Human Geranylgeranyl Diphosphate Synthase
cDNA CLONING AND EXPRESSION*

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Geranylgeranyl diphosphate (GGPP) synthase (GGPPSase) catalyzes the synthesis of GGPP, which is an important molecule responsible for the C20-prenylated protein biosynthesis and for the regulation of a nuclear hormone receptor (LXR-RXR). The human GGPPSase cDNA encodes a protein of 300 amino acids which shows 16% sequence identity with the known human farnesyl diphosphate (FPP) synthase (FPFSase). The GGPPSase expressed in Escherichia coli catalyzes the GGPP formation (240 nmol/min/mg) from FPP and isopentenyl diphosphate. The human GGPPSase behaves as an oligomeric molecule with 280 kDa on a gel filtration column and cross-reacts with an antibody directed against bovine brain GGPPSase, which differs immunologically from bovine brain FPPSase. Northern blot analysis indicates the presence of two forms of the mRNA.

Since Schmidt et al. (1) first detected the incorporation of a mevalonate-derived intermediate into protein in Swiss 3T3 cells, a number of prenylated proteins have been found in various organisms (2–6). The prenylation of these proteins is essential for their function in the cells (7–9), and the direct precursors of the prenyl moiety have been elucidated to be farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) by identifying protein-prenyltransferases in yeast and mammalian brains (10). Further, the number of geranylgeranyl-ylated proteins has been shown to be larger than that of farnesylated proteins (11–13). Recently, these shorter chain prenyl diphosphates also have been shown to be involved in the function of nuclear hormone receptors. Forman et al. (14) have isolated a mammalian orphan receptor, FXR, which forms a heterodimeric complex with RXR and identified farnesol and isolated a mammalian orphan receptor, FXR, which forms a heterodimeric complex with RXR and identified farnesol and farnesylated proteins has been shown to be larger than that of

Considering that GGPP plays a role in the function of a nuclear hormone receptor and of geranylgeranylated proteins, it would be very important to characterize GGPPSase. Our original interest is in characterizing GGPPSase and FPFSase that occur in the same mammal or organ because GGPPSase could be regulated differently from FPFSase for their functions, although both of them catalyze the prenyl(C20)-transferring reaction. It is also interesting to learn the tissue-specific expression of GGPPSase because geranylgeranylated proteins have been reported to be rich specifically in brain (13). In the present study, we purified the GGPPSase of bovine brain in large amounts, isolated cDNAs of bovine and human GGPPSases using information from the bovine brain GGPPSase amino acid sequence, expressed the human GGPPSase in Escherichia coli, and characterized it. These analyses revealed that GGPPSase was a considerably different protein from FPFSase. Northern blot analysis indicated the presence of two mRNAs in various tissues.

EXPERIMENTAL PROCEDURES

Materials—[1-14C]IPP (55 mCi/mmol) and [a-32P]dCTP (6,000 Ci/mmoll) were purchased from Amersham Pharmacia Biotech. [1-3H] GGPP (20 Ci/mmol), and [1-3H]iGeranylgeranyl monophosphate (15 Ci/mmol) were purchased from American Radiolabeled Chemicals Inc.
Ex-Taq DNA polymerase was obtained from Takara Shuzou. Z,E,E-Geranylgeraniol was given by the Kuraray Corp. IPP, geranyl diphosphate (GPP), E, E-FPP, and E,E,E-GGPP were prepared as described in a previous report (26). Potato germ acid phosphatase (type I) was obtained from Sigma. All other chemicals were of reagent grade. Butyl-Toyopearl was obtained from Toyo Soda Co. Mono Q and Superdex 200 HR 10/30 were obtained from Amersham Pharmacia Biotech. Affinity gels with a geranylmethyl phosphonophosphate ligand and with a farnesylmethyl phosphonophosphate ligand were prepared by the method of Bartlett et al. (27). Bovine brain was obtained from a local slaughterhouse. Goat anti-guinea pig IgG heavy and light chain (alkaline phosphatase conjugate) were obtained from Bethyl Laboratories Inc. Guinea pig polyclonal antibodies were obtained by directing against purified bovine brain GGPPSase and FPPSase.

Table 1

| Steps            | Units | Protein | Specific activity | Yield | Purification |
|------------------|-------|---------|------------------|-------|--------------|
|                  |       | neq/min | nmol/min/mg      |       | % fold       |
| GGPPSase         |       | 40–60%  | NH₄SO₄         | 3,630 | 24,200       |
| Butyl Toyopearl  |       | 412     |                 | 1.38  | 115          |
| F-affinity       |       | 87.3    | 0.297           | 341   | 2.130        |
| Mono Q           |       | 2,430   | 0.16            |       | 100          |
| FPPSase          |       | 30–40%  | NH₄SO₄         | 15,200|              |
| G-affinity       |       | 775     | 0.725           | 1,070 | 31.9         |

a F-affinity, farnesylmethyl phosphonophosphate affinity chromatography.

b G-affinity, geranyl-methyl phosphonophosphate affinity chromatography.

Enzyme Assay—Bovine brain was homogenized with a Polytron homogenizer in 50 mm Tris-HCl buffer (pH 7.5) containing 0.1 mm leupeptin, 0.2 mm phenylmethylsulfonyl fluoride, 1 mm EDTA, and 1 mm EGTA. The crude homogenate was centrifuged at 14,000 × g for 30 min. The supernatant was fractionated with ammonium sulfate. For purification of GGPPSase, the fraction precipitating between 40 and 60% saturation of ammonium sulfate was subjected to Butyl-Toyopearl chromatography. The elution from a Butyl-Toyopearl column was performed with a downward linear gradient from 15 to 0% saturation of ammonium sulfate. The fractions containing GGPPSase activities were pooled, concentrated, and dialyzed against 10 mM Tris-HCl buffer (pH 7.0) containing 10 mM 2-mercaptoethanol and 1 mM MgCl₂. The dialyzed fraction was then applied to a affinity column with a farnesylmethyl phosphonophosphate ligand. The GGPPSase was eluted with a linear gradient of FPP to 0.5 mm. The active fractions were pooled and then applied directly to a Mono Q column and eluted with a linear gradient of NaCl to 0.5 m. The GGPPSase was eluted at 0.15 m NaCl. For purification of FPPSase, the fraction precipitating between 40 and 60% saturation of ammonium sulfate was dialyzed against 10 mM Pipes buffer (pH 7.0) containing 10 mM 2-mercaptoethanol and 1 mM MgCl₂. The dialyzed fraction was then applied to an affinity column with a geranylgeranyl phosphonophosphate ligand. The FPPSase was eluted with 1 m inorganic diphosphate. The active fractions were purified further by Mono Q chromatography.

Enzyme Assay—The standard assay mixture contained, in a final volume of 0.1 ml, 50 mm potassium phosphate buffer (pH 7.0), 2 mm dithiothreitol, 5 mm MgCl₂, 20 µm [1-14C]IPP, 25 µm GPP or FPP, and an appropriate amount of enzyme fraction. The mixture was incubated at 37 °C for 15 min and terminated by the addition of 0.3 ml of a mixture of concentrated HCl:methanol (4:1) followed by a 15-min incubation at 37 °C. The hexane-soluble hydrolysates were analyzed in a liquid scintillation fluid. For product analysis, the reaction products were extracted with 0.1 ml of 1-butanol saturated with water. The extracts were treated with potato acid phosphatase (2.4 units, 2.2 mg) according to the method of Fuji et al. (28). After incubation at 37 °C for 6 h, the hydrolysates were extracted with hexane and analyzed on a silica gel plate and a reverse phase C₁₈ plate in a solvent system of toluene:ethyl acetate (9:1) and in a solvent system of acetonitrile:water (7:1), respectively. The hydrolysates were also analyzed by the two-plate thin layer chromatography (TLC) method (29). The positions of authentic standards were visualized with iodine vapor. The radioactivity of polypropylene developed on TLC was determined with a Fuji Bioimage Analyzer BAS 1000.

SDS-PAGE and Immunoblotting—Proteins were analyzed on 10 or 14% polyacrylamide gels (1.0 mm) containing 0.1% SDS and transferred to a nitrocellulose membrane. FPPSase and GGPPSase were visualized using the specific antibodies and the secondary antibody conjugated to alkaline phosphatase.

Analysis of Amino Acid Sequence of Bovine Brain GGPPSase—The bovine brain GGPPSase preparation purified by affinity chromatography as described above was subjected to 10% SDS-PAGE and electro-transferred to a polyvinyliden difluoride membrane. The membrane corresponding to a Ponsau Red-positive GGPPSase band was cut off and analyzed by automated Edman degradation after treatment of lysyl endopeptidase. The sequences obtained were as follows: KAYR (fragment 1), HKLSK (fragment 2), KMFK (fragment 9), KTGQY (fragment 14), and KQIDARGGPN (fragment 15).

Amplification of DNA by PCR—the primers used for PCR were commercially synthesized: primer-1 was TCCCATGGAGAAGACTCAA; primer-2, ACTCA/AGGA/AGACAGT/ACGCTCA; primer-3, TTCTCG/TAGAA/CTTCAG/TTCT/ACAC; and primer-4, TTACTTATTA/CAATTCGAAA. The PCR mixture (50 µl) consisted of 0.5 ng of cDNA, 100 pmol of primers, 0.20 µl each deoxynucleotide triphosphate, and 1.25 units of Ex-Taq DNA polymerase. Thirty-five cycles of amplification (0.5 min at 94 °C for denaturation, 1 min at 50 °C for annealing, and 1 min at 72 °C for extension) were carried out using a Perkin-Elmer thermalcycler. The amplified products were analyzed on 1.4% agarose electrophoresis and recovered from the gels by use of a GeneElmer thermalcycler. The amplified products were analyzed on 1.4% agarose electrophoresis and recovered from the gels by use of a GeneElmer thermalcycler. The amplified products were analyzed on 1.4% agarose electrophoresis and recovered from the gels by use of a GeneElmer thermalcycler. The amplified products were analyzed on 1.4% agarose electrophoresis and recovered from the gels by use of a GeneElmer thermalcycler. The amplified products were analyzed on 1.4% agarose electrophoresis and recovered from the gels by use of a GeneElmer thermalcycler.

Expression in E. coli—The 919-base pair DNA containing human GGPPSase cDNA was isolated from pT7 blue T vector after double digestion with NcoI and BamHI, and the fragment was ligated into the NcoI and BamHI sites of a bacterial expression vector pDG-39A. E. coli JM109 cells transformed with the vector alone or with the plasmid containing human GGPPSase cDNA (pT7-HGG) were grown in 50 ml of LB medium to an A₆₅₀ of 0.5. Isopropyl 1-thio-ß-ß-galactosidase (IPTG) was added to a final concentration of 0.4 m, and after 2.5 h culture the cells were collected and suspended in 2.0 ml of TE buffer (pH 8.0). The cells suspensions were sonicated with a Branson sonifier before centrifugation at 14,000 × g to remove unbroken cells. The supernatants were frozen in aliquots for protein determination, SDS-PAGE, and GGPPSase assays.

RESULTS

Purification of Bovine Brain GGPPSase and No Cross-reactivity of an Anti-GGPPSase Antibody with FPPSase—In a previous report (25) we described a one-step purification of GGPPSase from bovine brain. The GGPPSase fraction still contained several minor proteins. We tried to improve the purification procedure with a combination of several chromatographies including affinity chromatography. As a result, Butyl-Toyopearl chromatography completely separated GGPPSase from FPPSase and IPP isomerase. This enabled us to prepare the purified GGPPSase with large amounts. Table I shows the entire purification steps of GGPPSase and FPPSase. The former enzyme (297 µg) and the latter enzyme (725 µg) were purified with specific activities of 294 and 1,070 nmol/min/mg from 6.01 kg.

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and 10.8 kg of bovine brain, respectively. Fig. 1A shows SDS-PAGE of the purified GGPPSase and FPPSase. The GGPPSase (35.0 kDa) migrated faster than the FPPSase (37.5 kDa), suggesting that the entire polypeptide chain length of GGPPSase is shorter than that of FPPSase unless a post-translationary modification is present. A polyclonal antibody directed against the GGPPSase did not recognize the FPPSase (Fig. 1B), and a polyclonal antibody directed against the FPPSase did not recognize the GGPPSase (Fig. 1C). The Western blot analysis of crude extracts did not show any positive protein bands with the anti-GGPPSase antibody. Only affinity-purified GGPPSase was recognized by the antibody, suggesting that the GGPPSase content in the brain is low and that the current antibody is either of low affinity or titer.

Cloning of GGPPSase cDNA—Edman analysis of the purified bovine brain GGPPSase suggested that the NH₂-terminal amino acid was blocked. Treatment of the enzyme protein with lysyl endopeptidase gave partial amino acid sequences of five fragments (fragments 2, 4, 9, 14, and 15). We searched various cDNAs registered in GenBank and found 22 partial human cDNA fragments as sequence homologs to a *Neurospora crassa* GGPPSase gene. Based on the sequence homology, we arranged the human cDNA fragments as shown in Fig. 2. The amino acid sequences KTQETVQRILLEPY (fragment 14) and KHLSKMFK (fragments 4 and 9) identified in bovine brain GGPPSase were found in the deduced amino acid sequence of these fragments. Based on the partial amino acid sequences of the bovine brain GGPPSase described above, we synthesized nucleotide primers (sense primer-2 and antisense primer-3).

PCR with primers-2 and -3 using human testis cDNA (CLONTECH) as a template gave an 875-base product. Sequence analysis revealed that this fragment contained the sequence of the partial human cDNA fragments picked up in Fig. 2. We synthesized sense primer-1 and antisense primer-4 con-
taining a starting codon (ATG) and a stop codon (TAA), respectively. As expected, PCR of human testis cDNA with primers-1 and -4 gave a 919-base product that covered an entire coding sequence of human GGPPSase.

On the basis of the similarity of the peptide sequence between NH2-terminal and COOH-terminal human and bovine GGPPSases, we performed a PCR with a combination of primers-1 and -4 using bovine liver cDNA as a template. Multiple PCR products were obtained in this case. However, further amplification of the multiple PCR products with a combination of the inner primers (primers-2 and -3) gave an 875-base product on agarose electrophoresis. The deduced amino acid sequence of human testis GGPPSase. The amino acid sequence of human testis GGPPSase and the similar properties of human GGPPSase to bovine GGPPSase in the substrate specificity as characterized previously (25). It should be emphasized that the cDNA sequence and the deduced amino acid sequence of human testis GGPPSase gene corresponds with a combination of FPP and [1-14C]IPP was enhanced about 40-fold in the expressed cells compared with the control cells as shown in Table II. The reaction products comigrated not only with the spot of geranylgeranyl monophosphate but also with that of GGPP on normal phase silica gel TLC (not shown). However, the hydrolysates by acid phosphatase treatment of the products were developed in isopropyl alcohol:ammonia:water (6:3:1). GGOH, geranylgeranyl (C20); FOH, farnesol (C15); and GPP, dimethylallyl diphosphate + [1-14C]IPP; lane 3, GPP + [1-14C]IPP; lane 4, FPP + [1-14C]IPP.

Expression of human GGPPSase in E. coli—To confirm that the human 919-base PCR product codes GGPPSase, we tried to express the protein and assay the enzyme activity. E. coli JM109 cells were transformed with the expression plasmid pTrc 99A containing the GGPPSase cDNA, and the cell extracts were subjected to SDS-PAGE. As shown in Fig. 3A, a polypeptide corresponding to 33 kDa was induced by the addition of IPTG. Western blot analysis showed that this polypeptide was specifically recognized by anti-bovine brain GGPPSase antibody (Fig. 3B). To determine whether the induced protein is active, the bacterial cell extracts were used as the enzyme source in GGPPSase assays. The enzymatic activity with a combination of FPP and [1-14C]IPP was enhanced about 40-fold in the expressed cells compared with the control cells as shown in Table II. The reaction products comigrated not only with the spot of geranylgeranyl monophosphate but also with that of GGPP on normal phase silica gel TLC (not shown). However, the hydrolysates by acid phosphatase treatment of the products were developed in isopropyl alcohol:ammonia:water (6:3:1). GGOH, geranylgeranyl (C20); FOH, farnesol (C15); and GPP, dimethylallyl diphosphate + [1-14C]IPP; lane 3, GPP + [1-14C]IPP; lane 4, FPP + [1-14C]IPP.

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to a polypeptide chain with molecular mass of 34,870 Da. The human GGPPSase only showed 16% sequence identity with the known human FPPSase (Fig. 4B) but 49.2% identity in the consensus sequences (I, II, III, IV, and V) proposed for trans-prenyltransferase. The total homology between the nucleotide sequences was 27.8%. The GGPPSase had three potential N-glycosylation sites.

### DISCUSSION

The original aim of our study was to learn how FPPSase and GGPPSase are different from each other because these enzymes catalyze a common isoprenyl-transferring reaction. Polyclonal antibodies directed against the two enzymes of bovine brain did not show any cross-reactivity with each other (Fig. 1). The immunochemical difference between these two proteins was confirmed with amino acid sequence comparison between the GGPPSase determined in this study and the known human FPPSase (30) (Fig. 4B). Five conserved amino acids were boxed.

### TABLE II

**GGPPSase activity in E. coli extracts**

| Plasmid      | IPTG | FPP | Specific activity | Relative specific activity |
|--------------|------|-----|------------------|---------------------------|
| pTrc 99A     | +    | +   | 1.80             | 1.00                      |
| pTrc-HGG     | +    | +   | 8.345            | 44.15                     |
| pTrc-HGG     | −    | +   | 0.340            | 1.80                      |
| pTrc-HGG     | −    | −   | 0.036            | 0.19                      |

**TABLE III**

**Distribution of enzymatic products**

| Allicy substrate | GPP | Products FPPa | GGPP |
|------------------|-----|---------------|------|
| DMAPP + [14C]IPP | 0   | 0             | 380  |
| GPP + [14C]IPP  | 0   | 561           | 2616 |
| FPP + [14C]IPP  | 0   | 0             | 2244 |

* β-Ray intensity (PSL) of the radioactivity corresponding to GPP (C15), FPP (C15), and GGPP (C20) on the normal-phase plate of Fig. 3E, was measured with a Fuji BAS 1000 bioimage analyzer.
* DMAPP, dimethylallyl diphosphate.

**FIG. 4.** Primary structure of human GGPPSase. Panel A, cDNA and deduced amino acid sequences of human GGPPSase. The stop codon is TAA (*). The potential N-glycosylation sites are underlined. Panel B, comparison between human GGPPSase and FPPSase (P14524). Five conserved amino acids in the two enzymes are boxed. Asterisks indicate the potential N-glycosylation sites in the GGPPSase sequence.
Acid motifs (I, II, III, IV and V) common to trans-prenyltransferases reported (18) were observed between the two enzymes, but the entire identity was only 16%. The human GGPPSase has three potential N-glycosylation sites, whereas human FPPSase has no such sites (30). Any potential N-glycosylation site is also not observed in the amino acid sequences of FPPSase of rat (19). It is not clear at present whether the GGPPSase is N-glycosylated. Human GGPPSase shows extremely high homology to other GGPPSases of bovine, Drosophila, and N. crassa (Fig. 6). Three potential N-glycosylation sites observed in human GGPPSase are also found in the bovine enzyme, and two of them are conserved in the insect enzyme, although two Asn-X-Ser/Thr motifs are found at different sites in the N. crassa enzyme.

Sheares et al. (20) reported that the expression of human FPPSase in Hep G2 cells is transcriptionally regulated: lovastatin, a potent inhibitor of HMG-CoA reductase, increased the level of the mRNA, whereas cholesterol in its 25-hydroxylated form or in low density lipoprotein particles reduced the amount of the mRNA, and mevalonate also decreased the mRNA levels. Similar regulation of HMG-CoA synthase, HMG-CoA reductase and low density lipoprotein receptor had been already reported (16), suggesting a common control mechanism for these enzymes. Recently, Guan et al. (31) described differential, transcriptional regulation of the human gene encoding squalene synthase, which accepts FPP as a substrate, by variation in the level of cellular cholesterol. Concerning GGPPSase, which also accepts FPP as a substrate, the study on regulation is far behind those of the enzymes described above. Lutz et al. (32) have demonstrated that GGPP synthesis is specifically inhibited by GGPP, suggesting that the cellular GGPP pool may be regulated by product inhibition of GGPPSase. Our in

FIG. 5. Tissue distribution of mRNA for human GGPPSase. Northern blots (CLONTECH) containing approximately 2 μg of poly(A)+ RNA/lane from 16 different human tissues were used. Hybridization was done at 68 °C for 1 h with a 32P-labeled cDNA probe (2 × 106 cpm/ml) for human GGPPSase. Filters were washed three times in 2 × SSC containing 0.05% SDS at room temperature for 10 min and twice in 0.1 × SSC containing 0.1% SDS at 50 °C for 20 min and exposed to an imaging plate overnight at room temperature. As a loading control, the same filters were then reprobed with random primed 32P-labeled oligonucleotides of human β-actin cDNA (2 × 106 cpm/ml). The reprobed filters were exposed for 4 h at room temperature (lower panel). Size markers are indicated by the arrows. Muscle, skeletal muscle; Intestine, small intestine; Leukocyte, peripheral blood leukocyte. kb, kilobases.

FIG. 6. Comparison of the deduced amino acid sequence of the human GGPPSase with several other GGPPSases. Human, human GGPPSase; Bovine, a partial sequence of bovine brain GGPPSase (T. Kuzuguchi, and H. Sagami, unpublished results); Drosophila, Drosophila melanogaster GGPPSase (AF049659); Neurospora, N. crassa GGPPSase (P24322). Consensus sequences shown below the five highly conserved sequence domains (I–V) proposed for trans-prenyltransferase by Chen et al. (18) are underlined. The numbers of amino acids are indicated on the left. Boxes enclose identical amino acids in at least three of the four sequences compared. Asterisks indicate the potential N-glycosylation sites in the human GGPPSase sequence.
vitro experiments using a purified bovine brain GGPPSase supported this regulation (25).

GGPP acts as a precursor for the C20 lipid moiety in prenylated proteins and also as a modulator for nuclear hormone receptor (LXRα-RXR) complex formation. In the present study two mRNAs of GGPPSase were identified. Arrangement of several partial cDNA sequences registered in GenBank (Fig. 2) further implies the presence of more than two GGPPSase mRNAs. Therefore, the GGPP synthesis might be regulated at the transcriptional level of GGPPSase. Rilling et al. (11) have reported the content of geranylgeranylated proteins in mouse tissues; these proteins are severalfold rich in brain compared with those in liver, kidney, and lung. In human tissues, two GGPPSase mRNAs were also observed in those tissues. Although they did not describe the content of geranylgeranylated proteins in mouse heart, skeletal muscle, and testis, much higher contents of human GGPPSase mRNAs were detected in these tissues. Two kinds of protein geranylgeranyl transferase responsible for the synthesis geranylgeranylated proteins have been found and characterized in mammals. On Northern blot analysis for rat protein geranylgeranyl transferase II consisting of α and β subunits (component B) and an escort protein (component A) (33, 34), the α and β subunits mRNAs are abundant in heart and much lower muscle and testis. The escort protein mRNA is also abundant in heart, much lower in muscle, and not detectable in testis. In the case of the α subunit of rat protein geranylgeranyl transferase I (35), which is the same protein as that of protein farnesyl transferase, the amount of the mRNA is rather low in heart and muscle, whereas the amount in testis is severalfold higher than in any other tissue. It would be difficult at present to explain the reason why the mRNA levels of these enzymes directly related to GGPP metabolism are regulated differently especially in these tissues.

It is very important to establish how many genes encode GGPPSase, which gene is transcribed in each tissue, and how each gene is regulated. FPPSase has been reported to be encoded by multiple genes (19, 20). Further analysis of mammalian GGPPSase will contribute to the understanding of a finely tuned mechanism of mevalonate pathway. Sepp-Lorenzino et al. (36) reported cell cycle-dependent differential prenylation of proteins. LXRs has been reported to display constitutive transcriptional activity that is negatively regulated with GGPP (15). It remains unclear how GGPP inhibits LXRs and contributes to signaling pathways. It would be also very interesting to know the regulation of GGPP formation in the process of embryonic development and cell differentiation.