Characterization of Recombinant Human Farnesyl-Protein Transferase: Cloning, Expression, Farnesyl Diphosphate Binding, and Functional Homology with Yeast Prenyl-Protein Transferases

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ABSTRACT: We have isolated cDNAs encoding the α and β subunits of human farnesyl-protein transferase (FPTase). The proteins encoded by these two cDNAs are 93–95% identical to the corresponding subunits of bovine and rat FPTase and show regions of homology with proteins encoded by *Saccharomyces cerevisiae* prenyl-protein transferase genes. Human FPTase expressed in *Escherichia coli* from a translationally coupled operon had kinetic properties similar to those of FPTase isolated from bovine brain. Examination of farnesyl diphosphate binding indicated that while neither individual subunit was capable of isoprenoid binding, a radiolabeled farnesyl diphosphate analog could be specifically photo-cross-linked to the β subunit of FPTase holoenzyme. To further analyze subunit structure–function and to detect functional similarities with yeast prenyl-protein transferases (FPTase and two geranylgeranyl-protein transferases), amino acid changes homologous to those found in mutant yeast prenyl-protein transferase subunits were made in the subunits of human FPTase. Substitutions in either the α or β subunits that decrease the activity of yeast prenyl-protein transferases were also observed to impair human FPTase. Kinetic analyses showed that these mutant human FPTases have K_m and k_{cat} values that are altered with respect to wild-type human FPTase.

Farnesyl-protein transferase (FPTase)[1] catalyzes the addition of the isoprenoid farnesyl, from farnesyl diphosphate, to a cysteine residue of a protein substrate such as Ras (Reiss et al., 1990; Schaber et al., 1990; Manne et al., 1990). Farnesylation of Ras facilitates its membrane binding, which is essential for efficient cell transformation by oncogenic forms of Ras (Willumsen et al., 1984). Thus inhibitors of FPTase may be antitumor agents (Goldstein & Brown, 1990; Gibbs, 1991). In vivo protein substrates for FPTase, which in addition to Ras (Casey et al., 1989) include nuclear lamin B (Farnsworth et al., 1989) and the γ subunit of transducin (Lai et al., 1990; Fukuda et al., 1990), have in common a carboxyl-terminal motif known as a Ca_1a_2X box in which C is the cysteine that is to be farnesylated, a_1 and a_2 are usually aliphatic amino acids, and X can be Ser, Met, Gln, Cys, or (in yeast) Ala (Reiss et al., 1991a; Moores et al., 1991). Ca_1a_2X tetrapeptides are substrates for FPTase with kinetic properties similar to those of polypeptide substrates, indicating that the critical determinants required for enzyme recognition are contained within the Ca_1a_2X box (Reiss et al., 1991a,b; Moores et al., 1991; Goldstein et al., 1991; Pompliano et al., 1992).

Mammalian FPTase is an $\alpha\beta$ heterodimer (Reiss et al., 1990, 1991b; Pompliano et al., 1992). By SDS–PAGE the α subunit has an apparent molecular mass of 47–49 kDa and the β subunit has an apparent molecular mass of 45–47 kDa (Reiss et al., 1990, 1991b; Pompliano et al., 1992). Complementary DNAs encoding part or all of the α subunit of FPTase from bovine and rat brain (Kohl et al., 1991; Chen et al., 1991b) have been isolated. The proteins that they encode share >95% amino acid sequence identity with one another and 30% identity with the *RAM2*-encoded subunit of *Saccharomyces cerevisiae* FPTase (He et al., 1991). A cDNA encoding the β subunit of rat brain FPTase has been isolated (Chen et al., 1991a), and the protein it encodes shares 37% amino acid sequence identity with the *DPR1/RAM1* (*RAM1*) encoded subunit of *S. cerevisiae* FPTase (Goodman et al., 1988).

Biochemical studies of mammalian FPTase have led to a model in which Zn^{2+}-dependent binding of the Ca_1a_2X-containing protein substrate occurs to the FPTase β subunit (Reiss et al., 1992). The α subunit of FPTase has been proposed to bind farnesyl diphosphate, which is transfered to the cysteine residue of the Ca_1a_2X peptide in a Mg^{2+}-requiring reaction (Reiss et al., 1992). Kinetic and biochemical evidence indicates that the reaction proceeds through a random-order, sequential mechanism (Pompliano et al., 1992; Reiss et al., 1992).

Additional prenyl-protein transferases, including at least two geranylgeranyl-protein transferases, have been identified in extracts from both mammalian and yeast cells (Moores et al., 1991; Horiuchi et al., 1991; Casey et al., 1991; Joly et al., 1991; Yokoyama et al., 1991; Seabra et al., 1992). One

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[1] Abbreviations: FPTase, farnesyl-protein transferase; GGPTase I, geranylgeranyl-protein transferase type I; GGPTase II, geranylgeranyl-protein transferase type II; Ca_1a_2X, C is Cys, a_1 and a_2 are usually aliphatic amino acids, and X is any amino acid; kDa, kilodalton(s); M_r, molecular weight; SSC, 0.15 M NaCl and 0.015 M sodium citrate; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; PBS, 0.15 M NaCl and 6 mM sodium phosphate, pH 7.2; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DATFP-GPP, (2-diazo-3-trifluoropropionyloxy)-geranyl diphosphate.

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geranylgeranyl-protein transferase (termed GGPTase I) utilizes protein substrates such as Rap1B and the γ subunit of bovine brain heterotrimeric G proteins (Moores et al., 1991; Casey et al., 1991; Joly et al., 1991; Yokoyama et al., 1991; Yamane et al., 1990, 1991). GGPTase I substrates have a Ca_1a_2X box in which X is Leu (Moores et al., 1991; Casey et al., 1991; Joly et al., 1991; Yokoyama et al., 1991). Similar to FPTase, Ca_1a_2X tetrapeptides compete for protein substrate binding to GGPTase I by serving as alternative substrates (Moores et al., 1991). The *S. cerevisiae* Cdc43/Cal1 protein (Ohya et al., 1991; Finegold et al., 1991) is one subunit of GGPTase I (Kohl et al., 1991; Finegold et al., 1991). The *S. cerevisiae* Ram2 protein, which is a subunit of FPTase, is the other subunit of GGPTase I (Kohl et al., 1991; Mayer et al., 1992). Immunological evidence indicates that mammalian GGPTase I also shares a similar, if not identical, subunit (α) with FPTase (Kohl et al., 1991; Seabra et al., 1991).

A second geranylgeranyl-protein transferase activity (GGPTase II), identified in both yeast and bovine brain (Moores et al., 1991), catalyzes the geranylgeranylation of the two carboxyl-terminal cysteine residues of protein substrates such as YPT1 or Rab1B (Kinsella & Maltese, 1991). In contrast to FPTase and GGPTase I, GGPTase II appears to require determinants in addition to the CC motif for proteins to act as substrates because short peptides do not act as competitive inhibitors (Moores et al., 1991). An enzyme termed "Rab GGPTase" may be the same enzyme as GGPTase II since it can geranylgeranylate both CC and CXC carboxyl-terminal motif-containing proteins (Seabra et al., 1992). In *S. cerevisiae*, the Bet2/Orf2 protein (Rossi et al., 1991; Peterson-Bjorn et al., 1990) is a subunit of GGPTase II (Kohl et al., 1991). The Cdc43/Cal1 (GGPTase I) and Bet2/Orf2 proteins share 30–40% amino acid identity with the *S. cerevisiae* FPTase β subunit protein Dpr1/Ram1 (Goodman et al., 1988) and the β subunit of mammalian FPTase (Chen et al., 1991a), indicating that there is significant homology among the known prenyl-protein transferase β subunits.

We are interested in elucidating the structure–function relationships of the two subunits of FPTase, including their involvement in catalysis and in binding of the two substrates. Furthering our knowledge of how FPTase works may be instrumental in identifying inhibitors of FPTase which may be useful in treating cancers involving oncogenic *ras*. With this in mind, we have cloned and functionally expressed in *Escherichia coli* the two subunits of human FPTase. By using information from mutations in genes encoding *S. cerevisiae* prenyl-protein transferase subunits, we have made and characterized mutations in homologous amino acids of the two subunits of human FPTase. Our results demonstrate the functional homology between yeast prenyl-protein transferases and human FPTase and define mutations that affect the kinetic parameters of human FPTase. Furthermore, a radiolabeled farnesyl diphosphate analog was specifically photolinked to the β subunit of FPTase, which indicates a role for this subunit in isoprenoid binding.

MATERIALS AND METHODS

Materials. Ras proteins used in this study were expressed from the plasmid [Leu68]RAS1(term.)-SLKCVLS (Temeles et al., 1985; Gibbs et al., 1989) or its derivatives that express Ras proteins with different Ca_1a_2X boxes (Moores et al., 1991). These proteins are denoted generically as Ras-Ca_1a_2X, of which specific examples are Ras-CVLS and Ras-CVIM. [^{3}H]-Farnesyl diphosphate (26.9 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO) or Du Pont-NEN (Boston, MA). [^{32}P]dNTPs (3000 Ci/mmol) were obtained from Amersham (Chicago, IL). Chemicals used were purchased from Sigma, Aldrich, and Fisher. The antibody YL1/2 (Kilmartin et al., 1982) was obtained from Harlan Bioproducts for Science (Indianapolis, IN).

cDNA Cloning. A bovine brain oligo(dT)-primed cDNA library in λgt10 (Vogel et al., 1988) or a human placenta cDNA library in λgt11 (Clonetech) was screened using the plaque hybridization method (Maniatis et al., 1982). To isolate a cDNA encoding the β subunit of bovine FPTase, two overlapping, complementary oligonucleotides, 5′-ATCCAG-GCCACCACCCACTTCCTGCAGAAGCCT 3′ and 5′-CTCCTCAAAGCCAGGCACAGGCTTCTGCAGGAA-3′, were made on the basis of codon preference usage (Lathe, 1985) for the codons of the peptide IQATTHFLQKPVPGFEE from the β subunit of rat brain FPTase (Reiss et al., 1991b). These two oligonucleotides were annealed and filled in with the Klenow fragment of DNA polymerase and all four ^{32}P-labeled deoxynucleotide triphosphates for use as probes. Filters from plaque lifts of the bovine brain cDNA library were prehybridized and hybridized in 5× SSC, 10× Denhardt's solution [1× Denhardt's solution is 0.2 mg/mL bovine serum albumin, 0.2 mg/mL poly(vinylpyrrolidone), and 0.2 mg/mL Ficoll], and 0.1% (w/v) SDS at 50 °C overnight (Kohl et al., 1991). The filters were washed in 5× SSC at 50 °C and autoradiographed. To isolate cDNAs for the α and β subunits of human FPTase, DNA fragments from cDNAs encoding the α (Kohl et al., 1991) and β subunits of bovine FPTase cDNAs were labeled and used to probe a human placenta cDNA library. The filters were prehybridized and hybridized as above except at 65 °C. The filters were washed as above at 65 °C.

DNA Sequencing, DNA/Protein Sequence Analysis, and Recombinant DNA Methodology. DNA sequencing was performed using the Sequenase II dideoxy sequencing kit (U.S. Biochemical Corp.) as described by the manufacturer using plasmid DNAs as templates. DNA and protein sequence analysis was performed using the Genetics Computer Group software package (Devereux et al., 1984) and the multiple sequence alignment program CLUSTAL (Higgins & Sharp, 1988). DNA subcloning, PCR, and other DNA manipulations were performed as described (Maniatis et al., 1982; Saiki et al., 1988). The DNA sequences of the FPTase cDNAs were submitted to GenBank and were given Accession Numbers L00633 (bovine β subunit), L00634 (human α subunit), and L00635 (human β subunit).

Characterization of ram1.1, ram1.2, and ram2.1 Mutations in S. cerevisiae. DNA from *S. cerevisiae* strains RS40-4C (*MATα, ram1-1, his3, ade8, trp1, ura3, can1*), RS 41-2A (*MATα, ram1-2, his3, ade8, trp1, ras1::ura3, leu2, can1*), and RS51-3A (*MATα, ram2-1, his3, ade8, trp1, ura3, can1*) was isolated as follows. Yeast cells were pelleted, resuspended in water, and repelleted in a microcentrifuge tube. The cells were resuspended in 0.2 mL of 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 M NaCl, 1% SDS, and 2% Triton X-100. To this tube were added 0.2 mL of phenol and 0.3 g of 0.4–0.5-mm glass beads, and the cells were vigorously vortexed for 3 min. After addition of 0.2 mL of 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, the tube was spun in a microcentrifuge for 2 min. The supernatant was removed, and nucleic acids were precipitated with ethanol.

The mutant *RAM1* or *RAM2* genes were isolated from the DNA by PCR (Saiki et al., 1988) and then sequenced. The amino acid changes that these mutant alleles encode were abbreviated using the following notation: "D143K" denotes an Asp to Lys change at amino acid 143.

Table I: Human FPTase Nucleotide Sequences Used to Create Expression Plasmids

plasmid	insert DNA sequence[a]
pFPTase-$\beta\alpha$	*Eco*RI-ATG-β codons aa_{2-437}-GAG-GAG-TTT-TAA-ATTC-ATG-α codons aa_{2-379}-TAA-*Hin*dIII
pFPTase-$\beta\alpha_{opt1}$	*Eco*RI-TAAGGAGGA_8-ATG-β codons aa_{2-437}-GAG-GAG-TTT-TAA-CT-ATG-GCT-GCT-ACT-GAA-GGT-GTT-GGT-GAA-GCT-GCA-CAG-GGT-α codons aa_{14-379}-TAA-*Hin*dIII
pFPTase-$\beta\alpha_{met39}$	*Eco*RI-TAAGGAGGA_8-ATG-β codons aa_{2-437}-GAG-GAG-TTT-TAA-CTT-ATG-α codons aa_{40-379}-TAA-*Hin*dIII
pFPTase-β	*Eco*RI-ATG-β codons aa_{2-437}-GAG-GAG-TTT-TAA-TTAA-*Eco*RI-*Eco*RV-*Hin*dIII
pT5T-FPTase-α	*Bam*HI-A-TTG-GAG-GAT-GAT-TAA-ATG-GCT-GCT-ACT-GAA-GGT-GTT-GGT-GAA-GCT-GCA-CAG-GGT-α codons aa_{14-379}-GAG-GAG-TTT-TAA-TTAA-GAATTC-*Hin*dIII

[a] The indicated fragments are the sequences inserted into pBTac1 (pFPTase-$\beta\alpha$, pFPTase-$\beta\alpha_{opt1}$, pFPTase-$\beta\alpha_{met39}$, or pFPTase-β) or pT5T (pT5T-FPTase-α).

Expression of Human FPTase in E. coli. To obtain heterodimeric human FPTase in *E. coli*, the two subunits were expressed as a translationally coupled (Schoner et al., 1990) operon from the tac promoter of the plasmid pBTac1 (Boehringer-Mannheim, Indianapolis, IN). Translation coupling was achieved by placing the β-subunit coding sequence upstream of the α-subunit coding sequence. The codons for Glu-Glu-Phe, which were designed to contain an *E. coli* ribosomal binding site necessary for translational coupling and facilitate purification of the enzyme (see below), were incorporated at the C-terminus of the β-subunit coding sequence. Expression plasmids were made by inserting an *Eco*RI–*Hin*dIII fragment of the following general organization into pBTac1:

*Eco*RI-β codons aa_{1-437}-GA<u>G-GAG</u>-TTT-TAA-N_n-
Glu- Glu- Phe- stop
α codons aa_{1-379}-*Hin*dIII

The underlined GGAG sequence in the Glu-Glu-Phe codons is the ribosomal binding site (Shine & Dalgarno, 1974) for the α subunit. The DNA fragments for the plasmids were made by a combination of PCR (Saiki et al., 1988) and double-stranded oligonucleotide replacement of DNA sequences. The sequences of the *Eco*RI–*Hin*dIII fragments used to create these plasmids are shown in Table I. Expression levels of approximately 0.1–1.0% were achieved from the *E. coli* FPTase expression strains.

Site-directed mutations of specific codons of the α and β subunits of FPTase were introduced into pFPTase-$\beta\alpha$ by recombinant PCR (Higuchi, 1990). All PCR-generated mutations were completely sequenced to ensure that only the intended mutation was present. To express human FPTase, the derivatives of pBTac1 in Table I were transformed into *E. coli* DH5α and grown in Luria broth (Maniatis et al., 1982) plus 100 μg/mL ampicillin at 37 °C until the cultures were in the late log to early stationary phase of growth. The cells were harvested, and FPTase was purified as described below.

The individual β subunit of human FPTase, with the Glu-Glu-Phe epitope, was expressed from pFPTase-β, which is pFPTase-$\beta\alpha$ without the α coding sequence. The individual α subunit was expressed by putting the α-subunit coding sequence with the Glu-Glu-Phe epitope into the bacteriophage T7 promoter expression vector pT5T (Eisenberg et al., 1990), creating pT5T-FPTase-α (Table I). To make the α protein, the plasmid pT5T-FPTase-α was transformed into *E. coli* BL21(DE3) and grown in Luria broth plus 100 μg/mL ampicillin to A_{600} = 1.0, and the culture was induced with 1 mM isopropyl β-D-thiogalactoside for 3–4 h.

Protein Purification. Bovine brain FPTase was isolated as described (Pompliano et al., 1992). Wild-type and mutant human FPTase and the individual subunits were isolated by a modification of an immunoaffinity procedure using the antibody YL1/2 (Stammers et al., 1991). An *E. coli* cell pellet was resuspended in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 μg/mL leupeptin, 2 mg/mL antipain, and 10 μg/mL aprotinin (approximately 5 g of cells/10-mL solution). The resuspended cells were broken by sonication, and the cell debris was pelleted by centrifugation at 30000*g* at 4 °C for 30 min. The soluble fraction was diluted with an equal volume of 0.15 M NaCl and 6 mM sodium phosphate, pH 7.2 (1× PBS), and applied at a flow rate of approximately 0.5 mL/min to a 2-mL column of the monoclonal antibody YL1/2 coupled to cyanogen bromide activated Sepharose (4 mg of antibody/mL of resin). After the protein was loaded onto the column, the column was washed with 10–20 mL of $^1/_2$× PBS containing 2 mM DTT, 0.1% Tween-20, 1 mM PMSF, 2 μg/mL leupeptin, 2 μg/mL antipain, and 10 μg/mL aprotinin. The column was then washed with 100–200 mL of $^1/_2$× PBS containing 2 mM DTT. FPTase was eluted with 3 × 3 mL of 5 mM Asp-Phe dipeptide (Sigma), 100 mM Tris-HCl, pH 7.5, and 2 mM DTT. To further purify the FPTase, protein eluted from the antibody column was chromatographed by HPLC on a Mono Q HR10/10 column (Pharmacia), where buffer A was 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, and 5 mM DTT and buffer B was buffer A plus 1 M NaCl. The column was run at 1 mL/min with a gradient of 0–20% buffer B, 0–10 min; 20–40% buffer B, 10–40 min; and 40–100% buffer B, 40–60 min. FPTase holoenzyme eluted at approximately 30–35% buffer B. Recombinant human FPTase prepared in this way was of 70–90% purity as estimated by Coomassie blue stained SDS–PAGE.

SDS–PAGE analysis of proteins was performed as described (Laemmli, 1970). Protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin as a standard (Bradford, 1976).

Transferase Assays. FPTase activity was assayed in 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, 10 μM $ZnCl_2$, and 0.1% (w/v) poly(ethylene glycol) (av mol wt 20 000) with [^{3}H]farnesyl diphosphate (1–3000 nM) and Ras-Ca_1a_2X protein (20–10 000 nM) as substrates as described (Pompliano et al., 1992). Since some lot to lot variability of both protein and farnesyl disphosphate substrates was seen, the same lot of each substrate was used when different enzyme preparations are compared to one another. Measurements of reaction rates at low farnesyl diphosphate concentrations (1–20 nM) were difficult to determine accurately because the total amount of labeled substrate was low. Thus the error limits for FPP K_m determinations below 20 nM are minimum estimates. Steady-state kinetic parameters were calculated using the nonlinear analysis program k·CAT (Europa Scientific Software Corp., Hollis, NH) assuming a molecular mass of 93 kDa for FPTase and 21 kDa for Ras-Ca_1a_2X.

Isolated subunits of FPTase were reconstituted by mixing for 30 min at 37 °C in 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 10 μM $ZnCl_2$, 2 mM DTT, and 0.2% (w/v) *n*-octyl β-D-glucopyranoside.

Farnesyl Diphosphate Binding Assay, K_d Determinations, and Cross-Linking with the Photoaffinity Label [^{3}H]-(2-Diazo-3-trifluoropropionyloxy)geranyl Diphosphate. To analyze binding of farnesyl diphosphate to FPTase, the enzyme was incubated at 37 °C for 10 min in 100 μL of 50 mM HEPES, pH 7.5, 0.1 M NaCl, 5 mM $MgCl_2$, 2 mM DTT, and 0.2% (w/v) *n*-octyl β-D-glucopyranoside plus 500 nM [^{3}H]-farnesyl diphosphate. Protein-bound farnesyl diphosphate was determined by a modification of the method of Reiss et al. (1991b), in which separation of free from protein-bound farnesyl diphosphate was achieved by loading the binding reaction on a 0.9-mL Sephadex G-50 fine spin column equilibrated in 50 mM HEPES, pH 7.5, 0.1 M NaCl, 5 mM $MgCl_2$, 2 mM DTT, and 0.2% *n*-octyl β-D-glucopyranoside. The column was centrifuged at 2000*g* for 2 min. The protein-bound farnesyl diphosphate, eluted in the excluded volume, was counted after being mixed with 3 mL of ReadiSafe scintillation fluid (Beckman, Palo Alto, CA).

To determine the binding constant (K_d value) for farnesyl diphosphate, equal amounts of enzyme (3–10 nM) were incubated with varying concentrations (1–3000 nM) of [^{3}H]-farnesyl diphosphate, and the amount of enzyme-bound farnesyl diphosphate was determined as above. From a plot of bound vs free concentrations for farnesyl diphosphate and by use of the nonlinear regression program Enzfitter (Biosoft, Cambridge, U.K.), the K_d value for farnesyl diphosphate was determined.

The farnesyl diphosphate analog [^{3}H]-(2-diazo-3-trifluoropropionyloxy)geranyl diphosphate (DATFP-GPP) (Baba et al., 1985) was photo-cross-linked to purified human FPTase. Human FPTase (10 μg) was incubated in 200 μL of 50 mM HEPES, 1 mM DTT, 5 mM $MgCl_2$, and 10 μM $ZnCl_2$, pH 7.5, with 5 μM [^{3}H]DATFP-GPP (51 mCi/mmol). Some of the incubations also contained farnesyl diphosphate (12.5 μM) and/or the peptide CVFM (5 μM). Photo-cross-linking of the [^{3}H]DATFP-GPP to protein was performed by irradiating the enzyme mixtures for 5–10 min at 4 °C in a quartz cuvette with a 6-W germicidal lamp essentially as described (Baba & Allen, 1984). After irradiation, 15 μg of BSA was added to each enzyme preparation, the total protein was precipitated with 20% trichloroacetic acid, and the proteins were separated by SDS–PAGE on a 10% acrylamide gel. The gel was lightly stained with Coomassie blue, destained, treated with Enlightening (Du Pont, Boston, MA), and autoradiographed.

RESULTS

Cloning of the β Subunit of Bovine FPTase and Both Subunits of Human FPTase. Complementary DNAs encoding the complete coding regions of the β (deduced M_r of 48 800) subunit of bovine FPTase and both the α (deduced M_r of 44 400) and β (deduced M_r of 48 800) subunits of human FPTase were isolated as described under Materials and Methods. The amino acid sequence derived from the cDNA encoding the full-length α subunit of human FPTase was 97% identical to the homologous regions derived from a partial cDNA of the α subunit of bovine FPTase (Kohl et al., 1991) and 93% identical to the amino acid sequence of the full-length α subunit of rat FPTase (Chen et al., 1991b). The amino acid sequences derived from the cDNAs encoding the β subunits of human and bovine FPTase were >95% identical to the amino acid sequence of the β subunit of rat FPTase.

To look for regions that might be functionally conserved, the deduced amino acid sequences of the α subunits of human, bovine, and rat FPTase were aligned with the amino acid sequence of the *RAM2*-encoded subunit of FPTase from *S. cerevisiae* (Figure 1A). Similarly, the deduced amino acid sequences for the β subunits of human, bovine, and rat FPTase were aligned with the amino acid sequences of the *S. cerevisiae DPR1/RAM1*-encoded subunit of FPTase, the *CDC43/CAL1*-encoded subunit of GGPTase I, and the *ORF2/BET2*-encoded subunit of GGPTase II (Figure 1B). These alignments showed regions of cross-species homology among the subunits of mammalian and yeast prenyl-protein transferases. Some of these areas of homology may be important for enzyme activity as will be examined below.

Expression of Human FPTase in E. coli. Both subunits of human FPTase were expressed in *E. coli* from a single plasmid in a translationally coupled operon (Materials and Methods). The codons for the C-terminal epitope Glu-Glu-Phe were appended onto the β-subunit coding sequence to facilitate purification of human FPTase with the antibody YL1/2 (Stammers et al., 1991) and to provide a ribosomal binding site for translational coupling (Schoner et al., 1990; Shine & Dalgarno, 1974) of expression of the α subunit to that of the β subunit. Full-length heterodimeric human FPTase was initially expressed from pFPTase-βα (Table I). Although the α subunit has a predicted molecular weight (44 400) that is smaller than the predicted molecular weight of the β subnit (48 800), on SDS–PAGE the α subunit of recombinant human FPTase, like that of FPTase isolated from bovine or rat brain (Pompliano et al., 1992; Reiss et al., 1991b), migrated more slowly than the β subunit (Figure 2). The α subunit of the human FPTase expressed from pFPTase-βα was found to be composed of two species with apparent molecular weights of 49 000 and 48 000 as determined by SDS–PAGE (Figure 2, lane 3) and immunoblotting with subunit-specific antibodies (data not shown). N-Terminal sequence analysis showed that the larger species of the α subunit (α′) contained five additional N-terminal amino acids (C. A. Omer, D. Soderman, and K. Thomas unpublished results). These amino acids were derived from a fortuitous translational start near the C-terminus of the β-subunit coding sequence. The smaller species of the α subunit had the authentic N-terminus. To eliminate this heterogeneity, another expression plasmid was made. This plasmid, pFPTase-$\beta\alpha_{opt1}$, yielded a single α subunit (Figure 2, lane 4) and optimized expression of the α and β subunits by changing the ribosomal binding site for the β subunit and decreasing the G + C content of the first 13 amino acid codons of the α subunit (Table I). A plasmid that expressed FPTase with a shortened α subunit starting at Met_{39} was also made (pFPTase-$\beta\alpha_{met39}$) (Table I; Figure 2, lane 5). The smaller α subunit of the FPTase expressed from pFPTase-$\beta\alpha_{met39}$ migrated on SDS–polyacrylamide gel electrophoresis with an apparent molecular weight of 40 000, similar to its predicted molecular weight of 40 500.

The kinetic parameters (K_m and k_{cat}) of the different, recombinantly expressed human FPTases and FPTase isolated from bovine brain were determined (Table II). The values were within 2-fold of one another, indicating that the *E. coli*-expressed FPTase was similar to FPTase isolated from a natural source and that the N-terminus of the α subunit of FPTase does not contain determinants essential for activity.

Reconstitution of Human FPTase from Purified Subunits and Characterization of Farnesyl Diphosphate Binding. The function of individual human FPTase subunits was analyzed

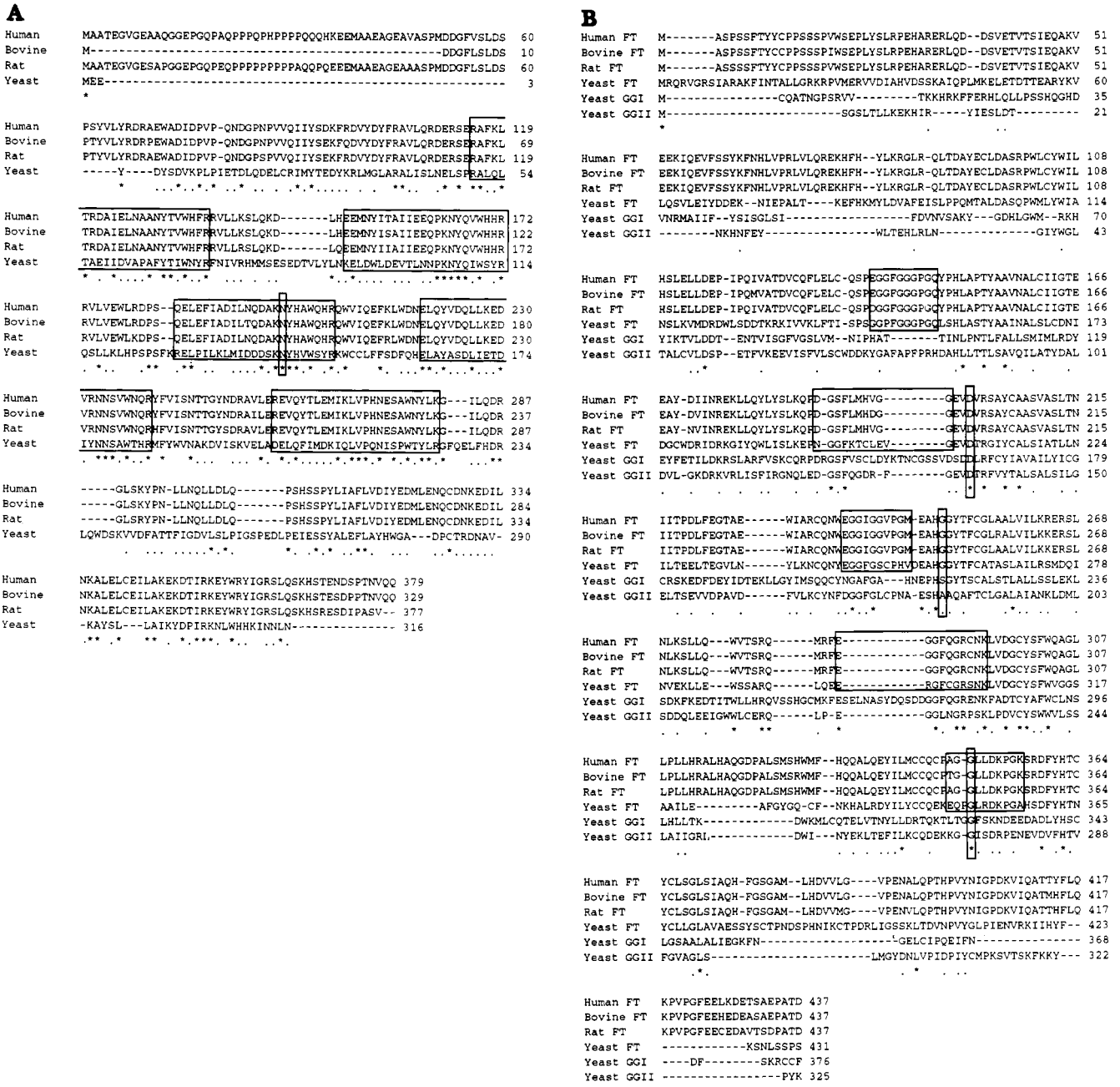

FIGURE 1: Alignment of prenyl-protein transferase subunit amino acid sequences. The amino acid sequences of the indicated proteins were aligned using the multiple sequence alignment program CLUSTAL (Higgins & Sharp, 1988). The single, boxed amino acids indicate positions where mutations have been identified in yeast prenyl-protein transferase subunit encoding genes (see Table IV). The boxed regions of the amino acid sequences indicate the core regions of repeats previously identified (Boguski et al., 1992). Asterisks indicate amino acids that are identical in the aligned sequences, and dots indicate amino acids where conservative changes are found. (A) The amino acid sequences of the human, bovine (Kohl et al., 1991), and rat (Chen et al., 1991b) α subunits of FPTase as deduced from their corresponding cDNAs were aligned with the amino acid sequence of the Ram2 subunit (yeast) of *S. cerevisiae* FPTase (He et al., 1991). (B) The amino acid sequences of the human FT, bovine FT, and rat FT FPTase β subunits (Chen et al., 1991a) as deduced from their corresponding cDNAs were aligned with amino acid sequences of the proteins encoded by the *DPR1/RAM1* (yeast FT) (Goodman et al., 1988), *CAL1* (yeast GGI) (Ohya et al., 1991), and *ORF2* (yeast GGII) (Peterson-Bjorn et al., 1990) genes.

by separately expressing and isolating the α and β subunits (Figure 2). Each protein was expressed with the C-terminal Glu-Glu-Phe epitope attached to facilitate its purification. The β subunit of FPTase was found to copurify with a major contaminant protein from *E. coli* of approximately 55–60 kDa that cochromatographed with the β subunit on both the immunoaffinity column and Mono Q (Figure 2). The contaminant protein did not react with specific antisera to either subunit of FPTase or to the YL1/2 antibody (data not shown). N-Terminal amino acid sequencing of the *E. coli* protein showed it to be the chaperonin GroE(L) (Hemmingsen et al., 1988) (P. Cameron and C. A. Omer, data not shown). Neither individual subunit bound farnesyl diphosphate (Table IIIA). When the two subunits were combined in vitro, they were capable of binding farnesyl diphosphate, indicating that the individual subunits were intact. As expected, neither FPTase subunit alone was catalytically active (Table IIIB). Incubating the isolated subunits together resulted in the formation of active enzyme; the small amount of activity of the isolated β subunit in the experiment shown was not seen reproducibly. Comparison of activity of FPTase reconstituted from isolated subunits with recombinant human FPTase holoenzyme showed that FPTase reconstitution was about 10% efficient (Table III).

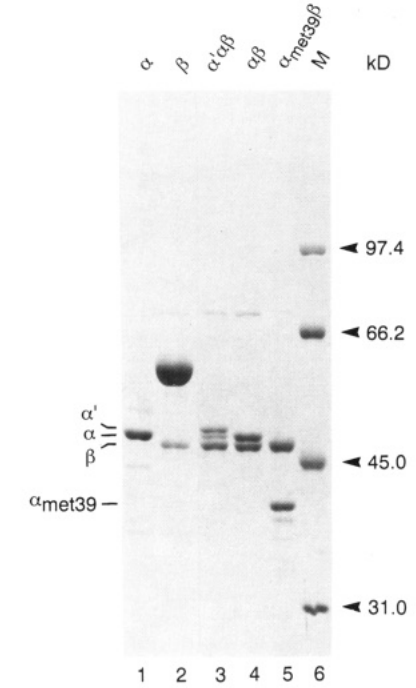

FIGURE 2: Human FPTase subunits and holoenzyme expressed in *E. coli*. Human FPTase holoenzyme and its subunits were isolated from *E. coli* strains by chromatography on a YL1/2 column and subsequent HPLC on Mono Q and then run on an 8% polyacrylamide SDS–PAGE. The human FPTase holoenzyme or subunits from strains containing the indicated plasmids are shown: lane 1, pT5T-FPTase-α (α); lane 2, pFPTase-β (β); lane 3, pFPTase-$\beta\alpha$ ($\alpha'\alpha\beta$); lane 4, pFPTase-$\beta\alpha_{opt1}$ ($\alpha\beta$); lane 5, pFPTase-$\beta\alpha_{met39}$ ($\alpha_{met39}\beta$); lane 6, molecular mass standards. The positions of the human FPTase subunits (α', α, α_{met39}, and β) are indicated as determined from immunoblot analysis with subunit-specific antibodies (data not shown). The approximately 55–60-kDa protein that copurified with the FPTase β subunit in lane 2 is the *E. coli* GroE(L) protein (Hemmingsen et al., 1988) (P. Cameron and C. A. Omer, data not shown). The molecular masses of the protein standards are indicated in kilodaltons (kD).

Table II: Kinetic Parameters for Recombinant Human FPTase

FPTase source[a]	FPP K_m (nM)	Ras-CVLS K_m (nM)	k_{cat} (s^{-1})
bovine brain	9.3 ± 5.8	620 ± 30	0.013
E. coli pFPTase-$\beta\alpha$	9.8 ± 1.7	392 ± 20	0.0096
E. coli pFPTase-$\beta\alpha_{opt1}$	12 ± 1.0	310 ± 44	0.011
E. coli pFPTase-$\beta\alpha_{met39}$	4.9 ± 0.7	420 ± 30	0.011

[a] FPTases were purified as described under Materials and Methods. Recombinant FPTases were as shown in Figure 2.

To further characterize farnesyl diphosphate binding to FPTase, we incubated the photoreactive farnesyl diphosphate analog [^{3}H]DATFP-GPP with FPTase-$\alpha_{met39}\beta$, a form of the enzyme in which the two subunits are readily resolved by SDS–PAGE (Figure 3). [^{3}H]DATFP-GPP has been shown to be an inhibitor of FPTase (Das & Allen, 1991) and has been used previously to photoaffinity label the farnesyl diphosphate utilizing enzyme undecaprenyl diphosphate synthetase (Baba et al., 1985). DATFP-GPP labeling of FPTase was observed on the β subunit (Figure 3). Labeling of the FPTase β subunit was dependent upon exposure to UV light and was competed by addition of nonradioactive farnesyl diphosphate. Labeling of the β subunit of FPTase by [^{3}H]-DATFP-GPP also occurred in the presence of the nonsubstrate inhibitor tetrapeptide CVFM (Figure 3, lane 5). Thus it appears that even though the β subunit of FPTase by itself cannot bind farnesyl diphosphate (Table III), the β subunit

Table III: Enzymatic Activity and FPP Binding of Isolated Subunits of Human FPTase

(A) Farnesyl Diphosphate Binding

α[a] (ng)	β[b] (ng)	FPP bound[c] (pmol)
900		<0.01
	900	<0.01
900	900	0.34 ± 0.05
200 ng of human FPTase $\alpha\beta$[d]		0.33 ± 0.06

(B) Farnesyl-Protein Transferase Activity

α[a]	β[b] (ng)	FPP incorpd[e] (pmol)/25 min
300		0.017 ± 0.013
	300	0.13 ± 0.01
300	300	13.9 ± 2.6
30 ng of human FPTase $\alpha\beta$[d]		10.2 ± 0.2
no enzyme		0.005 ± 0.001

[a] α subunits of human FPTase purified by immunoaffinity chromatography. [b] β subunits of human FPTase purified by immunoaffinity chromatography. [c] Net picomoles of farnesyl diphosphate (FPP) bound with blank value subtracted; mean of two determinations ± standard error. [d] Human FPTase isolated from *E. coli* DH5α pFPTase-$\beta\alpha_{opt1}$ ($\alpha\beta$). [e] Indicated proteins were incubated for 25 min at 30 °C in a 50-μL reaction volume with 0.5 μM [^{3}H]farnesyl diphosphate and 1 μM Ras-CVLS and assayed for [^{3}H]farnesyl diphosphate incorporation into Ras-CVLS; mean of two determinations ± standard error.

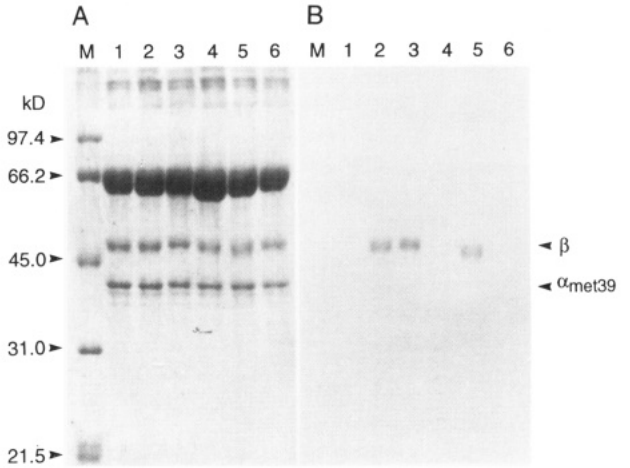

FIGURE 3: Photo-cross-linking of [^{3}H]-(2-diazo-3-trifluoropropionyloxy)geranyl diphosphate to FPTase. The $\alpha_{met39}\beta$ form of FPTase (10 μg, 0.56 μM) was photo-cross-linked to [^{3}H]DATFP-GPP (51 mCi/mmol, 5 μM) in the presence or absence of unlabeled farnesyl diphosphate (12.5 μM) and/or the peptide CVFM (1 μM) and acid precipitated in the presence of bovine serum albumin (15 μg), and then the subunits were separated by SDS–PAGE on a 10% polyacrylamide gel and autoradiographed. (A) Coomassie blue stained SDS–PAGE. (B) Autoradiograph of the gel in (A). Lanes: M, molecular mass standards; 1, no UV treatment; 2, 5-min UV; 3, 10-min UV; 4, 10-min UV in the presence of 12.5 μM farnesyl diphosphate; 5, 10-min UV in the presence of 1 μM CVFM; 6, 10-min UV in the presence of 1 μM CVFM and 12.5 μM farnesyl diphosphate. The molecular masses of the protein standards in kilodaltons (kD) and the positions at which the α_{met39} and β subunits of FPTase migrated are indicated.

of FPTase in the holoenzyme is associated with at least one end of farnesyl diphosphate.

Identification of Mutations in Yeast FPTase Subunit Encoding Genes and Kinetic Analysis of Homologous Mutations in Human FPTase. With the similarities seen among mammalian and *S. cerevisiae* prenyl-protein transferases (Figure 1), it seemed reasonable to model human FPTase site-directed amino acid changes after amino acid changes observed in mutant yeast prenyl-protein transferases. We sequenced two mutant alleles of *RAM1*, *ram1-1* and *ram1-2*, and the only known mutant allele of *RAM2*, *ram2-1* (Goodman

Table IV: Amino Acid Changes Identified in Mutant Alleles of the *S. cerevisiae* FPTase Encoding Genes *RAM1* and *RAM2*

allele	codon change	*S. cerevisiae* amino acid change	human FPTase homologous amino acid change
ram2-1	AAT → AAA	N143K	αN199K
ram1-1	GAT → AAT	D209N	βD200N
ram1-2	GGA → GTA	G259V	βG249V
cal1-1[a]	GGC → AGC	G328S	βG349S

[a] *cal-1* previously described (Ohya et al., 1991).

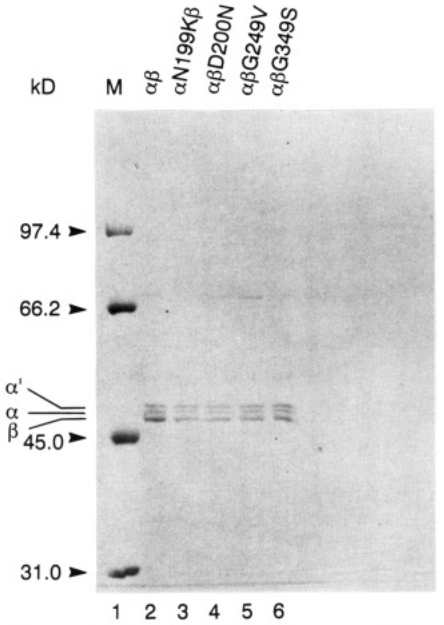

FIGURE 4: Expression of mutant forms of human FPTase. Human FPTase was isolated from *E. coli* strains expressing wild-type and mutant forms of FPTase. Approximately 0.5–1 μg of each purified FPTase was subjected to 7.5% SDS–polyacramide gel electrophoresis. Lanes: 1, molecular mass standards (M); 2, wild-type FPTase (αβ); 3, αN199Kβ FPTase (αN199Kβ); 4, αβD200N FPTase (αβD200N); 5, αβG249V FPTase (αβG249V); 6, αβG349S FPTase (αβG349S). The identity of the subunits (α', α, and β) is indicated as determined from immunoblot analysis with subunit-specific antibodies (data not shown). The molecular masses of the protein standards are indicated in kilodaltons (kD).

et al., 1990; Powers et al., 1986). A mutation in the *S. cerevisiae CAL1/CDC43* gene encoding the β subunit of GGPTase I had been previously sequenced (Ohya et al., 1991). The mutations in the yeast genes and homologous changes in the corresponding human FPTase subunits are shown (Figure 1, Table IV). As can be seen in the sequence alignments, the amino acids changed in the *ram1-1*, *cal1-1*, and *ram2-1* mutations are at residues conserved among the homologous protein-prenyl transferase subunits. The amino acid changed in the *ram1-2* mutation is conserved in the β subunit of the mammalian and yeast FPTases but not in the two yeast GGPTases.

Four mutant human FPTases were constructed by making amino acid substitutions homologous to those found in mutant yeast prenyl-protein transferases and were expressed in *E. coli* from pFPTase-βα. This plasmid expressed the complete α subunit of human FPTase as a doublet (α, α') along with the complete β subunit (Figure 4). All four mutant enzymes were purified on a YL1/2 antibody column, followed by HPLC on a Mono Q column (Figure 4). Gel electrophoresis of the purified enzymes showed all to be heterodimers similar to the wild-type enzyme. Since the Glu-Glu-Phe epitope for the YL1/2 antibody is only on the β subunit of the FPTase, the presence of the α subunit in the purified enzymes indicated that the two subunits were able to associate with one another. Additionally, when the two subunits were coexpressed in vitro in wheat germ extracts, the mutant subunits could be coimmunoprecipitated by antibody to the nonmutant subunit (data not shown). Thus the identified point mutations do not appear to impair subunit association.

The steady-state kinetic properties of wild-type human FPTase and the four mutant enzymes were analyzed (Table V). Michaelis constants (K_m), k_{cat} values, and catalytic efficiencies (k_{cat}/K_m) were determined for all enzymes for the substrates farnesyl diphosphate, Ras-CVLS (Harvey-Ras Ca_1a_2X), Ras-CVIM (Kirsten-Ras Ca_1a_2X), and Ras-CVLQ. For wild-type and the αN199Kβ human FPTase additional kinetic values were determined for the substrates Ras-CAIC, Ras-CAIM, and Ras-CAIS. All the mutations reduced FPTase catalytic efficiencies (k_{cat}/K_m) for protein or farnesyl diphosphate substrates (Table VC).

Human FPTase with an αN199K mutation had farnesyl diphosphate K_m values similar to those of the wild-type enzyme. For this mutant the K_m values for Ras-Ca_1a_2X proteins varied from near wild type (Ras-CVLQ) to greater than 20-fold higher than wild type (Ras-CAIC). The farnesyl diphosphate K_m was also determined in the presence of the two protein substrates (Ras-CAIC and Ras-CAIS) that showed K_m values the furthest from their wild-type values. In both cases the farnesyl diphosphate K_m value was similar to wild type, indicating that the mutation in αN199Kβ FPTase primarily affected Ras-Ca_1a_2X utilization.

Mutant FPTases containing either βD200N or βG349S were affected in their K_m values for Ras-Ca_1a_2X protein substrates, but the K_m values for farnesyl diphosphate were similar to the wild-type enzyme (Table VA). For the βD200N FPTase mutant, K_m values for protein substrates were approximately 6-fold (Ras-CVLS) to greater than 30-fold (Ras-CVIM) higher than the values observed with the wild-type enzyme. The βG349S FPTase mutant had K_m values for protein substrates 3-fold (Ras-CVIM) to 7-fold (Ras-CVLS) higher than the corresponding values for the wild-type enzyme.

Human FPTase with a βG249V mutation had K_m values for farnesyl diphosphate 10–100-fold higher than those for the wild-type enzyme, depending on the Ca_1a_2X substrate used (Table VA). The protein substrate K_m values for this mutant enzyme were also higher than those for wild-type human FPTase. The βG249V mutation, therefore, appears to affect utilization of both substrates by FPTase. To determine whether the increased farnesyl diphosphate K_m value observed in the αβG249V human FPTase was attributable to altered farnesyl diphosphate binding, we determined the farnesyl diphosphate K_d values for wild-type and mutant FPTases (Table VI). The K_d value for wild-type FPTase was similar to its K_m value. The farnesyl diphosphate K_d values determined for the four mutant human FPTases, including αβG249V, were all similar to that for wild-type FPTase. Thus the increase in the farnesyl diphosphate K_m value for the αβG249V FPTase was not due to decreased binding affinity and probably reflects a catalytic defect.

DISCUSSION

We have functionally expressed human FPTase in *E. coli* from plasmids that couple translation of the two subunits (Table I, Figure 2). The k_{cat} value for the *E. coli*-expressed enzyme was similar to that for FPTase isolated from bovine or rat brain (Reiss et al., 1990), and K_m values were similar to those determined for bovine brain FPTase (Table II). The

Table V: Kinetic Parameters for Wild-Type and Mutant Human FPTases[a]

substrate[c]	enzyme				
	$\alpha\beta$	αN199Kβ	$\alpha\beta$D200N	$\alpha\beta$G249V	$\alpha\beta$G349S
	(A) K_m (nM)[b]				
FPP_{CVLS}	9.5 ± 2.3	3.6 ± 0.8	4.9 ± 1.0	90 ± 14	10 ± 2
FPP_{CVIM}	6.2 ± 1.4	ND[d]	ND[d]	1700 ± 580	ND[d]
FPP_{CVLQ}	10 ± 3	5.9 ± 1.1	ND[d]	140 ± 40	ND[d]
FPP_{CAIC}	17 ± 3	8.6 ± 2.4	ND[d]	ND[d]	ND[d]
FPP_{CAIS}	13 ± 3	9.5 ± 1.7	ND[d]	ND[d]	ND[d]
Ras-CVLS	360 ± 50	840 ± 160	2000 ± 200	1400 ± 600	2600 ± 300
Ras-CVIM	140 ± 20	580 ± 90	4500 ± 600	630 ± 100	460 ± 90
Ras-CVLQ	350 ± 30	640 ± 120	11000 ± 700	2200 ± 800	1600 ± 100
Ras-CAIC	220 ± 30	4800 ± 600	ND[d]	ND[d]	ND[d]
Ras-CAIM	60 ± 10	240 ± 40	ND[d]	ND[d]	ND[d]
Ras-CAIS	330 ± 70	3700 ± 300	ND[d]	ND[d]	ND[d]
	(B) $10^2 k_{cat}$ (s^{-1})[b]				
Ras-CVLS	1.3 ± 0.5	0.062 ± 0.022	0.11 ± 0.032	0.048 ± 0.025	0.14 ± 0.04
Ras-CVIM	0.50 ± 0.25	0.21 ± 0.02	2.1 ± 0.1	0.092 ± 0.031	0.10 ± 0.004
Ras-CVLQ	0.85 ± 0.23	0.098 ± 0.042	0.66 ± 0.02	0.14 ± 0.01	0.16 ± 0.01
Ras-CAIC	1.4 ± 0.35	0.54 ± 0.01	ND[d]	ND[d]	ND[d]
Ras-CAIM	0.44 ± 0.02	0.33 ± 0.01	ND[d]	ND[d]	ND[d]
Ras-CAIS	1.6 ± 0.065	0.40 ± 0.14	ND[d]	ND[d]	ND[d]
	(C) $10^{-4} k_{cat}/K_m$ (mol^{-1} s^{-1})				
FPP_{CVLS}	140	17	22	0.53	14
FPP_{CVIM}	81	ND[d]	ND[d]	0.055	ND[d]
FPP_{CVLQ}	85	16	ND[d]	1.0	ND[d]
FPP_{CAIC}	82	63	ND[d]	ND[d]	ND[d]
FPP_{CAIS}	120	42	ND[d]	ND[d]	ND[d]
Ras-CVLS	3.7	0.074	0.055	0.034	0.054
Ras-CVIM	3.6	0.36	0.47	0.15	0.22
Ras-CVLQ	2.4	0.15	0.058	0.064	0.097
Ras-CAIC	6.2	0.11	ND[d]	ND[d]	ND[d]
Ras-CAIM	7.3	1.4	ND[d]	ND[d]	ND[d]
Ras-CAIS	4.9	0.11	ND[d]	ND[d]	ND[d]

[a] Enzymes used were wild-type ($\alpha\beta$) and four mutant human FPTases (αN199Kβ, $\alpha\beta$D200N, $\alpha\beta$G249V, and $\alpha\beta$G349S). [b] For all enzymes except the $\alpha\beta$G249V the error limits shown are derived from the nonlinear regression analysis program k-CAT. Since more variability was seen in kinetic values determined for the $\alpha\beta$G249V enzyme, the error limits shown are the mean error for two to four data sets. [c] Substrates tested: protein substrates (Ras-CVLS, Ras-CVIM, Ras-CVLQ, Ras-CAIS, Ras-CAIC, and Ras-CAIM) and farnesyl diphosphate (FPP). Protein substrates used in the farnesyl diphosphate K_m determinations are indicated as subscripts (i.e., $[FPP_{CVLS}]$). [d] ND, not determined.

Table VI: Farnesyl Diphosphate Binding Constant (K_d) Values for Wild-Type and Mutant Human FPTases

FPTase	farnesyl diphosphate K_d (nM)[a]
wild type $\alpha\beta$	11.1 ± 2.6
αN199Kβ	10.8 ± 3.6
$\alpha\beta$D200N	6.8 ± 2.5
$\alpha\beta$G249V	9.3 ± 3.0
$\alpha\beta$G349S	14.7 ± 2.8

[a] ±standard error as determined by the linear regression program EnzFitter for αN199Kβ and $\alpha\beta$G249V (one trial). For wild-type FPTase the error shown is from the average of three separate determinations; for $\alpha\beta$G349S and $\alpha\beta$D200N it is the mean of two determinations.

K_m and k_{cat} values of human FPTase were not affected by alterations of the N-terminus of the α subunit, including deletion of the amino-terminal 38 amino acids that are not present in the *S. cerevisiae* Ram2 protein (Table II; He et al., 1991). The anomalously slow migration of the complete α-subunit protein on SDS–polyacrylamide gel electrophoresis appeared to be due to the first 38 amino acids which contain a proline-rich region (Figure 2). Although the function of the region is presently unknown, its presence in both the rat (Chen et al., 1991b) and human α subunits of FPTase suggests it may have physiological importance in mammalian cells.

The homology among the prenyl-protein transferases was further demonstrated by conservation in the mammalian FPTase subunits of amino acid residues that were altered in mutant yeast prenyl-protein transferases (Figure 1, Table IV). Moreover, human FPTases with substitutions homologous to these yeast prenyl-protein transferase mutations showed kinetic defects (Table V). Mutations in either the α (N199K) or β (D200N, G249V, G349S) subunits of human FPTase affect K_m values for protein substrates. In addition, the βG249V mutation affected the K_m for farnesyl diphosphate. However, the increased farnesyl diphosphate K_m for the βG249V mutation appears to be a kinetic effect since farnesyl diphosphate binding of the $\alpha\beta$G249V mutant FPTase was similar to that of wild-type FPTase (Table VI).

The *S. cerevisiae ram1-1* and *ram1-2* alleles were selected in a screen involving yeast Ras2 which has a Ser in the X residue of the Ca_1a_2X box (Goodman et al., 1990; Powers et al., 1986). The temperature-sensitive phenotype at 37 °C of *ram1-1* but not *ram1-2* mutants of *S. cerevisiae* can be suppressed by overexpression of *RAS2* protein (Powers et al., 1986; S. Powers, unpublished results). This biological observation correlates with kinetic results seen with the homologous mutant human FPTases, since the βD200N (*ram1-1* like) human FPTase has increased K_m values for protein substrates and might be expected to be suppressed by overexpression of protein substrate. In contrast, the βG249V (*ram1-2* like) human FPTase has increased K_m values for both protein and farnesyl diphosphate substrates (Table VA). Thus overexpressing protein substrate alone might not be sufficient for suppression of the *ram1-2* mutation. An alternative possibility that we cannot rule out, however, is that the differences seen in suppressibility of *ram1-1* and *ram1-2* strains may also involve their ability to farnesylate other proteins, such as YDJ1 (Caplan et al., 1992), which in addition to Ras are necessary for high-temperature growth.

The k_{cat} values for the mutant human FPTases were generally 5–50-fold lower than for the wild-type enzyme (Table VB). An interesting exception, however, was the βD200N enzyme. With Ras-CVLS as the protein substrate the k_{cat} value was 10-fold slower than for wild-type FPTase, while with Ras-CVIM as the protein substrate the k_{cat} value was four times faster than for wild-type despite a K_m value 30 times higher than that observed with wild-type FPTase. The βD200N FPTase is, therefore, catalytically faster than wild-type FPTase when Ras-CVIM is used as the protein substrate, but the increased rate of catalysis is at the expense of a much higher Ras-CVIM K_m, resulting in an overall decreased catalytic efficiency (k_{cat}/K_m). We speculate that the βD200N mutation alters the Ca_1a_2X binding pocket of FPTase such that it is now in a better position or conformation for catalysis with Ras-CVIM but that this substrate binding pocket is less favorable for binding free Ras-Ca_1a_2X substrates or for catalysis with Ras-CVLS.

Although it cannot be totally discounted that the mutations examined disrupted protein structure over large regions of the proteins, we do not believe this to be the case. None of the mutations prevented subunit interaction since the purification of these heterodimeric proteins utilized an epitope tag present only on the β subunit, and the purified enzymes all contain similar relative amounts of the α and β subunits (Figure 4). Additionally, the four mutant FPTases have residual catalytic activity that would not be expected if large areas of protein structure were disrupted. We would, therefore, predict that the amino acids altered in these four mutant enzymes are at or near the substrate binding sites or the catalytic site of FPTase.

Recently, internal repeats have been identified in the amino acid sequences of the α and β subunit of FPTase and other prenyl-protein transferases (Boguski et al., 1992; Figure 1). The repeats in the two subunits of FPTase are distinct from one another, but each repeat contains a hydrophobic amino acid (Trp in α; Phe, Leu, or Ile in β) that by analogy to other gene families was implicated in protein–protein interaction of the two subunits (Boguski et al., 1992). The mutations examined here, although not in the hydrophobic amino acids thought to facilitate subunit association, are near or within the identified repeats. If the hypothesis that the hydrophobic amino acids within these repeats are responsible for subunit association in FPTase is correct, it would predict that the amino acids shown here to affect substrate utilization might also be near the interface of the α and β subunits.

Previous biochemical studies have led to a model for FPTase action in which the β subunit binds the Ca_1a_2X protein substrate and the α subunit binds farnesyl diphosphate (Reiss et al., 1991b, 1992). Our kinetic analysis of FPTase mutants suggests roles for both the α and β subunits in Ca_1a_2X protein substrate utilization. However, since kinetic (K_m) parameters were examined and not direct binding, we cannot unequivocally state that both subunits are involved in Ca_1a_2X recognition.

Photo-cross-linking of the farnesyl diphosphate analog [^{3}H]-DATFP-GPP to the β subunit of FPTase indicates a previously undetected role for this subunit in isoprenoid binding (Figure 3). Association of both subunits, however, is required for detectable binding of farnesyl diphosphate (Table III). Therefore, either both subunits are directly involved in isoprenoid binding or αβ subunit association is required for the β subunit to be in the proper conformation for farnesyl diphosphate binding. With our data we are not able to directly evaluate the role of the α subunit in farnesyl diphosphate binding. The involvement of the β subunit in isoprenoid binding, however, is consistent with the differential utilization of farnesyl diphosphate and geranylgeranyl diphosphate by FPTase and GGPTase I, which appear to share a common α subunit and have similar but distinct β subunits (Seabra et al., 1991; Moores et al., 1991; Kohl et al., 1991).

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SUPPLEMENTARY MATERIAL AVAILABLE

Detailed information about the construction of the plasmids used to express FPTase and individual FPTase subunits (6 pages). Ordering information is given on any current masthead page.

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