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Mechanism of interaction of PITPα with membranes: Conformational changes in the C-terminus associated with membrane binding

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Abstract

Eukaryotic phosphatidylinositol transfer proteins (PITPs) are composed predominantly of small (~32 kDa) soluble proteins that bind and transfer a single phospholipid, normally phosphatidylinositol or phosphatidycholine. Two forms, PITPα and PITPβ, which share approximately 80% amino acid sequence similarity, are known. Rat PITPα was labeled at specific single reactive Cys residues with I-AEDANS and used to examine PITP–membrane interactions. Upon binding to phospholipid vesicles, PITP labeled with AEDANS at the C-terminus, a region postulated to be involved in membrane binding, shows significant decreases in both steady-state and dynamic fluorescence anisotropy. In contrast, PITPs labeled with AEDANS at sites located distal to the C-terminus show increases in both steady-state and dynamic anisotropy. These results suggest that interaction of PITP with membrane surfaces leads to significant alterations in conformation and perhaps melting of the C-terminal helix.
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Eukaryotic phosphatidylinositol transfer proteins (PITPs)[1] are a family of proteins that have the ability to bind and in some cases transport phospholipids, in particular PtdIns and PtdCho. They are composed predominantly of small monomeric globular proteins with molecular weights around 32 kDa. PITPs[1] were first purified by Helmkamp et al. [1] from bovine brain. In 1989, the sequence of rat PITP was determined by analysis of cDNA [2]. It encoded a protein of 271 amino acids, which showed no sequence similarity with other known proteins. Subsequently, amino acid sequences have been determined from the cDNAs for a large number of PITPs in organisms ranging from humans to yeast [3–7]. Most higher eukaryotes contain genes encoding two related soluble proteins termed PITPα and PITPβ which exhibit 75–80% sequence identity [2,8]. Yeast has PITPs that appear to have a different evolutionary origin and show no significant sequence similarity to PITPs of higher eukaryotes. However, mammalian PI TPs can partially complement a defect in yeast PITPs (*sec*14 gene) [5]. Interestingly, a PITP-related membrane protein of ~160 kDa was first identified in *Drosophila* which encoded a gene related to retinal degeneration termed rdgB [9,10]. The amino terminal 250 amino acids of this protein show sequence similarity to the soluble PITPs. Mammals have two related genes termed rdgBα and rdgBβ [11,12]. In contrast to rdgBα which is membrane bound, rdgBβ is a soluble protein of ~38 kDa.

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[1] *Abbreviations used:* ANS, 1-anilinonaphthalene-8-sulfonic acid; CD, circular dichroism; Hepes PITP buffer, 0.02 M Hepes, pH 7.5, 1 mM EDTA, 0.05 M NaCl; I-*AEDANS*, *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid; PITP, phosphatidylinositol transfer protein; PITPα*, PITPα-C188A; PtdCho, phosphatidyl choline; PtdEth, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid; SUV, small unilamellar vesicles; r, anisotropy; r_0, the limiting anisotropy; ϕ, rotational correlation time; τ, excited state lifetime; TCSPC, time-correlated photon counting.

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Soluble PITPs have the capacity to catalyze phospholipid exchange from one membrane to another, which is presumably related to their function in vivo. The phospholipid transport function of PITPs requires that they be able to rapidly associate and dissociate from membranes during the process of phospholipid exchange. Previous studies have shown that the C-terminus plays a crucial role in this process. Tremblay et al. [13] found that free PITPα was highly resistant to trypsin digestion. However, when bound to phospholipid vesicles, the C-terminus was rapidly cleaved at Arg253 and Arg259. Gel filtration studies showed that cleavage of the C-terminus greatly enhanced binding of PITP to membranes [14]. Subsequently, PITPs truncated at positions 253 and 259 were produced and studied in detail [14–16]. PtdCho transfer activity of PITPα-1–259 was unaltered when measured with vesicles containing 100 mol% PtdCho, however, transfer activity was significantly inhibited if donor or acceptor membranes contained high levels of PtdOH. PITPα-1–253 showed low but measurable transfer activity with vesicles containing 100 mol% PtdCho. HPLC gel filtration studies showed that both PITPα-1–259 and PITPα-1–253 were completely bound to vesicles containing 80:20 mol% PtdCho:PtdOH. It was concluded that the C-terminus played an important role in regulating the affinity of PITP for membranes, especially membranes containing acidic phospholipids. Analysis of the conformational properties of the truncated derivatives showed that both were similar and that they differed significantly from full-length PITP in terms of exposure of Tyr and Trp residues, reactivity of sulfhydryl residues, and binding of the nonpolar fluorescent probe, ANS [15]. Overall, the structures of the truncated proteins appeared to be much more open than the structure of full-length PITPα. Thus, the C-terminus plays a crucial role in maintaining the folded compact structure of PITP as well as acting to reduce the affinity for membranes containing acidic phospholipids.

Recently, the structures of PITPα containing bound PtdCho as well as the apo form have been determined by X-ray diffraction [17,18]. In overall folding, PITPs were found to be structurally related to proteins containing the START domain [19,20]. The basic structure of PITP involves an eight stranded β-sheet with three long and four shorter α-helices forming a pocket that completely encloses the bound phospholipid. Thus, there must be significant conformational changes for phospholipid exchange to occur. The C-terminus helps to enclose the cavity containing the phospholipid with the C18 methyl group of the Sn-1 acyl chain only about 4 Å away from the C-terminal penultimate amino acid (Asp270). Based on the structure of PITPα, we proposed that the C-terminal tail region of PITP binds to the membrane and that the binding cavity opens up to allow exchange of the bound lipid for another membrane lipid [7]. Schouten et al. [18] determined the structure of the apo form of PITPα by X-ray analysis and found significant conformational differences from the holo form. In particular, the C-terminus appeared to swing out from the rest of the protein accompanied by unwinding and/or unfolding of residues 254–257 and 258–271.

To understand the function of PITPs will require information about the kinetics and thermodynamics of interaction with membranes and the nature of the conformational changes occurring during binding to membranes and exchange of phospholipids. To that end we have inserted single reactive Cys residues at specific locations within PITPα* which are predicted to be very near or part of the region interacting with the membrane surface (amino acid 269 or an additional Cys at 272) or regions thought to be located distal to the membrane surface (amino acids 91 or 122). These proteins have been labeled with the fluorescent probe, I-*AEDANS*, and used for studies of steady-state and dynamic anisotropy as well as the kinetics of PITP-membrane binding. Results show that the interaction of the fluorescent-labeled proteins with membranes is readily monitored using both equilibrium and rapid kinetic techniques and that the mobility of the C-terminal region is greatly enhanced by binding to membranes, consistent with melting of a significant part of the C-terminal helix.

Materials and methods

Reagents

I-*AEDANS* was from Molecular Probes. Phospholipids were from Avanti: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, 1,2-dioleoy-*sn*-glycero-3-phosphate, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine. Other chemicals were of reagent grade.

Expression and purification of PITPs

The coding sequences for PITPs in the expression vector pET11C were expressed in *Escherichia coli* BL21 DE3 and the proteins were purified to homogeneity as described previously [13].

Construction of mutant PITPs

Rat PITPα contains four cysteine residues at positions 95, 188, 192, and 231 [21]. In the free protein, Cys188 reacts with several sulfhydryl reagents. Cys95 reacts with such reagents only when the protein is bound to membranes. Cys192 and Cys231 are unreactive, even when the protein is bound to membranes. Conversion, individually, of each of these four Cys to Ala was without effect on phospholipid transfer properties [21]. We therefore used PITPα-C188A(PITPα*) as the starting clone for further amino acid changes. Oligonucleotide-directed mutagenesis (Quikchange, Stratagene, La Jolla, CA) was used to construct derivatives of PITPα-C188A which contained a single reactive sulfhydryl residue at either the C-terminus (amino acid 269 or 272) or in regions thought to be well away from the membrane binding site (positions 91 and 122, Fig. 1) [17]. Sequences of the mutants were confirmed by sequence

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