Nuclear Phospholipase C β1 (PLCβ1) Affects CD24 Expression in Murine Erythroleukemia Cells*

Received for publication, October 18, 2004, and in revised form, April 7, 2005
Published, JBC Papers in Press, April 22, 2005, DOI 10.1074/jbc.M411833200

Robertetta Fiume‡, Irene Faenza‡, Alessandro Matteuccii, AnnaLisa Astolfii, Marco Viitali‡, Alberto Maria Martelli§§**, and Lucio Cocco‡ ‡‡

From the ‡Department of Anatomical Sciences, Cellular Signaling Laboratory, University of Bologna, 40126 Bologna, Italy, the **School of Pharmacy, University of Bologna, 40126 Bologna, Italy, §§IITJ-CNR, Unit of Bologna, 06 IOR 40136 Bologna, Italy, the ¶Department of Experimental Pathology, Cancer Research Section, University of Bologna, 40126 Bologna, Italy, and the ‡‡Department of Anatomy, Pharmacology and Forensic Medicine, Human Anatomy Section, University of Parma, 43100 Parma, Italy

Inositol-specific phospholipase C (PLC) β1 is a key enzyme in nuclear lipid signal transduction affecting cell cycle progression and may be directly involved in regulation of gene expression and hematopoiesis. By microarrays, we compared the effect of nuclear PLCβ1 overexpression with that of PLC M2b cytoplasmatic mutant, which is exclusively located in the cytoplasm, in murine erythroleukemia cells. Out of 9000 genes analyzed, the CD24 gene, coding for an antigen involved in differentiation and hematopoiesis as well, was up-regulated in cells overexpressing nuclear PLCβ1 as compared with both cells overexpressing the M2b cytoplasmatic mutant and the wild type cells. Here we show that nuclear PLCβ1 up-regulated the expression of CD24. The correlation was strengthened by the observation that when PLCβ1 expression was silenced by means of small interfering RNA, CD24 expression was down-regulated. We also demonstrated that PLCβ1-dependent up-modulation of CD24 was mediated, at least in part, at the transcriptional level, in that PLCβ1 affected the CD24 promoter activity. Moreover, the up-regulation of CD24 was higher during erythroid differentiation of murine erythroleukemia cells. Altogether our findings, obtained by combining microarrays, phenotypic analysis, and small interfering RNA technology, identify CD24 as an molecular effector of nuclear PLCβ1 signaling pathway in murine erythroleukemia cells and strengthen the contention that nuclear PLCβ1 constitutes a key step in erythroid differentiation in vitro.

Inositol-specific phospholipase C (PLC)¹ β1 is a key enzyme in the lipid signaling pathway. PLCβ1 hydrolyzes phosphatidylinositol 4,5-bisphosphate, giving rise to the two second messengers diacylglycerol and inositol 1,4,5-trisphosphate. Both of them are involved in signal transduction cascades that influence many cellular events, including cell cycle progression and differentiation (1).

PLCβ1 exists in two forms, generated by an alternative splicing of the same transcript, which differ in apparent mass: 150-kDa PLCβ1a and 140-kDa PLCβ1b. Both forms are present mostly in the nucleus of the cell (2, 3). The importance of this cellular localization has been analyzed by means of a PLCβ1 mutant (M2b) defective in nuclear localization. Previously, stable clones, obtained overexpressing PLCβ1a, PLCβ1b, and M2b in Friend erythroleukemia cells, have been analyzed to find out downstream targets of nuclear PLCβ1 signaling. As a result, it has been shown that the overexpression of PLCβ1 induces cell cycle progression targeting cyclin D3, along with its specific kinase (4).

Therefore, we have studied the effects of PLCβ1 signaling by using a broad pattern approach, taking advantage of microarray technology. DNA microarrays permit the simultaneous comparison of the expression levels of thousands of genes among different samples. We have analyzed the global transcription pattern in wild type Friend erythroleukemia cells as well as in overexpressing PLCβ1a, PLCβ1b, or the cytoplasmic mutant M2b. Out of 9000 genes represented in the chip, we focused on the gene encoding the murine CD24, whose pattern of expression was significantly different in cells overexpressing nuclear PLCβ1 in comparison with both wild type cells and M2b cytoplasmatic mutant.

Both mouse CD24, also named heat stable antigen, and its human homologue are small cell surface proteins, which are anchored to the membrane by means of glycosylphosphatidylinositol. They are heavily N- and O-glycosylated, accounting for their broad variation in apparent molecular weight, in a cell type-dependent fashion. The protein has been detected in a variety of cell types, including hematopoietic cells, neurons, regenerating muscle, or epithelial cells. In hematopoietic lines, it was initially discovered in K562 human erythroleukemia cells (5) and in immature B and T lymphocytes. CD24 is notably expressed on most immature hematopoietic lineages, whereas its expression is low or absent in terminally differentiated cells (6, 7). The first identified function was as a CD28-independent costimulatory molecule on activated B cells (8). In addition to B cells, it is expressed on immature T cells, where it promotes the activation of CD4⁺ and CD8⁺ T lymphocytes (9). CD24 also functions as a ligand of P-selectin, an adhesion receptor present on activated endothelial cells and platelets, thus contributing to the metastasizing capacity of CD24-expressing tumor cells (10). CD24 is expressed on differentiating neurons during development, negatively regulates cell proliferation in zones of secondary neurogenesis (11), and constitutes a genetic modifier for susceptibility and progression of multiple sclerosis (12). In addition, CD24 is an important prognostic
marker of different tumor types.

The up-regulation of CD24 transcription observed by microarray analysis and the role of CD24 in hematopoiesis prompted us to investigate whether the expression of CD24 is indeed up-regulated by nuclear PLCβ1. We report that overexpression of PLCβ1 in cells expressing CD24 resulted in the up-regulation of CD24 and, conversely, that siRNA silencing of PLCβ1 resulted in the down-regulation of CD24. The regulation was exerted, at least in part, at the transcriptional level, and CD24 up-regulation was higher during erythroid differentiation in vitro.

EXPERIMENTAL PROCEDURES

Cell Cultures and Treatment—Murine erythroleukemia cells (Friend cells, clone 707) were grown in RPMI 1640 (BioWhittaker) supplemented with 10% fetal calf serum (BioWhittaker). Erythroid differentiation was induced by the addition of 1.5% (v/v) dimethyl sulfoxide (MeSO) (Merck) to the culture medium for 3 days (72 h).

Construction of Expression Vectors—The full-length cDNA for rat PLCβ1a and PLCβ1b (13) and for the M2b mutant for the nuclear localization sequence, in which the lysine residues 1056, 1063, and 1070 of the COOH terminus were substituted with isoleucine by means of site-directed mutagenesis, were cloned into pRc/CMV (Invitrogen) expression vector plasmid as described elsewhere (14).

Northern Blot—Total RNA was extracted from the cells by using the denaturing guanidinium isothiocyanate method (RNeasy mini kit; Qiagen). Fifty micrograms of each RNA sample were resuspended in RNA loading buffer (50% formamide, 2.2 M formaldehyde, and 1× electrophoresis buffer) and electrophoresed through a 1% agarose gel with 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M NaCitrate) and 0.1% SDS as the electrophoresis buffer. RNAs were transferred from the gel onto a nylon membrane and UV cross-linked for 1 min. The blot was hybridized with a 32P-labeled 1.1-kb-long DNA 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/0.1% SDS and exposed to a Kodak image station 2000R overnight.

Reverse Transcription (RT)-PCR—Total RNA was extracted as described above for Northern blot. cDNA was synthesized from 2 μg of tRNA using 200 units of M-MLV reverse transcriptase, 0.5 mM oligo(dT) primers, 25 units of ribonuclease inhibitor, 10 mM each dNTP, and 1× RT buffer for 1 h at 42 °C. PCR was performed with 5 μl of cDNA using 1.5 mM MgCl2, 10 μM each dNTP, and 1 μM each primer, 2.5 units of TaqDNA polymerase (Perkin-Elmer Life Sciences), and 0.2 μCi of [α-32P]dCTP using the random priming method and the Klenow reaction. Membranes were hybridized at 42 °C with fresh prehybridization solution plus the denatured 32P-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/0.1% SDS and exposed to a Kodak image station 2000R overnight.

Preparation of Cytoplasmic Fraction—The cytoplasmic fraction was obtained by homogenizing 10 × 106 cells with 20 strokes in a Dounce homogenizer in 1 ml of 10 mM Tris·Cl (pH 7.8) and 2 mM MgCl2 plus protease inhibitors, as described above, and then pelleting the nuclei at 400 × g. This procedure allows the recovery of pure cytoplasmic fraction and avoids the possible risk of contamination by nuclear debris. Cytoplasmic proteins were precipitated by trichloroacetic acid and solubilized in 200 μl of electrophoresis sample buffer. To compare properly the nuclear fraction versus the cytosolic one, we have also used as a cytoplasmic fraction the supernatant of nuclear preparation described above. The purity of cytoplasmatic fraction was assessed by checking the absence of histone H3.

Preparation of Whole Cell Extract—Whole cell lysates were prepared by lysing 5 × 106 cells in 500 μl of radiolabeled precipitation buffer (50 mM Tris, pH 7.5, Nonidet P-40, 1% SDS, 100 mM NaCl, 50 mM NaF, 1 mM EDTA) supplemented with a set of protease inhibitors: 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium benzamidine, and 1 mM phenylmethylsulfonyl fluoride.

Immunohistochemistry—80 μg of proteins from the purified nuclei, cytoplasmatic fractions, and whole cell extracts were separated on 6% polyacrylamide-0.1% SDS gels, as specified in the figure legends. Proteins were transferred to nitrocellulose membranes for subsequent immunodetection with the specific antibodies, and detected by using ECL method (Amersham Biosciences) and visualized in a Kodak digital image station 2000R. The following antibodies were used: monoclonal antibody anti-PLCβ1a and -b and goat polyclonal anti-histone H3 from Santa Cruz Biotechnology; monoclonal antibody anti-CD24 from Pharmingen; monoclonal antibody anti-β-tubulin from Sigma. Densitometric analysis of the blots was performed by using a Kodak image station 2000R.

Hybridization and Microarray Scanning—For microarray experiments, Agilent (Agilent Technologies) mouse cDNA arrays were used. Total RNA was extracted using the denaturing guanidinium isothiocyanate method, as described for Northern blot and RT-PCR. Both RNA samples and hybridization labeling of microarray experiments were performed following the instructions recommended by Agilent Technologies. Equivalent amounts of Cs2-cDNA and Cy5-cDNA were quantified using NanoDrop spectrophotometer and then combined, vacuum-dried, resuspended in water with deposition hybridization buffer (Agilent), mouse Cot-1 DNA (Invitrogen), and deposition control target (Operon Technologies), and hybridized to microarray slides for ~17 h, according to the manufacturer's guidelines. The slides were washed once with 0.1% SDS for 10 min and with 0.06× SSC for 5 min. Hybridized slides were scanned using the ScanArray LITE confocal laser scanner (PerkinElmer Life Sciences), minimizing the total number of saturated spots for both channels. Image analysis was performed with QuantArray software (PerkinElmer Life Sciences). Spots showing evident blemishes were flagged and excluded from the analysis. For each spot, signal intensity for both channels was calculated by subtracting local background. For each array, a normalization factor was calculated dividing the total signal intensity of all the spots in the Cy3 channel by the signal of all the spots in the Cy5 channel. Spots whose measured area was higher than 50% of the average element size on the array in at least one channel were kept for further analysis. Ratios between the mean net fluorescence from the Cy3 channel and the mean net fluorescence from Cy5 channel were calculated for each spot. Cy3/Cy5 expression ratio was considered as (2). A cut-off filtering criterion was employed; only those genes with greater than 2-fold induction or repression in both comparisons were considered.

CD24 Promoter Activity and Transfection—CD24 promoter activity was analyzed by using a construct containing the 5′-promoter sequence of CD24 conjugated with a chloramphenicol acetyl transferase (CAT) reporter gene. Two plasmids were employed as controls and used to normalize the data. One plasmid contained the same CD24 promoter sequence, but in inverse orientation, the other plasmid was a pCAT basic control plasmid (Promega). Transient transfection of these vectors into Friend cells was performed using an Amaxa Nucleofector apparatus (Amaxa), according to the manufacturer's instructions. Briefly, 1 × 106 cells were resuspended in the specific electrophoration buffer R plus 5 μg of the plasmid. Two days later, cells were lysed, and the amount of protein present in cell extracts was determined using the Lowry method (Bio-Rad). CAT expression in control (Empty) and in duplicate using the CAT enzyme-linked immunosorbent assay kit (Roche Applied Science) as recommended by the supplier. Values of CAT are given as average of at least three independent experiments and are reported as percentage.

Concentration of Antisense RNA of the PLCβ1 Gene—For use as antisense RNA, a construction corresponding to bases 797–998, from the antisense strand of the genomic genomic DNA (GenScript). The construct was transfected as described above. The transforms were selected by limiting dilution in medium containing the neomycin analogue G418 at a concentration of 500 μg/ml. Colonies were harvested and expanded separately in the presence of G418. The silencing efficiency was detected by Western blot analyses using specific antibodies.
nuclear and cytoplasmatic fractions (45 and 55%, respectively).

High level of PLC1 expression was modulated during erythroid differentiation. Therefore, we focused on the PLC1-overexpressing cell lines, which were compared. The four stable clones overexpressed one of the following: PLC1a, PLC1b, or PLC1 M2b cytoplasmic mutant or the empty vector (mock-transfected), respectively.

Agilent mouse cDNA microarrays, which contain probes for about 9000 genes, were used. In each experiment, poly(A) RNA from two clones was competitively hybridized to the chip, and the distribution of fluorescence intensity ratio between the two samples was compared and analyzed using the standard methodology detailed under “Experimental Procedures.”

To identify new genes up- or down-regulated by the expression of PLC1, four independent cDNA microarray assays were performed. For these experiments, four different clones, all obtained from a parental Friend erythroleukemia cell line, were compared. The four stable clones overexpressed one of the following: PLC1a, PLC1b, or PLC1 M2b cytoplasmic mutant or the empty vector (mock-transfected), respectively.

RESULTS

To identify new genes up- or down-regulated by the expression of PLC1, four independent cDNA microarray assays were performed. For these experiments, four different clones, all obtained from a parental Friend erythroleukemia cell line, were compared. The four stable clones overexpressed one of the following: PLC1a, PLC1b, or PLC1 M2b cytoplasmic mutant or the empty vector (mock-transfected), respectively.

Agilent mouse cDNA microarrays, which contain probes for about 9000 genes, were used. In each experiment, poly(A) RNA from two clones was competitively hybridized to the chip, and the distribution of fluorescence intensity ratio between the two samples was compared and analyzed using the standard methodology detailed under “Experimental Procedures.”

To identify modifications in gene expression due to the nuclear localization of PLC1, the effect of nuclear PLC1 overexpression was compared with that of PLC1a M2b cytoplasmic mutant. Moreover, the global transcription profile of PLC1-overexpressing cells was compared with that of wild type cells (mock-transfected). First of all, we have checked the distribution of PLC1 in purified nuclei as well as in both the cytosolic and the nuclear supernatant fractions. Fig. 1 shows that in wild type cells, 90% of PLC1b was in the nuclear fraction, whereas PLC1a distributed between the nuclear and cytoplasmatic fractions (45 and 55%, respectively). When overexpressed, PLC1a localized mainly to the nucleus (5.3-fold higher level in nucleus than in the cytoplasm), whereas PLC1b localized exclusively to the nucleus. Notwithstanding these differences, because of the overall high level of PLC1a expression, the amount of nuclear PLC1b was 2.1-fold lower than that of PLC1a, as assessed by densitometric analysis of the Western blots. Of note, the relative amounts of overexpressed proteins present in the cytoplasmatic fraction were approximately the same, either whether the quantification is carried out in the cytoplasm obtained by Dounce homogenization of the cells or whether the quantification is carried out in the supernatant of nuclear purification (Fig. 1). Moreover, it is worthwhile mentioning that both cytoplasmatic fractions do not show histone H3, a marker of chromatin contamination, hinting that the two procedures employed do not give rise to nuclear lysis. When overexpressed, the M2b mutant was entirely cytoplasmic.

Out of the 9000 genes analyzed, one of them, the murine CD24 gene, was up-regulated in cells overexpressing PLC1 in the nucleus, as compared with cells overexpressing the mutant in the cytoplasm M2b or with the wild type cells. Specifically, the level of the nuclear CD24 gene expression in PLC1-overexpressing clone was on average 2.10-fold higher as compared with M2b-overexpressing clone and 2.80-fold higher as compared with wild type clone.

To confirm these data, both semiquantitative RT-PCR and Northern blot analysis were performed using the same RNA employed in the microarray studies and independently extracted RNAs. As can be seen in Fig. 2A, PLC1a and -1b overexpression increased CD24 mRNA level 3.0- and 2.5-fold relative to the wild type cells, respectively. By contrast, the M2b mutant exhibited a level of transcript undistinguishable from that of wild type cells. In Northern blot analysis, the amount of RNA loaded in each lane was similar, as assessed by the quantity of 28 S and 18 S ribosomal RNA (Fig. 2A). The results of RT-PCR experiments were substantially similar and showed a high increase of CD24 message particularly in PLC1a-overexpressing cells (data not shown). Both experiments validate the array-based up-regulation in CD24 gene expression.

To ascertain whether the increase of CD24 at the transcriptional level was accompanied by an increase in protein level, we performed Western blot analysis. Fig. 2B shows that CD24 protein, already present in the wild type cell line, was highly increased in both PLC1a- and -1b-overexpressing clones, confirming and extending the Northern blot and arrays results. The densitometric quantification of the increase indicates that the effect of PLC1a is 1.5-fold stronger than that of PLC1b, and this parallels the levels of the overexpression of the two PLC1 splicing variants (Fig. 1 and legend). Given that Friend erythroleukemia cells can be induced to differentiate toward erythrocytes, we next addressed the issue of whether the CD24 expression was modulated during erythroid differentiation. Therefore, we focused on the PLC1a-overexpressing cells that displayed the higher levels of CD24. In addition, the PLC1a clone exhibited the higher expression of the PLC1 in the nucleus, i.e., 2.1-fold higher than the PLC1b clone (Fig. 1). Cells were grown in medium containing 10% fetal calf serum (growth medium) or committed to differentiation by exposure to 1.5% Me2SO (differentiation medium) and harvested at different time points during 72 h of tissue culture. Fig. 2C shows that the level of CD24 increased dramatically during erythroid differentiation and reached a very high level of expression at 72 h of Me2SO treatment.

Next, we asked whether the up-regulation of CD24 observed during differentiation of PLC1a-overexpressing cells took place also in the other clones. As shown in Fig. 3, cells overexpressing PLC1b exhibited an up-regulation of CD24, whereas

---

**Fig. 1. Western blot of the wild type, PLC1a and -1b, and M2b cytoplasmatic mutant in Friend cells.** The densitometric analysis of the blots shows that in wild type cells, PLC1b is 90% nuclear, and PLC1a is both nuclear and cytoplasmatic (45 and 55%, respectively). PLC1a overexpression is mainly nuclear (5.3-fold more than in the cytoplasm) and 2.1-fold more expressed in the nucleus than overexpressed PLC1b. PLC1b overexpression is totally nuclear. The M2b mutant, when overexpressed, is only cytoplasmic. wt, Friend erythroleukemia clone; 1a, clone overexpressing PLC1a; 1b, clone overexpressing PLC1b; M2b, clone overexpressing the M2b cytoplasmatic mutant.
cells overexpressing M2b mutant or wild type cells exhibited no up-regulation. We infer that CD24 is specifically up-regulated in cells that overexpress the nuclear PLCβ1a and -1b. The higher level of CD24 expression observed in PLCβ1a-overexpressing clone relative to the PLCβ1b-overexpressing cells (240- versus 110-fold increase) most likely reflects the higher level of expression of the two isoforms, seeing that the PLCβ1a was 2.1-fold more expressed than PLCβ1b.

To provide further evidence that CD24 up-regulation is dependent on PLCβ1 expression, we silenced PLCβ1 mRNA and protein expression in wild type and PLCβ1a-overexpressing cells by means of siRNA. For this purpose, a 21-nucleotide-long fragment complementary to the common sequence of both PLCβ1a and -1b was cloned in pRNA-H1/Neo vector under H1 promoter. siRNA-expressing clones were generated and induced to differentiate so that the effect of siRNA-induced inhibition was tested under conditions of maximal CD24 up-regulation. As can be seen in Fig. 4, siRNA was effective in decreasing PLCβ1 expression in both WT and PLCβ1a-overexpressing clones, as detected at messenger level by RT-PCR (Fig. 4A) and at protein level by Western blot (Fig. 4B).
The overexpressed PLC-17). Indeed, as shown previously in the same cells, when M2b mutant localized exclusively to the cytoplasm (Fig. 1) (14, previous characterization of the M2b mutant and show that the activation of the CD24 promoter was decreased by about 50% in as compared with the effect of the M2b cytoplasmic mutant, to discriminate the different role of PLC-sense direction to a CAT reporter gene were transfected in wild type Friend erythroleukemia clone; H9252-1a-overexpressing clone, CD24 promoter activity nearly doubled as compared with wild type clone. The specificity of this activation was further confirmed by means of siRNA. When both endogenous and overexpressed PLC-1 was silenced, a significant reduction in CD24 promoter activity occurred, as compared with non-silenced ones. CAT activity was normalized to the CAT positive-control transfected cells, which were arbitrarily set at 100%. Data are reported as means ± S.D. of three independent experiments each performed in duplicate. wt: wild type Friend erythroleukemia clone; H9252-1a, clone overexpressing PLC-1a, si wt: clone silencing PLC-1; si 1a, clone silencing the overexpressed PLC-1a.

silencing induced a strong reduction of CD24 expression, in both cell lines (Fig. 4C). These data strongly support a role for PLC-1 as a regulator of CD24 protein expression.

The last series of experiments was designed to investigate whether the PLC-1-induced regulation of CD24 expression is exerted at the transcriptional level. Plasmids containing the 5'-flanking promoter region of CD24 fused in sense or antisense direction to a CAT reporter gene were transfected in wild type or PLC-1a-overexpressing cells and their respective silenced clones. The amount of CAT protein was quantified by enzyme-linked immunosorbent assay 48 h after transfaction. Fig. 5 shows the average of three independent experiments.

PLC-1a overexpression resulted in about a 2-fold increase of CD24 promoter activity. When PLC-1 was silenced, the activation of the CD24 promoter was decreased by about 50% in wild type clone and by about 30% in PLC-1a-overexpressing clones. These findings provide evidence that the up-regulation of CD24 expression induced by PLC-1 is mediated, at least in part, at the transcriptional level.

**DISCUSSION**

The initial aim of this study was to identify new genes regulated by PLC-1, given the role exerted by its signaling in the nucleus during cell growth and differentiation (3) and its potential involvement in the progression of myelodysplastic syndrome to acute myeloid leukemia (16). The novel finding that emerged by the microarray experiments was an up-modulation of CD24 in cells overexpressing PLC-1 in the nucleus as compared with the effect of the M2b cytoplasmic mutant, which did not induce any increase. It is worthwhile to recall that the M2b mutant induces a forced expression of PLC-1 in the cytoplasmatic compartment (4) and constitutes a good tool to discriminate the different role of PLC-1 as a function of its subcellular localization. Current results strengthen and extend previous characterization of the M2b mutant and show that the M2b mutant localized exclusively to the cytoplasm (Fig. 1) (14, 17). Indeed, as shown previously in the same cells, when PLC-1 M2b mutant is overexpressed in the cytoplasm, the endogenous PLC-1 in the nucleus is still present, but at a very low level with respect to the cytoplasmic one. The difference in subcellular localization is thought to inhibit the physiological role of the nuclear PLC-1 (4). As regards CD24 expression, the M2b accumulation in the cytoplasm inhibits CD24 expression in comparison with the effect of the overexpression of both PLC-1a and -1b in the nucleus (Figs. 1–3). The conclusion that up-modulation of CD24 is regulated by PLC-1 nuclear signaling stems from three lines of evidence. Firstly, the microarray studies clearly indicated an increase in CD24 transcription. Secondly, up-regulation of CD24 was quantitatively confirmed both at the RNA messenger level by Northern blot and at the protein level by Western blot. Both assays show that CD24 expression was augmented in PLC-1a- and -1b-overexpressing clones and was significantly not affected in the clone overexpressing the cytoplasmic mutant. It is worthwhile to take into account that although both PLC-1a and -1b reside in the nucleus, the overexpression of PLC-1a is higher than that of PLC-1b. When comparing their effect on CD24 expression over wild type cells, the effect is very similar in that the increase of CD24 level is proportional to the overexpression of the two PLC-1 isoforms in the nucleus. Thirdly, we took advantage of silencing PLC-1 by small interfering RNA (18). We have specifically knocked down PLC-1 expression by transfecting a vector encoding a 21-nucleotide-long RNA complementary to the sequence of PLC-1. This resulted in an almost complete ablation of the expression of both of the endogenous PLC-1s present in wild type Friend cells and of all the PLC-1 overexpressed forms. The silencing gives rise to a dramatic down-modulation of CD24, as compared with the non-silenced ones. We are keen to underline that siRNA acts on both PLC-1a and -1b as well as on the M2b mutant, that in target site of siRNA corresponds to bases 379–398, from the initiation ATG start site, a common sequence of all the three PLCs. Moreover, it is important to take into account that the nuclear location of PLC-1 is necessary to obtain the up-regulation of CD24 since both PLC-1a and PLC-1b splicing variants are present in the nucleus. On the contrary, when PLC-1 expression is completely switched to the cytoplasm (M2b mutant), the up-modulation of CD24 does not take place. Here it is important to point out that endogenous PLC-1 isoforms are still present in the nucleus when M2b mutant is overexpressed (Fig. 1). This fact clarifies the issue that forced expression of PLC-1 in the cytoplasm does not induce down-regulation of CD24, which on the contrary takes place when PLC-1a and -1b are knocked down (Fig. 4). Indeed, the presence of endogenous PLC-1 isoforms gives rise to a behavior of CD24 expression identical to that of wild type cells.

To elucidate the molecular basis of the up-modulation mediated by PLC-1 of CD24, we addressed the issue of whether the CD24 gene up-regulation is exerted at the transcriptional level. For this purpose, we studied the regulation of CD24 promoter, using a reporter construct in which the promoter is placed upstream of the CAT reporter. Interestingly, we found a significant increase in the promoter activity in the PLC-1a-overexpressing clone in comparison with the wild type clone. In both cases, the inhibition of enzyme expression by siRNA gave rise to a significant decrease of CD24 expression. Further studies are currently on the way aimed to find out whether the effect on the promoter of CD24 is dependent on the generation of the canonical second messengers diacylglycerol and inositol 1,4,5-trisphosphate, which in turn could target the promoter itself, or whether it is dependent on a more direct interaction between PLC-1 and CD24 promoter sequence. It is worthwhile to take into account that the CD24 promoter activity assay provides only indirect evidence that up-regulation occurs at the level of transcription. Indeed, the substrate of PLC-1, phosphatidylinositol 4,5-bisphosphate, has been implicated in mRNA splicing (19), and inositol polyphosphates, downstream...
products of PLC activity, have been shown to regulate in yeast both mRNA export (20) and transcription (21). The likelihood that inositol polyphosphates could be the players acting on the CD24 promoter is hinted at by the fact that inositol phosphate kinase Ipk2 has been found to be located in nucleus (22).

Our data suggest a direct effect of nuclear PLCβ1 in the up-regulation of CD24 in murine Friend erythroleukemia cells, which can be committed to cessation of growth and to differentiation toward erythrocytes, following exposure to MeSO4. Therefore, to investigate the pattern of CD24 expression during erythroid differentiation, we compared the pattern of CD24 expression in all the clones overexpressing PLCβ1 in the nucleus or in the cytoplasm. Our results show that during erythroid differentiation of Friend cells, CD24 expression increased dramatically in the PLCβ1a- and -b-overexpressing cell lines, whereas no changes occurred in the wild type cells nor in the cytoplasmatic mutant clone. Further evidence that the up-modulation of CD24 was dependent on the PLCβ1 expression is based on the finding that PLCβ1 silencing, obtained by siRNA, resulted in an almost complete abolition of CD24 expression. This is consistent with the notion that CD24 expression undergoes modulation during B cell differentiation (6). Moreover, CD24 is overexpressed in a number of leukemias, including Burkitt’s lymphoma and pre-B acute leukemia, and is employed to distinguish, with notable accuracy, acute from chronic leukemia or lymphomas (23, 24).

In addition to its role in differentiation, CD24 plays a role in cell adhesion, a function ascribed to the oligosaccharidic moiety that are able to interact with p-selectin (25, 26). Because of its role in cell adhesion, CD24 is more and more considered a critical molecule in the metastasizing capacities of solid tumors, including ovarian (27), colorectal tumors (28), adenocarcinomas (29), and aggressive breast cancers (30). In these tumors, CD24 represents a prognostic marker, generally associated with a poor prognosis, and is being evaluated as a critical marker in the follow-up of tumor chemotherapy and for a careful individualization of the most effective therapy protocols. Our results, which point at PLCβ1 nuclear signaling as a mechanism for CD24 up-modulation in murine erythroleukemia cells, raise the possibility that this mechanism is operative also in tumor cells. It is worth mentioning that CD24 antigens, as well as its promoter, are specifically expressed in small cell lung cancer but little expressed in non-small cell lung cancer (31). Interestingly, PLCβ1 is more abundant in small cell lung cancer than in non-small cell lung cancer (32). The effect of nuclear PLCβ1 signaling on the expression of a surface marker such as CD24, which is important in the early steps of erythroid differentiation (5, 6), appears interesting because of the role played by nuclear PLCβ1 in this process (3, 4, 17). The evidence that the depletion of PLCβ1 gene is linked to the progression of myelodysplastic syndrome to acute myeloid leukemia in humans (16) strengthens this contention. Altogether our findings, obtained by combining microarrays, phenotypic analysis, and siRNA technology, identify CD24 as an effector of nuclear PLCβ1 signaling pathway in murine erythroleukemia cells and reinforce the notion that nuclear PLCβ1 is a key player in erythroid differentiation (33).

Acknowledgment—We are grateful to Peter J. Nielsen for the generous gift of the two CAT reporter plasmids containing CD24 promoter sequence and CD24 promoter sequence in the inverse orientation.

REFERENCES

1. Ehee, S. G. (2001) Annu. Rev. Biochem. 70, 281–312
2. Irvine, R. F. (2003) Nat. Rev. Mol. Cell. Biol. 4, 349–360
3. Martelli, A. M., Manzoli, L., and Cocco, L. (2004) Pharmacol. Ther. 101, 47–64
4. Faenza, I., Matteucci, A., Manzoli, L., Bili, A. M., Alugi, M., Peruzzi, D., Vitale, M., Castorina, S., Suh, P. G., and Cocco, L. (2000) J. Biol. Chem. 275, 30520–30524
5. Wengen, R. H., Ayne, M., Bose, R., Kohler, G., and Nielsen, P. J. (1991) Eur. J. Immunol. 21, 1039–1046
6. Hough, M. R., Chappell, M. S., Sauvageau, G., Takei, F., Kay, R., and Humphries, R. K. (1996) J. Immunol. 156, 479–486
7. Wengen, R. H., Rochelle, J. M., Seldin, M. F., Kohler, G., and Nielsen, P. J. (1993) J. Biol. Chem. 268, 23345–23352
8. Hubbe, M., and Altevogt, P. (1994) Eur. J. Immunol. 24, 731–737
9. Liu, Y., Jones, B., Brady, W., Janeway, C. A., Jr., Linley, P. S., and Linley, P. S. (1992) Eur. J. Immunol. 22, 2855–2859
10. Aigner, S., Strobegeer, Z. M., Fogel, M., Weber, E., Zarn, J., Ruppert, M., Zeller, Y., Vestweber, D., Stahel, R., Sammar, M., and Altevogt, P. (1997) Blood 89, 3385–3395
11. Belvindrah, R., Rougon, G., and Chazal, G. (2002) J. Neurosci. 22, 3594–3607
12. Zhou, G., Rammohan, K., Lin, S., Robinson, N., Li, O., Liu, X., Bai, F., Xin, L., Scarr, B., Hu, P., You, M., Guan, K., Zheng, P., and Liu, Y. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 15041–15046
13. Bakh, Y. Y., Lee, Y. H., Lee, T. G., Seo, J., Ryu, S. H., and Suh, P. G. (1994) J. Biol. Chem. 269, 8240–8245
14. Kim, C. G., Park, D., and Rhee, S. G. (1996) J. Biol. Chem. 271, 21187–21192
15. Martelli, A. M., Gilmour, R. S., Bertagnolato, V., Neri, L. M., Manzoli, L., and Cocco, L. (1992) Nature 358, 242–245
16. Le Vason, V. R., Calabrese, G., Manzoli, L., Palka, G., Spadano, A., Morizio, E., Guanci-Franchi, P., Fantasia, D., and Cocco, L. (2004) Leukemia (Basingstoke) 18, 1122–1126
17. Faenza, I., Matteucci, A., Baveloni, A., Marmirino, S., Martelli, A. M., Gilmour, R. S., Sub, P. G., Manzoli, L., and Cocco, L. (2002) Biochem. Biophys. Acta 1589, 305–310
18. Dersett, Y., and Tusche, T. (2004) Nat. Rev. Drug Discov. 3, 318–329
19. Osborne, S. L., Thomas, C. L., Gschmeissner, S., and Schiavo, G. (2001) J. Cell Sci. 114, 2501–2511
20. York, J. D., Odum, A. R., Murphy, R., Ives, E. B., and Wente, S. R. (1999) Science 285, 90–100
21. York, J. D., Guo, S., Odum, A. R., Spiegelberg, B. D., and Stolz, L. E. (2001) Adv. Enzyme Regul. 41, 57–71
22. Seeds, A. M., Sandquist, J. C., Spana, E. P., and York, J. D. (2004) J. Biol. Chem. 279, 47222–47232
23. Valet, G., Repp, R., Link, H., Ehninger, A., and Gramatzki, M. (2003) Cytometry 53B, 4–10
24. Belov, L., de la Vega, O., dos Remedios, C. G., Mulligan, S. P., and Christophersen, R. I. (2001) Cancer Res. 61, 4483–4489
25. Kadom, G., Eckert, M., Sammar, M., Schachner, M., and Altevogt, P. (1992) J. Cell Biol. 118, 1245–1258
26. Sammar, M., Aigner, S., Hubbe, J., Schirrmacher, V., Schachner, M., Vestweber, D., and Altevogt, P. (1994) Int. Immunol. 6, 1027–1036
27. Kristiansen, G., Denkert, C., Schluss, K., Dahl, E., Pilarsky, C., and Hauptmann, S. (2002) Am. J. Pathol. 161, 1215–1221
28. DePrimo, S. R., Wong, L. M., Khatri, D. B., Nicholas, S. L., Manning, W. C., Smolich, B. D., O’Farrell, A. M., and Cherrington, J. M. (2003) BMC Cancer 3, 3
29. Friederichs, J., Zeller, Y., Hafezi-Moghadam, A., Grone, H. J., Ley, K., and Altevogt, P. (2000) Cancer Res. 60, 6714–6722
30. Kristiansen, G., Winzer, K. J., Mayordomo, E., Bellach, J., Schluns, K., Denkert, C., Dahl, E., Pilarsky, C., Altevogt, P., Guski, H., and Dietel, M. (2003) Clinical Cancer Res. 9, 4906–4913
31. Pass, M. K., Quintini, G., Zarn, J. A., Zimmermann, S. M., Sigrist, J. A., and Stahel, R. A. (1998) Int. J. Cancer 78, 496–502
32. Straschnitzki, D., Shaffer, S. H., Phelps, S. W., and Williams, C. L. (2000) Cancer Res. 60, 2730–2736
33. Cocco, L., Manzoli, L., Barnabei, O., Gilmour, R. S., and Martelli, A. M. (2003) Adv. Enzyme Regul. 43, 1–13