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Advances in Brief

Nuclear but not Cytoplasmic Phospholipase C β_1 Inhibits Differentiation of Erythroleukemia Cells[1]

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Abstract

A body of evidence has shown the existence of a nuclear phosphoinositide cycle in different cell types. The cycle is endowed with kinases as well as phosphatases and phospholipase C (PLC). Among the PLC isozymes, the β family is characterized by a long COOH-terminal tail that contains a cluster of lysine residues responsible for nuclear localization. Indeed, PLCβ$_1$ is the major isoform that has been detected in the nucleus of several cells. This isoform is activated by insulin-like growth factor I, and when this isoform is lacking, as a result of gene ablation, the onset of DNA synthesis induced by this hormone is abolished. On the contrary, PLCβ$_1$ is down-regulated during the erythroid differentiation of Friend erythroleukemia cells. A key question is how PLCβ$_1$ signaling at the nucleus fits into the erythroid differentiation program of Friend erythroleukemia cells, and whether PLCβ$_1$ signaling activity is directly responsible for the maintenance of the undifferentiated state of erythroleukemia cells. Here we present evidence that nuclear PLCβ$_1$ but not the isoform located at the plasma membrane is directly involved in maintaining the undifferentiated state of Friend erythroleukemia cells. Indeed, when wild-type PLCβ$_1$ is overexpressed in these cells, differentiation in response to DMSO is inhibited in that the expression of β-globin is almost completely abolished, whereas when a mutant lacking the ability to localize to the nucleus is expressed, the cells differentiate, and the expression of β-globin is the same as in wild-type cells.

Introduction

Autonomous nuclear signaling via the phosphoinositide cycle has raised more interest (1) after the initial observation that isolated nuclei were capable of synthesizing polyphosphoinositides, and that their metabolism was changed with cell differentiation or growth factor stimulation (2, 3). All of the enzymes involved in the inositol lipid cycle are present in the nuclei (4–6), although more attention has been focused on inositide-specific PLC[4] (3).

Indeed, subcellular localization and isozyme characterization have been carried out, and the β family appears to be the key nuclear PLC (7–14). Namely, PLCβ$_1$ has been found to be specifically localized in the nuclei of different cell types and to undergo changes in activity and expression during cell growth or differentiation (7, 8, 10–15).

The hypothesis that nuclei are endowed with an autonomous signaling based on lipid metabolism is strengthened by the presence of phospholipases with substrate specificity for phosphatidylcholine (16) in addition to that of inositide-specific PLC.

Given the assumption that nuclear PLCβ$_1$ is down-regulated when Friend erythroleukemia cells treated with DMSO differentiate and synthesize β-globin (15), we have attempted to add insight on the function of nuclear PLC signaling and to determine the role of this peculiar signaling in erythroid differentiation. With this aim, we decided to overexpress PLCβ$_1$ using both wild-type cDNA and a mutant in the COOH-terminal region lacking the ability of localizing at the nucleus (13) to discriminate between the nuclear and cytoplasmic signaling activity of this PLC during erythroid differentiation.

Materials and Methods

Construction of Expression Vectors and Transfection. A 3.5-kb full-length cDNA for rat PLCβ$_1$ (17) and the mutant for the nuclear localization sequence in which lysine residues 1056, 1063, and 1070 in region 2 of the COOH terminus were substituted with isoleucine (13) by site-directed mutagenesis were cloned into pRc/CMV (Invitrogen) expression vector plasmid as described elsewhere (18). Friend cells were transfected using the following protocol. Briefly, a mixture of 2.5 μg of plasmid DNA and 10 μl of Transfectam (Promega) was added to 1×10^5 cells in one well of a 6-well plate for 24 h. The transformants were selected by limiting dilution in medium containing the neomycin analogue G418 at a concentration of 500 μg/ml. Clones were harvested and expanded separately in the presence of G418.

Cell Culture. Murine erythroleukemia cells (Friend cells, clone 707) were grown in RPMI 1640 supplemented with 10% FCS, and erythroid differentiation was induced by the addition of DMSO (1.5%, v/v) to the medium for 4 days.

Isolation of Nuclei. A hypotonic shock combined with nonionic detergent, essentially as described by Martelli *et al.* (19), was used. In addition to 0.5 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, 1 mM ethyleneglycol-bis(aminoethyl ether) tetraacetic acid, 10 μg/ml leupeptin, 0.3 μM aprotinin, 15 μg/ml calpain I inhibitor, and 7.5 μg/ml calpain II inhibitor were also added to the buffers. Nuclear purity was assessed by the detection of β-tubulin as described in Ref. 18, and only nuclei showing a complete absence of β-tubulin in Western blot were used in the reported experiments.

Preparation of Cytoplasmic Fraction. The cytoplasmic fraction was obtained by homogenizing cells with 20 strokes in a Dounce homogenizer with 10 mM Tris-Cl (pH 7.8) and 2 mM $MgCl_2$ plus protease inhibitors as described above and then pelleting the nuclei at 400 × *g*. This procedure allows the recovery of a pure cytoplasmic fraction and avoids the risk of contamination by nuclear debris that are present in the crude supernatant from nuclear purification.

PLC Assay. The assay was carried out exactly as described previously (7).

Flow Cytometry. Friend cells transfected with the empty vector, clones overexpressing wild-type PLCβ$_1$, and mutants for the nuclear localization sequence were analyzed by flow cytometry exactly as described previously (18).

Northern Blotting. Total cytoplasmic RNA was extracted from the cells using the denaturing guanidinium isothiocyanate method (Rneasy mini kit; Qiagen). Twenty μg of each RNA sample were resuspended in RNA loading buffer (50% formamide, 2.2 M formaldehyde, and 1× electrophoresis buffer)

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[4] The abbreviations used are: PLC, phospholipase C; $PtdInsP_2$, phosphatidylinositol 4,5-biphosphate; mAb, monoclonal antibody.

and electrophoresed through a 1% agarose gel with 2 mM 4-morpholinepropanesulfonic acid, 0.5 mM sodium acetate, and 0.1 mM EDTA as the electrophoresis buffer. RNAs were transferred from the gel onto a nylon membrane and UV cross-linked for 1 min. The membrane was prehybridized at 42°C in prehybridization mix (50% ultrapure formamide, 1 M NaCl, 1% SDS, 10% dextran sulfate, and 200 μg/ml sheared salmon sperm DNA) for over 4 h. A 500-bp segment of the β-globin insert (20) was digested from plasmid pRSV-βG with *Hin*dIII/*Bgl*II and radiolabeled with [α-^{32}P]dCTP using the random priming method. Membranes were hybridized overnight at 42°C with prehybridization solution plus the denatured ^{32}P-labeled probe. After hybridization, the blot was washed once for 30 min at room temperature with 3× SSC/1% SDS and twice for 30 min at 42°C with 2× SSC/1%SDS and exposed to X-ray film overnight. After analysis, the blots were stripped and reprobed with a ^{32}P-labeled β-actin plasmid (Ambion).

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared by resuspending the cells in 400 μl of swelling buffer [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, and 10 mM KCl], followed by incubation on ice for 10 min. Next the lysates were vortexed for 10 s before centrifugation for 1 min at 14,000 rpm at 4°C. The pelleted nuclei were resuspended in 50 μl of lysis buffer [20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, and 0.2 mM EDTA] and kept on ice for 20 min with occasional mixing. Subsequently, the lysates were centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatant was stored at −70°C. All buffers were supplemented with 0.5 mM DTT and 0.2 mM phenylmethylsulfonyl fluoride. A synthetic oligonucleotide containing the consensus binding site for erythroid transcription factor NF-E2, 5′-TGGGGAACCTGTGCTGAGTCACTGGAG-3′, was used as a probe for the assay. The probe was labeled with [α-^{32}P]dATP using T4 polynucleotide kinase. Nuclear extract (10 μg) was used in each binding reaction. Where appropriate, the extract was preincubated for 10 min at room temperature with either 0.1 μg of NF-E2 p45 antibody, 5 pM unlabeled NF-E2 oligonucleotide, or 5 pM unspecific competitor. Subsequently, binding to 1 μl of radioactive NF-E2 oligonucleotide was carried out in a reaction mixture containing 1 μg of poly(deoxyinosinic-deoxycytidylic acid), 20 mM HEPES (pH 7.6), 70 mM KCl, 5 mM $MgCl_2$, 0.05% NP40, 12% glycerol, 1 mg/ml BSA, and 0.5 mM DTT for 30 min at room temperature. DNA-protein complexes were separated on a 4% nondenaturing polyacrylamide gel and visualized by autoradiography.

Immunochemical Analysis. Proteins from the purified nuclei and cytoplasmic fractions (60 μg) and proteins from the whole cell homogenates (30 μg) were separated on 8% polyacrylamide-0.1% SDS gels and transferred to nitrocellulose paper, and PLCβ$_1$ and PLCγ$_1$ isoforms were detected by the enhanced chemiluminescence method (Amersham). mAbs against PLCβ$_1$ and γ$_1$ were as described in Ref. 21.

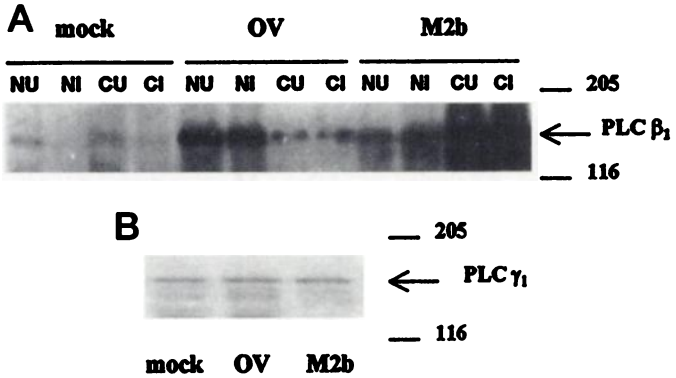

Fig. 1. Western blot analysis of the PLC isoforms in Friend erythroleukemia cells. *mock*, cells transfected with the empty vector; *OV*, cells transfected with wild-type PLCβ$_1$; *M2b*, cells transfected with the mutant lacking the nuclear localization sequence; *NU*, nuclei from uninduced cells; *NI*, nuclei from induced cells; *CU*, cytoplasm from uninduced cells; *CI*, cytoplasm from induced cells. Molecular weights (in thousands) are indicated on the *right*. *A*, immunochemical analysis with anti-PLCβ$_1$ mAb (60 μg of protein loaded for each lane); *B*, immunochemical analysis with anti-PLCγ$_1$ mAb (30 μg of protein loaded for each lane). The Western blot analysis reported in this figure is representative of 10 identical clones for each type of transfectant.

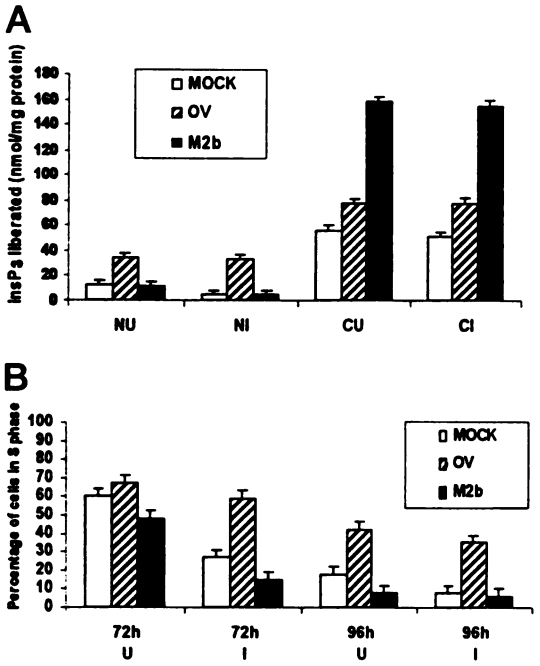

Fig. 2. Correlation between the nuclear PLC activity and DNA synthesis. *A*, a histogram showing PLC activity using [^{3}H]PtdInsP$_2$ as a substrate (expressed as nmol/mg protein inositol 1,4,5-trisphosphate liberated/30-min incubation). *B*, a histogram of the data obtained from a flow cytometric analysis of the cell cycle showing the percentage of cells in S phase. *Mock*, cells transfected with the empty vector; *OV*, cells transfected with wild-type PLCβ$_1$; *M2b*, cells transfected with the mutant lacking the nuclear localization sequence. The results shown in both panels are the average of seven separate experiments ± SD.

Results

An analysis of the PLC phenotype in the stable transfectants we obtained is shown in Fig. 1. The clone overexpressing the wild type and the M2b clone shown in this Western blot are representative of more than 10 identical clones for each type of transfectant. Cells transfected with the empty vector (*mock*) behave exactly as the original untransfected Friend cells do (15), in that PLCβ$_1$ is down-regulated upon treatment with DMSO. The overexpression of wild-type PLCβ$_1$ gives rise to a preferential distribution in the nucleus as compared with the cytoplasm (*i.e.*, 65 *versus* 35%, as determined by densitometric analysis). On the contrary, the M2b mutant, in which lysine residues 1056, 1063, and 1070 were substituted with isoleucine (13), shows an almost complete loss of nuclear localization of PLCβ$_1$, *i.e.*, less than 10% in the nuclear fraction (Fig. 1*A*). These findings agree with a previous report dealing with Rat-2 cells (13). The same panel shows that when overexpressed, PLCβ$_1$ is no longer down-regulated upon DMSO treatment. As an additional control, we checked the other PLC isoform detected in Friend erythroleukemia cells, *i.e.*, the γ$_1$ isoform (15), to evaluate whether or not the establishment of stable transfectants could affect the expression of this isoform and, in turn, induce changes in PLC activity due to the γ family. Fig. 1*B* shows that in both the wild-type PLCβ$_1$ and M2b transfectants, the amount of PLC γ$_1$ is unaffected. Fig. 2*A* shows the PLC activity in the nuclear and cytoplasmic fractions. The overexpression of wild-type PLCβ$_1$ produces a nearly 3-fold increase in nuclear PLC activity, whereas that of M2b mutant increases at the same magnitude, only in the cytoplasmic fraction. In addition, whereas the nuclear PLC activity in mock-transfected cells is dramatically reduced in the nuclei of fully differentiated cells, as reported previously (15), the overexpression of PLCβ$_1$ in the nucleus determines a resistance to DMSO treatment; nuclear PLC activity is not

affected at all by this differentiative inducer. The cytosolic PLC activity increases only in M2b mutants in which PLCβ_1 localizes in the plasma membrane. Because the differentiation toward erythrocytes of rapidly dividing Friend cells is accompanied by a reduction in proliferation, we checked the effect of wild-type and M2b mutant overexpression by measuring the number of cells in S phase actively incorporating 5-bromodeoxyuridine using flow cytometry. Fig. 2*B* shows that at the time at which DNA synthesis begins to decrease, *i.e.*, from 72 h as well as after 96 h of culture, only transfectants overexpressing PLCβ_1 in the nucleus are still actively synthesizing DNA, even in the presence of DMSO; in this latter condition, 58 and 30% of cells are in S phase at 72 and 96 h, respectively. This implies that nuclear PLCβ_1 signaling, which is essential for cell growth, could impair erythroid differentiation. This hypothesis has been sustained by Northern analysis of the expression of the β-globin gene, which is the only reliable marker for the differentiation of Friend cells. In fact, in the *upper panel* of Fig. 3, it is possible to see that DMSO treatment induces the expression of β-globin in control (*mock*) cells as well as in the M2b mutant, whereas transfectants overexpressing PLCβ_1 in the nucleus almost completely lack the ability to express β-globin. Because expression of the β-globin gene is dependent on the NF-E2 transcription factor acting as an enhancer-binding protein (22), namely, upon DMSO treatment that increases NF-E2·DNA complex formation, we have evaluated the activation of NF-E2 in transfectants overexpressing PLCβ_1 in the nucleus or at the plasma membrane by a DNA gel mobility shift assay. Fig. 4 shows that by overexpressing PLCβ_1 in the nucleus, the activation of NF-E2 is almost completely abolished, because the band detected with increased intensity in both the control and the M2b mutant upon DMSO treatment disappears in cells overexpressing PLCβ_1 in the nucleus. A further confirmation that this is a NF-E2·DNA complex comes from the fact that this band is supershifted by the addition of the antibody against the p45 subunit of NF-E2, which is the subunit restricted to hematopoietic lineages (23).

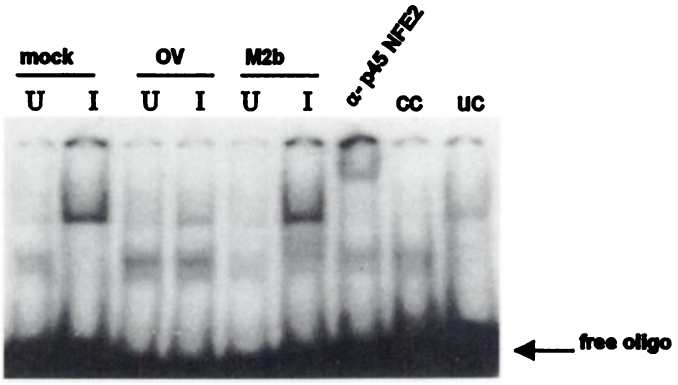

Fig. 4. Effect of PLCβ_1 overexpression on NF-E2·DNA complex formation after treatment with DMSO. NF-E2·DNA complex formation was determined by DNA gel mobility shift assay as described in "Materials and Methods." *CC*, preincubation with unlabeled NF-E2 oligonucleotide; *UC*, preincubation with unspecific competitor. Nuclear extract (10 μg) was used in each binding reaction. *mock*, cells transfected with the empty vector; *OV*, cells transfected with wild-type PLCβ_1; *M2b*, cells transfected with the mutant lacking the nuclear localization sequence. The mobility shift analysis reported in this figure is representative of 10 identical clones for each type of transfectant.

Discussion

The role of cytoplasmic PLCs has been widely investigated during the last 10 years (1). These studies have provided evidence that the hydrolysis of a minor membrane phospholipid, *i.e.*, PtdInsP$_2$, is one of the main events in the regulation of several cell functions including cell growth and differentiation. How the cell surface-generated signals impinge upon the nucleus has been also studied, and attention has mainly been focused on the phosphorylative cascades or on plasma membrane-generated second messengers that ultimately reach the nucleus (for a review, see Ref. 24 and the references therein). The discovery of a nuclear lipid metabolism (2–7, 24) hinted at a key role for some phospholipids in the nucleus in the regulation of nuclear functions. Indeed, the demonstration of the presence of an autonomous nuclear signaling via polyphosphoinositide hydrolysis has paved the way for studies that aimed to achieve the understanding of the significance of this cycle in comparison with the better known one at the plasma membrane. It appeared clear that the nuclear cycle is activated in conditions in which external stimuli do not trigger the hydrolysis of cytoplasmic inositol lipids (6). We have shown that PLCβ_1 can localize in the nucleus, having its own signaling activity in the insulin-like growth factor I stimulation of quiescent fibroblasts (7), and more recently, we presented evidence that nuclear localization depends on a cluster of lysine residues in the COOH-terminal domain (13), and that nuclear PLCβ_1 plays a crucial role in insulin-like growth factor I-induced mitogenesis (18). We have used the mutant M2b, which lacks the cluster of lysines responsible for nuclear localization, as a tool for discriminating between the biological role of the cytoplasmic and nuclear isozyme to analyze the effect of the overexpression of both the wild-type and mutated PLCβ_1 on the differentiation toward erythrocytes of Friend erythroleukemia cells upon DMSO treatment. One interesting point supporting this experimental approach is the previously reported evidence that nuclear PLCβ_1 is also a target for an antileukemic drug (15) and is therefore a good candidate to be a key molecule responsible for the establishment of a tumor phenotype. The results we have presented here indicate that only when PLCβ_1 is overexpressed in the nucleus there is an increase of continuously cycling cells even after a DMSO treatment of up to 96 h, and this inhibits the effect of the differentiative inducer. In Friend erythroleukemia cells, DMSO increases the activity of transcription factor NF-E2, which plays a crucial role and acts as an enhancer-binding protein for the expression of the β-globin gene (22). From our data, it appears that nuclear PLCβ_1 in Friend cells is responsible for a signal determined by the hydrolysis of PtdInsP$_2$ that maintains cell cycling and inhibits the activation of NF-E2, resulting in an almost complete abolition of the expression of the β-globin gene. Therefore, the function of nuclear PLCβ_1 assumes a key significance in that it is directly related to the maintenance of an indifferentiate state in the cells that lose the capability of ceasing to divide upon appropriate stimulus. All in all, these data assign a specific role to nuclear PLC signaling and strengthen the argument that nuclear PLCβ_1, acting as a negative regulator of differentiation, is a key element in

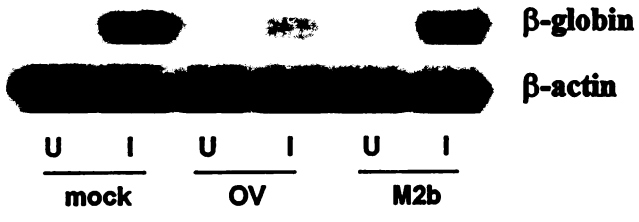

Fig. 3. Northern analysis for the expression of β-globin. RNAs extracted from uninduced (*U*) and induced (*I*) cells were separated electrophoretically, transferred onto a nylon membrane, hybridized with the labeled β-globin probe, and autoradiographed (*top panel*). The membrane was then stripped and reprobed with β-actin for normalization (*bottom panel*). For each lane, 20 μg of RNA were loaded. *mock*, cells transfected with the empty vector; *OV*, cells transfected with wild-type PLCβ_1; *M2b*, cells transfected with the mutant lacking the nuclear localization sequence. The Northern blot analysis reported in this figure is representative of 10 identical clones for each type of transfectant.

switching erythroleukemia cell programming from a proliferative to a differentiative state.

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