RESEARCH COMMUNICATION

Phospholipase C δ_1 requires a pleckstrin homology domain for interaction with the plasma membrane

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The structural requirements of phospholipase C δ_1 for interaction with the plasma membrane were analysed by immunofluorescence after microinjection into living cells. Microinjection of deletion mutants revealed that the region required for membrane at-

tachment and binding of inositol 1,4,5-trisphosphate *in vitro* corresponded to the pleckstrin homology domain, a structural module described in more than 90 proteins.

INTRODUCTION

A wide variety of extracellular signals stimulate cells to hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP₂) thus generating two second messengers, *myo*-inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol [1]. In addition to this well-defined function of PIP₂, new roles and different cellular locations where the hydrolysis could take place have recently been suggested [2]. These complex roles of PIP₂ and its hydrolysis are tightly regulated by the phospholipase C (PLC) enzymes, which comprise a superfamily of related proteins with different subcellular distribution, linked by diverse mechanisms to transmembrane receptors [2]. The three main families of PLC are designated as PLC β , PLC γ and PLC δ . Sequence similarity within the superfamily is mainly restricted to the catalytic domain, while each family has characteristic features implicated in interactions with upstream signalling components and targeting molecules. The structural features of PLC β and PLC γ involved in regulatory and targeting interactions have been studied extensively [2].

In contrast to PLC β and PLC γ isoenzymes, the physiological role and regulation of PLC δ remain poorly defined. Limited experimental evidence has suggested that calcium ions could play an important part in the regulation of this isoenzyme [3]. Consistent with this possibility is the presence of a calcium-binding motif, an EF-hand, as a unique feature of members of the PLC δ family [4]. PLC δ was originally regarded as a cytosolic enzyme. However, recent experimental evidence has demonstrated a distribution between the cytosolic and particulate fractions in several cell lines and tissues [3,5–7]. Furthermore, PLC δ_1 has been found in highly purified preparations of colonocyte plasma membrane [8], sarcolemma [9] and ascites hepatoma plasma membrane [10], demonstrating interaction with cellular membrane structures. Supporting evidence for interaction with membranes has also been obtained from studies of purified PLC δ_1 *in vitro*. It has been shown that PLC δ_1 can bind

specifically to lipid vesicles containing PIP₂; once bound this enzyme could subsequently catalyse many cycles of substrate hydrolysis, acting in a processive mode [11]. These studies have also shown that the attachment to lipid vesicles requires the N-terminal part of the protein. Interestingly, the N-terminal part of PLC δ_1 contains a pleckstrin homology (PH) domain, a sequence homology region of approximately 100 amino acids found in more than 90 proteins [12,13]. Despite limited sequence identity, the structural similarities recently reported for the PH domains of pleckstrin, spectrin and dynamin support the classification of these regions as discrete domains. Whether the PH domain of these proteins share common functions remains to be elucidated. Nevertheless, studies of several proteins *in vitro* suggest that their PH domains could provide a site for protein–lipid and protein–protein interactions [14,15].

In this study, we investigate the subcellular localization and targeting domains of PLC δ_1 . We describe the distribution of the enzyme in cell lines that endogenously express PLC δ_1 and in cells where it was introduced by transient transfection or microinjection. It is demonstrated that PLC δ_1 can interact with plasma membrane and, by microinjection of deletion mutants, that this attachment requires the PH domain. Since the PH domain represents a common module of many different proteins, this study provides the first example of its role in membrane association in the context of living cells.

MATERIALS AND METHODS

Plasmid construction

The cDNA encoding rat brain PLC δ_1 [16] was subcloned into eukaryotic expression vector pEXV3 using *Xma*I restriction sites; the resulting construct was designated pEXV3-PLC δ_1 . A bacterial expression vector, pGEX-2T (Pharmacia), was used to generate PLC δ_1 fusion proteins. The fragment from PLC δ_1

Abbreviations used: PIP₂, phosphatidylinositol 4,5-bisphosphate; IP₃, *myo*-inositol 1,4,5-trisphosphate; PLC, phospholipase C; PH domain, pleckstrin homology domain; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MDCK cell, Madin Darby canine kidney cell; β ARK, β -adrenergic receptor kinase.

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encoding amino acid residues 1–175 was amplified by PCR and subcloned between the *Bam*HI and *Eco*RI sites in the pGEX-2T vector; this construct was designated 1–175 PLC δ_1 . For the subcloning of full-length PLC δ_1 into pGEX-2T vector, the internal *Bam*HI site was first mutated by PCR without changing the amino acid sequence; the *Bam*HI/*Dra*III fragment from the 1–175 PLC δ_1 construct was then subcloned into a plasmid, encoding amino acids 58–756 of PLC δ_1 , as a glutathione S-transferase fusion [16], also digested with *Bam*HI and *Dra*III. The deletion of the fragment encoding residues 30–40 was introduced by PCR and the amplified DNA was subcloned into plasmid 1–756 PLC δ_1 using *Bam*HI/*Dra*III restriction sites, thus generating construct 1–756 Δ 30–40 PLC δ_1 . For construction of the plasmid 1–290 PLC δ_1 , the construct 1–756 PLC δ_1 was digested with *Acc*I/*Sma*I and re-ligated. The pGEX plasmid 135–756 PLC δ_1 has been described previously [16]. All fragments generated by PCR were verified by DNA sequencing.

Expression and purification of recombinant proteins

Expression and purification of recombinant PLC δ_1 protein lacking the first 57 amino acid residues has been described previously [16]. The same procedure was used to obtain the full-length protein and other proteins with deletions within the N-terminal part of PLC δ_1 . PLC δ_1 protein containing residues 1–290 was concentrated on a Mono Q column and further purified by chromatography on a Superdex 75 column (PC 3.2/30) equilibrated in PBS. The other deletion mutant containing only the N-terminal part of PLC δ_1 (residues 1–175) was purified on a Mono S column (PC 1.6/5); a linear gradient of NaCl in 50 mM Mes, pH 6.5, was used to elute the protein. The N-terminus of each protein was verified by protein sequencing.

Cell culture, transfection and fractionation

All cell lines were grown in Dulbecco's modified Eagles's medium (DMEM) containing 10% fetal calf serum (FCS). For transient transfection, COS1 cells were incubated with pEXV3-PLC δ_1 plasmid DNA–lipofectin (Gibco-Life Science) complex (1 μ g:25 μ l) for 5 h in the serum-free medium and then grown for 48 h in 10% FCS/DMEM. The efficiency of transfection was between 30 and 50%. For cell fractionation of all cell lines, 2–5 60-mm Petri dishes of cells grown to confluency were prepared. Preparation of cytosol and particulate (membrane containing) fractions was as described previously [17]. The particulate fraction was washed and resuspended in the buffer containing 0.2% Triton X-100 and the extraction was carried out for 15 min at 4°C. Samples of the different fractions (about 50 μ g of protein) were analysed by Western blotting using a pool of mouse anti-PLC δ_1 antibodies (Upstate Biotechnology) and an enhanced chemiluminescence detection kit (Amersham).

Assays of PLC activity and IP $_3$ binding

The assay of hydrolysis of PIP $_2$ used to analyse PLC δ_1 deletion mutants was the same as described previously [18]. The assay for the binding of IP $_3$ to PLC δ_1 was performed according to the method of Kanematsu et al. [5]. The assay mixture (0.5 ml) contained 0.1–0.8 μ g of PLC δ_1 proteins in 50 mM Tris/HCl, pH 8.3, 1 mM EDTA, 0.2% Triton X-100 and 1 nM [3 H]IP $_3$ (Amersham).

Microinjection and immunofluorescence analysis

Madin Darby canine kidney (MDCK) cells grown to confluency

on 60-mm Petri dishes were subjected to microinjection on a Zeiss Microinjection Workstation (Carl Zeiss, Oberkochen). The EXV3-PLC δ_1 plasmid (50 μ g/ml) was microinjected into the nucleus of selected cells. Samples of purified proteins (0.5–2 mg/ml) were microinjected intracytoplasmically. Immunofluorescence staining was carried out as described previously [19]. Primary antibody incubation with mouse anti-PLC δ_1 monoclonal antibodies (diluted 1:150) was for 1 h at room temperature. After a wash in PBS, the cells were incubated with fluorescein isothiocyanate–anti-mouse IgG antibody conjugate for 1 h at room temperature. For double staining, anti-ras rat monoclonal antibodies (Y13-238 plus Y13-259) were included in the primary incubation and Texas Red anti-rat fluorescence antibody conjugate was added to the second antibody incubation. Labelled preparations were mounted under glass coverslips and examined with a Bio-Rad MRC 600 confocal imaging system in conjunction with a Nikon Diaphot epifluorescence microscope.

RESULTS

Subcellular distribution of PLC δ

In our studies of the subcellular distribution of PLC δ_1 we first tested cell lines that endogenously express this PLC isoform. Rat adrenal pheochromocytoma (PC12), MDCK epithelial cells, rat glial cells (C6) and mouse fibroblasts (NIH-3T3) were selected for cell fractionation and detection of PLC δ_1 by Western blotting. The post-nuclear supernatant was used to obtain particulate (membrane containing) and cytosolic fractions [17]; the membranes were solubilized by extraction with 0.2% Triton X-100. In all four cell lines, monoclonal antibodies to PLC δ_1 reacted with an 85 kDa polypeptide in both the cytosolic (c) and particulate (p) fractions (Figure 1). Most of the PLC δ_1 present in the particulate fraction was extracted with 0.2% Triton X-100. The same result was obtained after fractionation of COS cells transiently transfected with the plasmid encoding PLC δ_1 (Figure 2A).

To further investigate subcellular localization of PLC δ_1 , we complemented the biochemical studies with immunocytochemical methods. In cells expressing PLC δ_1 endogenously, the level of detectable staining and the cellular morphology were not suitable for this experimental approach. Instead, the immunofluorescence analysis was carried out with MDCK epithelial cells microinjected with the plasmid encoding PLC δ_1 . MDCK cells

Figure 1 Distribution of PLC δ_1 between cytosolic (c) and particulate (p) fractions prepared from PC12, MDCK, C6 and NIH3T3 cells

The presence of PLC δ_1 in the different fractions was analysed by Western blotting. The fractions correspond to the cytosol (lanes 1, 4, 7 and 10), extract obtained from the particulate fraction using 0.2% Triton X-100 (lanes 2, 5, 8 and 11) and pellet remaining after the Triton extraction (lanes 3, 6, 9 and 12). The positions of molecular-mass markers (kDa) are indicated on the right-hand side.

Figure 2 Subcellular localization of recombinant PLC δ_1

The distribution of PLC δ_1 after transient transfection of COS cells with the plasmid (pEXV3-PLC δ_1) encoding full-length protein was analysed by Western blotting (A). The fractions correspond to the cytosol (lane 1), extract obtained from the particulate fraction using 0.2% Triton X-100 (lane 2) and pellet remaining after the Triton extraction (lane 3). The positions of molecular-mass markers (kDa) are indicated on the right-hand side. Immunofluorescence analysis of MDCK cells was performed 18 h after microinjection of the pEXV3-PLC δ_1 plasmid (B, left panel). The same cells were also stained with an anti-ras antibody (B, right panel). Immunofluorescence analysis of MDCK cells microinjected with purified PLC δ_1 protein (0.5 mg/ml), expressed in bacteria, was carried out 8 h after microinjection (C). PLC δ_1 endogenously expressed in MDCK cells could not be detected by immunofluorescence analysis. The scale bar (B, left panel) corresponds to 20 μ m.

present a tall, vertical stack of membranes at their basolateral margins and plasma membrane associated proteins, such as p21 ras (Figure 2B, right panel), can be visualized easily by immunofluorescence. Staining of the microinjected cells clearly shows that some of the PLC δ_1 associates with the plasma membrane (Figure 2B, left panel). When MDCK cells were microinjected with the bacterially expressed and purified PLC δ_1 protein, the same staining pattern was observed (Figure 2C).

For analysis of the structural requirements for membrane attachment, deletions were introduced into different regions of PLC δ_1 and the resulting recombinant proteins were expressed in bacteria. This enabled the same protein preparation to be used for biochemical studies *in vitro* and for analysis of subcellular localization after microinjection in MDCK cells.

Expression and characterization *in vitro* of PLC δ_1 deletion mutants

The full-length and deletion mutants of PLC δ_1 are represented schematically in Figure 3A. In construct 2, the entire PH domain (residues 22–134) and a short region preceding it (residues 1–21) were deleted. A smaller portion of the PH domain (residues 22–57) and the preceding region (residues 1–21) were deleted in construct 3. Within the PH domain of PLC δ_1 , the region between

residues 30 and 43 is highly charged (Figure 3B), and it has been shown that a synthetic peptide encompassing these residues can interact with IP $_3$ *in vitro* in a sequence-specific manner [20]. Part of this region (residues 30–40), corresponding to predicted variable loop 1, was deleted in construct 4. This loop varies in size (and can even be absent) in different PH domains [21]. Two constructs (5 and 6) that expressed only the N-terminal part of PLC δ_1 (residues 1–290 and 1–175) were also made. The levels of expression of all deletion mutants were comparable with that of the full-length protein. Furthermore, thrombin cleavage of each fusion protein produced a polypeptide of the predicted molecular size and correct sequence at the N-terminus (Figure 3C).

The integrity of the catalytic core of PLC δ_1 proteins with deletions within the N-terminal part of the molecule was confirmed in an enzyme assay *in vitro* with the substrate presented as sodium cholate/PIP $_2$ mixed micelles (Figure 4A, lanes 2, 3 and 4). This assay does not measure processive catalysis, and the specific activities of full-length PLC δ_1 and the deletion mutants were about 1000 μ mol/min per mg, consistent with previously reported values [16].

Several assays related to function(s) of the N-terminus of PLC δ_1 have been developed *in vitro*. The full-length protein can bind, specifically, phospholipid vesicles containing PIP $_2$ as well as IP $_3$, the water-soluble product of PIP $_2$ hydrolysis [20,22]. We used the binding to IP $_3$ to analyse the full-length and deletion mutants of PLC δ_1 (Figure 4B). The calculated value for IP $_3$ -binding to the full-length protein (350 ± 40 pmol/mg) was within the range of values reported previously [5,20]. All proteins containing a deletion within the N-terminal part of PLC δ_1 completely lost the ability to bind IP $_3$ (Figure 4B, lanes 2, 3 and 4). In contrast, the N-terminal part of PLC δ_1 , encoded by constructs 5 and 6, still bound IP $_3$ (Figure 4B, lanes 5 and 6); this binding was, however, reduced 4–5-fold. The reduction in binding was not due to deletion of a separate binding site, since it has been determined by Scatchard analysis that the full-length protein [5,20] and the N-terminal part of PLC δ_1 (results not shown) have a single binding site for IP $_3$. Analysis of the interaction between PLC δ_1 deletion mutants and large unilamellar vesicles containing PIP $_2$ was also performed. Results of these experiments, although not quantitative, were in agreement with measurements of IP $_3$ binding (results not shown).

Subcellular localization of PLC δ_1 deletion mutants

To study the ability of PLC δ_1 deletion mutants to interact with plasma membrane, the purified proteins were microinjected into MDCK cells. Detection of these proteins was based on their recognition by a pool of monoclonal antibodies raised against full-length PLC δ_1 . All deletion mutants shown in Figure 3 were recognized by the antibodies; the cross-reactivity with proteins containing residues 1–175 and 1–290 (constructs 5 and 6) was reduced only 2–3-fold.

The results of immunofluorescence analysis are summarized in Figure 5. PLC δ_1 protein with deletion of 134 residues from the N-terminus (construct 2) completely lost its ability to interact with plasma membrane (Figure 5B). A smaller deletion from the N-terminus (57 residues, construct 3) and the deletion of 10 residues within the PH domain (residues 30–40, construct 4) also completely abolished membrane attachment (Figures 5C and 5D). The subcellular distribution of the constructs consisting of the N-terminal part of PLC δ_1 (residues 1–290 and 1–175) was very similar to that observed with the full-length protein; plasma membrane staining could be detected for both of these proteins (Figures 5E and 5F).

Using immunofluorescence analysis, it is difficult to evaluate

Figure 3 PLCδ₁ and the deletion mutants

(A) Schematic representation of the structure of PLCδ₁ and the deletion mutants that were analysed. Within the diagram of the full-length protein (construct 1) the location of the PH domain and the EF-hand motif (EF) are indicated as stippled areas. The regions conserved within the PLC superfamily (X and Y) are denoted by white boxes. The deletion mutants (constructs 2–6) are represented by black bars and the numbers corresponding to the first and last amino acid residue encoded by each construct are shown at the right-hand side. (B) Sequence alignment of the predicted PH domain of PLCδ₁ and the PH domains (from dynamin, spectrin and pleckstrin) with determined 3-dimensional structure. The sequence alignment presented here is based on alignments of different PH domains reported previously [21]. Underlining in the pleckstrin sequence indicates the residues reported to be involved in PIP₂ binding [14]. The peptide (residues 30–43) underlined in the PLCδ₁ sequence has been shown to bind IP₃ *in vitro* [20]. The arrows indicate residues selected for construction of PLCδ₁ deletion mutants. (C) Analysis of the full-length and deletion mutants of PLCδ₁ by SDS/PAGE. Samples of purified proteins (about 8 μg) were analysed on a gel containing 10% (lanes 1–5) or 15% polyacrylamide (lane 6). Lane 1, construct 1; lane 2, construct 4; lane 3, construct 3; lane 4, construct 2; lane 5, construct 5; lane 6, construct 6. The positions of molecular-mass markers (kDa) are indicated on the right-hand side.

the relative amounts of PLCδ₁ present in the cytosol or bound to the membrane; the resulting data are therefore mainly qualitative. Within this limitation, the results obtained for the membrane attachment (Figure 5) and the measurements of IP₃-binding *in vitro* (Figure 4B) show good correlation for all tested deletion mutants of PLCδ₁.

DISCUSSION

In this paper we describe studies on the structural requirements of PLCδ₁ for its attachment to membrane. Our initial analysis of the subcellular localization of PLCδ₁ demonstrated a distribution between cytosolic and membrane-containing fractions (Figure 1). Similar observations have been reported for other tissue and cell types [3,5–7]. Studies in several systems [8,10] defined more precisely the plasma membrane as one of the cellular components

that contains PLCδ₁. It is therefore likely that the ability of PLCδ₁ to associate with cellular membranes represents a general property of this enzyme. Our immunofluorescence analyses of PLCδ₁ microinjected into MDCK cells (Figures 2B and 2C) are in agreement with the experiments using subcellular fractionation.

The results obtained from immunofluorescence analysis of PLCδ₁ deletion mutants (Figure 5) demonstrate that the intact N-terminal part, containing the PH domain, is required for membrane attachment. It has been pointed out [14] that many proteins with the PH domain require interaction with the membrane in order to function. Studies of the role of the PH domain in several proteins have suggested two functions that could determine or facilitate interactions with cellular membranes, namely, binding to βγ-subunits of G-proteins and binding of PIP₂. It has been found that the region of interaction with βγ-

Figure 4 Measurements of PIP₂ hydrolysis and IP₃ binding by the full-length and deletion mutants of PLC δ_1 .

Preparations of purified proteins were compared in two assays *in vitro*, hydrolysis of PIP₂ (A) and binding of IP₃ (B). The values for the specific enzyme activity and IP₃ binding of the full-length protein are stated in the text. The values determined for each deletion mutant are expressed as a percentage of the value obtained for the full-length protein. Three independent measurements were performed for each protein sample.

subunits in β -adrenergic receptor kinase (β ARK) overlaps with the C-terminal part of the PH domain [23]. These observations have been extended to PH domains from some other proteins [24]; the PH domain of PLC δ_1 was not included in these studies. The other function suggested for several PH domains (pleckstrin, T-cell-specific kinase, ras GTP-ase activating protein and β ARK) is binding to PIP₂ presented within phospholipid vesicles. Analysis of NMR chemical-shift differences upon binding to PIP₂ have shown that the residues within the N-terminal part of the pleckstrin PH domain are involved in this interaction [14]. Previous experiments with PLC δ_1 [11,20], and those presented here (Figure 4B), also demonstrated a requirement for the N-terminal part of the PH domain for binding of PIP₂ and its water-soluble product, IP₃. Thus, the two suggested functions (interaction with $\beta\gamma$ -subunits of G-proteins and binding to PIP₂) may be related to different regions of the PH domains. It is, however, unlikely that the N-terminal and C-terminal halves can be structurally independent [13], and mutations affecting the structure of the PH domain could interfere with both functions.

The mutations affecting the PH domain of PLC δ_1 abolished

IP₃-binding *in vitro* and also abolished the membrane attachment within living cells. Provided that the structural integrity of the PH domain was preserved after deletion of the region directly implicated in binding to the inositol head group (residues 30–40), these data suggest that interaction with PIP₂ may be involved in membrane attachment. This is consistent with a model proposed by Harlan et al. [14], that an interaction site for proteins with the PH domain could be provided by PIP₂ molecules present in cellular membranes.

We also attempted to define regions of PLC δ_1 that are sufficient for its attachment to membrane. The smallest construct recognized by the antibodies and found to interact with the plasma membrane (Figure 5F) consisted of the first 175 residues. This region included the entire PH domain and an additional 41 residues at its C-terminus (construct 6, Figure 3A). Thus, at present, involvement of other interaction sites within the C-terminal part of this construct cannot be ruled out. Recent experiments have shown that translocation of β ARK requires interaction with PIP₂ and $\beta\gamma$ -subunits of G-proteins [15]. If the PH domain of PLC δ_1 is involved in both protein–lipid and protein–protein interactions, $\beta\gamma$ -subunits of G-proteins may not be the interacting partner. Consistent with previous finding [25], these subunits could not activate PLC δ_1 in the same system *in vitro* where substantial activation of PLC β_2 was achieved. A role for the EF-hand motif as a membrane-attachment site also seems unlikely, since the point mutations that abolished binding of calcium *in vitro* did not change the subcellular distribution of PLC δ_1 protein between the cytosolic and particulate fractions (results not shown).

The importance of membrane attachment for the regulation of PLC δ_1 *in vivo* has yet to be assessed experimentally. Nonetheless, existing evidence suggests that activation of PLC δ may involve steps additional to association with the membrane. It has been shown that overexpression of PLC δ_1 in Chinese hamster ovary cells led to a substantial increase in the amount of PLC δ_1 present in the membrane fraction, but the production of IP₃ in those cells was only marginally increased. Only further stimulation of these cells (e.g. using a calcium ionophore or thrombin) resulted in a 5–7-fold increase in IP₃ production [3]. Another line of ex-

Figure 5 Immunofluorescence analysis of the full-length and deletion mutants of PLC δ_1 after microinjection of the purified proteins into MDCK cells

MDCK cells were microinjected with the purified proteins (1.5 mg/ml) and stained after 2 h with a pool of monoclonal antibodies to PLC δ_1 , followed by fluorescein isothiocyanate–anti-mouse IgG antibody conjugate. The fluorescence micrographs correspond to: (A) construct 1; (B) construct 2; (C) construct 3; (D) construct 4; (E) construct 5; and (F) construct 6.

perimental evidence obtained from studies of PLC δ_1 *in vitro* indicates that membrane attachment could be the site of down-regulation rather than activation. Since IP $_3$ can compete with PIP $_2$ binding *in vitro* [22], it has been suggested that an increase in IP $_3$ concentration upon stimulation of the enzyme could inhibit membrane attachment and thus limit access to its substrate.

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