Phospholipase C-δ1 Contains a Functional Nuclear Export Signal Sequence*

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We have previously observed, using a green fluorescent protein (GFP) fusion system, that PLC-δ1 is localized mainly at the plasma membrane and in the cytosol, whereas little is present in the nucleus in Madin-Darby canine kidney cells (Fujii, M., Ohtsubo, M., Obara, T., Kamata, H., Hirata, H., and Yagisawa, H. (1999) Biochem. Biophys. Res. Commun. 254, 284–291). Herein, we demonstrate that PLC-δ1 has a functional nuclear export signal (NES) sequence in amino acid residues 164–177 of the EF-hand domain. The fluorescence of NES-disrupted GFP/PLC-δ1 expressed in Madin-Darby canine kidney cells was present not only at the plasma membrane and in the cytosol but also in the nucleus. Moreover, treatment with leptomycin B, a specific inhibitor of NES-dependent nuclear export, resulted in the accumulation of GFP/PLC-δ1 in the nucleus. A site-directed mutant containing a pleckstrin homology domain, which does not bind inositol 1,4,5-trisphosphate and cannot hydrolyze phosphatidylinositol 4,5-bisphosphate in vitro, accumulated in the nucleus to a much greater extent than wild-type GFP/PLC-δ1 after treatment with leptomycin B. These results suggest that PLC-δ1 is shuttled between the cytoplasm and the nucleus; its nuclear export is dependent on the leucine-rich NES sequence and its active nuclear import is regulated by an unidentified signal(s).

Phosphatidylinositol-specific phospholipase C (PI-PLC) is one of the key enzymes in the intracellular signal transduction pathway (1, 2). PI-PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) generating inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) and diacylglycerol (DG), both of which act as second messengers in cells. Ins(1,4,5)P₃ participates in intracellular Ca²⁺ mobilization and DG activates certain protein kinase C isoforms (3, 4). At present, there are three main families of PI-PLC: PLC-α, PLC-γ, and PLC-β (1, 2, 5, 6). PLC-β is activated by α- or β-subunits of the heterotrimeric Gq protein (7–11). PLC-γ, which contains two Src homology 2 domains and one Src homology 3 domain, is activated by both receptor and nonreceptor tyrosine kinases (12–17). The regulatory mechanisms of the PLC-δ isoform family, however, are not well understood. In mammalian δ type PLC, four isoforms have been cloned (5, 18–22). The δ family is thought to be the earliest evolutionary form of mammalian PLCs, because the structure of the isoforms is the simplest, and cloned PLCs of yeast, slime molds, and plants show the highest homology with the δ isoform (23–28). PLC-δ1, which has been purified from rat brain, is an 85-kDa soluble protein (29). Recent x-ray crystallographic analysis of PLC-δ1 (30) has revealed that the molecule essentially consists of four domains: from the N to the C terminus a pleckstrin homology (PH) domain, four closely associated EF-hand motifs, a catalytic X and Y domain, and a C2 domain.

We have previously demonstrated that PLC-δ1 is predominantly localized at the plasma membrane and in the cytosol (31, 32). The PH domain is essential for binding to the plasma membrane. A novel δ4 isoform has recently been cloned from regenerating rat liver (21) and from rat brain (22) and its localization in the nucleus has been reported (21). Although the structure of the δ4 isoform is similar to that of δ1 (45% identity, 65% similarity) (33), its intracellular distribution is quite different. Expression of nuclear PLC-δ4 dramatically increases at transition from the G1 to S phase, and the high expression continues to the end of the M phase (21). PLC-β1 is constantly present in the nucleus of Swiss 3T3 cells, in which polyphosphoinositides are hydrolyzed quickly in response to insulin growth factor 1 (34). Although there is no evidence that this breakdown is caused by nuclear PLC-β1, it has been reported that treatment of Swiss 3T3 cells with insulin growth factor-1 induces activation of nuclear PI-PLC, a decrease in the levels of phosphatidylinositol monophosphate and PtdIns(4,5)P₂, and an increase in nuclear DG levels that causes the translocation of protein kinase C-α (35, 36). PLC-β1 and -γ1 were also detected in the nucleus of rat liver cells (37), and the β2, β3, γ1, and y2 isoforms have recently been identified in the nucleus of HL-60 promyelocytic leukemia cells (38). These data support the idea that at least some PLC isoforms are responsible for intranuclear polyphosphoinositide turnover during cell proliferation and differentiation (39–41).

The nuclear export signal (NES), a short leucine-rich sequence motif, has recently been identified as a transport signal that is necessary to mediate active nuclear export (42, 43). It was reported that the intracellular localization of many proteins, including the human immunodeficiency virus, type 1-coded Rev protein (44), an inhibitor of cAMP-dependent protein kinase (45), and mitogen-activated protein kinase kinase (46) (Fig. 1), is regulated by NES. Moreover, leptomycin B (LMB), an antifungal antibiotic and an inhibitor of the cell cycle in mammalian and fission yeast cells (47), has been
shown to inhibit NES-dependent nuclear export (48) by binding to CRM1, a NES receptor in the nuclear pore complex (49, 50).

Here, we have identified residues 164–177 of PLC-δ1 as a putative NES. We have demonstrated that disruption of the putative NES results in the nuclear localization of PLC-δ1 in MDCK cells using a GFP fusion system and that LMB induces the nuclear accumulation of GFP/PLC-δ1. These results suggest that the NES motif in PLC-δ1 is functional.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—MDCK and NRK cells were grown in Dulbecco’s modified Eagle’s medium containing 5% heat-inactivated fetal calf serum (FCS).

**DNA Constructs for the Expression of GFP-fused PLC-δ1 and Mutants**—Plasmids for the GFP-fused wild-type enzyme, pGFP/PLC-δ1, and its site-directed mutant, pGFP/PLC-δ1 R40A, were as described previously (32). Plasmids for the site-directed mutant, pGFP/PLC-δ1 L172A/I174A in which Leu172 and Ile174 are replaced by alanines, were synthesized using the Fmoc (9-fluorenylethoxycarbonyl) cleavage method on an Applied Biosystems 431A peptide synthesizer and further purified by reverse phase high pressure liquid chromatography (HPLC). Critical hydrophobic residues (Leu165, Leu169, Leu172, Ile174, and Val176) in the closest similarity with PLC-δ1 among the PLC-δ subforms (33), which shows the closest similarity with PLC-δ1 tagged with GFP, in which A

**Electroinjection of Plasmids**—The GFP expression plasmids were purified by the Trition-CsCl method (51). Each plasmid was introduced into MDCK cells by electroporation as described (32). Briefly, growing cells were trypsinized and resuspended in an electroporation buffer (25 mM HEPES-NaOH (pH 7.2), 140 mM KCl, 0.75 mM Na$_2$HPO$_4$). An aliquot (400 μl) of cell suspension (10$^7$ cells/ml) was incubated with 20 μg of plasmid solution for 5 min on ice, transferred to an electroporation cuvette, and then electroporated at 150 V for 20 ms using an Electro Square Porator (BTX, T-820). One day after electroporation, cells transiently expressing a construct were analyzed by fluorescence microscopy (Carl Zeiss Axiovert 135) at room temperature. Transfected MDCK cells stably expressing GFP/PLC-δ1 were cloned as described (32).

**Peptide Preparation**—A L-peptide corresponding to residues 164–177 of rat PLC-δ1 (PLC-δ1 L164–177), ELKDFKLERKIQVD, and its mutant peptide (A-peptide), EAKDKFAKENAQAD, in which all critical hydrophobic residues (Leu166, Leu169, Leu172, Ile174, and Val176) were replaced by alanines, was synthesized using the Fmoc (9-fluorenylethoxycarbonyl) cleavage method on an Applied Biosystems 431A peptide synthesizer and further purified by reverse phase high pressure liquid chromatography (HPLC) by essentially the same method of Fukuda et al. (46). Briefly, Sulfo-SMCC-activated OV was purified by gel filtration column, Bio-Gel P-10 (15 mm × 160 mm), in phosphate-buffered saline (pH 8.0) and rotating for 1 h at room temperature. Excess cross-linker was removed by passage through a gel filtration column, Bio-Gel P-10 (15 mm × 160 mm), in phosphate-buffered saline (pH 7.4). The absorbance at 280 nm of each fraction (1 ml) was measured using a spectrophotometer (Beckman DU-7400), and fractions containing OV were recovered.

**Microinjection of Synthetic Peptides**—MDCK cells were plated onto CELLocate coverslips (Eppendorf, Inc.) and cultured in Dulbecco’s modified Eagle’s medium with 5% FCS overnight. Microinjection was performed using the Transjector 5246 (Eppendorf, Inc.). The synthetic peptide (1.5 mg/ml) was added to sulfo-SMCC-activated OV. After gentle rotation for 3 h at room temperature, free peptide was removed by a gel filtration column, Bio-Gel P-10 (15 mm × 160 mm), in phosphate-buffered saline (pH 7.4). Each fraction was measured by its absorbance at 280 nm, and fractions containing L- or A-peptide-conjugated OV (L- or A-OV) were recovered and concentrated by Centricon 30 (Amicon).

**Immunofluorescent Cell Staining**—The cells were fixed with 4% formaldehyde in 0.1 M potassium phosphate buffer (pH 7.4) for 1 h at room temperature. Then they were permeabilized and blocking solution (0.1% Triton X-100, 2% FCS in phosphate-buffered saline) for 30 min at room temperature. Then the coverslips were incubated with primary antibody (rabbit polyclonal) for 1 h at room temperature and then with Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) for 1.5 h at room temperature. The primary antibody was used as a rabbit anti-ovalbumin antibody (Cosmo Bio) or a rabbit anti-rat PLC-δ1 antibody (52).

**RESULTS**

**NES in PLC-δ1**—It was demonstrated that PLC-δ1 is predominantly localized at the plasma membrane and in the cytosol (31, 32, 53), whereas the δ4 isoform is localized in the nucleus during most of the stages of the cell cycle (21). To examine why the intracellular distribution of these PLC isoforms are different, we compared the amino acid sequence of PLC-δ1 with that of PLC-δ4 and found that PLC-δ1 has a typical NES sequence (residues 164–177) in its EF-hand domain (Fig. 1). The corresponding domain in PLC-δ4 does not contain such a sequence. Interestingly, PLC-δ3, which shows the closest similarity with PLC-δ1 among the δ subforms (33), also fulfills the Leu-rich NES consensus. Neither the corresponding domain of PLC-β1 or of PLC-γ1 has the Leu-rich NES-like sequence in their EF-hand domains (data not shown).

**Cytoplasmic Localization of GFP/PLC-δ1 Requires Its NES Sequence in the EF-hand Domain**—To test whether this putative NES is important for the cytoplasmic localization of PLC-δ1, we constructed a mutant PLC-δ1 tagged with GFP, in which two critical hydrophobic residues (Leu172 and Ile174) were replaced by alanines (GFP/PLC-δ1 L172A/I174A) (Fig. 2). It has been previously reported that these substitutions make the canonical NES sequence nonfunctional (42–45). We purified a plasmid encoding GFP/PLC-δ1 L172A/I174A, electroporated it into MDCK cells. Wild-type GFP/PLC-δ1 was localized at the plasma membrane and in the cytosol (Fig. 2, left panel), whereas the L172A/I174A mutant was present at the plasma membrane, in the cytosol, and also in the nucleus (Fig. 2, right panel). These results indicate that the cytoplasmic localization of GFP/PLC-δ1 is determined by one putative NES sequence in the EF-hand domain of PLC-δ1.

An NES Sequence in the EF-hand Domain of PLC-δ1 Is
Independently Functional—To show that the putative NES sequence of PLC-δ1 is autonomously functional, the peptide corresponding to residues 164–177 of PLC-δ1 (L-peptide) and its mutant peptide, in which four important hydrophobic amino acids (Leu<sup>165</sup>, Leu<sup>169</sup>, Leu<sup>172</sup>, and Ile<sup>174</sup>) and an additional hydrophobic amino acid, Val<sup>176</sup>, were replaced by alanine (A-peptide), was synthesized (Fig. 3A) and chemically conjugated to OV. Each synthetic peptide conjugate was injected into the nucleus of MDCK cells. L-OV was excluded from the nucleus almost completely within 1 h after nuclear injection and detected in the cytosol (Fig. 3B, lower left panel). In contrast, co-injected fluorescein isothiocyanate-bovine serum albumin remained in the nucleus (Fig. 3B, upper left panel). Unlike L-OV, A-OV remained in the nucleus 1 h after injection (Fig. 3B, lower center panel). Moreover, this nuclear export of L-OV was inhibited by LMB, a specific inhibitor of NES-dependent nuclear export (Fig. 3B, lower right panel). These results suggest that a sequence of residues (164–177) of PLC-δ1 acts as a functional NES independently.

**LMB Induces Nuclear Accumulation of PLC-δ1 and Its Mutant**—To examine the effect of LMB on the distribution of PLC-δ1, MDCK cells that stably expressed GFP/PLC-δ1 were incubated in medium containing LMB (5 ng/ml) (Fig. 4, upper panels). Within 3 h of LMB treatment, GFP/PLC-δ1 was observed in the nucleus, and after 24 h it had accumulated almost exclusively in the nucleus. However, fluorescence was still visible at the plasma membrane. Then, to examine the influence of the plasma membrane targeting of PLC-δ1 on its nuclear accumulation, the MDCK cells expressing GFP-fused PLC-δ1 R40A, a PH domain mutant that does not bind the PH ligand in vitro or the plasma membrane in vivo (31, 32), were treated with LMB. GFP/PLC-δ1 R40A accumulated more rapidly and exclusively in the nucleus than wild-type GFP/PLC-δ1 (Fig. 4, lower panels). This result indicates that the more GFP/PLC-δ1 is localized in the cytosol, the more nuclear translocation occurs.

Moreover, to test whether LMB treatment induces endogenous PLC-δ1 to accumulate in the nucleus, NRK cells were treated with LMB. After 30 h, the cells were fixed, and the intracellular distribution of endogenous PLC-δ1 was determined by immunocytochemistry using an anti-PLC-δ1 antibody. Endogenous PLC-δ1 was localized in the cytosol before treatment with LMB (Fig. 5, left panel). After treatment with LMB, endogenous PLC-δ1 accumulated in the nucleus (Fig. 5, right panel). These results suggest that blocking NES-dependent nuclear export results in the nuclear accumulation of PLC-δ1 that is transported into the nucleus by some mechanism(s).

Although GFP-PLC-δ1 or endogenous PLC-δ1 was found predominantly in the nuclei of transfected MDCK cells (Fig. 4) or of NRK cells (Fig. 5), respectively, after treatment with LMB, in some cells (but not all cells) the enzyme also showed perinuclear distribution. LMB has been shown to bind directly and irreversibly to CRM1 and inhibits NES-mediated active nuclear export (50). Because the concentration of LMB used (5 ng/ml) was as low as the minimum required for the inhibition, it is plausible that LMB cannot bind to newly synthesized CRM1 resulting in a partial restoration of the nuclear export.
regulated by two mechanisms, an NES-dependent nuclear export, PLC-δ1 imported into the nucleus is exposed to CRM1. This would result in the immediate export of PLC-δ1 from the nucleus.

**DISCUSSION**

In this study, we found that PLC-δ1 has a functional NES sequence in its EF-hand domain. The EF-hand domain consists of four units of a helix-loop-helix structure (30), and a canonical NES sequence is present in the most N-terminal region (EF-hand 1). It has been previously reported that the EF-hand 1 contains a calcium binding motif, although there are no evidence that Ca\(^{2+}\) binds to this region. An x-ray crystallographic study (54) and a binding study using isothiocyanate alizarin (55) or atomic absorption\(^{2}\) have suggested that there are four Ca\(^{2+}\) binding sites in PLC-δ1, one in the catalytic domain and three in the C2 domain but none in the EF-hand domain.

According to the molecular coordinates for a crystal of the Δ1-132 deletion variant of rat PLC-δ1 (Protein Data Bank code 2IDJ), the N terminus of EF-hand 1 lacks the expected electron density for E1α (the N-terminal o-helix of EF-hand 1) and the loop between E1α and F1α (the C-terminal o-helix of EF-hand 1) (30), indicating that EF-hand 1 is flexible. Furthermore, there is a slight difference in the F1α structure of the crystal in the presence of Ca\(^{2+}\) (Protein Data Bank code 1DJJ), suggesting that the conformational change of the EF-hand motif would expose the sequence that could be recognized by CRM1, a receptor and carrier protein for the NES sequence.

We have also shown in this study that treatment with LMB induces the nuclear accumulation of exogenous GFP/PLC-δ1 and endogenous PLC-δ1. Because GFP/PLC-δ1 and PLC-δ1 have an apparent molecular mass of ~110 and ~85 kDa, respectively, nucleocytoplasmic shuttling by the passive diffusion is unlikely. Molecules with such molecular mass cannot cross the nuclear pore complexes by simple diffusion (56). Active nucleocytoplasmic translocation mechanisms must exist. Our results suggest that the intracellular distribution of PLC-δ1 is regulated by two mechanisms, an NES-dependent nuclear export mechanism and an unidentified active nuclear import mechanism. In ordinary conditions, PLC-δ1 would enter the nucleus as the result of certain stimuli such as growth factors, stress, and cell cycle-regulated signals, but the nuclear PLC-δ1 would be immediately exported from the nucleus by NES-dependent nuclear export (Fig. 6). Because LMB blocks this NES-dependent nuclear export, PLC-δ1 imported into the nucleus is not exported from the nucleus resulting in the nuclear accumulation of PLC-δ1 (Fig. 6).

The rate of entering the nucleus may be at least partly determined by the concentration of free PLC-δ1 in the cytosol, because GFP/PLC-δ1 R40A, which cannot bind the plasma membrane and is distributed mainly in the cytosol, accumulates more rapidly and intensely in the nucleus than the wild-type fusion protein by treatment with LMB (Fig. 4). It is noteworthy that the GFP fluorescence remains at the plasma membrane with the L172A/I174A mutant (Fig. 2) or with the wild-type fusion protein after treatment with LMB (Fig. 4). It is noted that the GFP fluorescence remains at the plasma membrane with the L172A/I174A mutant (Fig. 2) or with the wild-type fusion protein after treatment with LMB (Fig. 4) indicating strong affinity between PLC-δ1 and the membrane.

Signals responsible for translocation to the nucleus, such as a nuclear localization signal, however, have not been identified in PLC-δ1 yet. To elucidate the nuclear localization of PLC-δ4, Liu et al. (21) have suggested that the positively charged residues in the PH domain serve as the nuclear localization signal sequence. This hypothesis, in the case of PLC-δ1, is unlikely. When NIH3T3 cells were transfected to express the GFP-PLC-δ1 PH domain (1–170) and treated with ionomycin, fluorescence released from the plasma membrane appeared only slowly in the nucleus (57), suggesting that this nuclear accumulation was because of simple diffusion of the 40-kDa molecule into the nucleus. On our hands, neither the GFP-PLC-δ1 PH domain containing the NES sequence (1–224) (32) nor that which lacks the NES sequence (1–140) (not shown) enters the nucleus of MDCK cells even when the cells were stimulated by Ca\(^{2+}\)-mobilizing agents.

The function of PLC-δ1 in the nucleus has not been clarified. However, it has previously been demonstrated that other PLCs are responsible for nuclear polyphosphoinositide turnover. Neri et al. (36) reported that, after insulin growth factor-1 stimulation of 3T3 cells, nuclear PI-PLC is activated and causes increased intranuclear DG levels and nuclear translocation of protein kinase C-α. This nuclear PI-PLC activity is essential for G1/S phase transition. From such evidence, it is possible that PLC-δ1 is also activated in the nucleus and hydrolyzes nuclear PtdIns(4,5)P\(_2\) generating Ins(1,4,5)P\(_3\) and DG. This nuclear PLC-δ1 activity, however, may be transient. The increase in Ca\(^{2+}\) generated in the nucleus by Ins(1,4,5)P\(_3\) may cause a conformational change in the EF-hand domain of PLC-δ1, exposing the NES sequence to CRM1. This would result in the immediate export of PLC-δ1 from the nucleus (Fig. 6). The NES-dependent nuclear export mechanism would act as a rapid down-regulation system of nuclear PI-PLC. Nevertheless, it has not been revealed what induces the nuclear translocation of PLC-δ1. It appears that targeting to the plasma

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\(^2\) H. Yagisawa, Y. Tamaoka, and H. Hirata, unpublished data.
membrane is not necessary for the signaling pathway by which PLC-δ1 enters the nucleus, because the R40A mutant, which cannot be localized at the plasma membrane, is more potently translocated into the nucleus. It is possible that a novel association molecule(s) mediates this signaling pathway.

In the future, it will be important to identify any novel association molecule(s), and also it would be particularly intriguing to reveal the physiological meaning of this nucleocytoplasmic translocation of PLC-δ1.

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