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Advances in Brief

Essential Role for Nuclear Phospholipase C β_1 in Insulin-like Growth Factor I-induced Mitogenesis[1]

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Abstract

The nucleus has been shown to be a site for the inositol lipid cycle that can be affected by treatment of quiescent cells with growth factors such as insulin-like growth factor I (IGF-I). Indeed, the exposure of Swiss 3T3 cells to IGF-I results in a rapid and transient increase in nuclear phospholipase C (PLC) β_1 activity. In addition, several other reports have shown the involvement of PLC β_1 in nuclear signaling in different cell types. Although the demonstration of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate hydrolysis by nuclear PLC β_1 established the existence of nuclear PLC signaling, the significance of this autonomous pathway in the nucleus has yet to be thoroughly clarified. By inducing both the inhibition of PLC β_1 expression by antisense RNA and its overexpression, we show that this nuclear PLC is essential for the onset of DNA synthesis following IGF-I stimulation of quiescent Swiss 3T3 cells.

Introduction

The involvement of polyphosphoinositides in cellular signaling has been widely documented (1), and many reports focused attention on the PLC[3] specific for inositol lipids as a key step of the inositol lipid cycle (2). PLC β_1 is of particular interest because of its reported nuclear localization (3–8) in addition to its presence at the plasma membrane (2). Previous investigations from our laboratory and others have shown the existence in several cell types of an autonomous intranuclear inositide cycle endowed with both conventional lipid kinases and PLC (reviewed in Ref. 9). It has also been shown that activation of nuclear inositol polyphosphate 1-phosphatase, which is exclusively responsible for the metabolism of inositol 1,4-bisphosphate, resulted in a 50% decrease in DNA synthesis (10). The activity of the nuclear PLC β_1 increases 2–3-fold within 5 minutes of stimulation of Swiss 3T3 cells by IGF-I, and this can be verified with measurement of the breakdown of polyphosphoinositides in the cell nucleus (11). However, the final evidence was still lacking that the requirement of nuclear PLC β_1 was essential for Swiss 3T3 cells to be stimulated to grow by IGF-I. Here we present evidence that activation of nuclear PLC β_1 is essential for IGF-I mitogenicity because the inhibition of PLC β_1 expression by AS RNA abolishes the mitogenic effect of IGF-I, whereas the overexpression of this isoform dramatically enhances DNA synthesis upon treatment with the same growth factor.

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[3] The abbreviations used are: PLC, phospholipase C; PDGF, platelet-derived growth factor; MoAb, monoclonal antibody; CMV, cytomegalovirus; IGF-I, insulin-like growth factor I; AS, antisense; BrdUrd, 5-bromodeoxyuridine.

Materials and Methods

Construction of Expression Vectors for Both Sense and AS PLC β_1. A 3.5-kb full-length cDNA for rat PLC β_1 (12) was cloned as a *NotI/Bam*HI fragment into the multiple cloning site of the cytomegalovirus promoter-driven eukaryotic expression vector pRc/CMV (Invitrogen Corp., San Diego, CA) as described elsewhere (13). To obtain an AS expression construct, a 0.505-kb *NotI/Hin*dIII fragment containing the beginning of the PLC β_1 coding region was cloned in the reverse orientation into the equivalent sites of pRc/CMV.

Transfection and Screening of Transformants. Subconfluent 3T3 cells were transfected with sense or AS PLC β_1 essentially as described previously (13). Briefly, a mixture of 2.5 μg of plasmid DNA and 10 μl of Transfectam (Promega Corp., Madison, WI) was added to 1×10^5 cells in one well of a six-well plate for 24 h. Then transformants were selected in medium containing the neomycin analogue G418 (Sigma Chemical Co., St. Louis, MO) at a concentration of 500 μg/ml. Clones were harvested and expanded separately in the presence of G418.

Growth Factor Stimulation and Isolation of Nuclei. Swiss 3T3 cells were grown exactly as described by Manzoli *et al.* (14). Briefly, cells grown to complete confluence in DMEM containing 10% fetal bovine serum were washed twice with serum-free medium containing 1% BSA and then incubated in the same serum-free medium containing 20 ng/ml IGF-I or 1 μM bombesin for the times indicated. In some experiments, PDGF (10 ng/ml) was also used. Nuclei were obtained by hypotonic shock in the presence of detergent exactly as described previously (3). The detection of β-tubulin, as a marker for cytoplasmic contamination, was performed using an anti-β-tubulin MoAb assay exactly as reported before (15). Only nuclei showing a complete absence of β-tubulin were used in these experiments.

Preparation of Nuclear Lysates and PLC Assay in the Presence or Absence of Neutralizing MoAb. All procedures were exactly as described by Martelli *et al.* (3). Characterization of the anti PLC β_1 MoAb was described previously (16).

Immunochemical Analysis. Proteins (30 μg) from whole cells and purified nuclei were separated on 8% polyacrylamide-0.1% SDS gels and transferred to nitrocellulose paper, and PLC isoforms were detected exactly as described elsewhere (3). Characterization of the antibodies against PLC γ_1 and PLC β_1 have been described previously (16, 17).

Flow Cytometric Analysis of the Cell Cycle. Cells were grown as described above, and when completely confluent, they were treated in serum-free medium with IGF-I (20 ng/ml) or bombesin (1 μM), or in some experiments with PDGF (10 ng/ml), for up to 20 h and then labeled for 60 min with 100 μM BrdUrd. Cells (10^6) were then trypsinized, washed twice with PBS, and resuspended in 100 μl of the same buffer. To this was added 900 μl of ice-cold 70% ethanol, washed in the same buffer, incubated for 30 min in 4 N HCl, washed once more, and treated with Triton X-100 for 3 min. For analyzing the samples by fluorescence-activated cell sorting, the cells were washed in PBS (three times in 1 ml), resuspended in the same buffer, and incubated with FITC-conjugated anti-BrdUrd MoAb (Becton Dickinson, San Jose, CA) for 30 min at 4 C° and counterstained with propidium iodide (5 μg/ml). The flow cytometric analysis was then carried out with a FACStar Plus flow-cytometer (Becton Dickinson, San Jose, CA; Ref. 18).

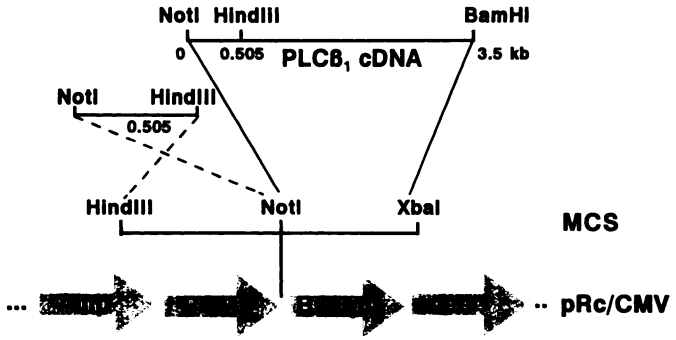

Fig. 1. Schematic representation of the multiple cloning site of the expression vector pRc/CMV and insertion of sense (*solid lines*) and AS (*hatched lines*) cDNA for PLC β_1.

Results and Discussion

Fig. 1 shows the cloning strategy for both sense and AS cDNA for PLC β_1. The full-length cDNA for PLC β_1 was cloned in the eukaryotic expression vector pRc/CMV, and to obtain an AS expression vector, a *Not*I/*Hin*dIII fragment (505 bp) containing the 5′ PLC β_1 coding sequence was inserted into the same vector in the opposite orientation. By using both sense and AS constructs, we obtained, after selection, several clones that were screened for the expression of PLC β_1.

The Western Blot analysis of whole-cell proteins from stable transfectants carrying the AS cDNA reveals that two clones, named clone 3 and clone 9, show a complete absence of PLC β_1 (Fig. 2*a*). As a control, we have checked the expression of the other isozyme detected in wild-type 3T3 cells, *i.e.*, the PLC γ_1 isoform (19). In whole-cell homogenates, the amount of this PLC is unaffected (Fig. 2*c*), and neither does its localization change (data not shown), remaining completely cytoplasmic as occurs in wild 3T3 and several other cells (3, 4, 8, 19). A comparison of the nuclear proteins solubilized from isolated nuclei of wild-type 3T3 cells with those from clones 3 and 9 shows clearly that nuclei from the AS clones are completely lacking in PLC β_1 (Fig. 2*b*). For cells transfected with the full-length sense cDNA for PLC β_1, the Western Blots show an increased expression of PLC β_1 in both whole cells and isolated nuclei (Fig. 3, *a* and *b*). Also in this case, the expression of PLC γ_1 is unaffected (Fig. 3*c*). Previous studies showed that within 2 min of treatment of confluent Swiss 3T3 cells with IGF-I, there is a rapid and transient 100% increase in nuclear PLC β_1 that can be inhibited by a neutralizing isoform-specific monoclonal antibody (3). Fig. 4*a* shows that in nuclei from wild-type 3T3 cells, treatment with bombesin does not affect the basal PLC β_1 activity, and this is in agreement with previous observations showing that bombesin does not alter the mass of nuclear polyphosphoinositides (20, 21). IGF-I, on the other hand, stimulates nuclear PLC β_1 activity, and this is abolished in the presence of neutralizing antibody. Fig. 4*a* also confirms that the two AS clones lacking in PLC β_1 do not have any appreciable nuclear PLC activity. In nuclei from transfectants overexpressing PLC β_1, the basal PLC activity is dramatically increased compared to wild-type 3T3 cells, is stimulated by IGF-I but not by bombesin, and is neutralized completely by anti-PLC β_1 antibody. A further confirmation that nuclear PLC β_1 activation is linked to IGF-I comes from experiments in which PDGF has been used instead of IGF-I. Indeed, in nuclei from both AS and overexpressing clones, 25 nmol of inositol 1,4,5-phosphate/mg protein are liberated upon PDGF stimulation, *i.e.*, the control values. This agrees with previous reviewed data (2) showing that cytosolic PLC γ_1 is targeted by PDGF. The cell cycle analysis has been carried out by incorporation of BrdUrd to discriminate the living cells actually synthesizing DNA in S phase. Flow cytometric analysis (Fig. 4*b*) shows that in wild-type 3T3 cells, both IGF-I and bombesin as well as PDGF stimulate the onset of DNA synthesis. The AS clones lacking in PLC β_1 show an abolition of IGF-I-induced DNA synthesis and more than 50% reduction following bombesin treatment. By contrast, overexpression of PLC β_1 increases dramatically the number of cells in S phase actively incorporating BrdUrd after both IGF-I and bombesin treatment. In both cases (*i.e.*, AS and overexpressing clones), PDGF does not affect the uptake of BrdUrd respect to control.

Autonomous nuclear PLC β_1 signaling appears to be one of the earliest events following exposure of Swiss 3T3 cells to IGF-I (3, 9, 11) as well as of human osteosarcoma SaOS-2 cells to interleukin 1α (5) and is also implicated in the differentiation of murine erythroleukaemia cells (6, 7, 22, 23). PLC β_1 differs from the PLC γ and δ isozymes in that it has a long COOH-terminal sequence that contains a cluster of lysine residues that are critical for association with the nucleus (8). The data reported here suggest that the activation of nuclear PLC β_1 is required for the onset of IGF-I-dependent DNA synthesis. The finding that overexpression of nuclear PLC β_1 increases the capacity of this response also suggests that PLC concentration, and hence signal strength, is an important determinant of the nuclear signaling response. The demonstration that bombesin stimulates DNA synthesis in wild-type 3T3 cells but fails to activate nuclear PLC β_1 (Fig. 4, *a* and *b*) confirms that its effects are mediated via cytoplasmic PLC β isozymes given that bombesin receptor is a G-protein-coupled receptor (2). In this regard, it is worth noting that although AS treatment effectively reduces nuclear PLC β_1 to undetectable levels, less than 5% of total cellular PLC β_1, as evaluated by densitometric analysis of the blots, is still associated with the cytoplasmic membrane fraction (data not shown). This could explain the substantial but not complete reduction in the stimulation of DNA synthesis seen in clones 9 and 3 on exposure to bombesin, although we cannot exclude the possibility of activation of other members of the PLC β family. The corresponding IGF-I response, however, is abolished to quiescent levels, suggesting that there could also be an

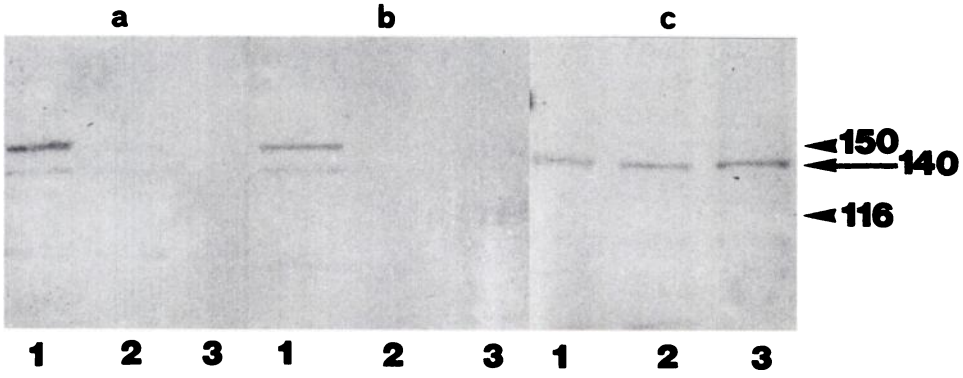

Fig. 2. Western Blot analysis of PLC isoforms in 3T3 cells transfected with the AS cDNA for PLC β_1. *a*, whole-cell proteins reacted with anti-β_1 isoform antibody; *b*, nuclear proteins reacted with anti-β_1 isoform antibody; *c*, whole-cell proteins reacted with anti-γ_1 isoform antibody. For each panel, the lanes are as follows: *Lane 1*, wild-type 3T3 cells; *Lane 2*, clone 3 carrying the PLC β_1 AS; and *Lane 3*, clone 9 carrying the PLC β_1 AS. *Arrows*, molecular weight (in thousands).

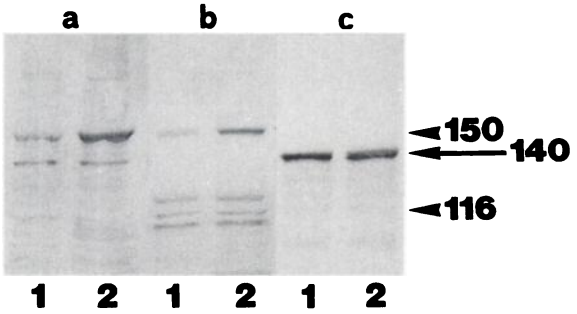

Fig. 3. Western Blot analysis of PLC isoforms in 3T3 cells transfected with the sense cDNA for PLC β_1. *a*, whole-cell proteins reacted with anti-β_1 isoform antibody; *b*, nuclear proteins reacted with anti-β_1 isoform antibody; *c*, whole-cell proteins reacted with anti-γ_1 isoform antibody. For each panel, *Lane 1* refers to wild 3T3 cells, and *Lane 2* refers to transfected cells. *Arrows*, molecular weight (in thousands).

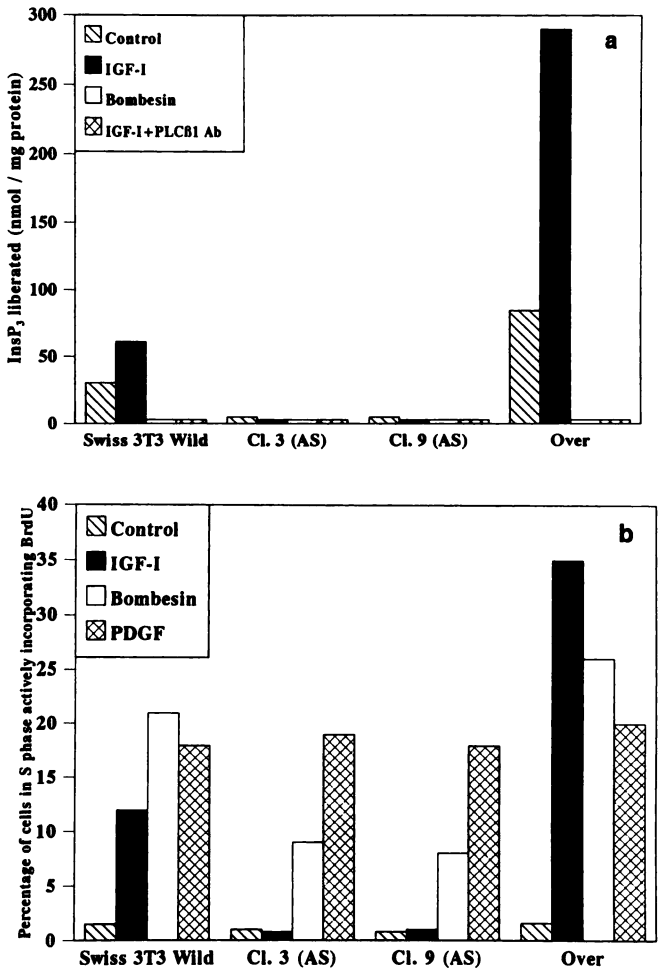

Fig. 4. Correlation between the activity of nuclear PLC β_1 and DNA synthesis after growth factor stimulation of quiescent wild-type 3T3 (*Swiss 3T3 Wild*), clones carrying the PLC β_1 AS [*Cl. 3 (AS)* and *Cl. 9 (AS)*], and clones overexpressing the PLC β_1 (*Over*). *a*, PLC activity after stimulation with IGF-I or bombesin for 2 min (*i.e.*, the time of maximal activation of nuclear PLC upon growth factor stimulation; Refs. 3 and 11). [^{3}H]Phosphatidylinositol 4,5-bisphosphate was used as substrate, and the activity measured in the presence of neutralizing anti-PLC β_1 antibody was carried out exactly as before. *b*, histogram of data obtained from flow cytometric analysis of the cell cycle carried out by BrdUrd incorporation in wild-type Swiss 3T3, clones carrying the PLC β_1 AS, and clones overexpressing the PLC β_1 after growth factor stimulation. The results in both panels are the average of five separate experiments (SD $\leq$ 7% for each point).

inherent difference in stability of the cytoplasmic and nuclear PLC β_1 pools. We conclude, therefore, that nuclear PLC β_1 plays an essential role in the initiation of cell division. Although no naturally occurring oncogenic forms of PLC isozymes have been found, it is interesting to note that in the rat, the PLC β_1 gene localizes to chromosome 3q 35–36, a region that is known to undergo frequent rearrangement in a number of chemically induced tumors (24).

Acknowledgments

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