High Level Expression of Mammalian Protein Farnesyltransferase in a Baculovirus System

THE PURIFIED PROTEIN CONTAINS ZINC*

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The mammalian enzyme protein farnesyltransferase is a heterodimeric protein that catalyzes the addition of a farnesyl isoprenoid to a cysteine in ras proteins. Since oncogenic forms of ras proteins require the farnesyl group for transforming activity, the structure and mechanism of this enzyme are important to define. However, such studies have been difficult to approach because of the low abundance of the enzyme in mammalian tissues and hence the problems of obtaining large quantities of the enzyme, particularly in its homogenous form. In the present study, we report here the expression of two subunits of protein farnesyltransferase by Sf9 cells infected with a recombinant baculovirus containing the coding sequences of both polypeptides. This results in the production of milligram quantities of enzyme which can be readily purified by conventional chromatographic methods. The individual subunits of the enzyme can also be expressed in the Sf9 cells, but the ability to reconstitute active enzyme from extracts containing individual subunits is quite low. In contrast, the enzyme produced by co-expression of the two subunits is fully active and retains the properties of the mammalian form, including the specificity for the COOH-terminal amino acid of substrate proteins and the ability to bind short peptides encompassing the prenylation site of a ras protein. Furthermore, through atomic absorption analysis of the purified protein, we have confirmed the previous tentative assignment of protein farnesyltransferase as a zinc metalloenzyme by demonstrating that it contains an essentially stoichiometric amount of zinc. The ability to produce and purify milligram quantities of protein farnesyltransferase readily will allow detailed mechanistic and structural studies on this enzyme.

The enzyme protein farnesyltransferase (PFT) catalyzes the formation of a thioether linkage between a farnesyl group donated by farnesylyl pyrophosphate and a cysteine residue at the COOH terminus of protein substrates. Previously identified substrates include the ras proteins, oncogenic forms of which require the farnesyl group for transforming ability, the nuclear lamins A and C, and the γ subunit of GTP-binding protein transducin (1–3). The cysteine that is farnesylated is the 4th residue from the COOH terminus in the consensus sequence termed Cys-AAX, where A can be any of several amino acids, and X is generally either methionine or serine (1, 4, 5).

PFT has been purified to homogeneity from both rat brain and bovine brain cytosol using peptide affinity chromatography (2, 5). The enzyme is composed of two nonidentical subunits, designated α and β, with apparent molecular weights of approximately 48,000 and 46,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The complete amino acid sequence of both subunits has recently been deduced from their cloned cDNAs (6–8). The cDNA sequences revealed that the α and β subunits are the mammalian homologues of the yeast RAM2 (9) and the DPR1/RAM1 (10, 11) gene products, respectively, which have been implicated in the processing of ras proteins in this organism. When the cDNAs encoding the PFT subunits were introduced into human embryonic kidney 293 cells by transfection, neither of the individual subunit cDNAs produced significant PFT activity. However, the enzymatic activity was markedly enhanced when the cells were simultaneously transfected with cDNAs encoding both the α and β subunits, suggesting that both subunits are required for enzyme activity (6, 7). These same studies demonstrated that co-expression of both subunits is also necessary for the cells to accumulate detectable quantities of either subunit as assessed by immunoblot analysis. These data suggest that either subunit alone is unstable in the cells in the absence of its counterpart.

PFT is apparently a zinc metalloenzyme. Both enzymatic activity and binding of the protein substrate to the enzyme are abolished by EDTA treatment of the enzyme, and both activities are restored by the addition of Zn²⁺ (12, 13). However, it has been difficult to demonstrate directly that the purified enzyme contains zinc. The mechanistic properties of PFT and its individual subunits are just beginning to be elucidated. A recent steady-state kinetic analysis has shown the reaction mechanism to be random order sequential, with either substrate (protein, farnesyl diphosphate) able to bind independently to the enzyme (14). This property is consistent with the ability of the protein to form a complex with farnesyl diphosphate in the absence of protein substrate (12) and the above-mentioned successful application of peptide substrate affinity chromatography. The binding site for protein (and peptide) substrates has been assigned to the β subunit on the
basis of cross-linking experiments (15). The function of the α subunit is not known but has been proposed to play a role in the binding of prenyl diphosphate (15). Interestingly, the α subunit of the PPT appears to be identical to that of the closely related protein geranylgeranyltransferase, the enzyme responsible for the addition of the 20-carbon geranylgeranyl isoprenoid to candidate proteins containing the Cys-AAX motif (13, 16).

Efforts to study the detailed mechanism and structure of the PPT have been hampered by the limited availability of purified enzyme. To overcome this obstacle, we have employed a baculovirus expression system to produce the enzyme. The baculovirus expression system has been used to produce a variety of mammalian proteins (17), including multisubunit proteins by multiple infection with recombinant viruses (18).

In this report, we describe the production and characterization of fully active PPT in insect Sf9 cells infected with a single recombinant baculovirus that simultaneously expresses both the α and β subunits of the enzyme. The recombinant protein was subsequently used to determine definitively that the PPT is a zinc metalloenzyme.

Experimental Procedures

Plasmid Construction—Plasmids pUC-PFTA and pUC-PFTB containing the entire coding region of the rat PPT α and β subunits, respectively, were obtained from American Tissue Culture Collection (ATCC 63134 and 63127). Baculovirus expression vector pAcUW51 was purchased from PharMingen (San Diego, CA). pAcUW51 contains a copy of the p10 gene promoter and SV40 transcription termination signals inserted in the opposite orientation upstream of the polyhedrin gene promoter. A 1.5-kilobase DNA fragment containing the entire coding region of the PPT α subunit was excised from pUC-PFTA, blunt ended with DNA polymerase Klenow fragment, and ligated into BamHI-cut and blunt-ended pAcUW51. The resulting plasmid is designated pAcUW-A. Similarly, a 2.4-kilobase EcoRI fragment from pUC-PFTB containing the entire coding region for the PPT β subunit was removed and inserted into EcoRI-cut pAcUW51 to create pAcUW-B. Insertion of this 2.4-kilobase EcoRI fragment from pUC-PFTB into EcoRI-cut pAcUW-A created pAcUW-A+B. In this latter construct, expression of the α subunit is controlled by the polyhedrin promoter, whereas β subunit expression is independently controlled by the p10 promoter.

Production of Recombinant Virus—The Sf9 cell line was obtained from the American Type Culture Collection, maintained in Grace's medium (GIBCO), supplemented with 3.3 μg/ml lactalbumin hydrolysate (Difco), 3.3 pg/ml yeastolate (Difco), 10% fetal calf serum (HyClone Laboratories, Logan, UT), 50 μg/ml gentamicin (GIBCO), and 0.1% Pluronic F-68 (GIBCO) in 125-ml Spinner flasks (Techne, Princeton, NJ).

To generate recombinant baculovirus, Sf9 cells (2 × 10⁷) were transfected with 0.5 μg of BaculoGold wild-type viral DNA (PharMingen) and 2 μg of either pAcUW-A (for PPT α subunit expression), pAcUW-B (for PPT β subunit expression), or pAcUW-A+B (for expression of the PPT dimer) using a cationic liposome transfection kit according to the manufacturer's instructions (Invitrogen; San Diego, CA). The virus from each transfection was harvested after 4 days and screened using a plaque assay as described by Summers and Smith (19). Recombinant viruses obtained from this screen were subjected to two further rounds of plaque purification.

Analysis of PPT Subunit Expression in Sf9 Cells—The three purified recombinant viruses were used to infect three independent cultures of Sf9 cells at a multiplicity of infection of 2. Cells were harvested ~48 h postinfection by centrifugation at 800 × g for 15 min. The cells were washed once with phosphate-buffered saline and the resulting cell pellet flash-frozen in a dry ice/ethanol bath. Cell extracts were prepared by thawing the cell suspension in 5 volumes of 20 mM Tris-Cl, pH 8.0, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, incubating the suspension on ice for 1 h, followed by disruption using four strokes of a Dounce homogenizer. The resulting extract was centrifuged for 1 h at 30,000 × g, and the supernatant (density gradient fraction) was stored at −80 C. Protein concentrations were determined using the immuno blot experiments described in Figs. 1 and 2.

Production and Purification of PPT—The purified recombinant virus obtained containing the cDNA sequences for both PPT subunits (i.e. that produced from pAcUW-A+B, see above) was used to infect 1 liter of Sf9 cells at a multiplicity of infection of 2. Cells were harvested ~60 h postinfection, and an extract was prepared as described above for the smaller cultures. The soluble extract obtained from this procedure was loaded onto a 5.0 × 10.0-cm column of Sephacryl S-200 HR and processed previously for the partial purification of bovine brain PPT (13). The two subsequent steps, Q-HP and phenyl-Sepharose chromatography, were also performed as for the bovine brain preparation except that the buffers contained no added ZnCl2. The peak fractions obtained from the phenyl-Sepharose step were concentrated and flash-frozen in aliquots containing ~3 mg of protein for the final processing step, which involved chromatography on a high resolution phenyl-Superose HR 5/5 column (Pharmacia LKB Biotechnology Inc.). A 3-mg aliquot from the phenyl-Sepharose fraction was thawed, supplemented with 4 mM KCl to a final concentration of 1.5 M, and loaded onto the phenyl-Superose column. The column was then washed with 5 ml of 20 mM Tris-Cl, pH 8.0, 1 mM dithiothreitol (buffer A) containing 1.5 M KCl, and the enzyme eluted with a 20-ml linear gradient of KCl decreasing to zero in buffer A, followed by a 10-ml wash with buffer A. Fractions of 0.5 ml were collected. The PPT eluted as a single peak from the KCl gradient and in the first few fractions of the buffer A wash. Fractions were monitored both by PPT activity and by SDS-PAGE analysis. The peak fractions, containing essentially homogeneous PPT, were concentrated with buffer exchange into buffer A using a CentriPrep 30 concentrator (Amicon) to a protein concentration of ~1 mg/ml, flash-frozen in aliquots, and stored at −80 C.

Materials—Unlabeled farnesyl diphosphate was obtained from Du Pont-New England Nuclear. The zinc standard solution (1 mg/ml) was purchased from Perkin-Elmer Cetus Instruments (Boston, MA). A 3-mg aliquot from the phenyl-Sepharose fraction was thawed, supplemented with 4 mM KCl to a final concentration of 1.5 M, and loaded onto the phenyl-Superose column. The column was then washed with 5 ml of 20 mM Tris-Cl, pH 8.0, 1 mM dithiothreitol (buffer A) containing 1.5 M KCl, and the enzyme eluted with a 20-ml linear gradient of KCl decreasing to zero in buffer A, followed by a 10-ml wash with buffer A. Fractions of 0.5 ml were collected. The PPT eluted as a single peak from the KCl gradient and in the first few fractions of the buffer A wash. Fractions were monitored both by PPT activity and by SDS-PAGE analysis. The peak fractions, containing essentially homogeneous PPT, were concentrated with buffer exchange into buffer A using a CentriPrep 30 concentrator (Amicon) to a protein concentration of ~1 mg/ml, flash-frozen in aliquots, and stored at −80 C.

Miscellaneous Methods—SDS-PAGE was carried out as described (22). Molecular mass determinations were made by calibration of the gel with protein standards (Bio-Rad). Immunoblot analysis was performed using antisera Y-536, a polyclonal rabbit antibody directed against a peptide sequence—(WENQ[W])—of the bovine PPT β subunit (a gift from C. Omer, Merck). Immunoblot analysis was performed as described previously (23), except that detection was via a commercial alkaline phosphatase-based second antibody method (Promega).

Protein assays were routinely performed by the Bradford method (24) using a commercial dye preparation (Bio-Rad). For the determination of the zinc stoichiometry, the protein was also assayed using the Amido Black dye-binding method (25) and the values obtained from the two methods averaged. Bovine serum albumin was the protein standard in both cases.

Materials—Unlabeled farnesyl diphosphate was obtained from USF Research Foundation. [1-3H]Farnesyl diphosphate (15–20 Ci/mmoll was obtained from Du Pont-New England Nuclear. The zince standard solution (1 mg/ml) was purchased from Perkin-Elmer Cetus Instruments. Peptides were synthesized on the Applied Biosystems Synergy synthesizer and purified by high performance liquid chromatography prior to use.
RESULTS

Infection of Sf9 cells with the recombinant baculovirus containing DNA sequences encoding both PFT subunits resulted in a dramatic enhancement of the PFT activity measured in soluble extracts prepared from transfected cells (Fig. 1). Comparison of the PFT activity in the soluble fraction of extracts from either uninfected cells or those infected with wild-type baculovirus showed a >200-fold increase in the enzymatic activity toward farnesylation of H-ras substrate. We also infected Sf9 cells with recombinant viruses containing the coding sequences of either the α or β subunits, respectively, of PFT. Cells infected with the virus containing individual subunits accumulated the encoded subunit to a level similar to that seen in cells infected with the virus containing both subunits (Fig. 2, upper panel). However, the PFT activity in extracts of cells expressing individual subunits was essentially identical to that of cells infected with wild-type virus, i.e. there was no appreciable increase in PFT activity by expression of only one subunit of the enzyme (Fig. 2, lower panel). Notably, however, when extracts of Sf9 cells expressing the α and β subunits were mixed, a ~5-fold enhancement of PFT activity was observed (Fig. 2, lower panel inset), providing evidence that the functional oligomer can be reconstituted from the individual subunits, albeit much less efficiently than by co-expression of the two subunits.

Previous purification strategies for the PFT from extracts of mammalian brain (the tissue with the highest activity of the enzyme) required the use of peptide affinity columns because of the low abundance of the enzyme; the typical yield from this approach is 5–10 μg of enzyme from 150 g of brain tissue (2). The high level expression of PFT by the recombinant baculovirus-infected Sf9 cells resulted in the production of enzyme that could be purified readily from the high speed supernatant of cell extracts by use of commercially available resins, without having to synthesize an affinity resin. Chromatography of the soluble extract from these cells expressing both subunits of the enzyme on a series of four ion-exchange and hydrophobic resins resulted in the purification of the characteristic PFT heterodimer, with subunits of 48 and 46 kDa visualized on SDS-PAGE analysis (Fig. 3). The purification protocol is summarized in Table I and shows that ~5 mg of purified PFT can be obtained from a 1-liter culture of Sf9 cells co-expressing the two subunits of the protein. We have recently scaled this procedure up to a 5-liter culture and have purified ~20 mg of the enzyme from this preparation (results not shown).

The recombinant PFT purified from the Sf9 cells retains
the specificity properties of the native enzyme. The specific activity of the purified enzyme (Table I) is essentially identical to that reported for the rat brain enzyme (2). It should be noted that this is the first time that the specific activity of this enzyme has been determined using a standard protein assay to determine protein concentration; previous estimates of the enzyme’s specific activity were based on protein determination from intensity of silver staining on SDS-PAGE, as the quantities of protein obtained from native tissue did not allow direct assay of the protein concentration (2).

Mammalian PFTs (and those from other sources) exhibit a high degree of specificity for the COOH-terminus residue of their substrate proteins. The most prevalent are serine and methionine, and both of these residues are found at the COOH terminus of members of the ras protein family. Fig. 4a shows that the Sf9-produced PFT retains this specificity, showing high activity toward the H-ras protein (which has a serine at the COOH terminus) while exhibiting very low activity toward the same protein with a substitution of leucine for serine at this position (ras-CVLL). Instead, we have shown previously that the ras-CVLL protein is a good substrate for the protein geranylgeranyltransferase, which prefers substrates containing the COOH-terminal leucine residue (21).

The Sf9 produced PFT also retains the ability to recognize short peptides encompassing the Cys-AAX motif of ras proteins, an observation first noted for the rat brain enzyme (2). Fig. 4b shows that the hexapeptide that corresponds to the COOH terminus of the K-ras protein is a potent competitor for the reaction, with an $EC_{50}$ of $\sim 0.3 \mu M$, whereas the same peptide containing a switch of leucine for methionine at the COOH terminus or a switch of cysteine for serine at the prenylation site is essentially inactive in competing for PFT activity. Also, the tetrapeptide Cys-Val-Phe-Met, a potent inhibitor of PFT which is inactive as a substrate for the enzyme (26), also potently inhibits PFT produced in Sf9 cells.

Taken together, the activity and specificity data shown in Fig. 4 demonstrate that the recombinant PFT produced in the Sf9 cells retains the specificity properties of the enzyme purified from mammalian tissues.

Mammalian PFT has been shown to be dependent on zinc atoms for activity (12, 13). Demonstration of the zinc dependence of the enzyme requires dialysis against, or purification in the presence of, buffers containing EDTA. Based on this evidence, PFT has been designated a zinc metalloenzyme. However, because of the limited quantities of enzyme available from mammalian tissue, there has been as yet no direct demonstration that the purified enzyme actually contains zinc. Such a demonstration is important as it remains possible that the enzyme contains a metal other than zinc but that zinc can reconstitute the activity of the metal-depleted enzyme. Consistent with previous results with PFT purified from mammalian sources, treatment of the Sf9-produced PFT with EDTA essentially abolished the activity of the enzyme in a fashion that could be restored by the addition of micromolar levels of zinc (Fig. 5). Two other divalent metals, calcium and cobalt, were ineffective in restoring the activity of EDTA-treated PFT when tested under the same conditions. We then analyzed the purified PFT produced in the Sf9 cells by atomic absorption spectroscopy to determine whether in fact it did contain zinc. Fig. 6 shows the results of the analysis, which reveals a zinc content of the enzyme of $>0.9 \text{ mol/mol}$ enzyme. In contrast, the EDTA-treated enzyme contains essentially none of this metal. These results demonstrate that purified PFT does contain essentially a stoichiometric amount of zinc, and they confirm the importance of this metal in activity of the enzyme.

**DISCUSSION**

We have demonstrated the utility of the baculovirus expression system in the production of heterodimeric PFT. The availability of milligram quantities of this enzyme will make possible detailed structural and mechanistic analysis of this important enzyme. This expression system is also ideally suited for analysis of mutant forms of the protein produced for structure-activity studies, as it should allow ready production and purification of the altered proteins for detailed studies. Such analysis of the PFT is important, as results from a number of studies suggest that specific inhibitors of enzymatic farnesylation of the ras proteins may be useful in therapeutic intervention of ras-connected human cancers (27, 28).

Expression of cDNAs encoding the individual subunits of PFT in Sf9 cells also resulted in production of substantial quantities of these polypeptides. This is in contrast to results of similar expression studies in mammalian cells, in which the individual subunits could not be produced, apparently because each was unstable in the absence of its partner (6). However, co-incubation of extracts of Sf9 cells expressing the individual subunits showed only marginal reconstitution of

**TABLE I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein</th>
<th>PFT activity (nmol h$^{-1}$)</th>
<th>Yield (%)</th>
<th>Specific activity (nmol h$^{-1}$ mg$^{-1}$)</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble extract</td>
<td>660</td>
<td>7,660</td>
<td>100</td>
<td>11.6</td>
<td>1.0</td>
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<tr>
<td>DEAE-Sepharose</td>
<td>120</td>
<td>4,610</td>
<td>60</td>
<td>38.4</td>
<td>3.3</td>
</tr>
<tr>
<td>Q-HP</td>
<td>27.4</td>
<td>3,230</td>
<td>42</td>
<td>118</td>
<td>10</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>8.2</td>
<td>2,500</td>
<td>33</td>
<td>276</td>
<td>24</td>
</tr>
<tr>
<td>Phenyl-Superose</td>
<td>1.3*</td>
<td>660*</td>
<td>28</td>
<td>510</td>
<td>44</td>
</tr>
</tbody>
</table>

* 2.5 mg of the phenyl-Sepharose pool was processed over the phenyl-Superose column.

**Fig. 3. SDS-PAGE analysis of PFT purification from Sf9 cell extracts.** Aliquots of the pools obtained from each of the processing steps in purification were resolved on a 9% cross-linked gel. Lane 1, 35 μg of soluble extract; lane 2, 25 μg of the DEAE pool; lane 3, 25 μg of the Q-HP pool; lane 4, 5 μg from the phenyl-Sepharose pool; lane 5, 2 μg of the final phenyl-Superose step; lane 6, molecular mass standards. The gel was stained with Coomassie Blue.
active PFT. Envisioned possibilities for this include: 1) only a small fraction of one or both of the individual subunits have adopted a reconstitution-competent conformation, or 2) the PFT dimer can form but has a very poor enzymatic activity. If the former possibility is the case, it may be possible to resolve the "active" species by chromatographic or other methods. This would allow analysis of the individual subunits with regard to such properties as isoprenoid and protein substrate binding and zinc content. Cross-linking studies with radiolabeled substrate peptides have suggested that the primary interaction with this substrate is with the \( \beta \) subunit (15), but no information is yet available on the isoprenoid binding site of the enzyme. The finding that the \( \alpha \) subunit of PFT is apparently identical to that of the related enzyme, protein geranylgeranyltransferase (13, 16), has prompted...
speculation that this subunit may play a regulatory role in the activities of both these enzymes. Further information on the properties of the individual subunits of PFT would be useful both in understanding the mechanism of this enzyme and in potential modulation of PFT activity by subunit interactions.

The unambiguous identification of PFT as a zinc metalloenzyme provides the necessary base to begin efforts to ascertain the role of the metal ion in catalysis by this enzyme. Approaches combining metal substitution, spectroscopic analysis, and mutagenesis analysis have proven quite useful in analyzing other zinc metalloenzymes such as carboxypeptidase A (29). Our initial results show that cobalt, the initial candidate for metal substitution analysis, cannot appreciably restore activity to zinc-depleted enzyme when examined under the same conditions in which added zinc does restore activity. More detailed analysis will be required to determine if other metals, other experimental conditions, or a combination of the same conditions in which added zinc does restore activity.

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REFERENCES