RESEARCH COMMUNICATION

Deletion of 24 amino acids from the C-terminus of phosphatidylinositol transfer protein causes loss of phospholipase C-mediated inositol lipid signalling

Simon PROSSER*, Robert SARRA†, Philip SWIGART*, Andrew BALL* and Shamshad COCKCROFT*‡

*Department of Physiology, Rockefeller Building, University College London, University St., London WC1E 6JJ, U.K., and †Department of Crystallography, Birkbeck College, University of London, Malet Street, London WC1E 7HX, U.K.

Phosphatidylinositol transfer protein α (PITPα) is a 32 kDa protein of 270 amino acids that is essential for phospholipase C-mediated phosphatidylinositol bisphosphate hydrolysis. In addition, it binds and transfers phosphatidylinositol and phosphatidylcholine between membrane compartments *in vitro*. Here we have used limited proteolysis of PITPα by subtilisin to identify the structural requirements for function. Digestion by subtilisin results in the generation of a number of slightly smaller peptide fragments, the major fragment being identified as a 29 kDa protein. The fragments were resolved by size-exclusion chromatography and were found to be totally inactive in both *in vivo* PLC reconstitution assays and *in vitro* phosphatidylinositol transfer assays. N-terminal sequencing and MS of the major 29 kDa fragment shows that cleavage occurs at the C-terminus of PITP at Met^{246}, leading to a deletion of 24 amino acid residues. We conclude that the C-terminus plays an important role in mediating PLC signalling *in vivo* and lipid transfer *in vitro*, supporting the notion that lipid transfer may be a facet of PITP function *in vivo*.

INTRODUCTION

Phosphatidylinositol transfer protein (PITP) has recently been identified as playing essential roles in phospholipase C (PLC)-mediated signalling [1–3], in regulated exocytosis [4,5] and in vesicle budding [6] in mammalian cells. PITP was first purified from bovine brain and named because of its ability to transfer phosphatidylinositol (PI) and to a lesser extent phosphatidylcholine (PC) between membrane compartments [7]. Recent studies have identified two mammalian forms of PITP, referred to as α and β [8–10]. The complete primary sequences of mammalian PITPα and PITPβ consist of 270 amino acid residues and are 77 % identical and 94 % homologous. Both forms have the capacity to bind and transfer PI and PC between membrane compartments, and, in addition, PITPβ can also transfer sphingomyelin [9,10].

The involvement of PITPα in PLCβ signalling was first demonstrated when examination of PLC activation by G-protein-coupled receptors in permeabilized HL60 cells was found to be dependent on a cytosolic factor. This factor was identified as PITPα [1]. Application of a similar strategy revealed that PLCγ activation by the epidermal-growth-factor receptor and by cross-linking of the IgE receptor was also dependent on the presence of PITP [2,3]. Both mammalian forms are able to participate in PLC signalling in addition to SEC14, the yeast PITP [3]. Yeast PITP, like the mammalian PITP, can be used interchangeably, despite the lack of similarity in the primary sequence, in both the reconstitution of PLC signalling and in exocytosis [3,4]. Because the PI binding/transfer activities of PITP/SEC14 are the common features shared by all three transfer proteins, it must be a relevant activity that determines their ability to restore inositol lipid-mediated signalling and exocytosis.

In addition to PI binding/transfer activity, it has been proposed that PITP participates as a cofactor in the synthesis of phosphatidylinositol 4,5-bisphosphate (PIP_2), the substrate for the PLC [2,11]. The model speculates that the PI bound to PITP is a preferential substrate for PI kinase and subsequently phosphatidylinositol phosphate 5-kinase. The function of PITP in regulated exocytosis is to participate in the synthesis of PIP_2, but here PIP_2 functions as an intact structure akin to a second messenger [4,12].

We have used limited proteolysis of PITP to identify the structural requirements for inositol lipid signalling and PI transfer. While the present study was in progress, it was reported that deletion of the C-terminus of PITP by 12 and 18 residues reduced PC transfer function [13]. However, no measurements of PLC signalling or PI transfer function were reported. We report that deletion of 24 amino acid residues from the C-terminus eliminates PLC signalling and PI transfer activity in parallel.

MATERIALS AND METHODS

Materials

All sources of materials used here were as described previously [1]. Subtilisin (P5380) was purchased from Sigma. The gel-filtration column HiLoad™ 26/60 Superdex 75 prep grade (S-75) was purchased from Pharmacia Biotech (catalogue no. 17-1070-01).

Purification of recombinant PITP

PITPα was expressed in *Escherichia coli* as a His-tagged protein and purified exactly as described [3], except that the His-tag was removed by thrombin cleavage prior to gel filtration. After cleavage, the recombinant PITP has three additional amino acids, Gly-Ser-His, at the N-terminus. These three residues remain after thrombin cleavage and have been taken into

Abbreviations used: PITP, phosphatidylinositol transfer protein; PI, phosphatidylinositol; PC, phosphatidylcholine; PLC, phospholipase C; GTP[S], guanosine 5′-[γ-thio]triphosphate; SEC14, the yeast PITP; PIP_2, phosphatidylinositol 4,5-bisphosphate.

‡ To whom correspondence should be sent.

consideration when the masses of the PITP fragments have been determined.

Proteolysis by subtilisin

PITPα was incubated with subtilisin (PITP/subtilisin, 2:1, w/w) at pH 6.8 in 20 mM Pipes buffer/137 mM NaCl/3 mM KCl. Proteolysis was performed at 25 °C and stopped using 1 mM PMSF.

Purification of PITP fragments

The proteolysed fragments were resolved on an S-75 gel-filtration column in 20 mM Pipes/137 mM NaCl/3 mM KCl buffer and eluted at a flow rate of 1.5 ml/min. A total of 94 fractions (volume 1 ml each) were collected between 125 ml (fraction 1) and 219 ml (fraction 94), the region where the proteins were found to be eluted. The column was calibrated with standards. Blue Dextran (2000 kDa) was eluted in the void volume at 109 ml, BSA (67 kDa) was eluted at 135 ml (fraction 10), ovalbumin (43 kDa) was eluted at 151 ml (fraction 26), chymotrypsinogen A (25 kDa) was eluted at 178 ml (fraction 53) and RNA (13.7 kDa) was eluted at 197 ml (fraction 72). PITP was eluted at 185 ml (fraction 60).

Reconstitution of G-protein-mediated PLCβ activity in permeabilized HL60 cells

Reconstitution of PLC activity in cytosol-depleted cells was performed exactly as described previously [14]. Briefly, [^{3}H]inositol-labelled HL60 cells suspended in 20 mM Pipes/137 mM NaCl/3 mM KCl, pH 6.8, were permeabilized with streptolysin O (0.6 i.u./ml) for 10 min to deplete cytosolic proteins, including PITP. The cytosol-depleted cells (20 μl) were incubated in a 50 μl assay containing guanosine 5′[γ-thio]triphosphate (GTP[S]) (10 μl, 50 μM) and fractions were obtained after gel filtration (20 μl). The final assay also contained 10 μM GTP[S], 1 mM MgATP, 2 mM $MgCl_2$, pCa 6 (1 μM Ca^{2+}), 10 mM LiCl, 1 mg/ml glucose and 1 mg/ml BSA (buffered with 3 mM EGTA as previously described [14]). After incubation for 20 min at 37 °C, the reactions were quenched and the mixture assayed for inositol phosphates.

PI transfer activity

PI transfer activity was measured by the ability to transfer ^{3}H-labelled PI from donor microsomes to unlabelled acceptor liposomes and was determined exactly as described in [1]. Column fractions were diluted 5-fold prior to assay. Microsomes (50 μl), liposomes (50 μl) and column fractions (25 μl) were mixed and the assay mixture was incubated at 25 °C for 30 min and quenched using sodium acetate (50 μl, 0.2 M) and 0.25 M sucrose to cause aggregation of microsomes. The samples were then vortex-mixed thoroughly and sedimented at 15000 ***g*** at 4 °C for 15 min. A portion (100 μl) was removed and its radioactivity was determined.

SDS/PAGE and immunoblotting

After SDS/16 %-PAGE, proteins were either revealed by silver staining or analysed by immunoblotting with anti-PITP polyclonal and monoclonal antibodies. Rabbit polyclonal antibodies that recognize both PITPα and PITPβ isoforms were affinity-purified and used at 1:2000 dilution [11]. A mouse monoclonal antibody specific for PITPα (5F12) was used at a 1:10 dilution of hybridoma supernatant. Detection was by an enhanced chemiluminescence technique.

MS

The samples obtained after gel filtration (fractions 10–24) were pooled and concentrated in Centricon filters to 2 mg/ml. Mass spectra were collected on a VG Platform electrospray mass spectrometer (Micromass, Altringham, Cheshire, U.K.). Samples (10 μl) were injected under standard conditions (20 pmol/μl in 50 % acetonitrile/0.25 % formic acid) and the delivery solvent (50 % acetonitrile) pumped at 10 μl/min. Twelve 10 s scans were accumulated for each sample over the appropriate m/z range, usually 750–1150. The source temperature was set at 50 °C. Spectra were processed using the Masslynx software supplied with the instrument (version 2.1). Calibration of the mass spectrometer was with horse heart myoglobin (0.5 pmol/μl, m/z range 750–1150) using the calculated molecular mass of 16951.5 Da as determined by Ashton et al. [15].

RESULTS

Proteolysis with PITP leads to loss of PI transfer activity

We initially screened several proteolytic enzymes, including subtilisin, trypsin and staphylococcal V8 protease, for their ability to influence PI transfer activity and found that subtilisin caused a loss in transfer function (Figure 1A) and generated a number of smaller fragments with one major fragment at approx. 29 kDa (Figure 1B). PI transfer activity diminishes progressively as the full-length transfer protein is degraded (Figure 1A). Figure 1(B) shows a time course of digestion where PITP appears to be completely digested by 60 min.

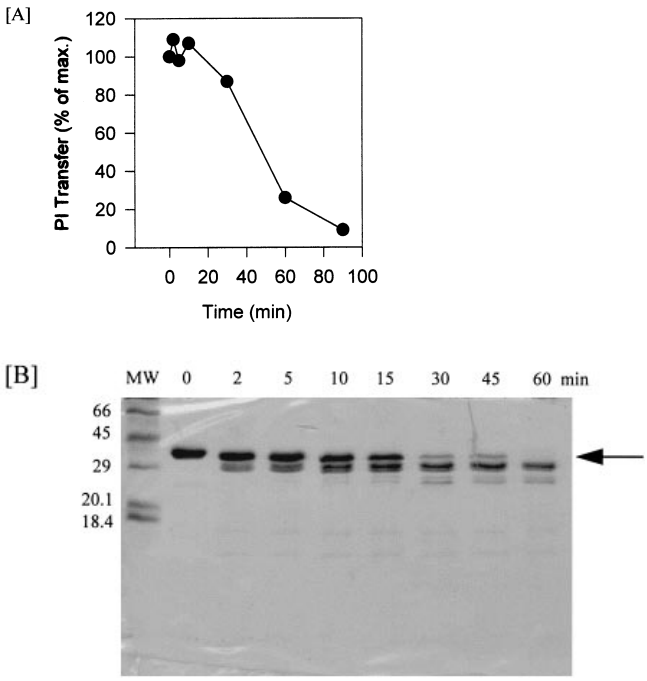

Figure 1 [A] PI transfer activity diminishes with time of incubation with subtilisin, and [B] protein profile during proteolysis of PITP by subtilisin

[**A**] A 60 μg portion of PITP was incubated with subtilisin in 100 μl and the reaction halted with PMSF at time points indicated. A 5 μl sample was used for monitoring transfer activity. The results are expressed as a percentage of maximum activity seen with undigested protein. Similar data were obtained in two other experiments. [**B**] PITP was digested with subtilisin and the samples analysed by SDS/PAGE. gels were stained with Coomassie Blue. The position of the full-length protein is indicated by the arrow. MW, molecular-mass markers.

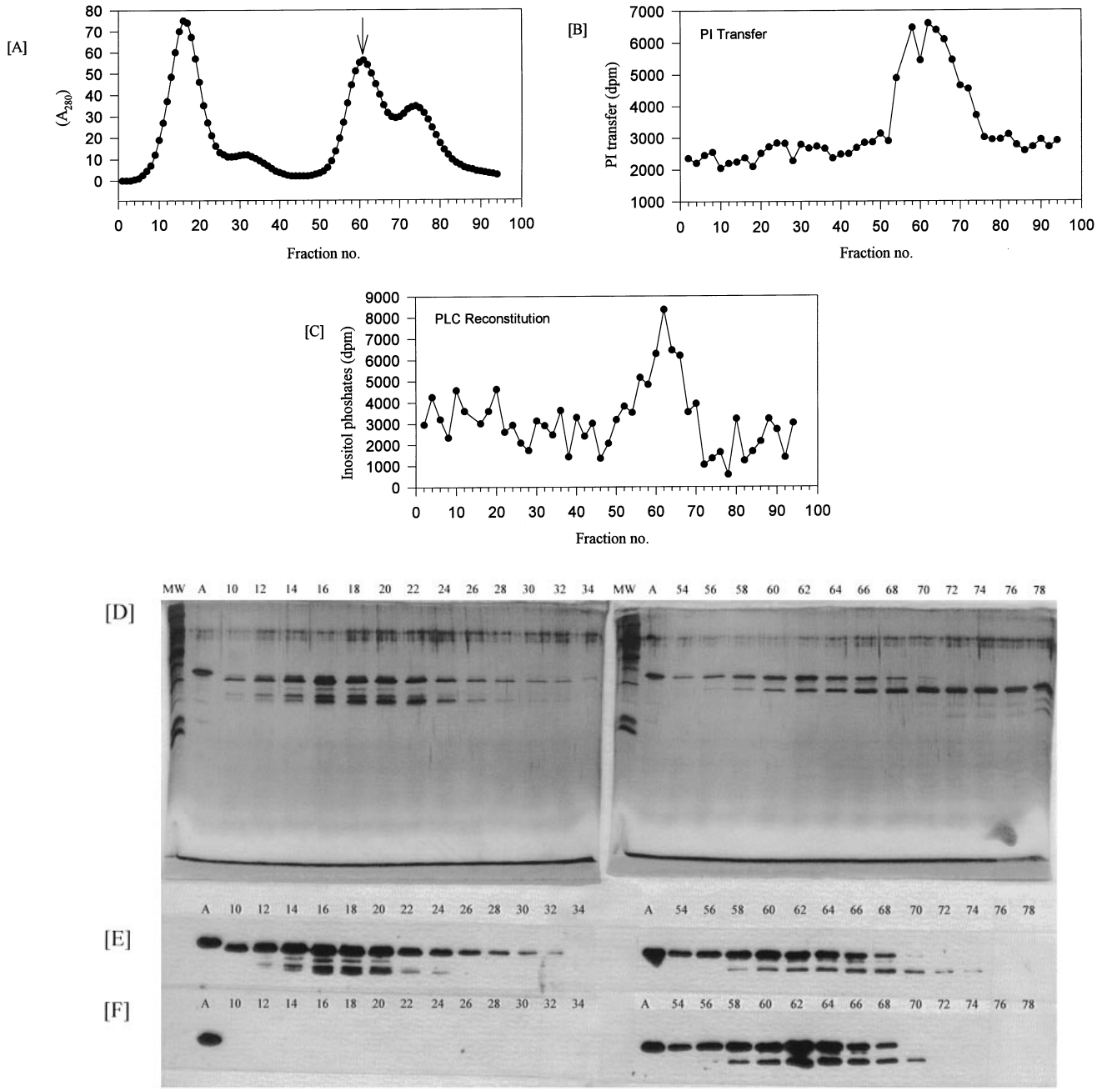

Figure 2 Separation of the truncated forms of PITP from the full-length species by gel filtration and analysis of PI transfer activity, PLC reconstitution, protein profiling and immunoblotting

[**A**] Protein trace after size-exclusion chromatography of proteolysed PITP. A 4.4 mg portion of PITP was digested for 10 min and loaded on a Pharmacia S-75 gel-filtration column eluted at a flow rate of 1.5 ml/min. The first 125 ml were not collected, as no protein was seen in this region. Fractions of volume 1 ml were subsequently collected. The arrow indicates the position where full-length PITP is eluted. [**B**] Transfer activity of column fractions after size-exclusion chromatography. Column fractions were diluted 5-fold prior to analysis of 25 μl samples in a 125 μl assay. [**C**] PLC reconstitution activity of column fractions after size-exclusion chromatography. Samples (20 μl) of column fractions were assayed in a 50 μl assay. [**D**–**F**] Analysis by SDS/PAGE and immunoblotting of column fractions after size-exclusion chromatography. Alternate fractions (3 μl) were analysed. Data from regions where the major proteins are eluted are shown. (fractions 10–34 and 54–78 inclusive). The fraction numbers are labelled, and lane A denotes the full-length PITP protein. 'MW' denotes the molecular-mass markers of 205, 116, 97.4, 66, 45, 29, 20.1 and 18.4 kDa. [**D**] Silver-stained SDS/PAGE gel; [**E**, **F**] Western-blot analysis with [**E**] polyclonal antibody and [**F**] monoclonal antibody 5F12.

To obtain sufficient material for the analysis of PLC activation in the reconstitution assay, conditions were optimized for scaling up the digestion. A 10 min incubation time was found to give suitably sized fragments with sufficient full-length PITP still available to act as an internal control. A 4.4 mg portion of PITP was digested with subtilisin and the fragments were separated by size-exclusion chromatography. The protein profile showed three distinct peaks (Figure 2A). The first peak was eluted between fractions 8 and 24 and contained fragments of PITP (Figure 2D). The elution of the full-length PITP is indicated by an arrow in

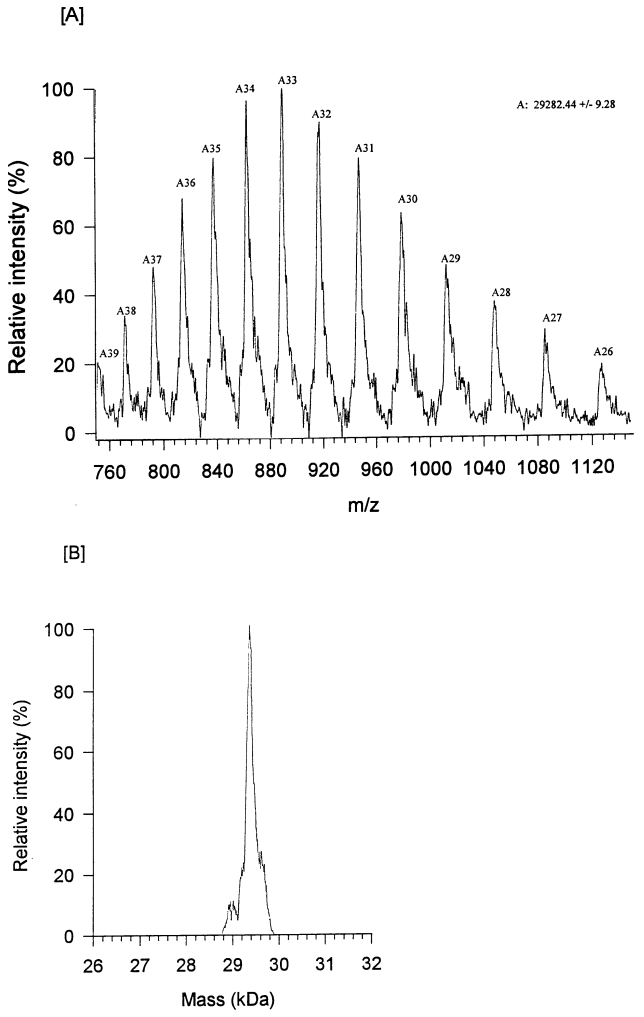

Figure 3 MS analysis of proteolysed fragments of PITP reveals the major fragment having a mass of 29282.4 ± 9.28

Fractions 11–26 inclusive were pooled and concentrated to 2 mg/ml for analysis by MS. [**A**] Electrospray mass spectrum of PITP fragments showing charge states from +26 to +39. [**B**] Reconstructed mass spectrum showing the mass of the neutral molecule.

Figure 2(A) and was independently verified using the full-length protein. As previously observed, PITP is eluted anomalously on gel filtration as a 20 kDa protein [7]. It is clear that the 29 kDa protein fragments are eluted on gel filtration earlier than the full-length PITP. This is most likely the result of dimerization, since calibration of the gel-filtration column indicates that they are eluted between the 43 and 67 kDa markers. Dimerization can be explained if a hydrophobic domain within the protein was exposed. Tremblay et al. [13] reported that cleavage of the C-terminus results in the association with lipid vesicles, again indicating that a hydrophobic region gets exposed. The third peak of protein, which was eluted in fraction numbers 68–80, is attributable to subtilisin.

Column fractions were also probed with polyclonal and monoclonal antibodies to PITP. The polyclonal antibody recognized a major band of 29 kDa in fractions 10–24, plus three other bands at lower molecular masses. Full-length PITP was detected in the expected fractions (54–66). A second minor band of PITP immunoreactivity was also present in fractions 58–70. The monoclonal antibody only identified the full-length PITP

Figure 4 C-terminal cleavage site of PITP by subtilisin

The arrow indicates the site of cleavage at Met^{246} of the major fragment produced by hydrolysis of PITP by the protease subtilisin.

and fragments which were eluted in fractions 58–68. No immunoreactivity was observed with the fragments in fractions 10–24. The lack of immunoreactivity by the monoclonal antibody would suggest that the epitope recognized by the monoclonal antibody had been cleaved.

Lipid transfer and PLC reconstitution activity of PITP fragments

A PI transfer assay and PLC reconstitution assay were performed on the fractions across the gel-filtration run. Figures 2(B) and 2(C) illustrate that PI transfer activity (Figure 2B) and PLC reconstituting activity (Figure 2C) is only associated with the full-length protein. The peak for transfer activity is somewhat wider than the PLC reconstitution peak, as much smaller amounts of PITP are required for the *in vitro* assay. No transfer activity or PLC reconstituting activity was observed in the region of the PITP fragments (fractions 10–24). Peak fractions in this region contained 150 μg/ml of protein, whereas maximal transfer is attained at 1 μg/ml full-length protein. In several runs, transfer activity was analysed without dilution of the sample, but no activity was found to be associated with the fragments.

Mapping of the cleavage site

To identify the site of cleavage by subtilisin of the major fragment in fractions 10–24, the samples from gel filtration were pooled and concentrated to 50 μl. Analysis by SDS/PAGE revealed a major band at 29 kDa with a few minor bands (results not shown). The sample was analysed by MS, and Figure 3 shows a clear major fragment of 29282 Da.

To identify whether cleavage had occurred at the N-terminus or the C-terminus, the concentrated pooled fractions were separated by SDS/PAGE and the major 29 kDa fragment was excised for analysis by Edman degradation. The resultant analysis showed that the N-terminus was completely intact. On the basis of the MS data and the finding that the N-terminus was intact, we calculate the 29 kDa fragment derived from proteolysis by subtilisin to have lost 24 amino acids from the C-terminus of full-length PITP. Cleavage occurred after Met^{246}. The site of cleavage is indicated in Figure 4.

DISCUSSION

Proteolysis of PITPα by subtilisin results in the formation of several fragments, including a major 29 kDa fragment that is 2–3 kDa smaller than the full-length protein. The 29 kDa fragment has lost 24 amino acids residues from the C-terminus of full-length PITP, with cleavage occurring after Met^{246}. Separation of the truncated species from remaining full-length PITP allowed investigation of the properties of these truncated versions. Deletion of the C-terminus led to loss of both PI transfer function and the ability to restore PLC signalling in permeabilized HL60 cells. We conclude that the C-terminus is crucial for the biological activity of PITP.

While the present study was in progress, Tremblay et al. [13] reported that proteolysis of PITP by trypsin also resulted in the deletion of the C-terminus. Their analysis revealed deletions of 12 and 18 amino acid residues respectively. The only analysis of function performed on these truncated forms was for PC transfer activity, and this was found to be substantially reduced with respect to the full-length protein. In a further study, using deletion mutants where 12 and 18 amino acids were deleted, it was reported that deletion of 18 amino acid residues completely eliminated PC transfer activity, whereas deletion of 12 amino acid residues had no effect [16]. PC transfer is an insensitive assay for PITP function and, in addition, it has been noted previously that PI transfer and PC transfer can be uncoupled [17]. In our study we analysed both PI transfer and PLC reconstitution as markers of PITP function, and our results, combined with those of Tremblay et al. [13], confirm the importance of the C-terminus.

A polyclonal antibody to PITP recognizes all the truncated species. However, the monoclonal antibody 5F12 fails to recognize the fragments in fractions 10–24. This allows us to conclude that the epitope recognized by the monoclonal antibody is in the last 24 amino acid residues of the C-terminus.

We have previously put forward a model whereby PI bound to PITP is sequentially presented to the lipid kinases involved in the production of PIP_2. The newly synthesized PIP_2 molecule is subsequently presented to PLC for hydrolysis [11]. It may be that the loss of the C-terminus is important in the role of PITP as a substrate supplier to the kinases. This function would require specifically the PI transfer activity of PITP and, in keeping with the above, this activity is eliminated by deletion.

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