Involvement of the α Subunit of Farnesyl-Protein Transferase in Substrate Recognition

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ABSTRACT: Using photoaffinity labeling, we have identified a region in mammalian farnesyl-protein transferase (FPTase) involved in substrate recognition. The photolabel used (Compound 1) is a peptide containing the photoactive amino acid *p*-benzoylphenylalanine (Bpa). Upon exposure to UV light, Compound 1 inhibits FPTase activity in a time- and concentration-dependent manner. Photoinhibition of FPTase activity by Compound 1 is prevented by adding H-Ras to the reaction mixture, indicating that labeling is targeted to the enzyme active site. We used peptide mapping by HPLC, Edman sequencing, and matrix-assisted time-of-flight (MALDI-TOF) mass spectrometry to identify the site of interaction with radiolabeled Compound 1. These experiments indicate that a specific region of the α subunit of the enzyme, Asp110−Arg112, is involved in substrate binding and suggest that Glu111 is likely to be the residue covalently modified by the photoaffinity label. Sequence alignments between yeast and mammalian FPTases reveal that Glu111 is conserved. The implications of this finding are discussed in light of previous mutagenesis studies on FPTase.

Prenylation is a form of posttranslational modification of intracellular proteins involving the covalent attachment of an isoprenoid group to carboxyl-terminal cysteine residues of protein acceptors. The addition of either a farnesyl (15-carbon) or a geranylgeranyl (20-carbon) group appears to be required for the biological activity of a variety of proteins involved in signal transduction and intracellular trafficking [reviewed in Clarke (1992)]. Protein prenylation may be involved in mediating protein−protein interactions and in the association of proteins with intracellular membranes (Glomset et al., 1990; Cox & Der, 1992; Casey & Seabra, 1996).

The enzymes that catalyze protein prenylation have been recently characterized: farnesyl-protein transferase (FPTase) (Reiss et al., 1990), geranylgeranyl transferase type I (GGTase I) (Moomaw & Casey, 1992; Yokoyama & Gelb, 1993), and geranylgeranyl transferase type II (GGTase II) (Seabra et al., 1992; Armstrong et al., 1993). FPTase and GGTase I are heterodimers with identical α subunits but distinct β subunits (Seabra et al., 1991). Both prenyl transferases recognize a sequence motif, designated the CAAX motif (C, Cys; A, usually aliphatic amino acid; and X, another amino acid), that is found at the C terminus of the protein substrate. CAAX tetrapeptides comprise the minimum region required for the interaction of the protein substrate with enzyme (Reiss et al., 1990). The last residue of the CAAX motif specifies which isoprenoid is attached. It has been shown, that if X is S, M, C, A, or Q, the protein is a substrate for FPTase and a farnesyl group is attached. There may be other C-terminal residues that dictate farnesylation that have not yet been identified. If X is L or F, a geranylgeranyl group is added by GGTase I (Moores et al., 1991; Casey et al., 1991; Yokoyama et al., 1991). Subsequent to prenylation of proteins containing the CAAX motif, other enzymes catalyze the proteolytic removal of the three C-terminal AAX amino acids and the carboxymethylation of the newly exposed prenylcysteine (Glomset & Farnsworth, 1994). In contrast, GGTase II catalyzes the transfer of a geranylgeranyl group to proteins containing CXC or CC motifs (Farnsworth et al., 1994).

Among the protein substrates of prenyl transferases, substantial attention has been focused on the small GTPases of the Ras superfamily. Mutated forms of Ras genes are among the most prevalent genetic abnormalities associated with human cancers (Rodenhuis, 1992). Because the farnesyl modification on Ras oncoproteins appears to be necessary for transforming activity (Casey et al., 1989; Jackson et al., 1990; Kato et al., 1992), there has been considerable interest in developing inhibitors of FPTase as anticancer agents. Indeed, specific FPTase inhibitors can reverse the transformation of cells by oncogenic forms of Ras (Kohl et al., 1993; James et al., 1993) and have been shown to suppress the growth of tumors arising from Ras-transformed cells in animal models (Kohl et al., 1994, 1995).

Mechanistic studies of FPTase have been facilitated by purification of the enzyme (Reiss et al., 1990) and by isolation of the cDNA sequences of both subunits (Chen et al., 1991a,b; Omer et al., 1993). Kinetic and biochemical studies indicate that the reaction proceeds through a random steady-state mechanism but with a strong tendency for farnesyl pyrophosphate (FPP) to bind to the enzyme prior to prenyl acceptor protein (Furfine et al., 1995). Both Mg^{2+} and Zn^{2+} are required to support farnesylation (Reiss et al.,

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1992), though it is not yet clear whether Zn^{2+} plays a structural or catalytic role. Previous cross-linking studies indicate that both protein−substrate (Ying et al., 1994; Reiss et al., 1991; Andres et al., 1993) and isoprenoid−donor (Omer et al., 1993; Bukhtiyarov et al., 1995) cross-link to the 46 kDa β subunit. Similarly, a photoaffinity analog of geranylgeranyl pyrophosphate (GGPP) specifically labels the β subunit of GGTase I (Yokoyama et al., 1995). The 49 kDa α subunit of FPTase is known to be important for catalysis (Andres et al., 1993), although the contribution of each subunit to substrate binding and catalysis is not completely understood. Presently, there is no information available as to which residues comprise the active site of the enzyme. Such knowledge could provide groundwork for the design of inhibitors with higher potency and/or specificity. In the present study, we used an active site-directed photoaffinity label to identify amino acid regions of FPTase involved in substrate binding and catalysis. We demonstrate that the photolabel specifically cross-links to the Asp110−Arg112 region in the α subunit.

MATERIALS AND METHODS

Materials. ScintiVerseII scintillation fluid was obtained from Fisher. [^{3}H]Farnesyl pyrophosphate (FPP) (15−20 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO) and DuPont NEN. Trypsin was purchased from Sigma. Glass fiber filters (#34, 2.5 cm) were obtained from Schleicher & Schuell. Ras-CVLS protein was expressed and purified as described previously (Moores et al., 1991).

Synthesis of Benzyl[1-[2-(4-Benzoylphenyl)-1-[[2-(benzyloxy)ethyl]carbamoyl]ethyl]carbamoyl]-2-(1H-imidazol-4-yl)-ethyl]carbamate (Compound 1). A solution of BOC-L-Phe-(4-Bz)-OH (Bachem; 0.25 g, 0.68 mmol), [2-(benzyloxy)-ethyl]amine hydrochloride (0.13 g, 0.68 mmol), dicyclohexylcarbodiimide (0.17 g, 0.81 mmol), 1-hydroxybenzotriazole (0.11 g, 0.81 mmol), and triethylamine (0.094 mL, 0.68 mmol) in dichloromethane (10 mL) was stirred at room temperature for 16 h. Ethyl acetate (12 mL) was added, and the resulting slurry was filtered. The filtrate was diluted with ethyl acetate (100 mL), washed with saturated sodium bicarbonate (1 × 35 mL), washed with brine (1 × 35 mL), dried ($MgSO_4$), and concentrated. The residue was chromatographed on silica gel (1:1 hexane−ethyl acetate) and concentrated. The resulting oil was triturated with ethyl acetate and filtered. The filtrate was concentrated to yield a semisolid, *N*-BOC-(4-Bz)L-Phe-NH[2-(phenylmethoxy)-ethyl] (0.29 g, 85% yield): 300 MHz ^{1}H NMR (CDC13) δ 7.76 (m, 4H), 7.60 (m, 1H), 7.47 (m, 2H), 7.31 (m, 7H), 6.39 (br s, 1H), 5.04 (br s, 1H), 4.46 (s, 2H), 4.37 (m, 1H), 3.45 (m, 4H), 3.15 (m, 2H), 1.41 (s, 9H). Without further purification, a solution of *N*-BOC-(4-Bz)L-Phe-NH[2-(phenylmethoxy)ethyl] (0.28 g, 0.56 mmol) in ethyl acetate [10 mL, saturated with HCl(g)] was stirred at room temperature for 16 h. The resulting solution was concentrated to an oil. Ether (20 mL) was added, and the resulting solution was concentrated to yield a foam, *N*-BOC-(4-Bz)L-Phe-NH[2-(phenylmethoxy)ethyl]·HCl (0.25 g, quantitative). Without further purification, a solution of *N*-BOC-(4-Bz)L-Phe-NH-[2-(phenylmethoxy)ethyl]·HCl (0.25 g, 0.57 mmol), CBZ-D-His-OH (0.17 g, 0.57 mmol), dicyclohexylcarbodiimide (0.14 g, 0.68 mmol), 1-hydroxybenzotriazole (0.092 g, 0.68 mmol), and triethylamine (0.079 mL, 0.57 mmol) in dichloromethane (5 mL) was stirred at room temperature for 16 h. The resulting solution was diluted with chloroform (100 mL), washed with saturated sodium bicarbonate (1 × 30 mL), washed with brine (1 × 30 mL), dried ($MgSO_4$), and concentrated. The residue was chromatographed on silica gel (99:1 chloroform−methanol and then 95:5 chloroform−methanol) to yield the title compound as a white powder (0.29 g, 76% yield): mp 113−115 °C; 400 MHz ^{1}H NMR ($CDCl_3$) δ 7.75 (d, J = 7.2 Hz, 2H), 7.61 (m, 3H), 7.46 (m, 2H), 7.40 (s, 1H), 7.29 (m, 13H), 7.05 (br s, 1H), 5.03 (d, J = 12.06 Hz, 1H), 4.70 (br s, 1H), 4.45 (br s, 1H), 4.43 (s, 2H), 3.49 (m, 2H), 3.38 (m, 2H), 3.15 (m, 3H), 2.86 (dd, J = 6.51 Hz, 14.95 Hz, 1H); ESMS 674 (MH^+). Anal. Calcd for $C_{39}H_{39}N_5O_6$: C, 69.52; H, 5.83; N, 10.39. Found: C, 69.11; H, 5.87; N, 10.36.

Synthesis of [^{3}H]Compound 1. CBZ-D-[^{3}H]His was prepared from [*ring*-^{3}H]His (Moravek Biochemicals) by treatment with dibenzyl dicarbonate and sodium hydroxide, as described (Geiger & König, 1981). [^{3}H]Compound 1 (molecular mass = 673.78 Da) was made by coupling CBZ-D-[^{3}H]His and 4-benzoyl-L-phenylalanine [2-(phenylmethoxy)-ethyl]amide with 1,3-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole hydrate in methylene chloride, as described above for the nonradiolabeled compound. The crude reaction product was purified by reverse phase HPLC on a C18 preparative column. The final material dissolved in DMSO had a specific activity of 22.9 mCi/mmol; the radiochemical purity was 97.3%.

Purification of FPTase. Recombinant rat brain FPTase was overexpressed in Sf9 cells using a baculovirus system (Chen et al., 1993; Scholten et al., 1996). Purification steps were carried out at 4 °C. Sf9 cell pellets were resuspended in homogenization buffer [20 mM Tris (pH 7.5), 50 mM NaCl, 20 μM $ZnCl_2$, and 1 mM DTT] and lysed in a French pressure cell at 700 kpsi. Following centrifugation for 45 min. at 100000*g*, proteins in the supernatant were precipitated with 55% ammonium sulfate. The ammonium sulfate precipitate was centrifuged for 20 min at 20000*g*. The pellet was then resuspended in homogenization buffer and dialyzed against 4 L of the same buffer. The sample was then subjected to ion exchange chromatography on a Mono Q HiLoad 26/20 (Pharmacia Biotech) column and affinity chromatography on a TKCVIM-agarose column as previously described (Reiss et al., 1990).

The affinity column for FPTase purification was prepared by mixing 12.8 mg of CNBr-activated CH-Sepharose with 26 mg of the peptide Thr-Lys-Cys-Val-Ile-Met in 43 mL of coupling buffer [100 mM $NaHCO_3$ and 500 mM NaCl (pH 8.2)] for 4 h with constant stirring at room temperature. The resin was then washed with 300 mL of buffer C (50 mM Tris-HCl, 100 mM NaCl, and 1 mM DTT) and poured into a column. The column was stored in 20 mM Tris-HCl (pH 7.5) and 0.02% sodium azide at 4 °C.

FPTase Assays. Activity assays were carried out according to previously described procedures (Omer et al., 1993; Pompliano et al., 1992). The assay medium contained 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 20 μM $ZnCl_2$, 5 mM DTT, 0.1% (w/v) polyethylene glycol (average molecular weight = 20 000), 1−2 μM H-Ras-CVLS, and 0.5 μM [^{3}H]-FPP. Reactions were initiated by adding FPTase (0.1 μg) to the assay medium (200 μL) and incubating at 37 °C. After 15−30 min, the reactions were stopped by addition of 1 mL of 10% HCl in ethanol. The samples were then incubated

for 10 min at room temperature, diluted into 2 mL of absolute ethanol, and vacuum filtered through glass fiber filter disks. Filters were rinsed four times with 3 mL of ethanol and counted in scintillation fluid.

Photoaffinity Labeling. Duplicate samples were irradiated in borosilicate tubes at 4 °C in a Rayonet RMR-500 instrument fitted with RMR 350 nm lamps (Southern New England Ultraviolet Co., Hamden, CT), as described previously (Miller, 1991). Samples containing FPTase (0.8 nM), Compound 1, 10−20% DMSO, and 50 mM HEPES (pH 7.5) in a volume of 50 μL were clamped 2 cm from the lamps. After various times of photolysis, 12.5 μL aliquots were withdrawn and assayed for FPTase activity. Graphs shown in the Results are representative of experiments performed at least twice with similar results.

Identification of Modified Sites. FPTase was purified to homogeneity as before except that PEG was omitted from the affinity column wash and elution buffers. Fractions eluting from the affinity column containing FPTase (20 mL) were pooled and concentrated using an analytical HR 5/5 Mono Q column (Pharmacia Biotech). The sample was loaded in 20 mM Tris (pH 7.5) and eluted using a 30 min gradient of 0 to 1.2 M NaCl in the same buffer. FPTase ($\alpha\beta$ heterodimer) eluted at approximately 0.5 M NaCl under these conditions. The resulting material, 1.1 mg (11 nmol) of FPTase in 2 mL, was subsequently dialyzed against 25 mM HEPES (pH 7.5) and 100 mM NaCl. The dialysate was adjusted to 23% DMSO, 5 mM EDTA, and 80 μM [^{3}H]-Compound 1, and the mixture was irradiated for 30 min. A portion of this sample was subjected to SDS−PAGE with Coomassie Blue staining. Bands corresponding to the α and β subunits of FPTase were excised, solubilized in H_2O_2 (55 °C, 2.5 h), and analyzed by liquid scintillation counting. The remainder of the reaction mixture was dialyzed to remove unreacted label and small photoproducts. Enzyme in the dialysate was precipitated by the addition of trichloroacetic acid to a final concentration of 10% (w/v) and resuspended in 8 M urea and 0.4 M NH_4HCO_3. After addition of DTT to a final concentration of 4 mM, the sample was incubated for 15 min at 50 °C, cooled to room temperature, and treated with iodoacetamide (8.3 mM final concentration). After 15 min at room temperature, the sample was diluted 2.3-fold and treated with trypsin (1% w/w) for 24 h (Matsudaira, 1989).

The resulting tryptic digest was analyzed by reverse phase HPLC on a Vydac C18 column with mobile phases of (A) 0.1% (v/v) TFA and (B) 75% (v/v) acetonitrile in 0.09% TFA. The flow rate was 1 mL/min. The following gradient program was used: 5 min at 2% B, 60 min at 2−38% B, 30 min at 38−75% B, 15 min at 75−98% B, 5 min at 98% B. The elution profile was monitored by absorbance at 220 nm. Fractions were collected and analyzed by scintillation counting. Fractions containing peaks of absorbance and radioactivity were analyzed by mass spectrometry, and then rechromatographed on a Vydac C4 column using the same gradient as above. Selected peaks were analyzed by mass spectrometry and by Edman sequencing using an Applied Biosystems Model 475 A pulsed liquid protein sequencer. Prior to sequencing, the samples were concentrated onto an Ultrafree device containing Immobilon CD membranes (Millipore) by centrifugation.

Mass Spectrometry. Determination of peptide masses was accomplished using a ProteinTOF matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Bruker Analytical Systems, Inc.). Data were acquired using the positive reflectron ion mode. Sinapinic acid and α-cyano-4-hydroxycinnamic acid in a mixture of 0.1% TFA and 30% acetonitrile were used as matrices.

Sequence Alignment. Sequence alignments of rat, human, bovine, and yeast α subunits of farnesyl-protein transferase were performed using the multiple-alignment program Clustal W (Thompson et al., 1994).

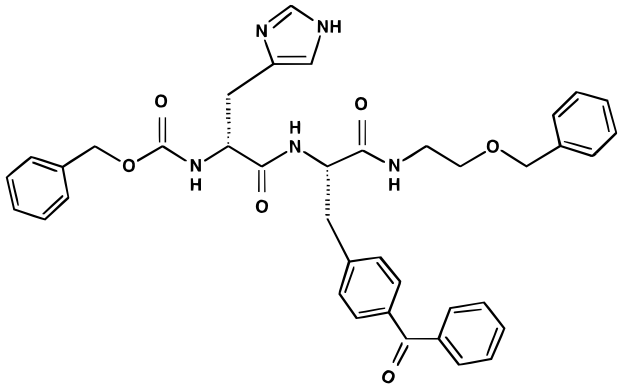

FIGURE 1: Structure of Compound 1.

RESULTS

Photoinhibition of FPTase by Compound 1. The structure of the photoaffinity label, CBZ-D-His-(4-Bz)L-Phe-NH[2-(phenylmethoxy)ethyl] (Compound 1), is shown in Figure 1. This peptide is a modified version of PD 083176, a pentapeptide inhibitor of FPTase (Scholten et al., 1996). Compound 1 contains the photoactivatable amino acid analog *p*-benzoylphenylalanine (Bpa) (Miller, 1991). Before photoaffinity labeling experiments were carried out, Compound 1 was tested as an inhibitor of FPTase *in vitro*. In HEPES buffer, Compound 1 acted as an inhibitor of FPTase with an IC_{50} (50% inhibitory concentration) of 13 μM (data not shown). In the presence of 5 mM phosphate in HEPES, the IC_{50} was shown to be 2.6 μM. This increased inhibitory potency in the presence of phosphate anion has also been observed for other peptidic inhibitors of FPTase (Scholten et al., 1996). The patterns of inhibition by Compound 1 versus peptide and FPP substrates were determined by kinetic experiments with varying substrate and inhibitor concentrations. Using nonlinear least-squares computer fits to the data, Compound 1 was found to be noncompetitive versus the hexapeptide substrate TKCVIM with a K_i of 11.1 ± 1.3 μM and mixed noncompetitive versus FPP with a K_i of 11.0 ± 1.0 μM (data not shown). A similar pattern of inhibition of FPTase has been observed for other peptidic inhibitors that act as mimetics of farnesylated CAAX peptides (Scholten et al., 1996; Scholten et al., in preparation). These results demonstrate that Compound 1 binds to FPTase in the absence of photoactivation.

FPTase was incubated with various concentrations of Compound 1 in 50 mM HEPES (pH 7.5). Aliquots from duplicate samples were withdrawn at the times indicated and assayed for enzymatic activity. As shown in Figure 2A, the photoinactivation of the enzyme proceeded in a time- and concentration-dependent manner. In the absence of photolabel, FPTase retained its activity throughout photolysis (Figure 2A), indicating that loss of activity was a result of photo-cross-linking of the probe to the enzyme.

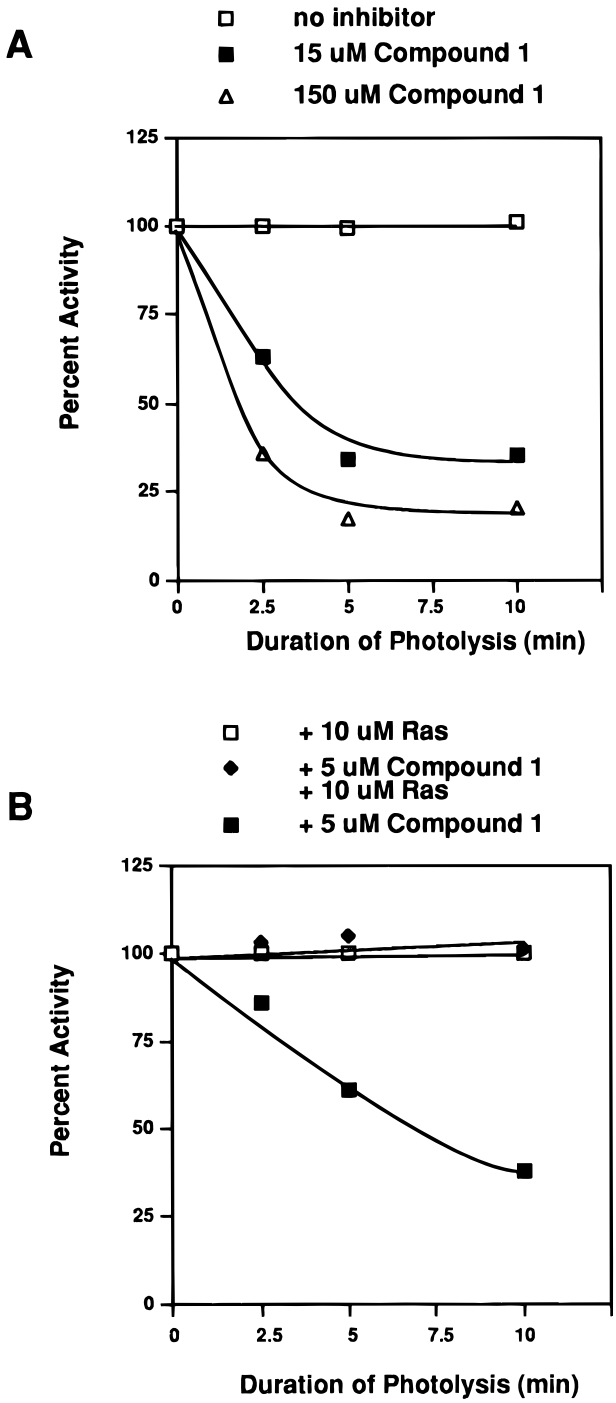

FIGURE 2: (A) Photoinactivation of FPTase by Compound 1. Enzyme (0.8 nM) and photoaffinity label (at 0, 15, or 150 μM) were combined in 50 mM HEPES (pH 7.4) and 10−20% DMSO and irradiated at 350 nm for up to 15 min. Aliquots were withdrawn and analyzed for FPTase activity. One hundred percent activity is defined as the activity of the enzyme−compound mixture before photolysis. (B) Protection from photoinhibition by Ras. Photoinactivation was carried out as above in the presence or absence of 10 μM Ras. The concentration of Ras in the enzyme activity assay was adjusted to 1 μM for each sample.

To show that photoinactivation of FPTase by Compound 1 was specific, the experiment was repeated in the presence of native Ras, a substrate of FPTase. The results, shown in Figure 2B, demonstrate that Ras protects FPTase from photoinactivation by Compound 1. Irradiation of FPTase in the presence of Ras, but in the absence of photolabel, showed no decrease in activity (Figure 2B).

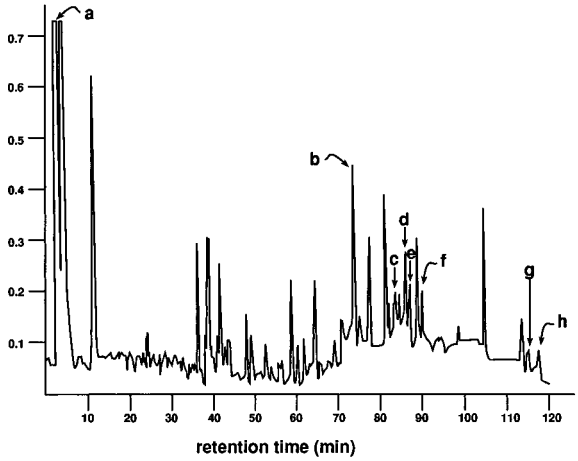

FIGURE 3: C18 HPLC analysis of trypsin-digested photoaffinity-labeled FPTase. Conditions for the digestion and for HPLC are given in Materials and Methods. Absorbance at 220 nm is shown in the *y*-axis. Peaks of absorbance with radioactivity ≥10-fold over background are indicated.

In contrast, addition of FPP to the photolysis mixture did not afford significant protection against photoinactivation (data not shown). These observations suggest that photocross-linking of Compound 1 occurs at the active site of FPTase. This efficient competitive protection by a low concentration of Ras establishes the use of Compound 1 as a valuable tool in identifying primary structure determinants of FPTase involved in substrate recognition.

Identification of FPTase Regions Modified by Compound 1. In a preliminary experiment, FPTase was irradiated in the presence of [^{3}H]Compound 1 and subjected to SDS−PAGE. Bands corresponding to the α and β subunits were excised from the gel, solubilized, and analyzed by scintillation counting. Approximately 70% of the radioactivity was associated with the α subunit in this experiment. In order to identify regions or amino acid residues of FPTase modified by the photolabel, we conducted a large scale cross-linking reaction using [^{3}H]Compound 1. Approximately 1 mg of pure FPTase was subjected to photolysis in the presence of [^{3}H]Compound 1 (80 μM) for 30 min. The sample lost 70% of its enzymatic activity after photolysis. A control experiment carried out in parallel with no photolabel showed no loss of activity under the conditions used. The photolysis reaction mixture was dialyzed to remove unreacted label. Cross-linked FPTase in the dialysate was precipitated by addition of TCA. The enzyme was then denatured and digested with trypsin as described in Materials and Methods. The choice of protease and digestion conditions were previously determined using unlabeled enzyme and by monitoring proteolytic fragments with reverse phase HPLC (data not shown).

Figure 3 shows the C18 HPLC chromatogram of tryptic fragments from labeled FPTase. Fractions containing absorbance peaks at 220 nm were collected, and 5% of each fraction was analyzed by scintillation counting. Eight fractions contained isolated peaks of radioactivity (≥10-fold over background) and are designated peaks a−h in Figure 3.

Because some radioactive peaks may have arisen from unreacted probe, chemical degradation of the probe, or probe cross-linked to buffer components that were not removed by dialysis, we analyzed all samples using the following

Table 1: Analysis of Radioactive Peaks from Trypsin-Digested Photolabeled FPTase

peak	retention time C18 (min)	yield[a] (%)	mass (Da)[b]	retention time C4 (min)	yield[a] (%)	mass (Da)[c]
a	3.7	*d*	< 675	–	–	–
b	73.3	66	1091.5, 1239.9	77.7	31	1091.5
				67.3	48	<675
c	83.1	*d*	2165.5, 2047.6	83.0	9	<675
d	85.5	*d*	651.0, 675.3	–	–	–
e	86.5	*d*	675.3	–	–	–
f	89.5	*d*	3072.9, 2623.7, 675.5, 1822.8	84.7	33	674.5
g	114.6	*d*	675.6, 551.8	–	–	–
h	116.8	*d*	551.0, 522.9	–	–	–

[a] Yields were calculated from the radioactivity of samples. [b] Molecular masses were determined by MALDI-TOF mass spectrometry and are shown in order of relative abundance within the fractions. [c] Radioactive peaks containing peptides with molecular masses higher than that of the probe were rechromatographed on a C4 column. Only samples containing radioactivity were analyzed using mass spectrometry. The sequence of the radioactive component of peak B was determined by Edman degradation. [d] Radioactivity in these samples was derived from the free photoaffinity label and/or photolysis products of the reaction, not from cross-linking to FPTase (see text for details).

general strategy. Fractions containing radioactivity ≥10-fold above background were analyzed by MALDI-TOF mass spectrometry to determine how many components were present in the fractions and the masses of each component. Since the molecular mass of Compound 1 is 673.8 Da, only fractions with higher molecular masses were chosen for further analysis. These fractions were rechromatographed using reverse phase HPLC on a C4 column. Radioactive fractions eluted from the C4 column were examined again by mass spectrometry, and those with molecular masses of >1000 Da were sequenced by Edman degradation. The results are summarized in Table 1. As shown, peaks b, c, and f from the C18 column contained components with a molecular mass higher than 674 Da and were rechromatographed on the C4 column (see below). In contrast, the most prominent constituents of the remaining fractions were identified as either small molecular mass components or as peaks identical in mass to the photolabel and were not analyzed further (Table 1).

To characterize potential byproducts of the photolysis reaction, we performed a photolysis reaction identical to the large scale cross-linking experiment described above, but in the absence of FPTase (data not shown). Examination of this photolyzed reaction mixture using HPLC revealed six peaks of absorbance with retention times on the C18 column of 64.2, 85.1, 85.9, 87.0, 87.6, and 89.1 min. The last peak corresponds to unreacted Compound 1, as previously determined during purification of the compound. The additional peaks probably represent Compound 1 nonspecifically cross-linked to DMSO, HEPES, or other components of the photolysis reaction. Because peaks d and e from the FPTase cross-linking reaction eluted at 85.5 and 86.5 min, respectively, it is likely that they contained such photoproducts. Furthermore, the most abundant components of these fractions had molecular weights of ≤675 Da (Table 1), and therefore, peaks d and e were not analyzed further.

As revealed by mass spectrometry, the remaining radioactive fractions from C18 HPLC (peaks b, c, and f) contained mixtures of components with masses higher than 674 Da (Table 1). To ensure that the radioactive components in these samples arose from tryptic fragments of FPTase labeled by Compound 1 (rather than from an unlabeled peptide that coeluted with a byproduct of photolysis), we rechromatographed the fractions on a C4 column. Only samples containing radioactivity during this second round of chromatography were analyzed by mass spectrometry. As summarized in Table 1, the radioactivity associated with peaks c and f was not due to a high-molecular mass peptide but instead to a low-molecular mass component. Furthermore, peaks c and f both had retention times on C18 HPLC that were similar to those arising from a control reaction mixture photolyzed in the absence of enzyme. However, when peak b was rechromatographed on a C4 column, a radioactive component with a molecular mass of 1091.5 Da was isolated. In addition, the retention time of peak b on a C18 column did not correspond to the retention times of the unreacted label or to those of the other peaks associated with photolysis, arguing that peak b is indeed a radioactive product derived from label-modified FPTase. The radioactivity of peak b corresponded to a 66% yield from the starting material (Table 1), arguing that this peak is the major protein-derived product from photo-cross-linking to FPTase.

Of all predicted tryptic fragments of the FPTase α and β subunits, one fragment has a mass of 418.4 Da, corresponding to the mass of peak b minus the mass of Compound 1 (no other predicted fragment produced by partial or complete trypsin digestion of FPTase has a mass within 13 Da of 418.4). This peptide fragment is derived from the sequence Asp110−Arg112 of the α subunit of FPTase, suggesting that photo-cross-linking occurred within this peptide. To confirm this assignment, we analyzed peak b by Edman sequencing after a second round of HPLC on a C4 column. The first cycle of Edman degradation yielded Asp as the N-terminal amino acid of peak b (yield of PTH-Asp, 281 pmol). No signal over background was observed at the second, third, fourth, or fifth cycles of Edman sequencing, nor was any radioactivity released from the filter during any of the sequencing cycles. These results suggest that the isolated 1091.5 Da radioactive product arises from the peptide Asp110−Arg112 in the α subunit of FPTase cross-linked to Compound 1.

DISCUSSION

Peptide-based photoaffinity labels containing Bpa have been useful in the identification of amino acid residues involved in enzyme−substrate recognition (Miller & Kaiser, 1988; Zhang et al., 1996) and noncatalytic binding sites, such as SH2 domains (Gergel et al., 1994; Williams & Shoelson, 1993). The triplet-state reactive intermediate generated upon irradiation of benzophenones inserts preferentially into C−H bonds (Helene, 1972). This confers certain advantages to the use of benzophenone-type photoaffinity labels such as Bpa; in particular, generally unreactive amino acids in proximity to the photoactivated Bpa may be labeled, and the efficiency of labeling of the biological molecule is high compared to that of solvent (Dormán & Prestwich, 1994). In the present study, we have used this technique to identify a region of FPTase involved in substrate recognition. The photoaffinity label used, Compound 1, cross-links specifically to the $\alpha_{110-112}$ region of FPTase. This region of the enzyme was not previously known to play a role in recognizing substrates.

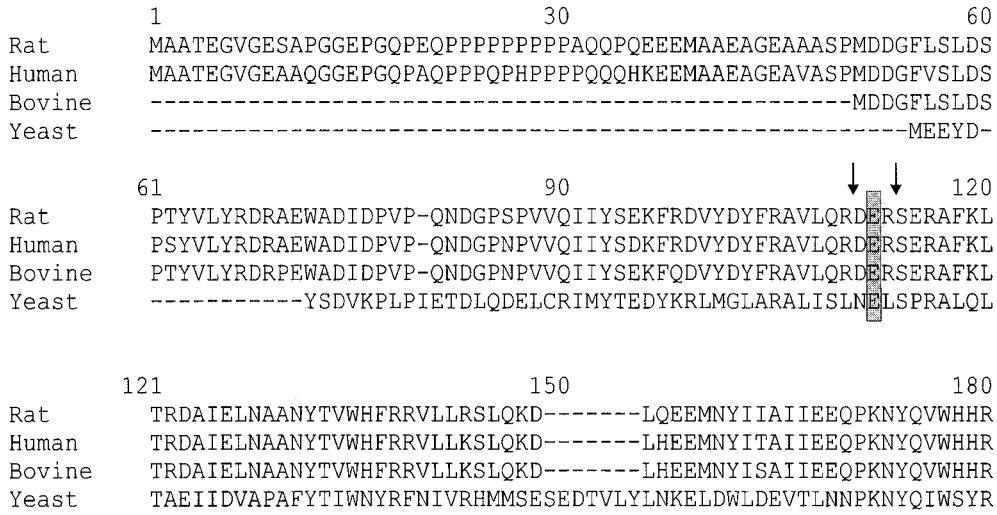

FIGURE 4: Comparison of the sequences of the amino-terminal 180 amino acids of mammalian and yeast FPTase α subunits. The position of Glu111 (numbered according to the sequence of the rat α subunit) is denoted by the shaded box. Arrows indicate the points of trypsin cleavage.

Although in principle any of the residues in Asp-Glu-Arg could have been modified by the label, several lines of evidence suggest that Glu111 is the most likely modification site. First, Edman sequencing of this modified peptide identified (unmodified) Asp as the N-terminal amino acid. Second, no phenylthiohydantoin-amino acid signal over background was observed in the second cycle or subsequent cycles. We (Miller, 1991) and others (Mourey et al., 1993; Dormán & Prestwich, 1994) have observed abrupt sequence termination for sequencing of photo-cross-linked sites with benzophenone labels. These results suggest that the covalent modification took place at the second position. Third, it is unlikely that derivatized Arg could be recognized as a site for trypsin cleavage. Finally, a homology sequence alignment between the α subunits of yeast and mammalian FPTases, shown in Figure 4, indicates that the sequence Asp-Glu-Arg is conserved among all mammalian FPTases identified to date. In contrast, examination of the corresponding yeast sequence, Asn-Glu-Leu, reveals that only Glu remains invariant. Thus, Glu111 may be involved in substrate binding and/or catalysis. The precise role of Glu111 in FPTase's catalytic mechanism may be clarified by site-directed mutagenesis.

Previous experiments have indicated that both the α and β subunits of FPTase are essential for enzymatic activity; the exact contribution of each subunit to substrate binding and catalysis, however, remains unclear. Cross-linking studies have shown that both protein−substrate (Ying et al., 1994; Reiss et al., 1991; Andres et al., 1993) and isoprenoid−donor (Omer et al., 1993; Bukhtiyarov et al., 1995) cross-link to the β subunit, suggesting that primary determinants of binding and specificity lie in this subunit. Furthermore, because GGTase I and FPTase share identical α subunits, the β subunit must play a decisive role in protein specificity. On the other hand, a bifunctional CAAX motif heptapeptide derivatized with two benzophenone groups was able to covalently tether both α and β subunits, suggesting that the catalytic domain of FPTase lies at the subunit interface (Ying et al., 1994). This is consistent with a model in which both substrates come into contact, during binding or catalysis, with both α and β subunits. Our observation of preferential cross-linking to the α subunit by Compound 1 implies that the α subunit participates in substrate binding. Furthermore, the pattern of inhibition observed for Compound 1 is consistent with photo-cross-linking to an activated form of the enzyme (Scholten et al., 1996).

Support for the involvement of the α subunit of FPTase in substrate recognition comes from mutagenesis studies. For example, Omer et al. (1993) reported that a point mutation in the α subunit (N199K) primarily affected protein−substrate utilization as revealed by an increase in the K_m value of the enzyme, although direct binding parameters were not obtained. Similarly, deletion of only five C-terminal amino acids in the α subunit, or deletion of the N-terminal 106 residues of the α subunit, abolishes enzyme activity and farnesyl pyrophosphate binding (Andres et al., 1993). A point mutation in the α subunit of FPTase, K164N, yielded an enzyme with reduced catalytic activity but with normal binding of the two substrates, leading to the suggestion that the α subunit is directly involved in the catalytic reaction.

Recently, it was reported that the FPTase α subunit interacts with the transforming growth factor-β receptor *in vivo* and is phosphorylated by the receptor on Ser/Thr residues *in vitro* (Kawabata et al., 1995; Wang et al., 1996). Although the phosphorylation site was not identified, nor were the functional consequences of phosphorylation determined, it is interesting to note that four out of the seven conserved Ser/Thr residues in the α subunit of FPTase lie in close proximity to Glu111 (within 21 amino acids). From results reported here, introduction of a phosphate near this position might be expected to alter the substrate binding properties, and potentially the activity, of mammalian FPTase.

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