was dried over 3 Å molecular sieves. *E. coli* strain DH5α was used for cloning, and strains JM101 and BL21 (DE3) were used for protein synthesis. *E. coli* strain CJ236 was purchased from Bio-Rad and used in site-directed mutagenesis experiments. Mutagenic primers were phosphorylated chemically during solid-phase synthesis. The bacterial expression plasmid pDR200 was obtained from pLM1 (Sodeoka et al., 1993) by removing the *Nde*I site and adding a new *Nde*I site downstream of the *Bam*HI site. A pUC19 multicloning site was then subcloned into the *Nde*I to *Hin*dIII region of the pLM1. The *E. coli* expression vector pARC306N was obtained from M. Bittner (Biotechnology Division, Amoco Research Corp.). The bacterial expression plasmid pTTQ18 was obtained from Amersham and modified to pTTQ18N by adding an *Nde*I site that overlaps the ATG codon for initiation of translation. The *BET4* (plasmid: pBC-KS background) and *BET2* (plasmid: pUC118 background) open reading frames (orf's) as well as the *YPT1* gene in expression plasmid pNRB429 were provided by Dr. Susan Ferro-Novick (Yale University) (Jiang et al., 1993). The *MSI4* gene (plasmid: pKF130) was obtained from Dr. Akio Toh-e (University of Toyko) (Fujimura et al., 1994).

Construction of the Expression Plasmid for Recombinant PGGTase-II (BET4-BET2). (See supporting information for a flowchart for construction of plasmids.) A ∼1.0-kb fragment from the pBC-KS plasmid containing the native *BET4* gene (Jiang et al., 1993) was ligated into pBlueScript II SK(+) using *Kpn*I and *Pst*I restriction sites. Site-directed mutagenesis was performed to delete the *Kpn*I site and add new *Kpn*I and *Pst*I sites downstream from the termination codon in *BET4* (pWGS-2-43). The *Kpn*I-*Pst*I fragment from the pUC118 derivative containing *BET2* (Jiang et al., 1993) was cloned into pWGS-2-43 to give pDJW-1-187. *Pst*I and *Kpn*I restriction sites were introduced downstream of the termination codon in *BET2*, and an *Nde*I site was added upstream of the initiation codon for *BET4* (pDJW-1-227/12). At the same time, the *BET4* and *BET2* orf's were coupled by deletion mutagenesis with the sense strand primer (5′-CTGGAGCAGCATA**AGGAGGA**GTTTTGATG-TCAGGATCTCTGACG-3′, which overlapped the translation termination codon for *BET4* with the initiation codon for *BET2* (underlined) and added a C-terminal Glu-Glu-Phe anti-α-tubulin epitope to Bet4p using codons that created a strong RBS (bold) upstream of the *BET2* orf. A ∼1.8-kb fragment containing the *BET4-BET2* cassette was subcloned into pTTQ18N (pDJW-1-245), pDR200 (pDJW-1-244/7), and pARC306N (pDJW-1-244/5) to place the synthetic operon under control of *tac*, *T*7, and *rec*7 promoters, respectively.

Construction of the Expression Plasmid for Recombinant MSI4 (5′-Poly-His). (See supporting information for a flowchart for construction of plasmids.) An *Xho*I-*Pst*I fragment containing the *MSI4* (Fujimura et al., 1994) gene from plasmid pKF130 was cloned into pBlueScript II SK(+). An *Nde*I restriction site was introduced upstream of the *MSI4*

[2] The following genes and (proteins) are also designated by a second name: *RAM1* (Ram1p) is *DPR1* (Dpr1p), *CDC43* (Cdc43p) is *CAL1* (Cal1p), *MSI4* (Msi4p) is *MRS6* (Mrs6p), and *BET4* (Bet4p) is *MAD2* (Mad2p).

initiation codon, and *Kpn*I and *Pst*I sites were added downstream of the *MSI4* termination codon to generate pDJW-1-93. Oligonucleotides 5′-TATG*CATCATCATCATCATCAT*ATAGAGGGGCG-3′ and 3′-AC*GTAGTAGTAGTAGTAGTA*TATCTCCCCGCAT-5′ were annealed and ligated into the *Nde*I site of pDJW-1-93. The resulting insert contained codons for six histidines (italics) and a Factor Xa recognition site (underlined). After the correct orientation of the insert was determined by a DNA electrophoresis gel (Metaphor, 3%) *via* restriction enzyme screening, the "poly-His"-tagged *MSI4* orf was cloned into pTTQ18N (pDJW-1-139), pDR200 (pDJW-1-141), and pARC306N (pDJW-1-140) to place the synthetic operon under control of *tac*, *T* 7, and *rec*7 promoters, respectively.

Overproduction and Purification of Recombinant PGGTase-II. *E. coli* BL21 (DE3)/pDJW-1-244/7 transformants were incubated overnight at 37 °C in LB (Luria-Bertani) (3 mL) containing 100 μg of ampicillin (Amp)/mL. A 40 μL portion was used to inoculate 40 mL of M9-CAGM media [40 mL of M9 salts (Sambrook et al., 1989), 50 mL of 20% (w/v) casamino acids, 6.4 mL of 40% (w/v) glucose, 1.2 mL of 1 M $MgSO_4$, 0.25 mL of 0.1 M $CaCl_2$, 0.25 mL of 0.1% (w/v) thiamine hydrochloride, 0.1 mL of trace minerals (0.8 g of $ZnSO_4$, 1.4 g of $CoCl_2$, 1.4 g of Na_2MoO_4, 1.6 g of $CuSO_4$, 0.4 g of H_2BO_3, 1.0 g of $MnSO_4$, all per 200 mL), 0.1 mL of 0.33 M $FeCl_3$, and 50 mg of AMP, all per L] in a 250 mL Erlenmeyer flask, and the mixture was incubated overnight at 250 rpm at 30 °C. A 5 mL portion was used to inoculate 500 mL of M9-CAGM in a 2.8 L Fernbach flask. Cells were grown with shaking at 250 rpm at 30 °C to an OD_{600} of 0.5−0.7 and were then induced with isopropyl thio-β-D-galactoside (IPTG), 0.2 mM (final concentration). Incubation was continued for 4 h at 30 °C with shaking at 250 rpm before the cells were harvested by centrifugation (4500*g* for 10 min) and either carried on for purification or stored at −80 °C.

Recombinant PGGTase-II was purified by a procedure similar to that described for recombinant yeast PFTase (Mayer et al., 1993). The cell pellet (∼4 g) was suspended in 40 mL of lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM BME, and 1 mM PMSF) and disrupted by sonication. The lysate was centrifuged at 17 400*g* for 15 min at 4 °C and chromatographed on a 3 cm × 12 cm DE52 column using a NaCl stepwise gradient in 50 mM Tris-HCl, pH 7.3, buffer containing 10 mM $MgCl_2$ and 10 mM BME. Active PGGTase-II eluted with 100 mM NaCl and was loaded onto an anti-α-tubulin immunoaffinity column (10 cm × 0.75 cm) (Mayer et al., 1993) preequilibrated with binding buffer (50 mM Tris-HCl, pH 7.3, 8 mM $MgCl_2$, 10 mM BME, and 150 mM NaCl). The column was washed with binding buffer (∼25 mL) and eluted with binding buffer containing 5 mM Asp-Phe. Fractions containing PGGTase-II were stored in elution buffer containing 20% glycerol at −80 °C until needed. Recombinant PGGTase-II was stable for several months at −80 °C and for several days at 0 °C.

Overproduction and Purification of Ypt1p. Ypt1p was overproduced in *E. coli* strain BL21 (DE3)/pNRB429 provided by Dr. Susan Ferro-Novick (Yale University School of Medicine). Transformants were incubated overnight at 37 °C in LB (3 mL) containing 100 μg of Amp/mL. A 40 μL portion was used to inoculate 40 mL of minimal medium (10 g of bactotrytone and 5 g of NaCl per L) in a 250 mL Erlenmeyer flask, and the suspension was incubated overnight at 250 rpm at 30 °C. A 5 mL portion was used to inoculate 500 mL of ZB in a 2.8 L Fernbach flask. Cells were grown with shaking at 250 rpm at 30 °C to an OD_{600} of 0.4−0.6 before the addition of IPTG to 0.2 mM (final concentration). Incubation was continued for 4 h at 30 °C with shaking at 250 rpm. The cells were harvested by centrifugation (4500*g* for 10 min), washed with 50 mM Tris-HCl, pH 8.0, and 100 mM NaCl, and repelleted. The cell paste was disrupted for purification or stored at −80 °C.

Ypt1p was purified using a modification of the procedure of Jiang et al. (1993). Cell paste (∼2 g) was suspended in 20 mL of buffer A (20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM NaN_3, 10 mM BME) containing 1 mM PMSF and disrupted by sonication. The lysate was centrifuged at 17 400*g* for 15 min at 4 °C and chromatographed on a 3 cm × 12 cm DEAE-Sephacel column with buffer A using a NaCl stepwise gradient. Ypt1p eluted with 100 mM NaCl and was concentrated to 1.3 mL using a Centriprep 10 (Amicon). The solution was loaded onto a 2 cm × 60 cm HiLoad 16/60 Superdex 200 column (Pharmacia) equilibrated in buffer A and eluted at a flow rate of 0.5 mL/min. The fractions containing Ypt1p were combined and chromatographed on a Poros HQ column (PerSeptive Biosystems) using column buffer using a 40 mL NaCl linear gradient of 0−150 mM. The fractions were analyzed by SDS−PAGE. Those containing Ypt1 protein were pooled and stored at −80 °C.

Overproduction and Purification of Recombinant Msi4p. *E. coli* strain BL21 (DE3)/pDJW-1-95 and pDJW-1-141 cells containing the *MSI4* constructs (3′-"EEF"-tail and 5′-histidine-tagged, respectively) were grown as described for recombinant PGGTase-II. Cell paste from BL21 (DE3)/pDJW-1-141 containing histidine-tagged Msi4p was resuspended in buffer B (50 mM NaH_2PO_4, pH 8.0, 5 mM BME, and 300 mM NaCl) containing 1 mM PMSF and disrupted by sonication. The cell-free homogenate was clarified by centrifugation (12 000*g* for 10 min) and chromatographed on a 0.7 cm × 5 cm Ni^{2+}-Sepharose affinity column according to the instructions of the manufacturer (QIAGEN). The column was washed with a 0−18 mM imidazole gradient, and Msi4p was eluted with 100 mM imidazole. The pooled fractions were loaded onto a 2 cm × 60 cm HiLoad 16/60 Superdex 200 column (Pharmacia) equilibrated in buffer C containing 50 mM Tris-HCl, pH 7.2, 5 mM $MgCl_2$, 10 mM BME, and 0.1 mM Nonidet P-40 and eluted at a flow rate of 0.5 mL/min. The fractions containing Msi4p were pooled, concentrated with a Centriprep 30 (Amicon) concentrator, and chromatographed on a Poros HQ column (PerSeptive Biosystems) with a 100−400 mM 40 mL linear NaCl gradient in buffer C. Fractions were analyzed by SDS−PAGE, and those containing Msi4p were stored at −80 °C. Recombinant Msi4p was stable for several months at −80 °C and for several days at 0 °C.

Recombinant Msi4p containing the "EEF"-tail was purified in a similar manner as the recombinant PGGTase-II, except the Msi4 protein in the pooled fractions from the DE52 column did not bind to an anti-α-tubulin immunoaffinity column. Work with this construct was discontinued.

Assay of Yeast GG Transferase Activity. Activity for PGGTase-II was determined by measuring incorporation of radioactivity into Ypt1p from [1-^{3}H]GGPP. Incubations, in a final volume of 50 μL, contained the following: 50 mM Tris-HCl, pH 7.3, 8 mM $MgCl_2$, 5 mM DTT, 1 mM Nonidet

P-40, and the indicated amounts of PGGTase-II, Ypt1p, [^{3}H]-GGPP, and Msi4p. After incubation at 30 °C for 15 min, the reaction was quenched with 1:10 (v:v) concentrated HCl/95% ethanol (1 mL). The samples were filtered on Whatman GF/C filters using a Brandell MR-24 cell harvester (Pompliano et al., 1992). The filters were washed four times with 95% ethanol (1.2 mL), vortexed in CytoScint (10 mL) for 40 s, and counted by liquid scintillation spectrometry. All determinations were made in the linear range of the assay.

Product Analysis of Modified Ypt1p. (a) Autoradiographic Analysis. A mixture containing the standard assay buffer, 1.6 μM Msi4p, 1.3 μM Bet4p/Bet2p, 6.7 μM Ypt1p, and 1.1 μM [^{3}H]GGPP (6.7 Ci/mmol) was incubated for 2 h at 30 °C. Two controls were run simultaneously: one without Msi4p and a second without Bet4p/Bet2p. After quenching with 25 μL of SDS loading buffer, 50 μL of each reaction was loaded onto a 12% SDS−polyacrylamide gel. The gel was treated with Entensify solution (NEN), dried, and exposed to XAR film for 24 h at −80 °C.

(b) Modified Ypt1p Digest and Analysis. The modified Ypt1p was digested and analyzed by a procedure similar to that described for Rab proteins (Farnsworth et al., 1994), except for the following changes. A reaction mixture containing standard assay buffer, 0.71 μM Msi4p, 0.66 μM Bet4p/Bet2p, 10 μM of Ypt1p, and 12 μM of GGPP in a final volume of 110 μL was incubated for 3 h at 30 °C. The proteins were digested as described by Farnsworth et al. The resulting sample was injected onto a 4.0 mm × 150 mm C_8 reverse-phase column (Varian) using a Waters model 501 HPLC system equipped with a Waters 441 detector. The column was washed at 1.0 mL/min with 0.06% trifluoroacetic acid (TFA) in water and eluted with a 45 mL gradient of 5%−100% acetonitrile (0.06% TFA). The retention time of the prenylated peptide was determined by injection of ^{3}H-labeled peptide obtained from an incubation with [^{3}H]GGPP. Fractions were collected manually and freeze-dried. The sample was resolubilized in 6 mM HCl/methanol and injected onto a 1.0 mm × 50 mm PLRP-S column (Michrom Bioresources Inc.) using a Michrom Bioresources UMA 600 HPLC system. The gradient was 2% B (B = 0.09% TFA/90% acetonitrile/10% water) in A (A = 0.1% TFA/2% acetonitrile/98% water) to 25% B in 0.02 min, then 25% B to 98% B in 25 min with a flow rate of 40 μL/min. The HPLC effluent was pumped into a Sciex API *III* electrospray triple-quadrupole mass spectrometer (Perkin-Elmer-Sciex Inc., Thornhill, Ontario) operated in the positive ion mode. The mass range was set for 500−2400 mass units. Mass calibration was performed by matching ions of polypropylene glycol to known reference masses.

pH Dependence for PGGTase-II. The standard assay was performed with assay buffer containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 40 μM $ZnCl_2$, 5 mM DTT, and 1 mM Nonidet P-40 over a pH range of 6.8−8.5. Each reaction contained 41 nM Bet4p/Bet2p, 65 nM Msi4p, 4.0 μM Ypt1p, and 3.6 μM [^{3}H]GGPP (1.0 Ci/mmol). A polybuffer containing 100 mM Tris, 50 mM MES, 50 mM acetic acid, 8 mM $MgCl_2$, 2 μM $ZnCl_2$, 5 mM DTT, and 1 mM Nonidet P-40 was also used over a pH range of 5−9. In this case, each reaction contained 27 nM Bet4p/Bet2p, 0.30 μM Msi4p, 5.0 μM Ypt1p, and 4.0 μM [^{3}H]GGPP (3.2 Ci/mmol).

Metal Dependence for PGGTase-II. (a) Mg^{2+} Assay. Bet4p/Bet2p, Ypt1p, and Msi4p were dialyzed against 50 mM Tris-HCl, pH 7.5, 20 mM EDTA, and 20% glycerol overnight and then against 50 mM Tris-HCl, pH 7.5, for 24 h. Assays with varying concentrations of $MgCl_2$ contained 50 mM Tris-HCl, pH 7.3, 10 μM $ZnCl_2$, 5 mM DTT, 1 mM Nonidet P-40, 49 nM Bet4p/Bet2p, 0.27 μM Msi4p, 4.1 μM Ypt1p, and 3.6 μM [^{3}H]GGPP (5.7 Ci/mmol).

(b) Zn^{2+} Assay. Bet4p/Bet2p, Ypt1p, and Msi4p were dialyzed against 50 mM Tris-HCl, pH 7.5, 6 mM EDTA, 2 mM, 1,10-phenanthroline, 1 mM DTT, and 10% glycerol for 4 h and then against 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.2 mM 1,10-phenanthroline, 1 mM DTT, and 10% glycerol for 4 h. The assay buffer was stirred for 8 h with pretreated Chelex-100 (Falchuk et al., 1988) before the addition of Mg^{2+} and Zn^{2+}. Assays with varying $ZnCl_2$ concentrations contained 50 mM Tris-HCl, pH 7.3, 8 mM $MgCl_2$, 5 mM DTT, 1 mM Nonidet P-40, 24 nM Bet4p/Bet2p, 0.13 μM Msi4p, 5.5 μM Ypt1p, and 4.0 μM [^{3}H]-GGPP (0.62 Ci/mmol).

(c) Zinc Content Determination. The zinc contents of dialyzed and non-dialyzed yeast PGGTase-II samples were determined on a Perkin-Elmer 305A atomic absorption spectrophotometer. Standard curves for analysis were produced using a zinc standard diluted in water pretreated with Chelex-100 (Falchuk et al., 1988). PGGTase-II was dialyzed in an identical manner as for the Zn^{2+} dependence assay. The dialyzed and non-dialyzed samples were diluted in water before analysis.

RESULTS

Cloning and Expression of PGGTase-II. BET4 and *BET2* were cloned into pBlueScript SK(+) and translationally coupled by deletion mutagenesis (Kunkel et al., 1987). The stop codon of *BET4* was fused to the start codon for *BET2* with a TGATG sequence. In addition, a strong RBS was embedded in the 3′-end of *BET4* by insertion of an AGGAGGAGTTT sequence just before the stop codon. The encoded amino acids, EEF, formed a C-terminal anti-α-tubulin epitope, which provided a convenient handle for rapid purification of the heterodimeric enzyme by immunoaffinity chromatography. To find a suitable system for protein synthesis, a variety of expression vectors and conditions were tested. The coupled *BET4-BET2* sequence was subcloned into pDR200, pARC306N, and pTTQ18N to create synthetic operons with the *BET4-BET2* cluster under control of *T* 7, *rec*7, and *tac* promotors, respectively. These plasmids were transformed into *E. coli*, and the resulting cultures were separated into supernatant and pellet fractions. A portion of each fraction was analyzed by SDS−PAGE. The gels revealed that >50% of the desired protein was retained in the pellet fraction. However, upon incubation of the pDR200 derivative (pDJW-1-244/7) in M9-CAGM at 30 °C, the ratio of recombinant enzyme in the supernatant increased to 7:3, and ∼8% of the total cytosolic protein was PGGTase-II (data not shown). A control run with *E. coli* BL21(DE3)/pDR200, which contained the expression plasmid without the PGG-Tase-II cluster, had no activity for geranylgeranylation of Ypt1p (data not shown).

The order of the *BET4* and *BET2* genes was inverted to see if the sequence in which the subunits were synthesized affected the levels of production. The *BET2-BET4* cluster was constructed in a manner similar to that used for the *BET4-BET2* coupled genes. After cloning *BET2* and *BET4* into pBlueScript SK(+), the two genes were translationally

Table 1: Comparison of Activities for PGGTase-II with an α-Tubulin Epitope in the Bet2p or Bet4p Subunits[a]

PGGTase-II	crude extract (nmol min^{-1} mg^{-1})	purified protein (nmol min^{-1} mg^{-1})
Bet4p-EEF/Bet2p	0.64 ± 0.02	11 ± 0.6
Bet2p-EEF/Bet4p	0.18 ± 0.03	1.9 ± 0.2

[a] Assays were performed with 0.13 μM Msi4p, 5.5 μM Ypt1p, 3.6 μM [^{3}H]GGPP (3.2 Ci/mmol), and 5.7 μg of crude proteins or 25 nM purified PGGTase-II as described in the Experimental Procedures.

coupled, and a C-terminal EEF anti-α-tubulin epitope was added to the 3′-end of *BET2*. The coupled *BET2-BET4* sequence was subcloned into pDR200. Crude cytosolic and purified preparations of PGGTase-II from both orientations were assayed. PGGTase-II from the *BET4-BET2* construct was 4−6-fold more active than enzyme from the *BET2-BET4* construct in the crude extract and purified samples (Table 1).

Cloning and Expression of MSI4. Two separate strategies were explored to facilitate the purification of recombinant yeast escort protein. The first involved addition of an EEF anti-α-tubulin epitope to the C-terminus of Msi4p to facilitate purification by immunoaffinity chromatography. The same general mutagenesis procedure used for the *BET4-BET2* construct was performed. Modified *MSI4* was then subcloned into expression vectors containing *T* 7, *tac*, and *rec*7 promoters. The highest level of expression was seen for a *T* 7 construct (pDJW-1-95). The second strategy involved appending an N-terminal polyhistidine tag to Msi4p. An oligonucleotide insert containing codons encoding for a polyhistidine sequence and a Factor Xa recognition site was ligated immediately downstream of the *MSI4* initiation codon. The recombinant *MSI4* gene was subcloned into the same three expression vectors used for the *MSI4*:EEF gene. From analysis of crude extracts by SDS−PAGE, inclusion bodies containing the desired protein were formed when the transformants were incubated at temperatures above 30 °C. The amount of protein in the pellet decreased to ∼40% of total Msi4p for incubations in M9-CAGM media at 30 °C. In addition, the expression levels were the highest (∼8% of total cytosolic protein) when *MSI4* was under the control of a *T* 7 promoter (pDJW-1-141). A control was performed using *E. coli* strain BL21 (DE3)/pDR200 which lacked the *MSI4* insert. No activity was detected when crude supernatant was added to samples containing GGPP, Ypt1p, and PGGTase-II (data not shown).

Purification of Recombinant PGGTase-II. Recombinant PGGTase-II was purified 12-fold to >95% homogeneity in two steps by ion-exchange chromatography on DE52 followed by immunoaffinity chromatography on an anti-α-tubulin column (Table 2). The protein was stored in elution buffer containing 20% glycerol at −80 °C until needed. After immunoaffinity chromatography, the sample gave only two bands of equal intensity on SDS−PAGE (Figure 1, lane 3) at ∼31 and ∼33 kDa, which is slightly lower than the calculated weights for Bet2p and Bet4p of ∼36 and ∼35 kDa, respectively. The two bands were sometimes difficult to resolve due to their nearly equivalent masses.

Purification of Msi4p. Recombinant Msi4p::EEF was obtained from induced cells of *E. coli* strain BL21 (DE3)/pDJW-1-141. Fractions from DE52 chromatography were analyzed by SDS−PAGE, and those containing a band for the 66 kDa protein were purified by immunoaffinity chromatography. No detectable amount of the Msi4 protein bound to the column, suggesting that the C-terminal end of the protein is sterically inaccessible for binding to the α-tubulin antibody. However, the N-terminal polyhistidine tag bound to a Ni^{2+}-Sepharose column, and recombinant yeast Msi4p was purified 7-fold to >95% homogeneity in a three-step purification protocol involving Ni^{2+}-Sepharose affinity chromatography, gel filtration on a HiLoad 16/60 Superdex 200 column, and ion-exchange chromatography on a Poros HQ column (Table 3). The purified escort protein was stored in elution buffer containing 20% glycerol at −80 °C until needed. An SDS−PAGE gel of samples from each stage of the purification is shown (Figure 2). Purified Msi4p gave a single band (Figure 2, lane 4) whose molecular weight (∼66 kDa) is similar to that reported previously (Benito-Moreno et al., 1994).

Enzymatic Properties of Recombinant PGGTase-II. To determine whether PGGTase-II and escort protein were both necessary for prenyltransferase activity, each component was incubated with varying concentrations of the other. The α,β-heterodimer of recombinant yeast PGGTase-II did not catalyze geranylgeranylation of Ypt1p above normal background unless supplemented with Msi4p (Figure 3A). Likewise, Msi4p was inactive in the absence of PGGTase-II. However, as the concentration of Msi4p was increased in the presence of the α,β-heterodimer, activity increased to a maximal value when the ratio of Msi4p:PGGTase-II was ∼10. When incubated with 130 nM Msi4p (Figure 3A), a plot of velocity versus the concentration of α,β-heterodimer was linear up to a saturating level of ∼40 nM. This profile suggests that the heterodimer is the catalytic component in the reaction. The onset of the plateau in activity seen at higher concentrations of PGGTase-II occurs when the ratio of Msi4p to enzyme falls below 3:1 and may reflect a limitation of the Msi4p·Ypt1p complex at high enzyme concentration. In contrast, a plot of velocity versus concentration of Msi4p in the presence of 24 nM Bet4p/Bet2p (Figure 3B) gave a typical hyperbolic Michaelis−Menten profile, suggesting Msi4p was a required cofactor for the geranylgeranylation of Ypt1p.

Mixtures of purified PGGTase-II (Bet4p-EEF/Bet2p) and Msi4p were used to determine the steady state kinetic constants for PGGTase-II, and care was taken to use PGGTase-II enzyme concentrations such that the measurements were performed in the linear range of the reaction. A suboptimal 1:1 ratio of PGGTase-II/Msi4p was first used to determine the Michaelis constants for Ypt1p and GGPP to allow comparison with previously reported values. Michaelis constants for yeast PGGTase-II were obtained from simple-weighted nonlinear regression fits with a curve-fitting program using the substrate saturation curves shown in Figure 4 (Leatherbarrow, 1992). The errors for the fitted parameters were obtained from the error matrix generated from the fitting routines. The apparent K_M (1.1 ± 0.1 μM) and V_{max} (1.7 ± 0.1 nmol min^{-1} mg^{-1}) when Ypt1p was the substrate were of the same magnitude reported for the mammalian PGGTase-II (Cremers et al., 1994). This K_M should be considered an apparent K_M for the Msi4p·Ypt1p complex, since the two proteins presumably associate before interacting with the α,β-heterodimer. The K_M for Ypt1p was also determined at various ratios of escort protein and catalytic component, summarized in Table 4. As previously

Table 2: Purification of Yeast PGGTase-II[a]

step	protein (mg)	specific activity (nmol min^{-1} mg^{-1})	total activity (nmol min^{-1})	purification (n-fold)	recovery (%)
supernatant	210	0.71	0.60	1	100
DE52	36	2.2	0.31	3	52
immunoaffinity	3.2	8.2	0.10	12	17

[a] PGGTase-II activity was measured with 0.13 μM Msi4p using conditions described in the Experimental Procedures.

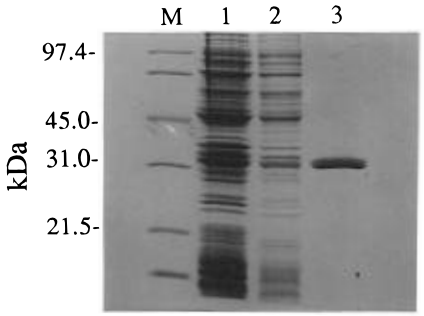


FIGURE 1: Purification of PGGTase-II. Samples from each step in the purification procedure were electrophoresed on an SDS−polyacrylamide gel and stained with Coomassie Blue. Lanes: M, molecular mass standards; 1, crude supernatant (~50 μg); 2, DE52 ion-exchange (~18 μg); 3, immunoaffinity chromatography (~6 μg).

noted (Figure 3), the rate of the reaction increased with increasing amounts of Msi4p, and the apparent V_{max} increased from 1.7 to 6.7 nmol min^{-1} mg^{-1} when the concentration of Msi4p was increased 10-fold. The apparent K_M for Ypt1p at 4 μM GGPP did not change, within experimental error, over the range of Msi4p concentrations. Likewise, the K_M for GGPP remained constant at 1.6 ± 0.3 μM.

It was necessary to carefully adjust the concentration of detergent (Nonidet P-40) during the measurement of K_M for GGPP. The GGPP dilution buffer required 2 mM Nonidet P-40 to prevent the loss of GGPP by absorption to pipette tips and Eppendorf tubes during manipulations. However, amounts of Nonidet P-40 greater than 3 mM in the assay buffer inhibited the prenylation reaction (data not shown).

A pH−rate profile for PGGTase-II has a maximum between pH 7.0 and 7.5 in the standard 50 mM Tris-HCl assay buffer and in a Tris/MES/acetic acid polybuffer that permitted measurements over a more extended range (data not shown).

Maximal activity for yeast PGGTase-II required the presence of Mg^{2+} and Zn^{2+}. PGGTase-II, Msi4p, and Ypt1p were dialyzed extensively against EDTA before the assays with added Mg^{2+} and Zn^{2+} were conducted. PGGTase-II showed an optimal activity at Mg^{2+} concentrations between 4 and 10 mM (Figure 5A) that decreased at higher concentrations. If non-dialyzed proteins were used during Zn^{2+} analysis, then the addition of Zn^{2+} was inhibitory, with ~90% inhibition at 100 μM Zn^{2+}, as reported for mammalian PGGTase-II (data not shown). When the three protein components were dialyzed with EDTA before analysis, activity was stimulated approximately 2-fold by addition of Zn^{2+} up to a concentration of 6 μM followed by a decline in activity at higher concentrations (data not shown). However, the dialyzed components retained half of their maximal activity in the absence of added Zn^{2+}. Since PFTase and PGGTase-I both contain a tightly bound Zn^{2+}, the residual activity in yeast PGGTase-II might result from difficulties in removing the metal from the catalytic heterodimer (Yokoyama et al., 1995). The retained activity, without the addition of Zn^{2+}, was decreased substantially by treating the assay buffer with Chelex-100 and dialyzing the protein components against EDTA and 1,10-phenanthroline (Figure 5B). In this case, a 10-fold stimulation of the activity for PGGTase-II was seen upon the addition of Zn^{2+} with an optimum at Zn^{2+} concentrations between 6 and 10 μM. Higher concentrations of Zn^{2+} were inhibitory (Figure 5B).

The Zn^{2+} content of PGGTase-II was analyzed by atomic absorption spectrometry. Dialyzed and non-dialyzed samples of PGGTase-II were diluted in Chelex-100-treated water, and the zinc content was measured by flame atomic absorption spectrometry. For the dialyzed sample, the stoichiometry was 0.12 ± 0.02 mol of Zn^{2+}/mol of PGGTase-II. For the non-dialyzed sample, the ratio was 0.7 ± 0.1 mol of Zn^{2+}/mol of PGGTase-II ($n = 2$ determinations) (data not shown). The analysis indicates that even prolonged treatment with EDTA and 1,10-phenanthroline did not completely strip Zn^{2+} from the enzyme and suggests that a single zinc atom is required for yeast PGGTase-II activity. The residual activity seen for dialyzed PGGTase-II is consistent with the results.

Product Analysis. Recombinant Ypt1p was isolated by a modification of the procedure described previously (Jiang et al., 1993). To test whether prenylated Ypt1p was the source of retained radioactivity in the PGGTase-II assay, samples were analyzed by SDS−PAGE. As shown in Figure 6 (lane 1), a band at ~23 kDa characteristic of geranylgeranylated Ypt1p was labeled with [^{3}H]GGPP. Furthermore, labeled Ypt1p was not detected in the absence of PGGTase-II or Msi4p (lanes 2 and 3, respectively).

In addition, modified Ypt1p was analyzed by three different procedures to determine whether one or two geranylgeranyl groups were added to the adjacent cysteines at its C-terminus. The first two involved purifying modified Ypt1p by gel filtration or HPLC, followed by mass spectrometry, and the third used a procedure similar to that described for Rab proteins (Farnsworth et al., 1994). A mixture of Msi4p, PGGTase-II, and similar concentrations of Ypt1p and GGPP was incubated. Following incubation, the sample was quenched with EDTA and injected directly onto a gel filtration column (Superdex 75, Pharmacia). Upon elution there was a loss in the intensity of the peak corresponding to Ypt1p; however, no clearly defined peak was seen for the geranylgeranylated product. SDS−PAGE of fractions collected during the gel filtration gave a band corresponding to a ~24 kDa protein; however, the material eluted over a broad range of 30−65 kDa. These results suggest that the geranylgeranylated Ypt1p eluted as a broad peak, perhaps due to self-association through the hydrophobic isoprenoid residues. In the second approach, the quenched sample was injected onto a C_4 reverse-phase column. Analysis of the HPLC trace showed a loss of intensity in the peak for Ypt1p and concomitant formation of a peak

Table 3: Purification of Msi4p[a]

step	protein (mg)	specific activity of PGGTase-II (nmol min^{-1} mg^{-1})	amount of protein used in specific activity assay (μg)	purification (*n*-fold)	recovery (%)
supernatant	180	0.10	2.0	1	100
Ni^{2+}-Sepharose	3.9	0.51	0.63	5	11
Superdex 200	2.4	0.92	0.63	9	12
Poros HQ	1.8	1.1	0.63	10	8.7

[a] PGGTase-II activity was assayed using 74 nM PGGTase-II, 5.5 μM Ypt1, 4.0 μM [^{3}H]GGPP (5.0 Ci/mmol), specified amount of protein from purification steps, and the standard assay conditions as described in the Experimental Procedures.

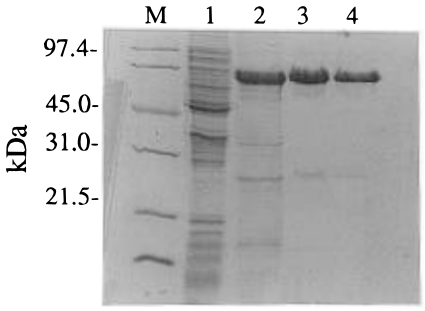


FIGURE 2: Purification of Msi4p. Samples (10 μL) from each step in the purification procedure were electrophoresed on an SDS−polyacrylamide gel and stained with Coomassie Blue. Lanes: M, molecular mass standards; 1, crude supernatant (~50 μg); 2, Ni^{2+}-Sepharose (~8 μg); 3, Superdex 200 (~9 μg); 4, Poros HQ (~6 μg).

characteristic of prenylated protein. The fractions containing Ypt1p and the putative modified Ypt1p were isolated and subjected to electrospray mass spectrometry. However, the intact prenylated protein did not produce a suitable electrospray mass spectrum. Finally, Ypt1p was prenylated in the normal manner, and the mixture of proteins in the incubation was treated with trypsin. A highly hydrophobic geranylgeranylated peptide was isolated by reverse-phase HPLC on a C_8 column and analyzed by electrospray mass spectrometry (see Experimental Procedures). The calculated monoisotopic masses of the mono- and digeranylgeranylated 12-mers resulting from the C-terminal tryptic peptide of Ypt1p are 1368.7 and 1640.9, respectively. The experimental mass of the digested reaction product (1641.0 ± 0.25) corresponds to the calculated mass of the C-terminal 12-mer of digeranylgeranylated Ypt1p. Thus, geranylgeranylation occurred at both of the C-terminal cysteines of the Ypt1p.

DISCUSSION

PGGTase-II is the least characterized of the protein prenyltransferases. The enzyme is a heterodimer whose α- and β-subunits have substantial sequence similarity with those of PFTase and PGGTase-I (Moores et al., 1991). Although all three enzymes catalyze prenylation of C-terminal cysteines, only a CaaX tetrapeptide is required for alkylation by PFTase and PGGTase-I, whereas the intact protein substrate is necessary for PGGTase-II. In addition, PGGTase-II requires an escort protein to present the substrate for geranylgeranylation and to remove the product. Until now, only rat PGGTase-II has been purified to homogeneity as a recombinant protein synthesized by a baculovirus-Sf9 insect cell system (Cremers et al., 1994). A similar approach was used to obtain recombinant rat REP-1 and human REP-2. Previously, recombinant yeast PGGTase-II activity was detected *in vitro* when both *BET2* and *BET4* genes were co-expressed in *E. coli*, and the activity increased >4-fold when crude extracts of recombinant Msi4p were added (Jiang & Ferro-Novick, 1994); however, the levels of recombinant proteins were low. Overexpression and purification of *S. cerevisiae* PGGTase-II, Msi4p, and Ypt1p on substantially larger scale allowed us to conduct kinetic and product studies with the yeast complex. The yeast genes for the α- and β-subunits of PGGTase-II were translationally coupled to maximize synthesis of active enzyme in a manner similar to yeast PFTase and PGGTase-I (*RAM2/RAM1* and *RAM2/CDC43*, respectively) (Stirtan & Poulter, 1995; Mayer et al., 1993). By proper arrangement of nucleotides in the ribosome binding site used to promote coupled translation of the two open reading frames, the recombinant Bet4p/Bet2p protein was modified to append a Glu-Glu-Phe anti-α-tubulin epitope on the C-terminus of the Bet4 protein. This allowed us to rapidly purify PGGTase-II to apparent homogeneity by immunoaffinity chromatography.

The yeast escort protein, Msi4p, is ~30% identical to both the mammalian REP-1 and Rab GDI proteins (Waldherr et al., 1993). Mutations in REP-1, the human CHM gene product, lead to deficient PGGTase-II activity and concomitant degeneration of the retina (Seabra et al., 1993). PGGTase-II activity is somewhat higher in other tissues, presumably because the escort function is partially restored by REP-2, a CHM-like gene product (Cremers et al., 1994). In yeast cells, Msi4p is thought to be the only escort protein for geranylgeranylation of Ypt1p and Sec4p (Jiang & Ferro-Novick, 1994). Msi4p was produced in *E. coli* (Fujimura et al., 1994), as well as a truncated version containing an N-terminal polyhistidine tag to facilitate purification on a Ni^{2+} column (Benito-Moreno et al., 1994). However, the full protein was not purified.

We purified recombinant Msi4p containing an N-terminal polyhistidine appendage by affinity chromatography on Ni^{2+}-Sepharose resin. As seen for the mammalian PGGTase-II, addition of the escort protein to the yeast catalytic heterodimer restored PGGTase-II activity. Maximal activity was achieved at a Msi4p:PGGTase-II ratio of 10:1. In the presence of a fixed concentration of Msi4p, the initial velocity for the geranylgeranylation of Ypt1p increased linearly with added catalytic heterodimer in a manner typically seen when the catalyst is rate limiting (Figure 3A). At high levels of the enzyme, where the molar ratio of carrier protein to catalyst dropped below 3:1, the rates reached a plateau, suggesting that the availability of substrate had become rate-limiting. The hyperbolic behavior seen in Figure 3B when the concentration of Msi4p was increased at a fixed concentration of enzyme is characteristic for saturation of enzyme binding by a substrate, in this case the Msi4p·Ypt1p complex. Similar behavior was seen for Ypt1p

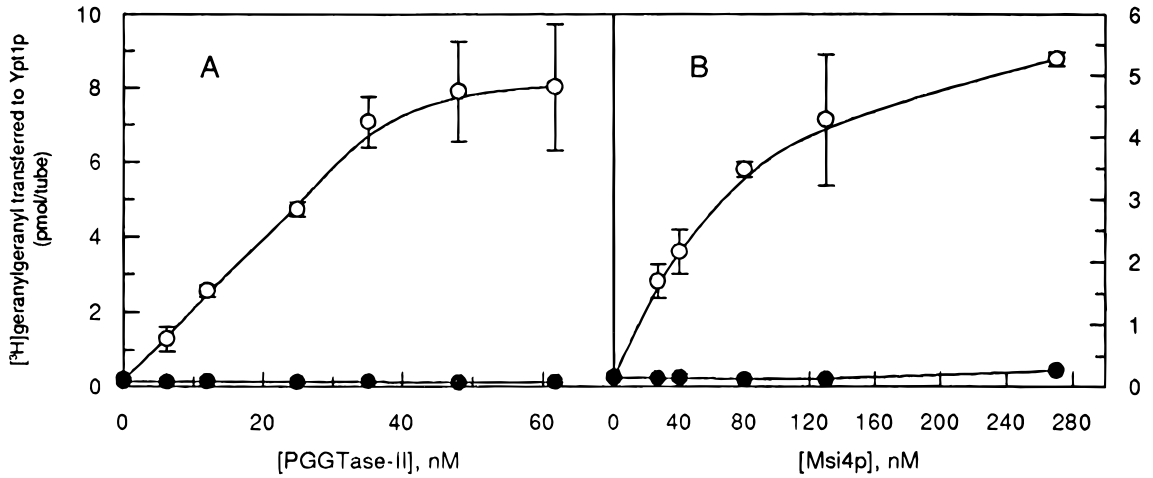


FIGURE 3: PGGTase-II activity requires Bet4p/Bet2p and Msi4p. (A) Geranylgeranylation of Ypt1p by varying amounts of PGGTase-II and fixed amounts of Msi4p, 124 nM (○), and 0 μM (●). (B) Geranylgeranylation of Ypt1p by varying amounts of Msi4p and fixed amounts of PGGTase-II, 23 nM (○), and 0 μM (●). Each assay contained 5.5 μM Ypt1, 3.6 μM [^{3}H]GGPP (3.2 Ci/mmol), and the indicated amounts of Msi4p and PGGTase-II. Assays were performed in triplicate, and blank values, carried out in the absence of Ypt1p, were subtracted from each value.

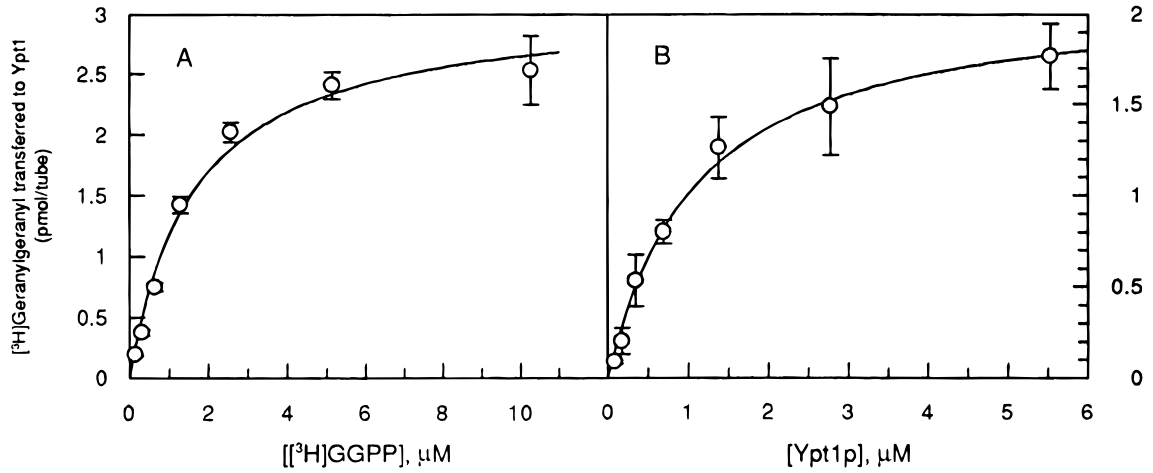


FIGURE 4: Substrate saturation curves for yeast PGGTase-II. (A) Varying concentrations of [^{3}H]GGPP. (B) Varying concentrations of Ypt1p. Assays contained 25 nM Msi4p, 23 nM PGGTase-II, varying concentrations of [^{3}H]GGPP (1.7 Ci/mmol), and 5.5 μM Ypt1p (A) or varying concentrations of Ypt1p and 4.0 μM GGPP (B). Assays were performed in triplicate, and blanks, determined without Ypt1p, were subtracted from each value.

Table 4: Comparing Kinetic Parameters for Ypt1p at Various Msi4p/PGGTase-II Ratios[a]

ratio of Msi4p/PGGTase-II (nM/nM) = ratio	apparent K_M (μM)	apparent V_{max} (nmol min^{-1} mg^{-1})	GGPP (μM)
(27/24) = 1.1	1.1 ± 0.1	1.7 ± 0.1	4
(89/24) = 3.7	1.1 ± 0.1	3.6 ± 0.1	4
(270/24) = 11.3	0.95 ± 0.2	6.7 ± 0.4	4

[a] Assays were performed with indicated amounts of various components as described in Experimental Procedures.

(Figure 4B). These results would suggest that the Msi4p·Ypt1p complex is the "true" substrate for the Bet4p/Bet2p heterodimer (Andres et al., 1993). One would predict that the Michaelis constant for Ypt1p is an apparent value which would depend on the concentration of Msi4p. The values of K_M we measured for Ypt1p at three different fixed concentrations of Msi4p were similar, suggesting that the dissociation constant of the Msi4p·Ypt1p complex is substantially smaller than the K_M for Ypt1p.

The K_M values for the allylic diphosphate substrates of yeast protein prenyltransferases are approximately an order of magnitude larger than for their mammalian counterparts (Stirtan & Poulter, 1995; Mayer et al., 1993). This phenomenon has been observed for other prenyltransferases and may reflect differences in the concentrations of isoprenoid metabolites in mammalian and fungal cells (Rilling et al., 1993).

Mg^{2+} is a required cofactor for a wide variety of prenyltransferases. In the case of farnesyl diphosphate synthase, which catalyzes the synthesis of farnesyl diphosphate from isopentenyl diphosphate and dimethylallyl diphosphate, King and Rilling (1977) proposed that the Mg^{2+} salts of the diphosphate esters were the true substrates for the enzyme. While several divalent cations can substitute for Mg^{2+}, including Zn^{2+} (Sagami et al., 1984), all are thought to serve a similar role. In contrast, the yeast and mammalian forms of PFTase and PGGTase-I require both Mg^{2+} and Zn^{2+} for activity. Zn^{2+} is tightly bound and may serve an important role in the binding of the peptide substrate. Based on the similarity in the amino acid sequences for the subunits in the catalytic heterodimers of the three protein prenyltransferases, one might anticipate similar metal ion requirements for the enzymes. However, Seabra et al. (1992b) reported that rat PGGTase-II only required Mg^{2+} and that

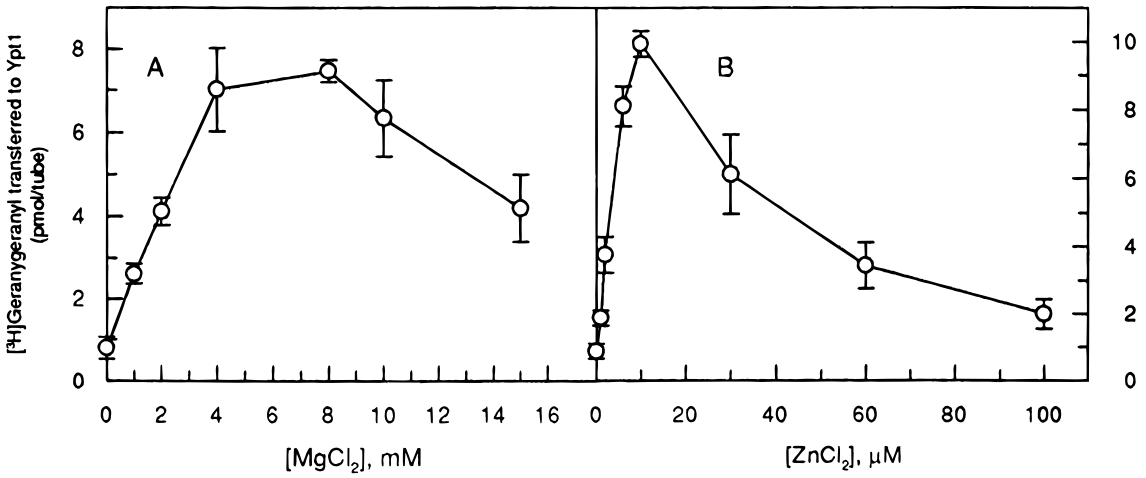
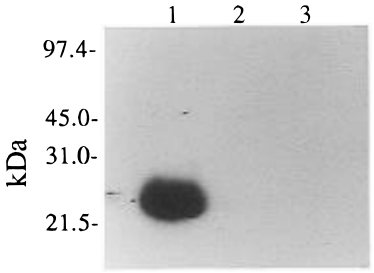


FIGURE 5: Metal ion dependence for yeast PGGTase-II. (A) Varying concentrations of $MgCl_2$. Assays contained 250 nM Msi4p, 49 nM PGGTase-II, 4.0 μM Ypt1p, and 3.6 μM [^{3}H]GGPP (5.7 Ci/mmol). (B) Varying concentrations of $ZnCl_2$. Assays contained 130 nM Msi4p, 24 nM PGGTase-II, 5.5 μM Ypt1p, and 4.0 μM [^{3}H]GGPP (0.62 Ci/mmol). All assays were performed in triplicate, and blank values, carried out in the absence of Ypt1p, were subtracted from each value.



Ypt1p	+	+	+
Bet4p-Bet2p	+	-	+
Msi4p	+	+	-

FIGURE 6: Prenylation of Ypt1p. Three separate incubations were performed simultaneously. The first contained PGGTase-II, Msi4p, Ypt1p, and [^{3}H]GGPP. Subsequent incubations were devoid of PGGTase-II and Msi4p, respectively. A portion (50 μL) of each reaction were loaded onto a 12% SDS−PAGE gel. The migration of geranylgeranylated Ypt1p on the gel was visualized by treating the gel with Entensify solution and exposing it to XAR film for 24 h at −80 °C. A control gel including molecular mass standards was run simultaneously to determine the positions of the molecular mass markers *via* staining with Coomassie Blue. Lanes: 1, Msi4p and PGGTase-II; 2, Msi4p only; 3, PGGTase-II only.

Zn^{2+} was inhibitory. In contrast, we found that PGGTase-II required both Mg^{2+} and Zn^{2+} for optimal activity and that these metal ion requirements were similar to those previously reported for yeast PFTase and PGGTase-I (Stirtan & Poulter, 1995; Mayer et al., 1993). In particular, the initial stimulation of activity by Zn^{2+}, followed inhibition at higher concentrations, was observed with the other two protein prenyltransferases (Stirtan & Poulter, 1995; Mayer et al., 1993). For yeast PGGTase-II the maximal velocity is seen at 8 μM Zn^{2+}, while the optimal Mg^{2+} concentration is 100 times higher.

Most prenyltransferases require a divalent metal for activity. In most cases Mg^{2+} is thought to be the physiologically important metal, although some enzymes show a distinct preference for Mn^{2+}. The metal ion requirements for prenyl transfer have been most extensively studied for farnesyl diphosphate (FPP) synthase (Sagami et al., 1984). The enzyme is not active in the absence of a divalent metal. While Mg^{2+} is preferred, several other metals, including Mn^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{2+}, promote the reaction. Interestingly, at submicromolar concentrations Zn^{2+} is an effective replacement of Mg^{2+}; however, it becomes inhibitory at higher concentrations, as seen for PGGTase-II. The catalytic roles for Zn^{2+} in PGGTase-II and FPP synthase are probably different. The protein prenyltransferases contain a tightly bound Zn^{2+} that is difficult to remove by dialysis and appears to promote binding of the peptide substrate. In contrast, FPP synthase is not a metalloprotein, and Zn^{2+} simply serves as a surrogate for Mg^{2+}. The inhibition seen at higher concentrations of Zn^{2+} for both enzymes may be related to the formation of Zn^{2+} complexes that render the diphosphate esters unsuitable as substrates.

Mammalian Rab proteins, Rab1A (XXCC), Rab3A (XCXC), and Rab5A (CCXX), are geranylgeranylated on both cysteines at the C-terminus (Farnsworth et al., 1994). The yeast counterparts, Ypt1p and Sec4p, have C-terminal XXCC sequences like Rab1A. Analysis of the 12-mer trypsin fragment from the C-terminus of Ypt1p by electrospray mass spectrometry showed that two geranylgeranyl groups were attached. Presumably yeast PGGTase-II alkylates both cysteines of the C-terminus of Sec4p as well. We did not detect formation of monogeranylgeranylated Ypt1p when similar concentrations of Ypt1p and GGPP were incubated with PGGTase-II and Msi4p under conditions that permitted multiple rounds of turnover. These results indicate either that the second geranylgeranylation reaction is substantially more efficient than the first or that the reaction proceeds *via* a processive mechanism.

In conclusion, PFTase, PGGTase-I, and PGGTase-II are closely related. They are all heterodimers which share a substantial degree of sequence similarity in both subunits. The enzymes catalyze similar reactions, have similar metal ion requirements, and have similar kinetic properties. The major differences are in the recognition of their respective isoprenoid and protein substrates with the added feature that PGGTase-II requires an escort protein to present the protein substrate to the catalytic heterodimer and to remove the geranylgeranylated product.

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SUPPORTING INFORMATION AVAILABLE

Flowcharts for the construction of the *BET2-BET4*, *BET4-BET2*, and *MSI4* plasmids (5 pages). Ordering information is given an any current masthead page.

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