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A Role for Nuclear Phospholipase Cβ_1 in Cell Cycle Control*

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Phosphoinositide signaling resides in the nucleus, and among the enzymes of the cycle, phospholipase C (PLC) appears as the key element both in *Saccharomyces cerevisiae* and in mammalian cells. The yeast PLC pathway produces multiple inositol polyphosphates that modulate distinct nuclear processes. The mammalian PLCβ_1, which localizes in the nucleus, is activated in insulin-like growth factor 1-mediated mitogenesis and undergoes down-regulation during murine erythroleukemia differentiation. PLCβ_1 exists as two polypeptides of 150 and 140 kDa generated from a single gene by alternative RNA splicing, both of them containing in the COOH-terminal tail a cluster of lysine residues responsible for nuclear localization. These clues prompted us to try to establish the critical nuclear target(s) of PLCβ_1 subtypes in the control of cell cycle progression. The results reveal that the two subtypes of PLCβ_1 that localize in the nucleus induce cell cycle progression in Friend erythroleukemia cells. In fact when they are overexpressed in the nucleus, cyclin D3, along with its kinase (cdk4) but not cyclin E is overexpressed even though cells are serum-starved. As a consequence of this enforced expression, retinoblastoma protein is phosphorylated and E2F-1 transcription factor is activated as well. On the whole the results reveal a direct effect of nuclear PLCβ_1 signaling in G_1 progression by means of a specific target, *i.e.* cyclin D3/cdk4.

It is demonstrated that an autonomous intranuclear inositide cycle exists and that nuclear PLCβ_1[1] is a key enzyme for cell proliferation and differentiation (1). The enzymes of polyphosphoinositide turnover occur in the nucleus of mammalian cells and yeast as well (Ref. references therein),and there is evidence for phosphatidylinositol bisphosphate (PIP_2) synthesis and degradation in the nuclear matrix (3). The evidence obtained with confocal and electron microscope analysis indicates that enzymes required for the synthesis and hydrolysis of phosphoinositides are localized at ribonucleoprotein structures of the inner nuclear matrix involved in transcript processing within the interchromatin domains (4). Although phosphatidylinositol cycle is activated only in nuclei from HeLa cells in S phase (5), striking changes occur mainly in PLCβ_1 activity a few minutes after growth factor stimulation (1). PLCβ_1 is composed of two subtypes, 150-kDa PLCβ_1a and 140-kDa PLCβ_1b, that are derived from a single gene by alternative RNA splicing (6). The two forms of the PLCβ_1 are detectable both in cytosolic and nuclear fractions although PLCβ_1b exists almost entirely in the nucleus (7), and the β_1a form localizes in equal amount in nuclei and plasma membrane (8). Previous investigations from our group have demonstrated that the nucleus-confined PLCβ_1 is directly involved in maintaining the undifferentiated state of Friend erythroleukemia cells even in the presence of inducers of erythroid differentiation, possibly due to a continuous stimulation of the cell cycle (9). With the above in mind, we sought to explore whether PLCβ_1 is actually involved in the regulation of cell cycle machinery.

In mammalian cells, proliferation is under the control of factors that regulate the transition between different cell cycle stages at two main checkpoints. The better described checkpoint is at the G_1-S phase transition for initiation and completion of DNA replication in S phase. The other checkpoint is at the G_2-M phase transition and controls mitosis and cell division (10). The cell cycle is primarily regulated by a family of structurally related serine/threonine protein kinases, which consist of a regulatory subunit, a cyclin, and a catalytic subunit, a cyclin-dependent kinase (cdk). In mammalian cells, cdk4 or cdk6, in combination with the D type cyclins (D1, D2, and D3), and cdk2 in association with cyclin E play key roles in regulating G_1 progression (Ref. 11 and references therein).

Furthermore, overexpression of E or D type cyclins is able to accelerate the transition through the G_1 phase of the cell cycle (12). Several lines of evidence suggest that the primarily function of D-type cyclins is to stimulate progression through G_1, rather than to promote the G_1/S transition. In addition it appears that in hematopoietic cell lines, overexpression of cyclin D2 or D3 prevents granulocyte differentiation, whereas overexpression of cyclin D1, which is normally not expressed in those cells, does not (13). Both cyclin D in complex with cdk4 or cdk6 and cyclin E-cdk2 phosphorylate the product of the retinoblastoma gene, the retinoblastoma protein (pRb), a well known tumor suppressor. This tumor suppressor activity relies on pRb role in gating S phase entry through its ability to repress genes activated by the E2F family of transcription factors. In fact phosphorylated pRb (ppRb) releases members of the E2F family that play an integral role in cell cycle progression by inducing the expression of gene required for S phase

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[1] The abbreviations used are: PLC, phospholipase C; IPs, inositol polyphosphates; IGF-1, insulin-like growth factor 1; PIP_2, phosphatidylinositol bisphosphate; cdk, cyclin-dependent kinase; Rb, retinoblastoma; pRb, retinoblastoma protein; ppRb, phosphorylated retinoblastoma protein; DTT, dithiothreitol; wt, wild type.

entry, including those involved in DNA synthesis such as S phase regulatory factors cyclin E, cyclin A, and cdk2 (14, 15). All five E2F family members bind the same DNA sequence, but additional levels of regulation have been observed as follows: E2F-1, E2F-2, and E2F-3 associate with pRb (p110); E2F·pRb complexes are found primarily in G_1; E2F-4 and E2F-5 preferentially bind to p107 and p130. Enforced expression by microinjection of E2F-1 induces starved cells to progress through G_1 and enter S phase (16). In order to understand the mechanism that couples nuclear PLCβ_1 and cell cycle machinery, we carried out a detailed analysis of the expression of the proteins involved in the regulation of the cell cycle. For this purpose we have overexpressed PLCβ_1a and -b in the nuclear compartment and the mutant M2b, which lacks the nuclear localization sequence (8, 9), in the cytoplasmic compartment in order to find out how nuclear localization of PLCβ_1 acts on cell cycle progression and on key signaling events that regulate the passage through G_1 phase.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors and Transfection—The full-length cDNA for rat PLCβ_1a (17), the full-length cDNA for rat PLCβ_1b (6), and the mutant for nuclear localization sequence, where by site-directed mutagenesis lysine residues 1056, 1063, and 1070 in region 2 of the COOH terminus were substituted with isoleucine (8), 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 1 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 ng/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% fetal calf serum. For experimental procedures cells were serum-deprived for 48 h and then stimulated with fresh serum for the indicated hours (figure legends).

Isolation of Nuclei—A hypotonic shock combined with non-ionic detergent, essentially described by Martelli *et al.* (19), has been used. In addition to 0.5 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, 1 mM EGTA, 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 detection of β-tubulin as described in Ref. 20, and only nuclei showing in Western blot a complete absence of β-tubulin 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 in 10 mM Tris-Cl, pH 7.8, 2 mM $MgCl_2$ plus protease inhibitors as above and then pelleting the nuclei at $400 \times g$. This procedure allows the recovery of pure cytoplasmic fraction and avoids the risk of contamination by nuclear debris that is present in the crude supernatant from nuclear purification.

PLC Assay—The assay was carried out exactly as described previously (20). Nuclear PLC activity *in vitro* has been checked by using [^{3}H]phosphatidylinositol bisphosphate and then analyzing the nanomoles of inositol 1,4,5-trisphosphate released per mg of protein/30 min of incubation.

Flow Cytometry—Friend cells transfected with empty vector, clones overexpressing PLCβ_1a and -b, and mutants for nuclear localization sequence were analyzed by flow cytometry. Briefly cells were grown with 10% fetal calf serum for 48 h and then starved for 24 h. Cells were washed in cold phosphate-buffered saline and fixed in cold 70% ethanol for 30 min. The pellet was resuspended in a solution containing 50 μg/ml propidium iodide, 1 mg/ml sodium citrate, 1 μl/ml Triton X-100 and 5 μg/ml RNase A and analyzed by a FACStar Plus flow cytometer (Becton Dickinson).

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 $MgCl_2$, 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 $MgCl_2$, 0.2 mM EDTA) and left 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 E2F-1, 5′-ATTTAAGTTTCGCGCCCTTTCTCAA-3′ (Santa Cruz Biotechnology), was used as a probe for the assay. The probe was labeled with [γ-^{32}P]dATP using T_4 polynucleotide Kinase (Promega). 10 μg of nuclear extract was used in each binding reaction. Where appropriate, the extract was preincubated for 10 min at room temperature with 5 pM unlabeled E2F-1 oligonucleotide and 5 pM unspecific competitor. For antibody perturbation supershift experiments, 0.25 μg of anti-pRb antibody (Santa Cruz Biotechnology) was added to the incubation 10 min prior to addition of oligonucleotide probe and incubated at room temperature. Subsequently, binding to 1 μl of radioactive E2F-1 oligo was carried out in a reaction mixture containing 1 μg of poly(dI·dC), 20 mM Hepes, pH 7.6, 70 mM KCl, 5 mM $MgCl_2$, 0.05% Nonidet P-40, 12% glycerol, 1 mg/ml bovine serum albumin, 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—Immunoblot analysis of 500 μg of nuclear and cytoplasmic fractions of wt were immunoprecipitated with 2.5 μg of anti-PLCβ_1, a + b isoform, (Transduction Laboratories) for 2 h at 4 °C with constant agitation, followed by 1 h of incubation with 50 μl of protein A-Sepharose (10% w/v). Pellets were washed three times in phosphate-buffered saline containing 1% Nonidet P-40, twice in 0.1 M Tris, 0.5 M LiCl, and twice in Tris, NaCl, EDTA (TNE). Pellets were resolved by SDS-polyacrylamide gel electrophoresis and blotted to nitrocellulose membrane for subsequent immunodetection with the same antibody, followed by detection using ECL (Amersham Pharmacia Biotech). For Western blot analysis, proteins from nuclear extract (20 μg) were separated on an SDS-0.1% polyacrylamide gel and transferred onto nitrocellulose paper. The following antibodies were used: polyclonal antibodies to cyclin D3, cyclin E, cdk2, and cdk4 from Santa Cruz Biotechnology; polyclonal antibodies to pRb and to ppRb for specific phosphorylation sites Ser-795, Ser-780, and Ser-807/811, from New England Biolabs.

Immune Complex Kinase Assay—Cells were suspended in lysis buffer containing 50 mM Hepes, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 5 μg of aprotinin per ml, 10 mM βglycerophosphate, 1 mM sodium orthovanadate, and 50 mM NaF, followed by a 10-s sonication and clearing by centrifugation at 14,000 rpm for 15 min. Supernatants were assayed for protein concentration by the Bradford assay (Bio-Rad); protein samples of 4 mg each were then immunoprecipitated for at least 2–4 h at 4 °C with protein A-agarose beads precoated with saturating amounts of the appropriate antibody (5 μg). Immunoprecipitated proteins on beads were washed three times with 1 ml of lysis buffer and twice with kinase buffer (50 mM Hepes, pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, plus protease inhibitors, as described above). The beads were resuspended in 50 μl of kinase buffer containing 2 μg of glutathione *S*-transferase (GST)-Rb-(769–921) fusion protein (Santa Cruz Biotechnology, Inc), 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 20 μM ATP, and 10 μCi of [γ-^{32}P]ATP (6000Ci/mmol, ICN). After incubation for 30 min at 30 °C, the samples were boiled in 2× Laemmli buffer and separated by SDS-polyacrylamide gel electrophoresis. Phosphorylated proteins were visualized by exposure to phosphorstorage screens. The antibodies used were as follows: for cyclin D3 and cdk4, polyclonal rabbit antibodies were obtained from Santa Cruz Biotechnology.

RESULTS

The subcellular distribution of the two subtypes of the PLCβ_1, a and b, was evaluated by Western blot analysis in wild type erythroleukemic Friend cells (Fig. 1*B*). Although the PLCβ_1b is almost entirely localized in the nucleus, the PLCβ_1a is nearly equally distributed among the two compartments. The Western blot analysis of the clones overexpressing the PLCβ_1a, PLCβ_1b, and the mutant for the nuclear localization sequence M2b, respectively, show that whereas the M2b mutant has lost the capacity for nuclear localization, the clones overexpressing the two subtypes β_1a and β_1b have the PLC in the nucleus, the b form being entirely nuclear and the a form distributed in both nucleus and cytoplasm, even though in the latter compartment it is less expressed (Fig. 1*A*). The expression of PLCβ_1 forms in wild type cells is very low since the immunoprecipitated PLC from 500 μg of both nuclear and

cytoplasmic protein gave rise to a signal even lower than the one obtained with 10 μg of protein from nuclei or cytoplasm of transfected cells. The PLC activity of the clones overexpressing PLCβ$_1$a and M2b mutant as well is the same reported in Ref. 9 (*i.e.* PLCβ$_1$a 38.0 ± 1.5, M2b 9.3 ± 0.7 nmol of IP$_3$/mg of protein/30 min of incubation). These values compared with nuclear PLC activity in wild type cells (9.8 ± 0.6 nmol of IP$_3$/mg protein/30 min of incubation) show a nearly 3-fold increase of the nuclear activity in the case of PLCβ$_1$a and PLCβ$_1$b (32.7 ± 2.0 nmol of IP$_3$/mg of protein/30 min of incubation) with no changes in the case of PLC M2b, which conversely is 3-fold higher in the cytoplasm.

These features characterize all the clones we have selected for each type of transfectant and show a complete agreement between the enzymatic activity and the level of expression of the PLCβ$_1$ forms. To determine whether PLCβ$_1$ is involved in cell cycle progression, we examined cyclin D3, cyclin E, cdk2, and cdk4 protein expression in serum-starved cells and after stimulation with serum. In erythroleukemic Friend cells cultured without inducer, the protein levels of cyclin D3 and E fluctuate during the cell cycle, whereas the level of cyclin D2 changes little. Cyclin D1 protein is not detected in these cells. Cyclin D3 increases as cells progress through G$_1$ phase and reaches a maximum level in S phase. Cyclin E peaks at the transition from G$_1$ to S phase (21). Fig. 2 shows that in nuclear extracts of Friend cells, cyclin D3 with its related cdk4 and cyclin E were expressed at barely detectable levels in serum-starved wild type cells and accumulated after stimulation with serum, whereas the level of cdk2 protein, whose activity is low in G$_1$ phase and increases during S phase (21), is constant after serum stimulation. On the contrary, the clones overexpressing in the nucleus both PLCβ$_1$a and PLCβ$_1$b show high levels of cyclin D3 and cdk4 in serum-starved cells, whereas M2b mutant clone and wild type cells as well do not. Since the expression of cyclin E does not increase in serum-starved transfectants overexpressing PLCβ$_1$a or PLCβ$_1$b, we thought that overexpression of enzymatically active forms of PLCβ$_1$ in the nucleus could exert a specific effect, via cyclin D3·cdk4 complex formation, in sustaining cell cycle progression even in the absence of serum. Therefore, we have evaluated the activity of the cyclin D3·cdk4 complex *in vitro* in order to establish whether the complex is enzymatically active. Cyclin D3 and cdk4 were immunoprecipitated from total cell lysates, and immune complex was then used for *in vitro* kinase assay by using a bacterially produced glutathione S-transferase-Rb fusion protein as a substrate. Fig. 2*C* shows that the activity of cyclin D3·cdk4 complex, which is almost absent in serum-starved wild type cells and M2b clone, increases after serum stimulation. On the contrary, in the clones overexpressing PLCβ$_1$a and -b in the nucleus the activity of the complex is present even after serum starvation, and its level is similar to that of both wild type cells and M2b clone after serum stimulation. This *in vitro* feature prompted us to analyze the *in vivo* phosphorylation status of pRb. We have evaluated pRb phosphorylation by using specific antibodies that detect specific phosphorylated sites as follows: Ser-780, Ser-795, and Ser-807/811 (New England Biolabs). When fresh medium with 10% serum was added for 12 h to serum-starved Friend cells (wild type cells), a significant increase of pRb Ser phosphorylation was observed on Ser-795, Ser-780, and Ser-807/811 as physiologically occurs (Fig. 3). Surprisingly transfectants overexpressing both forms of PLCβ$_1$ in the nucleus show high levels of pRb phosphorylation, only on

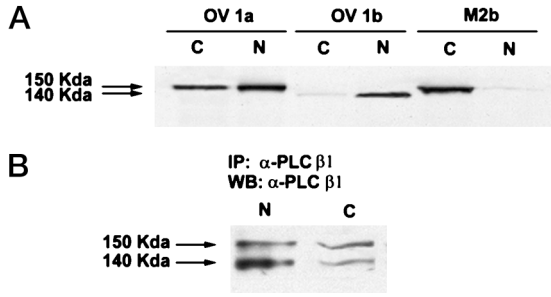

FIG. 1. **Analysis of PLCβ$_1$ phenotype in stable transfectants and Friend cells wt.** *A,* immunoblot analysis of 10 μg of nuclear and cytoplasmic lysate of the stable transfectants as follows: *Ov1a,* cells transfected with PLCβ$_1$a; *Ov1b*, cells transfected with PLCβ$_1$b; *M2b*, cells transfected with the mutant lacking the nuclear localization sequence. *B,* immunoblot analysis of 500 μg of nuclear (*N*) and cytoplasmic (*C*) fractions of wt immunoprecipitated (*IP*) with 2.5 μg of anti-PLCβ$_1$ (a and b) (Signal Transduction). The Western blots (*WB*) and PLC activities of stable transfectants reported here are representative of 5 other clones, which behave exactly as the ones in the figure.

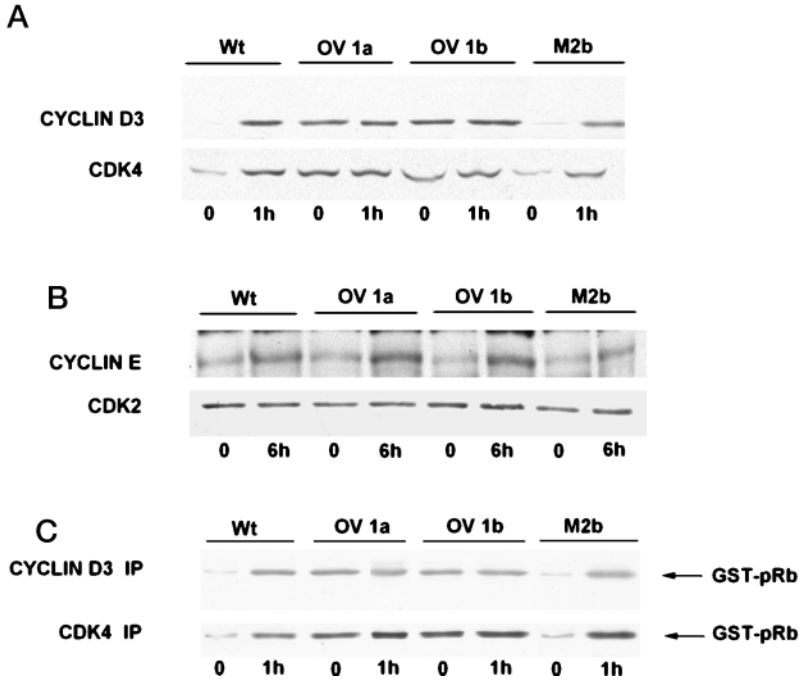

FIG. 2. **Effect of overexpression of PLCβ$_1$ subtypes (a and b) and the mutant M2b on both the expression and the activity of cell cycle proteins.** *A,* changes in the level of expression of cyclin D3 and cdk4 in the presence or absence of serum. *B,* changes in the level of expression of cyclin E and cdk2 in the presence or absence of serum. *C,* changes in the activity of cyclin D3·cdk4 complex in the presence or absence of serum. Cells transfected with PLCβ$_1$a (*Ov1a*), cells transfected with PLCβ$_1$b (*Ov1b*), cells transfected with the mutant lacking the nuclear localization sequence (*M2b*), and wild type cells (*Wt*) were serum-deprived for 48 h and then stimulated with fresh serum (10%) for the indicated time. The Western blots and the activity assay of stable transfectants reported here are representative of 5 other clones, which behaved in an identical fashion.

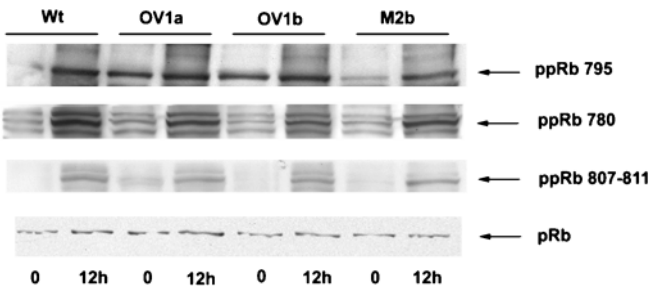

FIG. 3. **Effect of overexpression of PLCβ$_1$ subtypes and the mutant M2b on pRb phosphorylation.** Serum-starved cells, as described in the legend to Fig. 2, were diluted at 2×10^5 with fresh medium containing 10% fetal calf serum for 12 h after which time pRb phosphorylation was found to peak. Abbreviations for wild type cells and clones are as in the previous figures. The membrane was probed with anti-pRb (New England Biolabs) and with anti-ppRb for specific phosphorylation sites, *i.e.* Ser-795, Ser-780, and Ser-807/811 (New England Biolabs) in a 7% polyacrylamide gel. The phosphorylated form of Rb protein is indicated by the designation *ppRb,* the form not phosphorylated is indicated by *pRb.* The Western blots of stable transfectants reported here are representative of 5 other clones, which behaved in an identical fashion.

Ser-795, even during serum starvation, whereas this does not occur at all in both the M2b mutant and the wild type cells (Fig. 3). Indeed cells overexpressing PLCβ$_1$ in the nucleus show phosphorylated pRb (Ser-795) after serum starvation for 48 h. The two antibodies against phosphorylation sites Ser-780 and Ser-807/811 did not reveal any phosphorylation in serum-starved clones overexpressing PLCβ$_1$ in the nucleus. The phosphorylation in starved clones, overexpressing PLCβ$_1$ in the nucleus, is restricted to Ser-795 possibly indicating a specific effect of the nuclear localization of PLCβ$_1$. It is concluded that the overexpression of PLCβ$_1$a and -b in the nucleus up-regulates both the expression and the activity of the cyclin D3·cdk4 complex and affects pRb phosphorylation. The absence of these two events in cells overexpressing the same PLC in the cytoplasmic compartment indicates a very specific effect linked to the subcellular localization of this signaling PLC.

The Rb proteins bind to a number of cellular proteins like E2F that are involved in the regulation of transcription of genes relevant for the control of the cell cycle. E2F transcription factors regulate the transition from the G$_1$ to S phase, and its activity is regulated by members of pRb family (22) and appears to be a downstream step of the signaling cascade that takes place in G$_1$ phase. To assess whether overexpression of the two subtypes of PLCβ$_1$ could affect functional changes in E2F activity, we evaluated E2F DNA binding activity in resting cells and in cells after stimulation with serum. Fig. 4 shows the effect of the overexpression of PLCβ$_1$a and -b on E2F-1 binding (19). As expected, in serum-starved clones overexpressing the two nuclear subtypes of PLCβ$_1$ the binding activity was higher than in wild type cells and M2b mutant cells, suggesting that PLCβ$_1$a and -b when localized in the nucleus are indeed involved in the regulation of proteins responsible for the progression of cell cycle. To address whether, after serum starvation, pRb is bound to E2F-1 in both wild type cells and M2b clone and not in serum-starved cells expressing PLCβ$_1$a and -b in the nucleus, we have added anti-pRb antibody in the binding assay. Fig. 4 shows a change in the mobility of the E2F-1 complex only in serum-starved wild type cells and M2b mutant clone. These observations indicate that the E2F-1 transcription factor is already released from pRb in serum-starved Friend cells overexpressing PLCβ$_1$a or -b in the nucleus. Given that E2F transcription factors play an integral role in cell cycle progression by inducing the expression of genes required for S phase entry, we checked the effect of the overexpression of PLCβ$_1$a

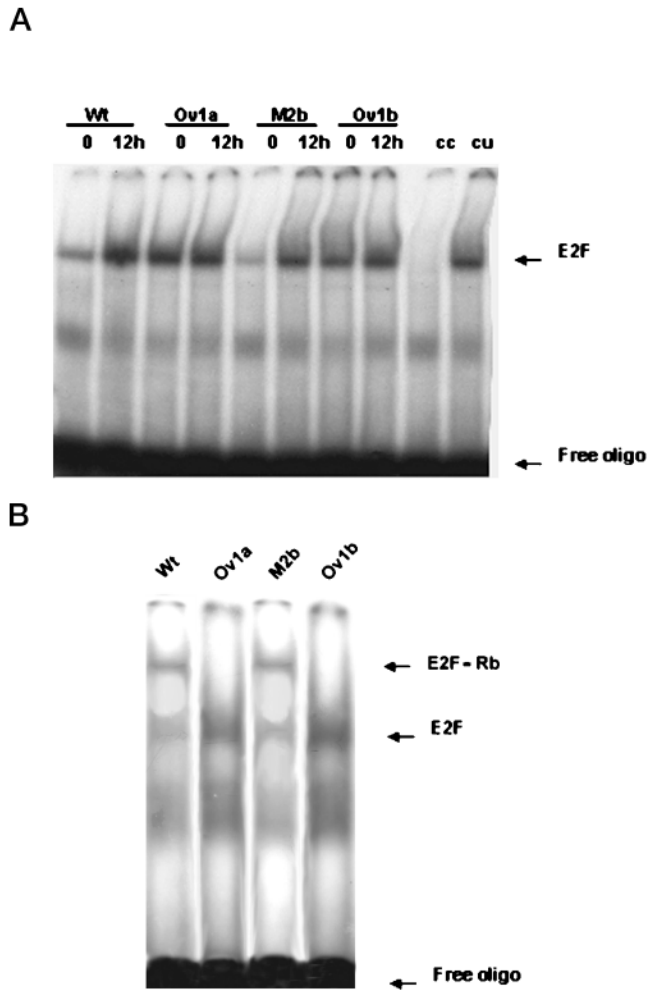

FIG. 4. **Effect of PLCβ$_1$a, PLCβ$_1$b, and PLCβ$_1$M2b overexpression on E2F-DNA binding activity.** Abbreviations for wild type cells and clones are as in the previous figures. Serum-starved cells (48 h) were stimulated by addition of fresh serum for 12 h. E2F-DNA complex formation was determined by DNA gel mobility shift assay (*A*). For antibody perturbation supershift experiments, anti-pRb antibody was added to the incubation in samples from serum-starved wild type cells and clones expressing PLCβ$_1$ (*B*). *cc*, preincubation with unlabeled E2F-1 oligonucleotide; *cu*, preincubation with nonspecific competitor. The stable transfectants reported here are representative of 5 other clones, which behaved in an identical fashion.

and -b and M2b mutant on the behavior of the cell cycle in these transfectants by means of flow cytometry. In the presence of serum the clones overexpressing PLCβ$_1$a and -b show a cell cycle profile characterized by a higher S phase compared with wt and the M2b mutant (Fig. 5). Moreover, when the clones overexpressing PLCβ$_1$a and -b in the nucleus are serum-starved, they still maintain a higher S phase compared with wt and M2b cells, the flow cytometric analysis of which shows an almost complete block in G$_1$ phase. Therefore, cells overexpressing the two subtypes of PLCβ$_1$ could at least in part effectively replace the requirement for serum as a mitogenic signal.

DISCUSSION

Nuclear PLCβ$_1$ signaling was suggested to be one of the earliest events following both exposure of human osteosarcoma Saos cells to interleukin-1α (23) and murine 3T3 cells to IGF-1 (18). A decrease in nuclear PIP_2 hydrolysis and down-regulation of nuclear PLCβ$_1$ have been detected during differentiation of erythroleukemia cells (24). Moreover, we have shown that nuclear PLCβ$_1$ is involved in maintaining the undifferen-

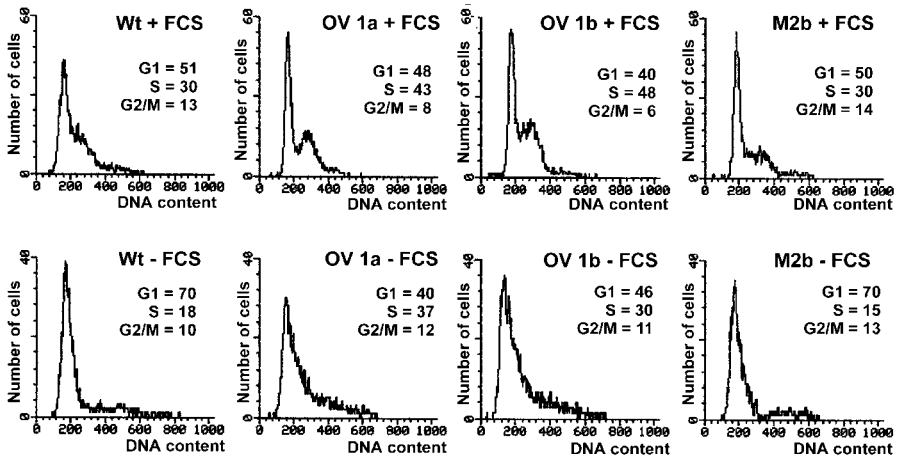

FIG. 5. **Enforced PLCβ_1 expression is sufficient to promote cell cycle progression.** Cells were grown with 10% fetal calf serum (*FCS*) for 48 h and then starved for 24 h. Abbreviations for wild type cells and clones are as in the previous figures. Both in the presence and absence of serum, clones overexpressing nuclear PLCβ_1 show a higher S phase as compared with wild type and M2b-transfected clones. The stable transfectants reported here are representative of 5 other clones, which behaved in an identical fashion.

tiated state of Friend erythroleukemia cells, possibly by opposing the inhibition of the cell cycle progression necessary for erythroid differentiation (9). Thus this isoform is a good candidate as a key element for the signaling cascade involved in cell growth and proliferation. The contention that subcellular localization of the inositol lipid cycle is central to the function of the cycle itself in controlling the signaling events has been strengthened by the discovery that in *Saccharomyces cerevisiae* a nuclear PLC, called PLC1, homologous in function to the mammalian PLCβ_1, and two inositol polyphosphate kinases constitute a signaling pathway that affects transcriptional control (25). Our study was intended to find out how nuclear PLCβ_1 is involved in the regulation of cellular proliferation. To address this issue we have tried to identify the targets of nuclear PLCβ_1 in the nuclear compartment. Therefore we first examined the expression of cyclins and cdks associated with G_1-S phase transition. Our evidence suggests that the overexpression of PLCβ_1 in the nucleus is directly responsible for the overexpression and activation of cyclin D3·cdk4 complex, which is known to stimulate progression through G_1 rather than to promote the G_1-S transition (11). This is indeed a specific target, given that the other cyclin, cyclin E, which is responsible for G_1-S transition (26) is not affected at all by nuclear PLCβ_1. The two splicing products of this isoform behave in the same fashion, even though the 1b is more specific for nuclear localization and 1a localizes also in the cytoplasmic compartment. This suggests that once PLCβ_1 is in the nucleus its role is different from its counterpart located at the plasma membrane. This is supported by the absence of an effect following overexpression of the M2b mutant in which PLC localizes exclusively to the cytosolic compartment (8, 9). In this case the effects on both cyclin D3 and cdk4 as well are totally absent. Only the clones overexpressing both PLCsβ_1a and -b, but not the M2b mutant, show a higher S phase compared with wild type and M2b-transfected clones both in cycling and serum-starved cells. This implies that, since cyclin E is not affected, both the overexpression and the activation of the cyclin D3·cdk4 complex are actually responsible for maintaining the cell cycle progression. Downstream events such as the phosphorylation of pRb and the subsequent release of E2F-1 transcription factor are also only observed in PLCβ_1a and -b clones after serum starvation.

It should be noted that the overexpression of PLCβ_1 in the nucleus gives rise to enzymatically functional PLC whose activity is 3-fold higher than the one of wt cells. This enhanced signaling capacity generates second messengers such as diacylglycerol, whose role in nuclear protein kinase C activation has been widely explored (19, 27, 28) and inositol trisphosphate, whose nuclear kinases, by producing the other inositol polyphosphates, are involved in mRNA export and transcription in yeast (25). Thus, data reported here appear to shed new light on the lipid-mediated signaling machinery residing in the nucleus, implicating nuclear PLCβ_1 as a key element for the specific regulation of the cyclin D3·cdk4 complex and its downstream targets such as pRb and E2F in facilitating cell cycle progression.

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