

# Limited Proteolysis of Rat Phosphatidylinositol Transfer Protein by Trypsin Cleaves the C Terminus, Enhances Binding to Lipid Vesicles, and Reduces Phospholipid Transfer Activity\*

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Jacqueline M. Tremblay, George M. Helmkamp, Jr., and Lynwood R. Yarbrough†

From the Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, Kansas 66160-7421

Rat phosphatidylinositol transfer protein (PITP) is a 32-kDa protein of 271 amino acids that transfers phosphatidylinositol and phosphatidylcholine between membranes. The  $\alpha$  isoform of rat PITP was expressed in *Escherichia coli* and purified in high yields. The purified protein contained 1 mol of phosphatidylglycerol and had a transfer activity for phosphatidylinositol and phosphatidylcholine equal to or greater than that of PITP purified from mammalian brain. Limited protease digestion was used to further define structure, activity, and function relationships in PITP. PITP alone is relatively resistant to digestion by chymotrypsin, trypsin, and *Staphylococcus* V8 protease but is readily cleaved by subtilisin. Phospholipid vesicles containing phosphatidic acid enhance susceptibility to digestion by all four proteases. In the presence of vesicles, PITP, which migrates as a 36-kDa protein in SDS-polyacrylamide gel electrophoresis, is cleaved rapidly by trypsin to a form that appears to be 2–3 kDa smaller than the native form. The tryptic fragment retains partial phospholipid transfer activity and shows an enhanced affinity for phospholipid vesicles containing phosphatidic acid. Analysis of the tryptic digestion products by immunoblotting, N-terminal sequencing, and electrospray mass spectrometry showed that trypsin cleaves the C terminus of PITP at Arg<sup>253</sup> and Arg<sup>259</sup>. Thus, removal of the C terminus enhances the affinity of PITP for vesicles and results in a diminution of transfer activity. Overall, the data show that PITP undergoes conformation changes and that the C terminus becomes more accessible to trypsin when bound to vesicles. Hence, the C terminus is not an essential component of the membrane binding site and may be located distal to it.

All eukaryotic cells contain proteins that are capable of binding phospholipids. Phosphatidylinositol transfer protein (PITP)<sup>1</sup> binds phosphatidylinositol and phosphatidylcholine

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† To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS 66160-7421. Tel.: 913-588-6960; Fax: 913-588-7440; E-mail: LYarbrou@KUMC.EDU.

<sup>1</sup> The abbreviations used are: PITP, phosphatidylinositol transfer protein; 98/2 PC/PA vesicles, vesicles containing 98/2 mol% phosphatidylcholine/phosphatidic acid; 80/20 PC/PA vesicles, vesicles containing 80/20 mol% phosphatidylcholine/phosphatidic acid; DTT, dithiothreitol; PtdCho, phosphatidylcholine; PtdGro, phosphatidylglycerol; rPITP, the  $\alpha$  isoform of rat PITP produced in *E. coli*; HPLC, high pressure liquid chromatography.

(PtdCho) with relatively high specificity and catalyzes their intermembrane transfer. PITP was first purified over 20 years ago from bovine brain, a tissue in which it is found in relatively high amounts (1). It contains a single binding site for phospholipid, and in SDS-PAGE it exhibits an apparent size of 36 kDa (2, 3).

cDNAs encoding PITP have been cloned and sequenced for rat, human, mouse, and yeast (4–8). The predicted PITPs of rat, mouse, and human consist of 271–272 amino acids, have very highly conserved sequences, and have molecular masses of approximately 32 kDa. More recently, a cDNA encoding another PITP form was isolated from rat brain by complementation in yeast (9). Although the predicted protein is similar in size to the predicted PITPs of rat, human, and mouse, it has only 70% amino acid sequence similarity with those previously reported. Thus, there appear to be two genes encoding PITP in rat and apparently also in bovines (10). PITPs having strong sequence similarity to that sequenced initially by Dickeson *et al.* (4) have been termed PITP- $\alpha$ . PITPs showing strong sequence similarity to that sequenced by Tanaka and Hosaka (9) have been termed PITP- $\beta$ . PITPs migrate anomalously in SDS-PAGE, which has given rise to incorrect mass assignments of 35–36 kDa (10). Most yeast proteins are slightly larger, around 300–310 amino acids, and show no apparent sequence similarity to the metazoan PITPs (4). Genetic studies have shown that in yeast, PITP is essential for cell viability (11). Interestingly, rat PITP- $\alpha$  can only partially complement defects in *SEC14*, the yeast gene encoding PITP (12).

The physiological role of PITP is becoming more clear. Recently, it has been found to be involved in both cell signaling and secretion (13–16, 18, 19). Hay and Martin (13) found that PITP was an essential component for ATP-dependent priming of calcium-activated secretion. Interesting, yeast PITP (SEC14 gene product) was able to substitute for PITP in this reaction. PITP also is required for signaling by epidermal growth factor and for phospholipase C-mediated inositol lipid signaling (14, 16, 20). It apparently stimulates the latter reaction by promoting synthesis of phosphatidylinositol 4–5-bisphosphate (20).

The structural information available about PITP is limited. Studies by Wirtz and co-workers (21, 22) suggest that the phospholipid binding site is hydrophobic and that there may be separate binding sites for the sn-1 and sn-2 acyl side chains. They also demonstrated that negatively charged phospholipids were inhibitors of the *in vitro* transfer reaction, apparently as a result of enhanced binding of PITP to the negatively charged vesicles (23). Transfer activity of PITP is inactivated by treatment with sulfhydryl reagents such as maleimides and iodoacetamide (12, 24). The location of the phospholipid binding site is unknown.

Limited protease digestion has proven to be a powerful tool for analysis of structure and function of proteins (25–27). We

have used this approach to characterize recombinant rat P1TP. In the absence of lipid vesicles, P1TP is highly resistant to digestion by trypsin. In the presence of vesicles, digestion by trypsin is greatly enhanced, and the C terminus is rapidly cleaved at Arg<sup>253</sup> and Arg<sup>259</sup> to generate a mixture of at least two truncated species. The truncated protein(s) have greatly reduced transfer activity, and the affinity for vesicles is significantly enhanced. The results show that structural changes in the C terminus significantly alter the affinity of P1TP for lipid vesicles.

#### EXPERIMENTAL PROCEDURES

**Materials**—PtdCho was purified from crude egg PtdCho (Sigma) by column chromatography on Silica Gel G. Bovine liver phosphatidylinositol and egg PtdCho-derived phosphatidic acid were purchased from Avanti Polar Lipids (Alabaster, AL). The following were purchased from Sigma: L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (T-8642),  $\alpha$ -chymotrypsin (C-3142), subtilisin (P-5380), *Staphylococcus aureus* V8 protease (P-2922), di-isopropylfluoro phosphate (D-0879), and phenylmethylsulfonyl fluoride (P-7626). Soybean trypsin inhibitor was from Cooper Biomed.

**Construction of a Recombinant Clone Expressing rP1TP**—A cDNA encoding rat P1TP  $\alpha$ -isoform was ligated into the pET-11C vector of Studier and co-workers (28, 29), which confers resistance to ampicillin, so that when the *lac* promoter regulating P1TP gene expression was induced by isopropyl  $\beta$ -D-thiogalactoside an unfused protein of 271 amino acids (31.9 kDa) would be produced. For protein expression, the resulting plasmid (pET-P1TP) was transformed into *Escherichia coli* BL21-DE3, which contained an additional plasmid encoding *groELS* (30). The plasmid encoding *groELS* had a different origin of replication and contained a gene conferring resistance to chloramphenicol. The expression of both P1TP and *groELS* were controlled by the *lac* repressor/operator.

**Purification and Characterization of rP1TP from E. coli**—Cells were grown in the presence of both ampicillin and chloramphenicol to an optical density of one at 595 nm and induced with 200  $\mu$ M isopropyl  $\beta$ -D-thiogalactoside for 18 h at 20 °C. Following induction, cells were harvested by centrifugation at 4 °C, and the resulting cell pellets were resuspended in buffer A (0.05 M Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride) and lysed in an SLM Aminco French pressure cell press (SLM Instruments, Inc.). The resulting lysate was centrifuged at 30,000 rpm for 30 min at 4 °C in a type 50.2 TI rotor (Beckman). The lysate supernatant from the centrifugation step (sample volume,  $\leq$ 5% of bed volume) was loaded on a Sephadex G-100 column (52 mm  $\times$  90 cm) that was equilibrated with buffer B (0.05 M sodium phosphate, pH 7.4, 0.1 mM EDTA, 0.02% sodium azide, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM DTT). The column was eluted with buffer B, and column fractions were analyzed by SDS-PAGE. The desired fractions were then pooled, dialyzed against buffer C (0.01 M sodium phosphate, pH 7.6, 0.1 mM EDTA, 0.1 mM DTT), and loaded on a column (25 mm  $\times$  85 mm) containing Q Sepharose Fast Flow strong anion exchange resin equilibrated with buffer C. The column was washed with buffer C and eluted with a linear gradient of NaCl (10–300 mM) in buffer C. The majority of the protein at this point appeared homogeneous with a yield of 25–30 mg of purified protein/liter of cells. In some cases, fractions containing impurities were dialyzed against buffer D (0.01 M sodium phosphate, pH 6.8, 0.1 mM EDTA, 0.1 mM DTT) and further purified on a Bio-Rad hydroxylapatite column (16 mm  $\times$  110 mm) equilibrated with the same buffer. The column was eluted with a linear gradient (10–200 mM) of sodium phosphate, pH 6.8, 0.1 mM DTT, and 0.1 mM EDTA. Protein concentrations were measured by the Bradford method using the Bio-Rad Protein dye assay or by absorbance at 280 nm using a measured molar extinction coefficient of 79,700 M<sup>-1</sup> cm<sup>-1</sup>. Phospholipid analysis was performed as described by Bartlett (31). Protein sequence was determined with an Applied Biosystems model 470A sequencer updated to pulse liquid chemistry fitted with a model 120 on-line phenylthiohydantoin-derivative analyzer.

**Purification with Lipid Exchange**—rP1TP purified from *E. coli* contains PtdGro, whereas that purified from mammalian cells or tissue contains PtdCho and phosphatidylinositol. To exchange the bound PtdGro for PtdCho, the pooled protein from the Sephadex column was dialyzed against buffer C and then concentrated to a volume of approximately 100 ml using an Amicon concentrator and Diaflo PM10 ultrafiltration membrane. Vesicles containing 100 mol% PtdCho were added to the protein to a final concentration of 1.44 mM and then incubated at room temperature for 18 h (total volume, 200 ml). This mixture was

then further purified by chromatography on Q Sepharose Fast Flow and hydroxylapatite as described above. Analysis of bound phospholipid following exchange showed  $0.90 \pm 0.02$  mol of PtdCho/mol of P1TP.

**Vesicle Preparation and Measurements of in Vitro Transfer**—Phospholipid transfer activities were measured between two populations of small unilamellar vesicles, as described by Kasper and Helmkamp (32). Vesicles used in assays were prepared by injection of chloroform solutions into buffer; vesicles used for binding studies were prepared by extensive sonication. Radiolabeled phospholipid substrates for assays were phosphatidyl[<sup>3</sup>H]inositol and 2-[<sup>3</sup>H]oleoyl-PtdCho. Control incubations were carried out in the absence of transfer protein. Activity is calculated as the percentage of donor phospholipid (40 nmol) transferred in a 30-min assay. Recovery of acceptor vesicles ranged between 94 and 100%; activity in the absence of transfer protein rarely exceeded 6%.

**Protease Digestion**—rP1TP (final concentration, 0.30 mg/ml) was digested with trypsin, chymotrypsin, subtilisin, and *S. aureus* V8 protease in the presence or the absence of 80/20 PC/PA vesicles. Digestions were performed in 10 mM HEPES, pH 7.4, 1 mM EDTA, 50 mM NaCl at 37 °C using the following ratios of protease to P1TP: trypsin and chymotrypsin (1:25 (w/w) ratio); subtilisin and *S. aureus* V8 protease (1:100 (w/w) ratio). Aliquots were removed at specific times, and the reaction was stopped by addition of 2  $\times$  SDS-PAGE sample buffer and boiling at 100 °C for 10 min. Samples were fractionated by SDS-PAGE and stained with Coomassie Blue.

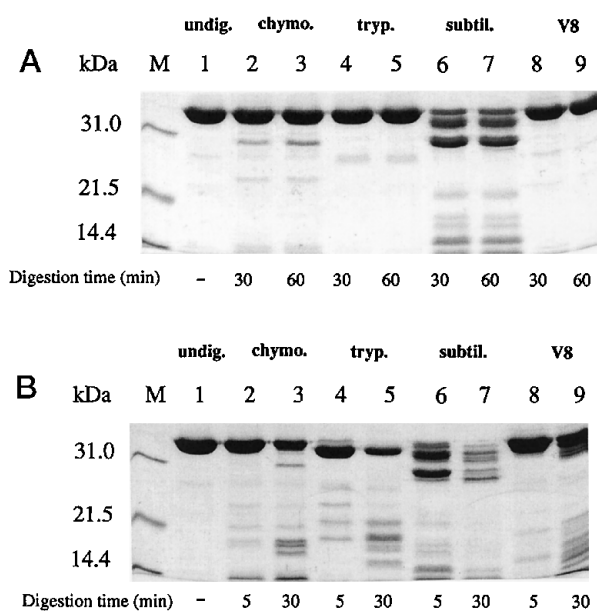
**Transfer Activity of Trypsin-digested rP1TP**—P1TP (final concentration in reaction, 0.25 mg/ml) was digested with a 1:150 (w/w) ratio of trypsin/P1TP for 5 min at 37 °C in the presence or the absence of 1 mM 80/20 PC/PA vesicles. Digestion was performed in 10 mM HEPES, pH 7.4, 1 mM EDTA, 50 mM NaCl. The reaction was stopped by addition of di-isopropylfluoro phosphate to 2 mM. Samples were left at room temperature for 1 h and then analyzed for transfer activity. Control experiments showed that transfer activity of undigested P1TP was not significantly affected by this treatment. Activity is expressed as the percentage of transfer of donor PtdCho during a 30-min assay using 98/2 PC/PA vesicles (40 nmol).

**HPLC Gel Filtration**—Gel filtration was performed using a Tosoh-Haas G2000SW<sub>XL</sub> 2000 column with a flow rate of 1 ml/min. Elution was monitored by fluorescence (excitation, 280 nm; emission, 340 nm) or absorbance at 280 nm. The buffer contained 10 mM HEPES, pH 7.4, 1 mM EDTA, and 50 mM NaCl. The void volume eluted at approximately 5.5 min. Samples of 20–30  $\mu$ l were injected.

**Digestion for Mass Spectral Analysis**—rP1TP (~1 mg in 0.7 ml) was digested for 5 min in 10 mM HEPES, pH 7.4, 1 mM EDTA, and 50 mM NaCl with a 1:25 (w/w) ratio of trypsin/P1TP at 37 °C in the presence of 1 mM 80/20 PC/PA vesicles. The digestion was stopped by addition of 1.3 molar equivalents of soybean trypsin inhibitor and placed on ice prior to loading onto a Sephadex G-100 column. SDS-PAGE confirmed that P1TP had been completely converted to the more rapidly migrating product(s). The trypsin-digested P1TP was purified at 4 °C using a 14-ml Sephadex G-100 column (12 mm  $\times$  170 mm). The column was equilibrated and protein eluted with 10 mM HEPES, pH 7.4, and 0.1 mM EDTA. Fractions were analyzed by SDS-PAGE, and peak fractions were pooled. The pooled trypsin-digested P1TP and a similar preparation of undigested P1TP were then dialyzed for 2 h at 4 °C against equimolar ratios of acetonitrile and water containing 1% acetic acid. The proteins were subsequently analyzed by electrospray mass spectrometry in the Biotechnology Center at Washington University (St. Louis, MO).

#### RESULTS

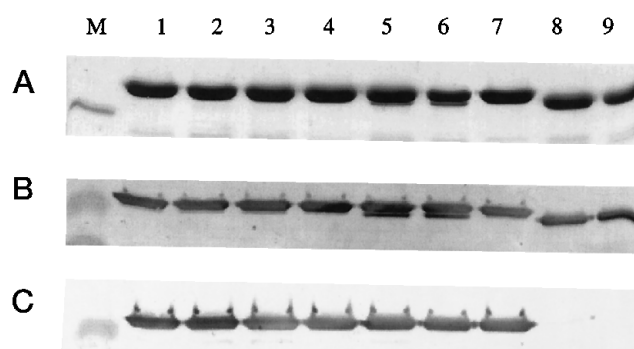
**Purification and Characterization of rP1TP Purified from E. coli**—The  $\alpha$  isoform of rat P1TP was expressed in *E. coli* using the procedures outlined under “Experimental Procedures.” Crude cell extracts were fractionated by gel chromatography and assayed by SDS-PAGE, and peak fractions were pooled. This material was then further purified by chromatography on Q Sepharose Fast Flow anion exchanger followed by chromatography on hydroxylapatite. After purification, P1TP appeared homogenous in SDS-PAGE. Yields normally ranged from 25–30 mg of purified P1TP/liter of cell culture, if *groELS* was overexpressed and cells were grown at 20 °C. P1TP was analyzed for bound lipid and found to contain  $0.92 \pm 0.08$  mol of PtdGro/mol of protein ( $n = 4$ ). P1TP purified from mammalian brain exists as isoforms containing PtdCho and phosphatidylinositol. Prior to determination of transfer activity, P1TP was incubated with



**FIG. 1. Effects of 80/20 PC/PA vesicles on digestion of PITP by proteases.** rPITP (75  $\mu$ g) was digested at 37  $^{\circ}$ C in the absence (A) or the presence (B) of 1 mM 80/20 PC/PA vesicles. For digestion by chymotrypsin and trypsin a 1:25 (w/w) ratio of enzyme to PITP was used. Digestions with subtilisin and V8 protease were performed with a 1:100 (w/w) ratio of enzyme to PITP. A, lane 1, undigested PITP; lanes 2 and 3, chymotrypsin (30 and 60 min); lanes 4 and 5, trypsin (30 and 60 min); lanes 6 and 7, subtilisin (30 and 60 min); lanes 8 and 9, *S. aureus* V8 protease (30 and 60 min). B, the time of digestion was 5 and 30 min instead of 30 and 60 min, and 1 mM 80/20 PC/PA vesicles were present; otherwise the lanes were the same as in A.

vesicles containing 100 mol% PtdCho to exchange PtdGro. Vesicles were removed, and the protein was shown to contain  $0.90 \pm 0.02$  mol of PtdCho/mol of protein. Transfer activities were then measured before and after lipid exchange. Both the PtdCho and PtdGro forms have approximately equal transfer activity in the standard assay, and the activity is equal to or greater than the activity of PITP purified from mammalian brain (data not shown).

**Vesicles Enhance Digestion of rPITP by Proteases**—Often the susceptibility to protease digestion is altered by interaction with ligands. We have therefore examined the digestion of PITP by chymotrypsin, trypsin, subtilisin, and *S. aureus* V8 protease in the presence or the absence of phospholipid vesicles. The products were analyzed by SDS-PAGE, and the results are shown in Fig. 1. In the absence of vesicles, PITP is resistant to digestion by chymotrypsin, trypsin, and V8 protease. Even after 1 h there is little digestion. In contrast, subtilisin produces two major fragments of approximately 25–30 kDa in size, and the digestion appears to be complete by 30 min. Thus, PITP is relatively resistant to protease digestion in the absence of vesicles, with the exception of subtilisin. Vesicles containing the anionic lipid, phosphatidic acid, enhance digestion by all proteases (Fig. 1B). Some new fragments are observed in the chymotrypsin-digested PITP and less of the undigested material remains. In the case of trypsin (lanes 4 and 5), conversion to a form that has an apparent size 2–3 kDa smaller than PITP is complete within 5 min. This product(s) then appears to be digested more slowly to smaller fragments. Subtilisin digestion is also enhanced; after 30 min little undigested material remains. V8 protease also digests PITP more readily in the presence of vesicles. Thus, PITP is more susceptible to digestion by a number of proteases if vesicles are present during the digestion. Moreover, both the PtdCho and PtdGro isoforms of PITP show the same patterns of protease sensitivity.



**FIG. 2. Effects of vesicle composition on digestion of PITP by trypsin as measured by SDS-PAGE and immunoblotting.** rPITP (15  $\mu$ g) was digested at 37  $^{\circ}$ C for 5 min or 15 min with 1  $\mu$ g trypsin in the absence of vesicles (A, lanes 1–3), with 1 mM 98/2 PC/PA vesicles (B, lanes 4–6), or with 1 mM 80/20 PC/PA vesicles (C, lanes 7–9). Digestion was stopped by the addition of sample buffer and heating for 10 min at 100  $^{\circ}$ C. Samples were run on 12% SDS-PAGE. Lanes 1, 4, and 7 are undigested controls. Lanes 2, 5, and 8 are 5-min time points. Lanes 3, 6, and 9 are 10-min time points. M indicates Bio-Rad unstained molecular weight marker for A (carbonic anhydrase,  $\sim$ 31 kDa) or pre-stained molecular weight marker (carbonic anhydrase) for B and C. The prestained marker migrates as a larger protein in SDS-PAGE. A, Coomassie Brilliant Blue staining. B, immunoblot with antibody to the N terminus of PITP. C, immunoblot with antibody to the C terminus of PITP. Gel lanes contained 2.4  $\mu$ g of PITP for protein staining and 0.6  $\mu$ g for immunoblots.

**Trypsin Digestion of rPITP Is Enhanced by Vesicles, Is Dependent on Vesicle Composition, and Cleaves the C Terminus**—To further characterize the digestion of PITP by trypsin, samples were digested in the absence of vesicles, with 98/2 PC/PA vesicles, or with 80/20 PC/PA vesicles, as described in Fig. 1. Samples were then fractionated by SDS-PAGE and stained with Coomassie Blue (Fig. 2A), immunoblotted with antibodies against the N terminus of PITP (Fig. 2B), or immunoblotted with antibodies to the C-terminal 12 amino acids of PITP (Fig. 2C). In the absence of vesicles (lanes 1–3), PITP is relatively resistant to digestion by trypsin, as described above. The immunoblots (Fig. 2, B and C) confirm that there is little if any digestion in the absence of vesicles. If, however, digestion is performed in the presence of vesicles containing 98/2 PC/PA, a band appears after 5 min of digestion that runs slightly below the main band in Coomassie-stained gels (lanes 4–6). This band reacts with antibody to the N terminus (Fig. 1B) but does not react with the antibody to the C terminus (Fig. 2C). When digestion is performed in the presence of vesicles containing 80/20 PC/PA the conversion to the slower migrating band is complete within 5 min (lanes 8 and 9). This slower migrating band, which appears to be smaller than PITP by about 2–3 kDa, reacts with antibody to the N terminus of PITP but shows no reaction with the antibody to the C terminus (lanes 8 and 9, Fig. 2C). Thus, the data suggest that trypsin cleaves rapidly the C terminus of PITP in the presence of 80/20 vesicles, thereby eliminating the antigenic determinant(s) associated with the C-terminal 12 amino acids of PITP. Conceivably, the C-terminal peptide produced by trypsin might remain associated noncovalently with the larger N-terminal fragment. Attempts to detect a small peptide of 13–18 amino acids by SDS-PAGE were not successful. Thus, we presume that the C-terminal peptide is released and degraded or that cleavage occurs from the C terminus in a sequential manner. Control experiments were performed to determine whether vesicles affected the action of trypsin on another protein, beef brain tubulin; no significant effects were observed upon the rate of digestion or the products produced. Thus, the enhanced digestion of PITP in the presence of vesicles is apparently a specific effect and not a general effect of vesicles on trypsin digestion of

TABLE I  
Effects of tryptic digestion on transfer activity of phosphatidylinositol transfer protein

P1TP was incubated with 80/20 PC/PA vesicles and digested with trypsin (1:150 (w/w) ratio) as described under "Experimental Procedures." Values given are averages of duplicate determinations after subtraction of blank values. SDS-PAGE showed that P1TP digested in the presence of vesicles was completely converted to the smaller form. Samples were assayed for transfer of PtdCho as described under "Experimental Procedures."

Trypsin	Vesicles	Transfer activity	
		% transfer/ $\mu\text{g}/30\text{ min}$	
-	-	8.5 $\pm$ 3.3	
+	-	8.5 $\pm$ 0.4	
+	+	2.1 $\pm$ 0.5	
-	+	8.8 $\pm$ 0.1	

proteins.

Fig. 2 shows that digestion of P1TP by trypsin (1:15 (w/w) ratio) is complete by 5 min. To further characterize the enhanced sensitivity to trypsin in the presence of vesicles, digestion was performed with 80/20 PC/PA vesicles using smaller ratios of trypsin to P1TP. Digestion with a ratio of trypsin to P1TP of 1:200 was complete by 5 min, as measured by SDS-PAGE, (data not shown). Thus, vesicles with a high content of phosphatidic acid enhance susceptibility to trypsin by orders of magnitude.

**N-terminal Analysis and Mass Spectrometry of Proteolytic Products**—The immunoblotting studies suggested that digestion by trypsin occurred at the C terminus of the molecule. To further characterize the digestion products, P1TP was digested briefly with trypsin in the presence of 80/20 PC/PA vesicles, which completely converted it into the smaller form, as measured by SDS-PAGE. The protein was then isolated, and N-terminal sequence analyses were performed. These studies showed that the majority of the digested protein retained the initiating Met residue, as was found in undigested controls (data not shown). Further characterization of the tryptic digestion products was performed using electrospray mass spectrometry. The molecular mass of P1TP, which had not been digested with trypsin, was determined to be 31,914 Da, in good agreement with a mass calculated for the predicted protein of 31,909, based on amino acid sequence. P1TP, which had been subjected to trypsin digestion displayed two components of lesser mass. One component had a mass of 30,607  $\pm$  3; a second had a mass of 29,846  $\pm$  4. These data were consistent with two components resulting from cleavage at Arg<sup>253</sup> and Arg<sup>259</sup>. The masses for these fragments were calculated to be 30,622 Da and 29,850 Da, respectively.

**Transfer Activity of Trypsin Digestion Products**—The *in vitro* transfer activity of control and trypsin digested P1TP was examined using standard assay conditions and 98/2 PC/PA donor and acceptor vesicles (Table I). P1TP showed about a 75% reduction in transfer activity following digestion with trypsin. Control experiments showed that this reduction in activity was dependent on digestion with trypsin. The addition of 80/20 PC/PA vesicles to undigested P1TP had no inhibitory effect in the assay. Thus, removal of the C-terminal 12–18 amino acids of P1TP greatly reduced but did not eliminate transfer activity as measured in the standard assay.

**Analysis of Binding of rP1TP and Its Trypsin Digestion Product to Lipid Vesicles by HPLC Gel Filtration**—Digestion of P1TP by trypsin occurred much more rapidly in the presence of 80/20 PC/PA than 98/2 PC/PA vesicles. This suggested differences in interaction of P1TP with vesicles of differing lipid composition. To explore the binding of P1TP to vesicles, P1TP was added to either 80/20 PC/PA or 98/2 PC/PA vesicles to give a final vesicle concentration of 1 mM and incubated for 5 min at

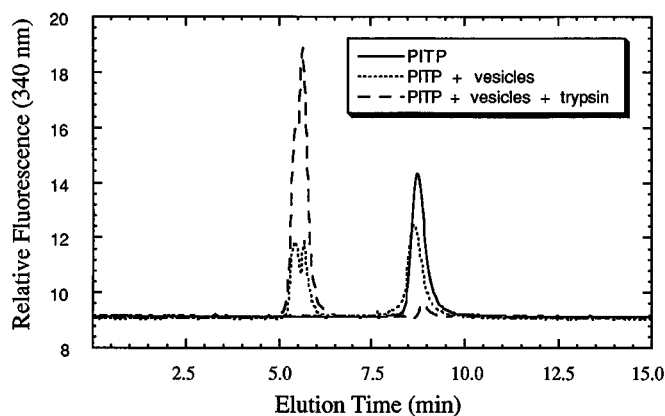


FIG. 3. Analysis of P1TP binding to vesicles by HPLC gel filtration before and after digestion with trypsin. rP1TP (25  $\mu\text{g}$ ) was mixed with 1 mM 80/20 PC/PA vesicles and incubated with or without 1  $\mu\text{g}$  trypsin for 5 min at 37  $^{\circ}\text{C}$ . Following incubation, 5- $\mu\text{g}$  samples were chromatographed on a TosoHaas TSK-GEL G2000SW<sub>XL</sub> column (0.78  $\times$  30 cm) in buffer containing 0.01 M HEPES, pH 7.4, 50 mM NaCl, and 1 mM EDTA with a flow rate of 1 ml/min. Elution of protein was monitored by intrinsic fluorescence with excitation at 290 nm and emission at 340 nm. A control sample without vesicles was also chromatographed. The column void volume was eluted at approximately 5.5 min.

37  $^{\circ}\text{C}$ . Following incubation, the solutions were analyzed by HPLC gel chromatography on a TosoHaas TSK-GEL G2000SW<sub>XL</sub> column. Elution was monitored by intrinsic fluorescence at 340 nm or absorbance at 280 nm. In the absence of vesicles, P1TP elutes as a homogeneous peak at about 8.7 min (Fig. 3). In the presence of 1 mM 80/20 PC/PA vesicles, about 30–40% of the fluorescence (P1TP) elutes within the void volume at about 5.5 min; the remainder elutes in the position of free P1TP (the total fluorescence intensity is not significantly affected by vesicles). With 98/2 PC/PA vesicles, in contrast, only a very small peak elutes in the void volume with vesicles (data not shown). Following digestion with trypsin, only traces of free P1TP (eluting at 8.7 min) are observed, and there is an increase in the material eluting in the void volume. Similar results were obtained if protein was monitored by absorbance at 280 nm (data not shown). Control experiments showed that vesicles alone eluted in the column void volume. Thus, it appears that removal of the C-terminal 12–18 amino acids of P1TP significantly enhances its affinity for vesicles so that essentially all P1TP remains bound to vesicles during gel chromatography. This has been confirmed by producing the truncated proteins (residues 1–253 and 1–259) in an *E. coli* expression system and examining their interaction with vesicles.<sup>2</sup>

#### DISCUSSION

Using the pET vector system, we have developed procedures for expression in *E. coli* and purification in large amounts of the  $\alpha$  isoform of rat P1TP. We have employed a combination of low cell growth temperature and overexpression of *groELS* to significantly enhance solubility of P1TP produced in *E. coli*. Previous studies by a number of investigators have shown that low cell growth temperature (33–35) and overexpression of molecular chaperones (17, 36, 37) can significantly enhance yields and/or solubility of proteins expressed in *E. coli*. As reported by Geijtenbeek *et al.* (6) for mouse P1TP, we find that if expressed at 37  $^{\circ}\text{C}$ , P1TP is mostly insoluble. In contrast, if expressed at 20  $^{\circ}\text{C}$  with elevated levels of *groELS*, approximately half the P1TP produced is in soluble form. Our yields (25–30 mg of P1TP/liter of cell culture) are about an order of magnitude greater than the 3 mg of P1TP/liter of cell culture reported for

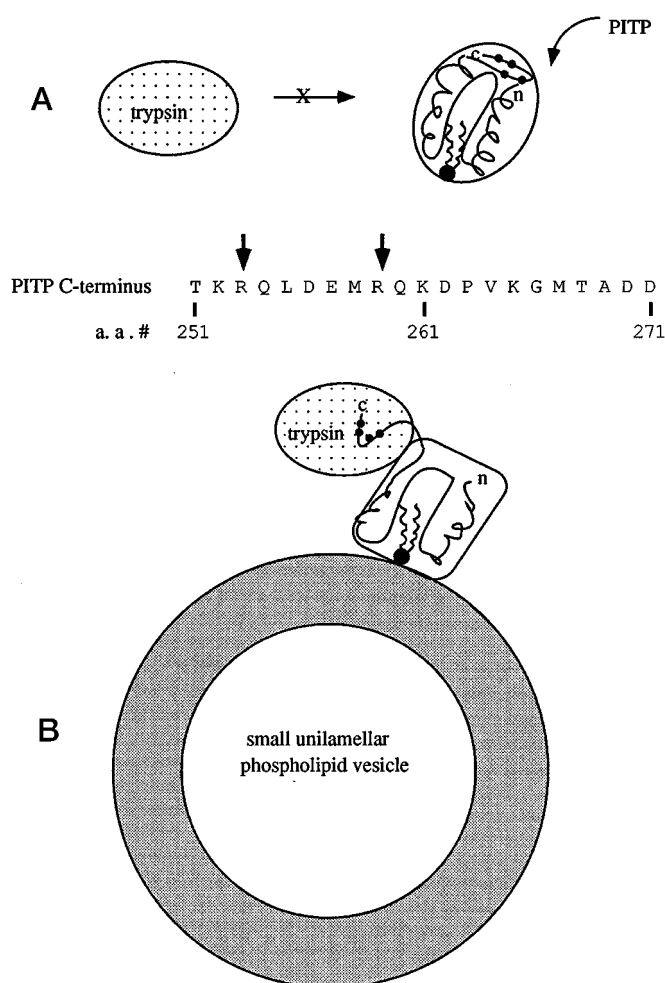
<sup>2</sup> J. M. Tremblay, G. M. Helmkamp, Jr., and L. R. Yarbrough, manuscript in preparation.

mouse P1TP (6). rP1TP is active and contains 1 mol of PtdGro as was previously reported for mouse P1TP (6). N-terminal sequencing and mass spectrometry analysis are consistent with the majority of the protein retaining its initiating Met residue.

The data we have obtained demonstrate that P1TP is highly resistant to digestion by trypsin, chymotrypsin, and V8 protease in the absence of phospholipid bilayer. However, in the presence of phospholipid vesicles, digestion by trypsin is greatly enhanced, and the conversion to a C-terminally truncated product is complete within 5 min, even at relatively low ratios of enzyme to P1TP. Under the conditions used for digestion, a significant fraction of P1TP is bound to vesicles. Moreover, increased binding to vesicles, as measured by gel filtration, is associated with a more rapid rate of digestion by trypsin. Thus, the C terminus of P1TP becomes highly susceptible to digestion by trypsin when bound to vesicles. This would appear to be due to changes in conformation of the C terminus. There may also be conformational changes in regions other than the C terminus of P1TP because the susceptibility to other proteases is also enhanced when it is bound to vesicles. Studies of sulfhydryl reactivity and tyrosine second derivative spectra show that binding of P1TP to vesicles is associated with increased exposure or reactivity of these residues. Overall, the data suggest that vesicle binding leads to a somewhat looser and more flexible conformation. Similarly, a more relaxed conformation has been described for two C-terminal truncated species (residues 1–253 and residues 1–259).<sup>3</sup> However, the changes in conformation of P1TP on binding to vesicles apparently do not involve significant changes in secondary structure because the far UV CD spectrum is not significantly altered by binding to vesicles.<sup>4</sup>

Why some proteins are subject to limited digestion by specific proteases is not clear. Conformational or segmental mobility and exposure or accessibility have been suggested previously as important determinants of proteolytic susceptibility (27). The interaction of trypsin with substrates and protein inhibitors has been studied extensively. X-ray crystal structures for several trypsin-protein inhibitor complexes have been determined. It has been suggested that the susceptible region of a protein that is cleaved by trypsin must assume a conformation similar to the conformation of that portion of the trypsin inhibitor (loop) that binds to the active site of the enzyme (26). Recent studies of tryptic cleavage sites of several native proteins using molecular modeling supports the conclusion that these "nick" sites are able to assume a loop-like conformation similar to that of the bound trypsin inhibitors (26).

Based on the results of these studies and the known specificity of trypsin, we have developed a model for the digestion of P1TP by trypsin (Fig. 4). In the absence of vesicles, the C terminus of P1TP is bound to the remainder of the protein and hence is resistant to digestion. When P1TP binds to vesicles the conformation and/or stability of the C terminus is altered resulting in weaker binding to the protein core. Consequently, the more flexible C terminus can then more readily assume a conformation, which is necessary for binding and cleavage by trypsin. Predictive methods based on the amino acid sequence of P1TP suggest that the C-terminal 18 amino acids are highly hydrophilic, located on the surface, and flexible. The model has the membrane binding site distal to the C terminus. This is based on the assumption that the C terminus would be unavailable to trypsin because of steric effects if it were a part of the membrane binding site.



**FIG. 4. Model for digestion of P1TP by trypsin in the presence of vesicles.** A, digestion of P1TP in the absence of vesicles. B, digestion of P1TP in the presence of vesicles. The four solid dots at the C terminus represent potential trypsin cleavage sites at Lys<sup>261</sup>, Lys<sup>265</sup>, Arg<sup>259</sup>, and Arg<sup>253</sup>. Bound phospholipid is shown by the two wavy lines connected to the filled circle.

The C-terminally truncated P1TP shows a significantly enhanced affinity for lipid vesicles (Fig. 3). Thus, the C terminus is not essential for binding to membranes. Indeed, the presence of the C-terminal amino acids apparently reduces the affinity of P1TP for vesicles. The mechanism by which this is accomplished is not clear. It is possible that the C terminus sterically alters the accessibility of a region(s) of P1TP that binds to membranes. Thus, removal of the C terminus could permit stronger or more extensive interactions with the vesicle. For example, removal of the C terminus of P1TP could expose positively charged residues on the trypsin-resistant core, which could enhance interaction with negatively charged vesicles. However, this mechanism envisions the C terminus as very near or a part of the membrane binding site. As noted above, we feel that this is unlikely. If the C terminus were part of the region that interacts with membranes, it appears that it would be unavailable for cleavage by trypsin when it is bound to membranes. Consequently, it appears more likely that there are global conformational changes induced by binding of P1TP to vesicles. These conformational changes lead to an enhanced affinity of P1TP for vesicles once the C terminus is removed. As shown in Fig. 3, P1TP that has been digested with trypsin binds much more avidly to membrane vesicles. However, because the mass spectral data suggest that the tryptic product is a mixture of species cleaved at Arg<sup>253</sup> and Arg<sup>259</sup>, a detailed analysis of

<sup>3</sup> P. Voziyan, J. M. Tremblay, L. R. Yarbrough, and G. M. Helmkamp, Jr., *Biochemistry*, in press.

<sup>4</sup> J. M. Tremblay and L. R. Yarbrough, unpublished data.

the effect of the C terminus on transfer activity and membrane binding requires the study of homogeneous truncated derivatives.

The increased affinity of trypsin-digested P1TP for vesicles is an important contributor to the decrease in transfer activity. Previous studies have shown that P1TP binds more tightly to negatively charged phospholipids and that transfer activity is reduced under these conditions (23). We have found that the transfer activities of derivatives of P1TP truncated at Arg<sup>253</sup> and Arg<sup>259</sup> are highly dependent on the lipid composition of the donor and acceptor vesicles and the position of truncation.<sup>2</sup> Both of these derivatives bind much more avidly to vesicles than full-length P1TP. Although membrane binding is a necessary condition for lipid transfer, if it becomes too tight, transfer activity may be greatly reduced or eliminated.

The *in vivo* function of P1TP is only now becoming clear. In view of the greatly enhanced binding of truncated P1TP derivatives to vesicles, it is possible that there might be significant biological effects if these derivatives were introduced into cells. Because of the greatly enhanced binding affinity they could form tight complexes with membranes, which might then affect interactions with the normal protein. Thus, the truncated P1TP derivatives may be useful in further elucidating the *in vivo* function of this protein.

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