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Cloning and functional analysis of the hematopoietic cell-specific phospholipase Cγ2 promoter

Jong Sun Kang[a], Franz Kohlhuber[b], Hubert Hug[c], Dieter Marmé[d], Dirk Eick[b], Marius Ueffing[b,*]

[a]*Mount Sinai Medical School, Department of Biochemistry, 1 Gustave Levy Place, New York, NY 10029, USA*
[b]*Institut für Klinische Molekularbiologie und Tumorgenetik, GSF, Marchioninistr. 25, 81377 München, Germany*
[c]*ZMBH, Im Neuenheimer Feld 282, 69120 Heidelberg, Germany*
[d]*Institut für Molekulare Medizin und Naturstofforschung, Klinik für Tumorbiologie, Breisacher Straße 117, 79106 Freiburg, Germany*

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Abstract **Phospholipase Cγ2 (PLCγ2) is a phospholipid-converting enzyme which, upon receptor stimulation, is activated within membrane-bound signalling complexes. In contrast to the highly ubiquitous PLCγ1, PLCγ2 is expressed predominantly in B-lymphocytes. Associated with antigen-coupling receptors it is activated by tyrosine phosphorylation after the triggering of B-cell surface immunoglobulin. We have cloned and sequenced the human PLCγ2 promoter. Primer extension analysis reveals the existence of a major transcriptional start site. The TATA-less promoter contains G+C-rich stretches with a cluster of contiguous SP1 consensus sites, an NF1, and an AP2 site between bp -220 to -70. A construct containing the region from -189 to +78 confers full promoter activity, as shown by fusion to a luciferase reporter gene construct. The distal part of the promoter between bp -662 to -293 containing an SRE, EBF and CACCC box contributed negatively to promoter activity in the B-cell line Raji but not in three adherent cell lines. In Raji cells, PLCγ2 mRNA is expressed at low levels with a half life greater than 4 h. After treatment with serum, TPA, retinoic acid, or with 5-azacytidine increased levels of PLCγ2 mRNA were induced in B-cells.**

Key words: Phospholipase Cγ2 (PLCγ2); Promoter; Gene regulation; SP1; B-cell

1. Introduction

PLCγ2 is a tissue-specific phospholipase, catalyzing the cleavage of phosphatidylinositole bisphosphate into the second messengers inositol 1,4,5-trisphosphate and diacylglycerol [1]. As these second messengers act as primary signal transducers for calcium mobilization as well as activators for protein kinase C, phospholipases of type C are essential components of many mitogen-induced signal cascades, especially of those involving receptor-associated tyrosine kinase activity [2]. Common to both isoforms of the PLCγ family are regions of homology to the *src* tyrosine kinase non-catalytic domain, SH2 and SH3 [3]. Ligand-induced receptor activation can result in tyrosine phosphorylation of a receptor or its associated molecules, thereby generating binding sites for the PLCγ SH2 domains. Binding through the SH2 domains leads to PLCγ activation through subsequent tyrosine phosphorylation. In contrast to the ubiquity of its closely homologous isozyme, PLCγ1, PLCγ2 expression is limited to the blood-drained tissues of lung and spleen and to cells of the hematopoietic lineage, predominantly B-cells and monocytes [4–6]. The protein is co-immunoprecipitated with components of the B-cell receptor [7] where it is likely to interact with associated tyrosine kinases through its SH2 and SH3 domains [8,9]. Further, it is rapidly hyperphosphorylated after stimulation of CD40, a receptor of the tumor necrosis factor gene family, expressed and activated during the clonal expansion of B-lymphocytes [10]. It is therefore thought to be implicated in the signal transduction of antigen-triggered B-cell responses, as well as in B-cell growth and differentiation [11]. Recently, the genetic locus of PLCγ2 has been mapped to the long arm of human chromosome 16 in band q24.1 [12].

To investigate the transcriptional regulation of the *PLCγ2* gene, we cloned and sequenced 1.5 kb of the PLCγ2 promoter region. In addition, we have identified the transcriptional start site, possible regulatory elements and characterized the promoter by deletional analysis in transfected B-cells and fibroblasts. Furthermore, the transcriptional activation of the *PLCγ2* gene in various B-cell lines was studied.

2. Materials and methods

2.1. Cloning and DNA sequencing

A human genomic EMBL 3A-λ-library derived from the Burkitt's lymphoma line Ly66 (a generous gift of M. Lipp, Max Delbrück Zentrum, Berlin Buch) was screened by plaque hybridization. λ-DNA preparation and screening were carried out using Qiagen reagents according to standard protocols [13]. Phage clones were screened using a 2.2 kb probe derived from human PLCγ2 cDNA [14] using the ECL direct nucleic acid labelling protocol from Amersham. Inserts were removed with *SalI*, cloned into Bluescript KS+ (Stratagene), and analysed by Southern blotting [13] using a 5′-fluorescein 11-dUTP-labelled oligonucleotide A (5′-GACTCGGGT-CACTCAGCGCC-3′) derived from nucleotide 7 to 26 of the published PLCγ2 cDNA [14] as the probe and using the ECL protocol as recommended by the manufacturer (Amersham). The DNA sequencing reaction carried out according to Sanger et al. [15], using 5′-fluorescein-labelled 17 mer-oligonucleotide primers derived from PLCγ2 cDNA or promoter sequences. Gel electrophoresis and sequence analysis were performed on an automated laser fluorescence (ALF) sequencer (Pharmacia).

2.2. Primer extension

The reaction was done essentially as described by McKnight and Kingsbury [16]. Using a Pharmacia gene assembler, a fluorescein-end-labelled oligonucleotide B (5′-CGGGAGAAATCAGGAAGCTG-3′) was synthesized complementary to nucleotide 103–122 of the PLCγ2 cDNA and hybridized overnight at 30°C with 25 μg of RNA isolated from Epstein-Barr virus transformed lymphoblastoid B-cells (a gift from I. Melchers, University of Freiburg). Reverse transcription was allowed to proceed for 2 h at 37°C using M-MuLV reverse transcriptase (Pharmacia), and the reaction products were then separated in a

*Corresponding author. Fax: (49) 89-7099-550.
E-mail: marius@haema112.gsf.de

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6% polyacrylamide gel containing 8 M urea [13]. The reaction products were analyzed using an ALF sequencer.

2.3. Expression vectors, cell culture and promoter assays

PLCγ^2 promoter fragments were ligated 5′ to the luciferase reporter vector PAH1409 [17] either blunt end or after a filling reaction into the filled-in *Xho*I or *Bam*HI sites of the vector as depicted in Fig. 3A. NIH3T3, 293, and Ltk$^-$ cells were grown in DMEM with high glucose (4.5 g/l), sodium bicarbonate (3.7 g/l) and 10% calf serum (Life Technologies). The cell lines BJA-B and Raji, as well as EBV transformed primary lymphoblastoid cells, were cultured in RPMI 1640 plus fetal calf serum (FCS) as indicated. All media were supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml; Life Technologies). NIH3T3, 293, and Ltk$^-$ cells were transiently transfected by the calcium phosphate method [18], each with 2 μg reporter plasmid DNA and 1μg of the β-galactosidase expression plasmid pRSV-lacZII (Pharmacia). The cell lines BJA-B and Raji were transfected by electroporation at 250 V, 960 μF using 25 μg of reporter DNA for each transfection and 1 μg of pRSV-lacZII. Cells were harvested 44–48 h later and luciferase assays performed as described [19]. Luciferase activity was normalized for transfection efficiency as determined by β-galactosidase activity [19]. For each reporter plasmid and cell line, a minimum of two independent transfections was performed.

2.4. RNA analysis

1×10^7 cells were either untreated left or stimulated with TPA (100 ng/ml), retinoic acid (10 μM) or actinomycin D (5 μg/ml) for an indicated time. The cells were then lysed in 4 M guanidinium isothiocyanate plus 0.5% lauroylsarcosin, 25 mM sodium citrate and 0.1 M 2-mercaptoethanol, pH 7.0. Total RNA was isolated by centrifugation through a cushion of 5.7 M CsCl [20]. RNAs (20 μg/sample) were separated in either 0.8% or 1% agarose-6% formaldehyde gels and blotted onto Hybond-N nylon membranes (Amersham) using 20× SSC. The RNA blots were hybridized to random prime ^{32}P-labelled probes derived from the PLCγ^2 cDNA [14], and then washed according to standard conditions [13]. The relative abundance of RNA per lane was checked by comparison of the ethidium bromide staining of the ribosomal RNA bands and by hybridization of RNAs with a probe of the housekeeping gene, glyceraldehyde-3-phosphate dehydro-

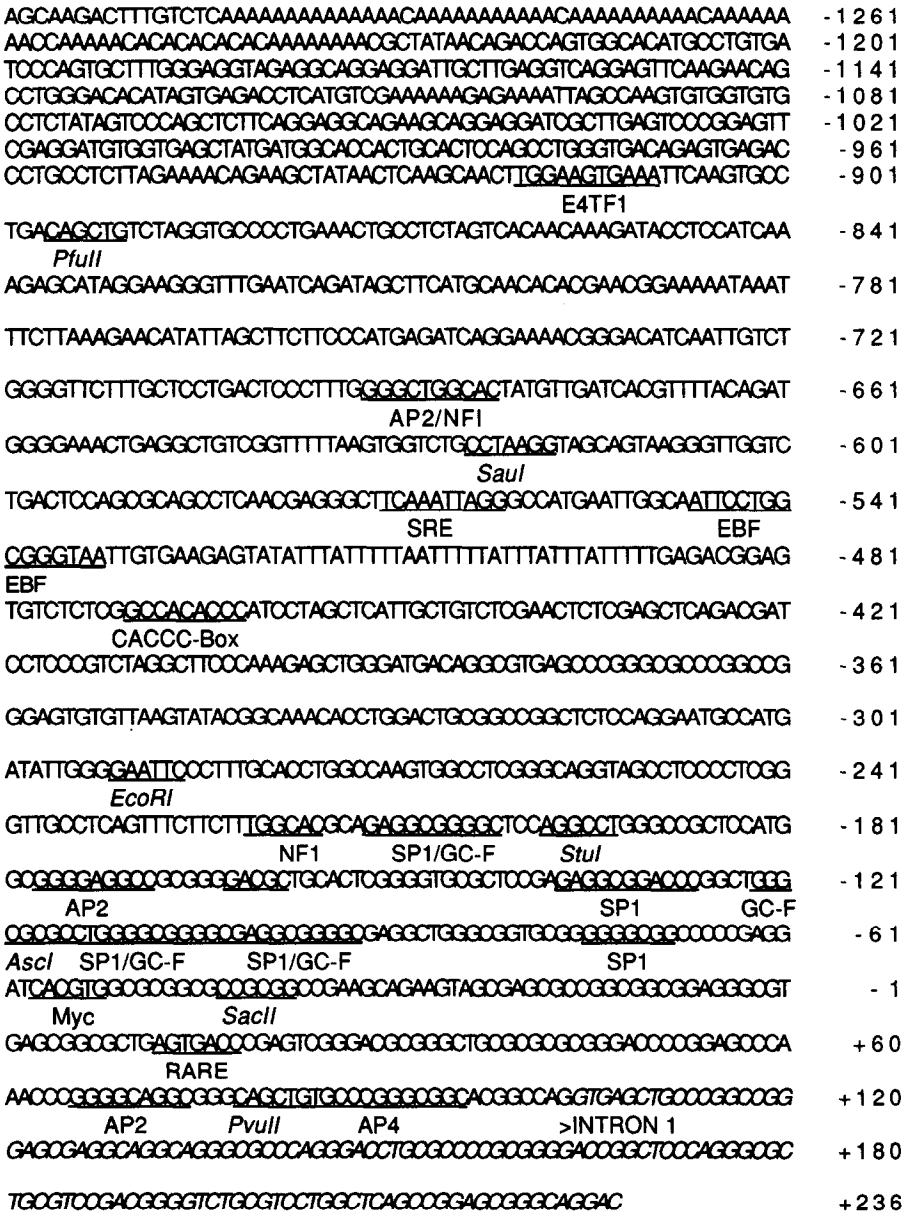

Fig. 1. Nucleotide sequence of the 5′ region of the *PLCγ^2* gene. Potential transcription factor binding sites and restriction sites are indicated (underlined). The arrow at position +1 indicates the main transcriptional start site determined by primer extension analysis. The sequence reported from this paper is available from the EMBL data library with the accession no. Z 22522.

genase (*GAPDH*) [21]. Blots were autoradiographed using Kodak X-OMAT AR5 films, exposed at -70°C.

3. Results

3.1. Cloning of the human PLCγ² promoter

A cDNA of PLCγ² from transformed human lymphocytes had been isolated previously [14]. For cloning of the PLCγ² promoter, DNA of a λ-EMBL-3A-library derived from the human Burkitt's lymphoma cell line Ly66 was screened with a 2.2 kb PLCγ² cDNA probe [14]. Fifteen λ-phage-clones were isolated.

These clones were cut with *Sal*I, *Eco*RI or *Bam*HI and monitored by Southern blot analysis using a 20mer oligonucleotide homologous to nucleotides +7 to +26 of the PLCγ² cDNA [14], resulting in two clones containing a positive 5.5 kb *Bam*HI-fragment (data not shown). The 5.5 kb *Bam*HI fragment and subfragments thereof were subcloned into Bluescript KS+ (Stratagene). The first exon (103 bp, see primer extension experiment), part of the first intron (124 bp) and the upstream region of the *PLCγ²* gene (1239 bp) were sequenced.

The major transcriptional start site was designated as +1 (Fig. 1). Several notable features were detected in the sequence 5′ of exon 1 within the putative promoter region. The proximal, GC-rich part of the promoter lacks a TATA box, but contains a cluster of five Sp1 motifs (positions -73, -100, -110, -134, -209, relative to the major transcriptional start site). Four GC-factor consensus sites [22] partially overlap the Sp1 sites at positions -98, -108, -112 and -207 (consensus GCGGGGC). In addition, a Myc/Max consensus site is located at position -55, AP-2 sites at positions -174 and -689, and a serum response element at position -567. For other, potential transcription factor binding sites, including those located at positions 3′ of the transcriptional start site (see Fig. 1).

3.2. Site of transcriptional initiation

The PLCγ² transcriptional start site was mapped by primer extension using a fluorescent-tag labelled 19mer oligonucleotide derived from nucleotides 97–115 of the PLCγ² cDNA [14]. Twenty-five μg RNA of a lymphoblastoid cell line were used for the reaction. Gel electrophoresis analysis was performed on an automated laser fluorescence sequencer (Pharmacia). As an internal marker, a sequence reaction for a *PKCζ* gene was co-electrophoresed. A major extension product of 119 bases was detected (Fig. 2). Primer extension with RNA from the T-cell-line Jurkat confirmed this extension product (data not shown). The band of 119 bases corresponds to position +1 in Fig. 1 and maps 4 bp upstream of the previously published PLCγ² cDNA [14].

3.3. Deletional analysis of the PLCγ² promoter

For a functional identification of the PLCγ² promoter, and in order to define the regions required for promoter activity, we cloned a series of progressively deleted DNA fragments of the putative promoter directly upstream of the firefly luciferase reporter gene (*luc*) (Fig. 3A). The resulting plasmids were transiently expressed in the murine fibroblast cell lines NIH 3T3, Ltk⁻, and the human embryonal kidney cell line 293. None of these cell lines express detectable amounts of RNA

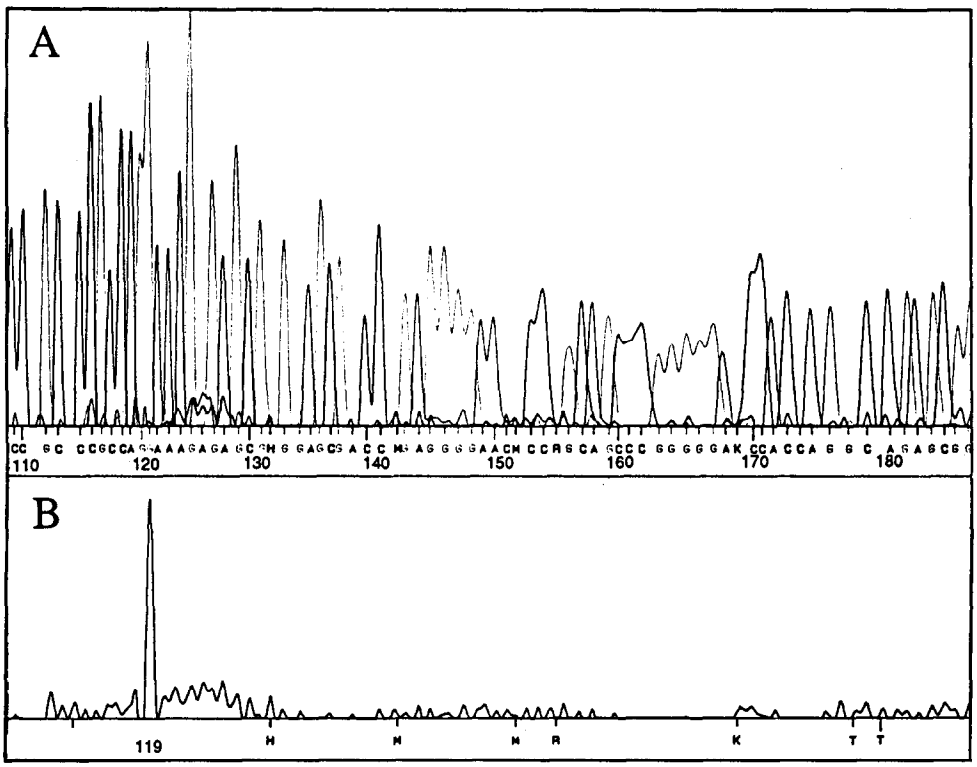

Fig. 2. Automated primer extension using fluorecein-labelled oligonucleotides. A: Sequenced PKC-zeta (without lane 4 for thymine) as a size marker sequence. B: Extended product at position 119 using oligonucleotide B as primer, derived from nucleotides 103–121 of the PLCγ² cDNA. The size of extension products increases from left to right. For details see Sections 2 and 3.

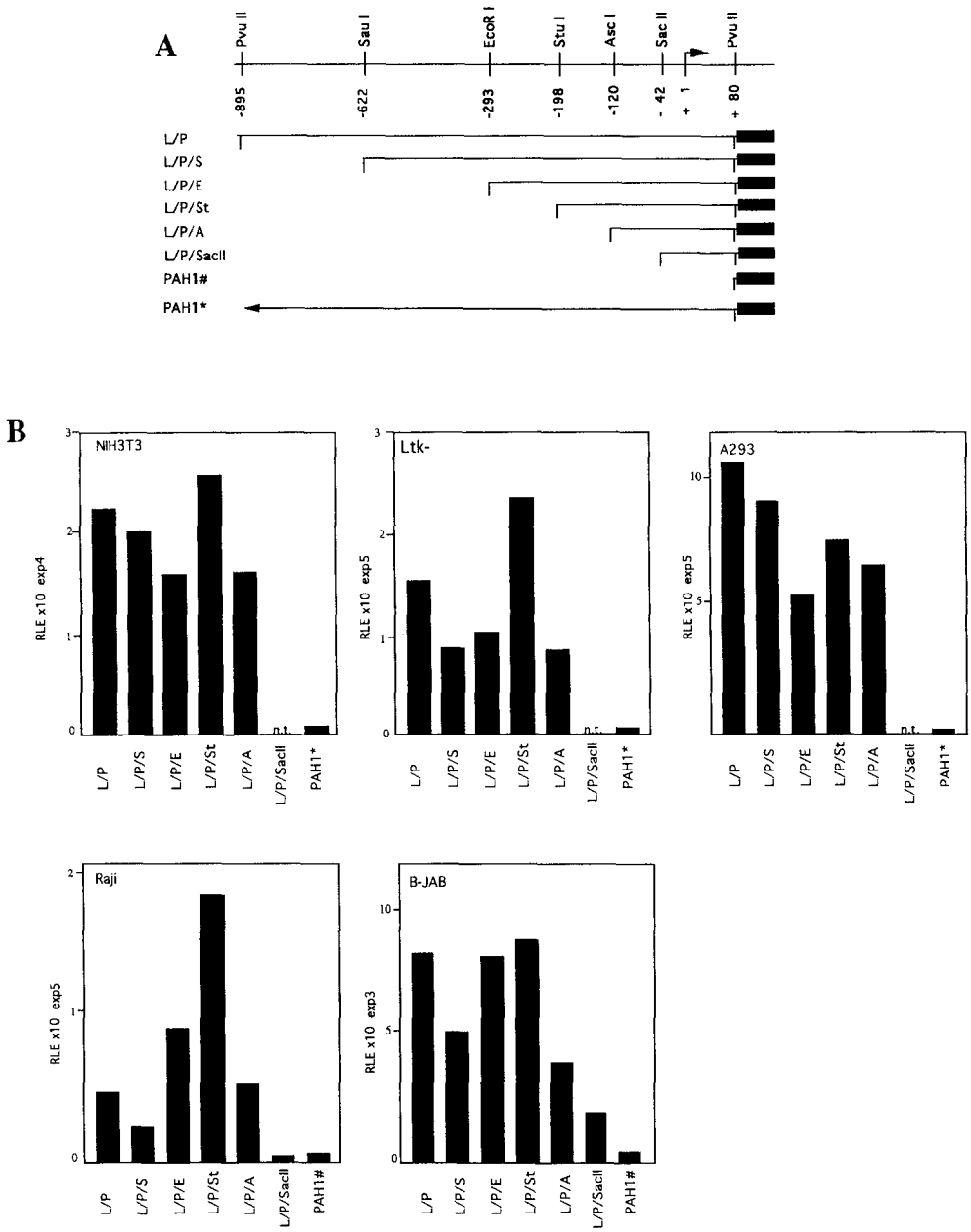

Fig. 3. Luciferase promoter-reporter assays using promoter fragments of the human *PLCγ²* gene. A: Promoter deletion constructs, generated as described in Section 2. B: Luciferase activity of the corresponding constructs after transfection into NIH3T3, Ltk⁻, 293, Raji or BJA-B cells. Data were obtained as described in Sections 2 and 3 and are expressed in relative light units (RLE). The high differences of RLE levels between different cell lines mainly reflect the cell-specific transfection efficiency of a respective cell line as determined by transfection experiments with a control expression plasmid for luciferase under the control of a CMV early promoter (data not shown).

of the endogenous *PLCγ²* gene. Two human B-lymphoma cell lines which both express low levels of PLCγ² RNA, Raji and BJA-B, were similarly transfected.

The pattern of activities determined for all constructs was similar for NIH 3T3, Ltk⁻, 293, and BJA-B cells but markedly different from the pattern detected for Raji cells. The two longest constructs, L/P and L/P/S, produced relatively strong signals except in Raji cells. Further 5′ deletion of the constructs, L/P/E and L/P/St, led to a 2- to 10-fold increase of activity in Raji cells, whereas only a slight or no increase was detected for the other cell lines. This identified a negative element in the distal part of the PLCγ² promoter between -895 bp and -198 bp which operates in Raji cells but not in, or only inefficiently in, the other tested cell lines. Deletion of the *StuI/AscI* fragment, corresponding to the region from -198 to -120, led to reduction of luc-activity. The decline was most pronouced in Raji cells, although barely evident in 293 cells. The further deletion of the *AscI/SacII* fragment corresponding to the region from -120 to -42, abolished luc-activity in Raji cells, but had only a minor effect in BJA-B cells. This result suggests that the PLCγ² promoter harbours at least two positive elements in the region -198 bp to -42 bp, and negative elements upstream of postion -198 bp.

To uncover the mechanisms of stimulus-dependent promoter regulation, we transfected NIH3T3, 293 and BJA-B cells with the promoter-luciferase constructs L/P, L/P/E and L/P/

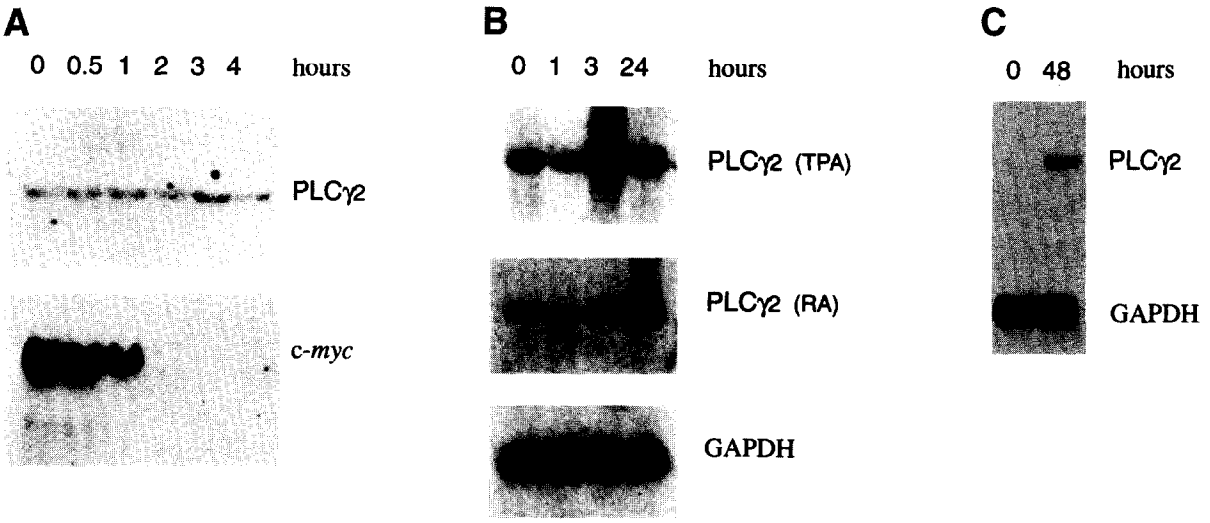

Fig. 4. Stability and expression of RNA in lymphoid cells. A: PLCγ² RNA stability in Raji. Northern blots were made with RNA from Raji cells treated with actinomycin D for the indicated hours as described in Sections 2 and 3. The upper panel shows the time course with PLCγ² RNA, the lower panel that of c-myc. B: Stimulus-dependent PLCγ² expression in BJA-B-lymphoma cells by TPA and retinoic acid (RA). Cells were kept under low serum conditions as described in Section 2 and stimulated for an indicated time. C: RNA induction in Raji cells by the DNA-demethylating agent 5 aza-cytidine. Raji were either untreated (0) or treated with 1 μM of 5-azacytidine for 48 h (48).

St. The transfected cells were then stimulated with TPA, 8-bromo-cAMP [23,24], or dexamethasone [25]. All three compounds elicited minor or no detectable effects compared to the untreated control (data not shown).

3.4. High stability of PLCγ² mRNA in B-cell-line Raji

The transcriptional block by actinomycin D has been shown to be a reliable tool for measuring RNA turnover in various cellular contexts [26]. RNA was isolated from Raji cells at different times after addition of the drug and studied by Northern analysis. RNA was hybridized either to a ^{32}P-labelled PLCγ² cDNA or, for control, to a labelled c-myc cDNA (Fig. 4A). The rapid decrease of c-myc RNA with a half life of less than 0.5 h upon addition of actinomycin D confirmed earlier reports [26,27]. In contrast, the PLCγ² RNA, even though expressed at low levels, was very stable and showed no decrease of steady-state levels during the time course of the experiment.

3.5. Activation of PLCγ² in B-cell lines

Through cloning and deletion analysis, various putative promoter elements and regions have been characterized which may regulate the expression of PLCγ² at its endogenous locus. We investigated the regulation of PLCγ² expression by Northern analysis using RNA from stimulated cell cultures of the lymphoma cell line BJA-B. BJA-B cells were stimulated with TPA or retinoic acid (Fig. 4B), substances associated with the induction of various B-cell responses. Treatment of BJA-B cells led to a marked increase of PLCγ² RNA after 3 h. RNA levels remained high for as long as 24 h. Retinoic acid treatment of BJA-B cells did not affect steady-state PLCγ² RNA levels within the first 3 h of treatment, but led to a strong induction after 24 h. The steady-state RNA levels of the *GAPDH* gene were not affected by TPA or retinoic acid. We have also observed a detectable increase in PLCγ² protein levels upon TPA and retinoic acid stimulation, although this was much more moderate compared to the increase in RNA (data not shown).

To investigate methylation as a possible mechanism of tissue-specific gene activation, Raji cells were treated for 48 h with 1 μM of the demethylating agent 5-azacytidine, and RNA isolated. Northern blots hybridized with a PLCγ²-specific probe reveal a sizable increase of PLCγ² expression with 5-azacytidine treated Raji cells (Fig. 4C). In NIH3T3 cells, however, which are negative for PLCγ², 5-azacytidine did not induce any detectable in PLCγ² RNA levels (data not shown). Thus demethylation of regulatory sequences of the *PLCγ²* gene or demethylation of a locus that positively regulates PLCγ² expression could at least partially enhance PLCγ² transcription in cells which are expressing detectable amounts of PLCγ².

4. Discussion

PLCγ² is expressed in a tissue-specific manner, suggesting a special role of this enzyme within lymphoid cell function. Cloning and analysis of a 1.5 kb sequence 5′ of the first exon of human PLCγ² revealed a promoter lacking both, TATA- and CAAT-boxes, with a stretch of highly GC-rich sequence motifs in its proximal part. A major RNA cap site was mapped by primer extension downstream of the GC-rich stretch with several consensus sites for the transcription factor SP1, including SP1 sites, which partly overlapped the consensus sites for the GC factor, reported to repress transcriptional activation of the *EGF* receptor gene [22]. Proximal to that cluster, an AP2 site, previously described as responsive to cAMP and TPA [23,24], was located. The promoter proximal part conferred maximal activity in a transient transfection assay. In lesser quantity, extension products of a similar length to the major product were detected, indicating a less precise transcriptional activation mechanism similar to that previously reported for a TATA-box deleted SV40 early-promoter [28,29]. Deletion of the two GC-rich regions abrogated the activity of the PLCγ² promoter in transient transfection assays. This finding indicates that the Sp1 clusters may possibly substitute for the missing TATA motif, which generally

confers basal transcriptional activation to most known core-promoters [30,31]. In accordance to previous reports [32,33], the cooperative binding of several Sp1 factors in combination with other factors might be neccessary for full transcriptional activity of the PLCγ² promoter. In case of the TATA-less promoter for human adenosine deaminase, deletion of the very proximal Sp1 site rendered the promoter inactive, while deletion of the more distal Sp1 motifs affected the transcription only modestly [31]. In case of the TATA-less promoter for the insulin-like growth factor binding protein-2 from rat [30], where Sp1/GC-factor binding sites are located more distant from the transcriptional start between positions -234 to -215 and from -189 to -125, deletion of these regions decreased promoter activity 25-fold, although it did not, however, render the remaining construct completely inactive. Thus, in addition to its compositional arrangement, the positioning of these elements seems to have a significant impact on promoter activity. Deletion of a PLCγ² promoter sequence carrying an AP2 site at position -174, and in addition that of one located within the first, untranslated exon at position +73, leads to some decrease of promoter activation in four out of five transfected cell lines, and to a major drop in activation in Raji cells. This suggests a major role for AP2 in Raji, whereas the effect of this motif could be minor in the four others. Deletion of a sequence carrying a serum response element (SRE) at position -567 also had a very moderate effect, except within the cell line BJA-B, where a more pronounced drop of activity could be seen. Thus, from the deletional analysis, the GC-rich region with its Sp1 and GC-factor motifs seems to be the most significant requirement for promoter function whereas other motifs, notably AP2 and SRE, seem to have a more moderately positive effect. The negative impact of a region between position -622 to -293 and nt -293 to nt -198 was not specifically investigated. This stretch seems to repress activity in all tested cell lines, most dramatic in Raji.

To our surprise, all promoter constructs showing activity in lymphoid cells did the same in adherent cell lines, where endogenous PLCγ² expression cannot be detected, suggesting that the tissue specificity of PLCγ² might not be defined by its promoter sequence. On the other hand, the amplitude of its transcriptional activity may be regulated by interaction of a subset of rather ubiquitous transcription factors, such as Sp1, GC factor, SRF, or factors binding to AP2 motifs that may be exogenously stimulated and regulated [2]. As these transcription factors are reported to be constitutively present, they may enhance transcription of PLCγ² only in those cells, where the transcriptional unit, and in particular, the promoter, is accessible for this type of transcriptional activation.

The rat promoter for the ubiquitously expressed *PLCγ¹* gene has been already isolated and characterized [34]. This promoter resembles that of PLCγ². It lacks a TATA box, shows GC-rich sequence stretches and contains clusters of Sp1 and GC-factor binding sites as well as an AP2-binding motif. The similarity of these promoters suggests that tissue specificity of PLCγ² expression is determined by means other than the promoter sequence. The promoter of PLA2, a phospholipase involved in the production of lysophospholipids, prostaglandins and leukotrienes, [35] lacks a TATA box, but shows no further similarity to the PLCγ² promoter.

As PLCγ² is suspected to be a signal transducer downstream of the B-cell receptor complex, an increase of its mRNA might be a stabilizing response to B-cell receptor-initiated signals which may finally lead to the clonal expansion of an antigen triggered B-cell. As the PLCγ² RNA is apparently quite stable compared to that of c-myc, the induction of RNA levels in BJA-B cells is likely to depend, at least in part, on the transcriptional activation of the gene. TPA and retinoic acid positively influence B-cell viability and differentiation, and could resemble inputs given to an antigen triggered B-cell in vivo. TPA and retinoic acid treatment in fact stimulated PLCγ² expression in the B-cell line BJA-B. The increase of PLCγ² mRNA in 5-azacytidine treated Raji cells might suggest the expression of this gene, especially when it is low or missing, to be directly or indirectly controlled by mechanisms of DNA methylation.

In summary, this study suggests a specific role for the transcription factor SP1 and its corresponding binding motif on the transcriptional activation of the *PLCγ²* gene. As promoter fragments fused to a *luc* reporter gene give rise to transcription of the reporter in cell lines which do not express detectable amounts endogenous of PLCγ², one may suggest that, once the gene is accessible, a rather abundant set of transcription factors, most prominently SP1, mediates transcriptional activation. The causality of tissue-specific PLCγ² expression, however, must rather be searched for within mechanisms of expressional regulation, which are not neccessarily confined to cell-specific factor/promoter interaction.

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