Expression of Phosphoinositide-Specific Phospholipase C Isoenzymes in Cultured Astrocytes

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Abstract Signal transduction from plasma membrane to cell nucleus is a complex process depending on various components including lipid signaling molecules, in particular phosphoinositides and their related enzymes, which act at cell periphery and/or plasma membrane as well as at nuclear level. As far as the nervous system may concern the inositol lipid cycle has been hypothesized to be involved in numerous neural as well as glial functions. In this context, however, a precise panel of glial PLC isoforms has not been determined yet. In the present experiments we investigated astrocytic PLC isoforms in astrocytes obtained from foetal primary cultures of rat brain and from an established cultured (C6) rat astrocytoma cell line, two well known cell models for experimental studies on glia. Identification of PLC isoforms was achieved by using a combination of RT-PCR and immunocytochemistry experiments. While in both cell models the most represented PI-PLC isoforms were β4, γ1, δ4, and ε, isoforms PI-PLC β2 and δ3 were not detected. Moreover, in primary astrocyte cultures PI-PLC δ3 resulted well expressed in C6 cells but was absent in astrocytes. Immunocytochemistry performed with antibodies against specific PLC isoforms substantially confirmed this pattern of expression both in astrocytes and C6 glioma cells. In particular while some isoenzymes (namely isoforms β3 and β4) resulted mainly nuclear, others (isoforms δ4 and ε) were preferentially localized at cytoplasmic and plasma membrane level. J. Cell. Biochem. 100: 952–959, 2007. © 2006 Wiley-Liss, Inc.

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Inositol phospholipids are involved in many key functions in signaling and membrane trafficking [Cocco et al., 1996, 2001a; Manzoli et al., 1996; Irvine, 2003]. Recently, a growing body of evidence highlighted the presence of nuclear signaling pathways based on lipid hydrolysis [Cocco et al., 2001b; Manzoli et al., 2005]. In the inositol lipid pathway, phosphatidylinositol 4,5-bisphosphate (PIP2) is hydrolyzed by means of a phosphoinositide-specific phospholipase C (PI-PLC) in response to a wide panel of stimuli, including growth factors, hormones, and neurotransmitters, which act on specific receptors localized at the plasma membrane. Such a reaction generates two second messengers: 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) [Cocco et al., 1996; Hisatsune et al., 2005]. The former activates DAG-dependent protein kinase C (PKC) isoforms or can be converted to phosphatidic acid, which has also signaling functions; the latter liberates Ca^{2+} from intracellular stores. The PI-PLC family includes a diverse group of enzymes differing for structure and tissue distribution. Ten mammalian isoforms have been identified so far and classified in four subfamilies. Four isoforms belong to PI-PLC β

sub family, two to PI-PLC γ, three to PI-PLC δ [Irino et al., 2004], and one to PI-PLC ε [Wing et al., 2003].

Many studies have been performed to detect the pathways in which PI-PLC isoforms are involved. In particular PI-PLC isoforms have been detected in many tissues, being preferentially expressed in hematopoietic cell lines (PI-PLC β2 and γ2) and in the nervous system (cerebrovisual cortex) (PI-PLC β4). Furthermore, PI-PLC δ1 is widely expressed in many tissues, but especially in cultured cells [Tanaka and Kondo, 1994; Adamski et al., 1999; Yamaga et al., 1999; Mao et al., 2000; Faenza et al., 2004]. PI-PLC δ3 also has been identified in many histotypes at low concentrations [Pawelczyk and Matecki, 1998]; PI-PLC δ4 is expressed in the brain as well as in regenerating tissues [Pawelczyk, 1999]; PI-PLC γ1 is expressed in keratinocytes and foetal cartilage [Ananthanarayanan et al., 2002] and has been found almost exclusively in the cytoplasm [McBride et al., 1991; Diakonova et al., 1997]; also PI-PLC ε occurs in many tissues [Wing et al., 2003]. Notwithstanding the growing interest for the role played by the PI-PLC family enzymes in the nervous system [Ledeen and Wu, 2004], no definitive studies have been accomplished in glial cells [Choi et al., 1989; Tallant and Higson, 1997; Wang and Reiser, 2003]. As a matter of fact the inositol lipid cycle seems to be involved in glial activation during neurodegenerative processes [Vitale et al., 2004]. Since a precise panel of glial PLC isoforms has not yet been determined, as a necessary background for future experimental studies, in the present experiments we analyzed the expression and the localization of specific PI-PLC isoforms in cultured primary astrocytes (obtained from neonatal rat cortex) and in an established glioma cell line (C6 rat astrocytoma cells), two very useful cellular models for studying in vitro glia biology and pathology.

MATERIALS AND METHODS

Cell Culture

Astroglial cultures were prepared from mixed primary glial cultures obtained from neonatal rat cortex as described previously [Levi et al., 1993], in accordance with national guidelines for care and use of laboratory animals. Glial cells were seeded into 75-cm^2 flasks at an initial density of $2 \times 10^6 – 3 \times 10^6$ cells per flask, maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Invitrogen, Carlsbad, CA), and cultured to confluence (14 days) in a 5% CO_2 incubator at 37°C with media changes every 2–3 days. Upon confluence, cells were lifted from the flask surface using 0.25% trypsin/0.02% EDTA. Cells were then washed, plated on coverslips (15 mm) or in 6-well plates, and grown 14 days until fixation for immunocytochemistry studies or suspended in TRIzol reagent for molecular biology analysis. Astroglial cultures were characterized for purity as previously described [Amruthesh et al., 1993] and consisted of >95% glial fibrillary acidic protein-positive cells. The rat astrocytoma cell line C6 (ATCC, Manassas, VA) was cultured in DMEM supplemented with 10% FCS.

Molecular Biology

Total RNA was isolated from samples by using TRIzol reagent (Invitrogen) following the manufacturer's instructions. The obtained RNA was quantitated by the UV absorption ratio 260:280 nm by using a UV/visible spectrophotometer (SmartSpec 3000, Bio-Rad Laboratories, Hercules, CA); 1 µg of total RNA was reverse transcribed by using SuperScript III reverse transcriptase according to manufacturer's instructions. Briefly, total RNA, oligo (dT), dNTP mix, and DEPC-treated distilled water were heated for 5′ at 65°C; then buffer (250 mM Tris pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) containing DTT and RNase Ribonuclease Inhibitor was added to the mixture. All reagents were obtained from Invitrogen. After incubation for 2′ at 42°C, SuperScript III reverse transcriptase (200 U) was added and incubation was continued for 50′. Reaction was stopped by heating for 15′ at 70°C. The final volume was 20 µl. For PCR reactions, the following primer pairs (M-Medical, Florence, Italy) were used: for PI-PLC β1 reverse 5′-CTG AGC TCA CCC ATG ACA GA-3′, forward 5′-TGC ATA CGT GTC TGG GAC AT-3′; for PI-PLC β2 reverse 5′-GAC ACA CTC GCT TTG GGA AG-3′, forward 5′-GGG CTC AGC TGC ATT TTA AG-3′; for PI-PLC β3 reverse 5′-GGA GGA GGA GGA TCA TAG GC-3′, forward 5′-CCC AGA AAG AGT TGG AGC TG-3′; for PI-PLC β4 reverse 5′-GGA TGC TCT ATC CGA TCC AA-3′, forward 5′-GCT CCT TTG CGT GTT TCT TC-3′; for PI-PLC δ1 reverse 5′-TCA TCC TGT CCC TGG AGA AC-3′, forward 5′-TCA GAC ACG TCA GTG GCT TC-3′; for

PI-PLC δ2 reverse 5′-TGG CTA CAG TCA GCC CTT CT-3′, forward 5′-AGG GTT CTG TGG CAT AGT GG-3′; for PI-PLC δ3 reverse 5′-GGG TTG GAA CAG CAG ATT GT-3′, forward 5′-AGT GGG TAC ACT CGG GTC AG-3′; for PI-PLC δ4 reverse 5′-CCT CTG GGA ACA AGA GCA AG-3′, forward 5′-GCT TCA TGG GAT TGA AGG AA-3′; for PI-PLC ε reverse 5′-CGG ATC AAA GGA GGA ATG AA-3′, forward 5′-GCC TCT GGT TGT CAG AAA GC-3′; for PI-PLC γ1 reverse 5′-TAC AGC AGG AAG GCC AGA CT-3′, forward 5′-CTC TCT GGG CCT TGT AGT CG-3′; for PI-PLC γ2 reverse 5′-GGT TCT GAT GGG CTG TGT TT-3′, forward 5′-GGC AAG ATG GTC TTC AGC TC-3′. The specificity of the primers was verified by searching in NCBI data base possible homology to cDNAs of unrelated proteins. Each PCR tube contained the following reagents: 0.2 μM of both sense and antisense primers, 3–5 μl template cDNA, 0.2 mM 4-dNTP mix (Invitrogen), 2.5 U Platinum Taq DNA polymerase (Invitrogen), and 1× reaction buffer (Invitrogen). MgCl$_2$ was added at variable (empirical determination by setting the experiment) final concentration. The final volume was 50 μl. The amplification was started with an initial denaturation step at 94°C for 2 min and was followed by 35 cycles consisting of denaturation (30 s) at 94°C, annealing (30 s) at the appropriate temperature for each primer pairs, and extension (1 min) at 72°C. The PCR products were analyzed by 1.5% TBE agarose gel electrophoresis (Submarine Agarose Gel Unit, Hoefer, San Francisco, CA). Gels were previously stained with ethidium bromide (50 ng/100 ml). A PC-assisted CCD camera (GelDoc 2000 System/ Quantity One Software; Bio-Rad) was used for gel documentation and quantification. Optical densities were normalized to the mRNA content of glyceraldehyde 3 phosphate dehydrogenase (GAPDH), a typical reference constitutive transcript. Gel electrophoresis of the amplification products revealed single DNA bands with nucleotide lengths as expected for all primer pairs. To exclude possible DNA contamination during the RT-PCR, RNA samples were amplified by PCR without reverse transcription. No band was observed, suggesting that there was no DNA contamination in the RNA preparation procedure (data not shown).

Immunocytochemistry

Both astrocytes and C6 cells were grown on coverslips and fixed in MetOH/Acetic acid 3/1.

After fixation, cells were washed several times in phosphate buffer (PBS) and first saturation was performed with 1% BSA in PBS; then incubation with primary antibodies was performed following the manufacturer's indications with regard to the dilutions overnight at +4°C; the used primary antibodies (purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were: mouse monoclonal anti-PI-PLC β1, rabbit polyclonal anti-PI-PLC β3, rabbit polyclonal anti-PI-PLC β4, mouse monoclonal anti-PI-PLC γ1, goat polyclonal anti-PI-PLC δ3, goat polyclonal anti-PI-PLC δ4, goat polyclonal anti-PI-PLC ε. After several washes with PBS, a second incubation was performed with the secondary fluorochrome-conjugated antibody 60′ at room temperature in humified dark chamber. Secondary antibodies were: anti-mouse and anti-rabbit conjugated with Cy3 (Sigma, St. Louis, MO), anti-goat conjugated with Texas Red (Santa Cruz Biotechnologies). Controls were performed by omitting the primary antibody (for the experiments testing PI-PLC β3, β4, δ3, and δ4) or by using normal goat IgG (for the experiments testing PI-PLC β1, γ1, and ε) (data not shown).

After several washes performed with PBS in dark chamber the coverslips were counterstained with DAPI, mounted on slides, and observed with a fluorescence microscope Nikon Eclipse 800 equipped with ACT image analysis and acquisition program (Nikon, Tokyo, Japan).

RESULTS

RT-PCR

Several isoform transcripts for PI-PLC were detected in cultured primary astrocytes by RT-PCR (Fig. 1A). In these cells transcripts for isoforms PI-PLC β4, δ4 and γ1, β3, δ1, δ3 and ε were expressed (Fig. 1 and Table I). Finally, specific mRNAs for PI-PLC isoforms β1, β2, and γ2 were not detected (Fig. 1A and Table I).

In C6 glioma cells (Fig. 1B and Table I) PI-PLC isoforms of β1, β3, β4, γ1, γ2, δ3, δ4, and ε were expressed. In contrast, transcripts for isoforms β2 and δ1 were absent (Fig. 1B and Table I).

Microscopy Immunofluorescence

All PI-PLC isoforms detected by RT-PCR were then examined for their localization by light microscope immunofluorescence using antibodies specific to each isoform.
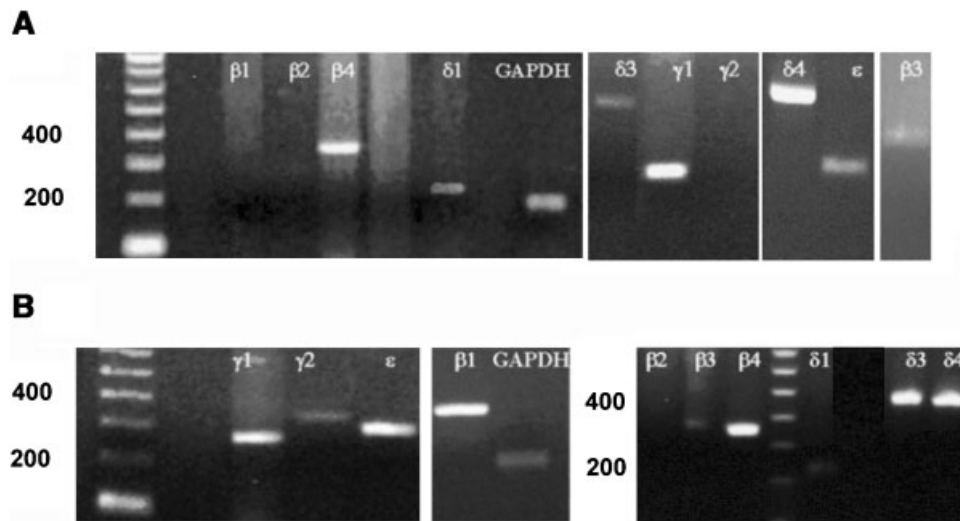
Fig. 1. RT-PCR analysis of PI-PLC isoform transcripts in cultured rat primary astrocytes (**A**) and astrocytoma (C6) cell line (**B**).

Interestingly, in cultured primary astrocytes most isoforms were localized at both cytoplasmic and nuclear level, with the exception of isoforms β3 which was almost exclusively nuclear (Fig. 2 and Table II). Confirming data obtained by RT-PCR, no specific immunoreactivity was visualized for PI-PLC β1 (Fig. 2, Tables I and II).

Similar results were obtained in rat (C6) astrocytoma cells, bearing specific immunoreactivity for most isoforms mainly distributed at cytoplasmic level (isoforms β1, β1, β3, β4, γ1, ε) (Fig. 3 and Table III). By contrast specific immunoreactivity for isoforms β3 and 4 was mainly or exclusively nuclear, even though the immunoreactivity for PI-PLC β3 is less intense as compared to primary astrocytes, which show also cytoplasmatic staining (Fig. 3 and

Table III). In contrast to primary astrocytes, which did not contain isoform β1 (Fig. 2), astrocytoma cells were clearly positive for this isoform, exclusively in the cytoplasm (Fig. 3). Finally, as in the case of primary astrocytes and in line with our RT-PCR data, no specific immunoreactivity for isoform β2 was detected in astrocytoma cells (Fig. 3 and Table III).

DISCUSSION

The occurrence of different PI-PLC isoforms has been detected in normal and pathological tissues, like, for example, the neural tissue (containing β isoforms) [Martelli et al., 1996; Vitale et al., 2004], smooth, skeletal and heart muscle cells (PI-PLC β1) [LaBelle et al., 2002; Faenza et al., 2004], primary human colon carcinoma cell lines (PI-PLC γ1, β1, and δ1) [Nomoto et al., 1995], liver (PI-PLC β1, γ1, and δ1) [Martelli et al., 2004]. Various PI-PLC isoforms have also been found in hepatoma cell lines (Morris hepatoma H7795 cells—HI and H3924A cells—HII) and different expression of some of them have been identified in slow or fast growing [Santi et al., 2003]. In the present study we investigated the presence of PI-PLC isoforms in astrocytes, a glial cell type which normally provide nutrient support to neurons, also playing a pivotal role in the response to brain injury (glia activation). Our present experiments show that resting (non-activated) astrocytes express a selected set of various

TABLE I. RT-PCR

PLC isoforms	Rat astrocytes	C6 (rat astrocytoma) cells
β1	−	+
β2	−	−
β3	+	+
β4	+	+
γ1	+	+
γ2	−	+
δ1	+	−
δ3	+	+
δ4	+	+
ε	+	+

Occurrence of PI-PLC isoform transcripts in cultured rat primary astrocytes and astrocytoma (C6) cell line.
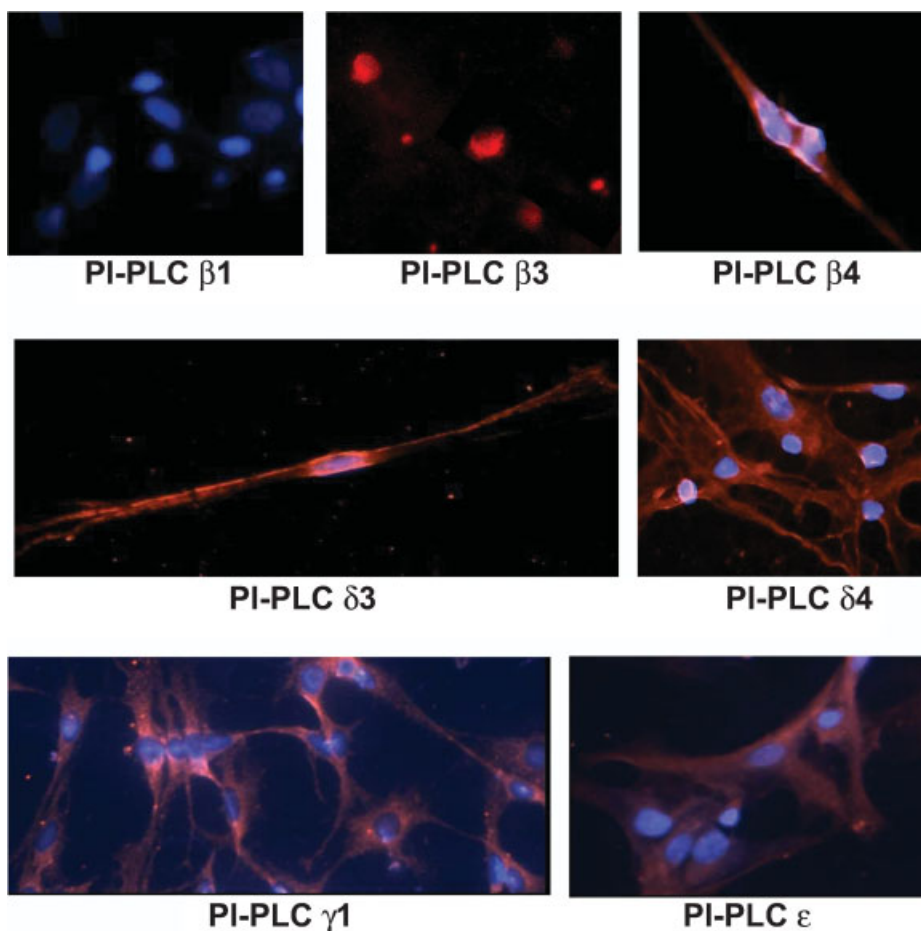+, evidence of signal; −, no signal.

Fig. 2. Immuncytochemical distribution of specific immunoreactivity for various PI-PLC isoforms in cultured rat primary astrocytes (40×). After staining with anti-PI-PLC isoform-specific antibodies, cells were counterstained with DAPI in order to label nuclei.

PI-PLC isoforms, suggesting that these enzymes may play an important role in the regulation of glial functions. In particular our experiments indicate that a common panel of specific isoforms is present in both rat

TABLE II. Immunocytochemical Distribution of PI-PLC Isoforms in Cultured Rat Primary Astrocytes

PI-PLC isoenzymes	Nucleus	Cytoplasm
β1	−	−
β3	++	−
β4	+	++
δ3	+	++
δ4	+	++
γ1	+/−	++
ε	+/−	+

++, strong signal; +, weak signal; +/−, very weak signal; −, no signal.

astrocytes and C6 cells. This panel primarily included PI-PLC isoforms β3, β4, γ1, δ1, δ4, and ε, which were expressed by both cell types. As far as the β and γ families may concern, according to previous studies on rat brain [Tanaka and Kondo, 1994] and cerebellar cortex [Choi et al., 1989; Vitale et al., 2004], our data indicate that in astrocytic cells the isoforms, usually present in the hematopoietic tissue (β2, γ2) [Mao et al., 2000; Kurosaki and Okada, 2001], were not found or were only slightly expressed. In this context it is not surprising that some isoforms are not expressed in astrocytes and that some of the expressed ones are differently represented in the neoplastic counterpart. This was the case of PI-PLC δ1, which was specifically associated to primary astrocytes, while PI-PLC isoforms β1 and γ2 were expressed exclusively by C6 cells (Fig. 1 and
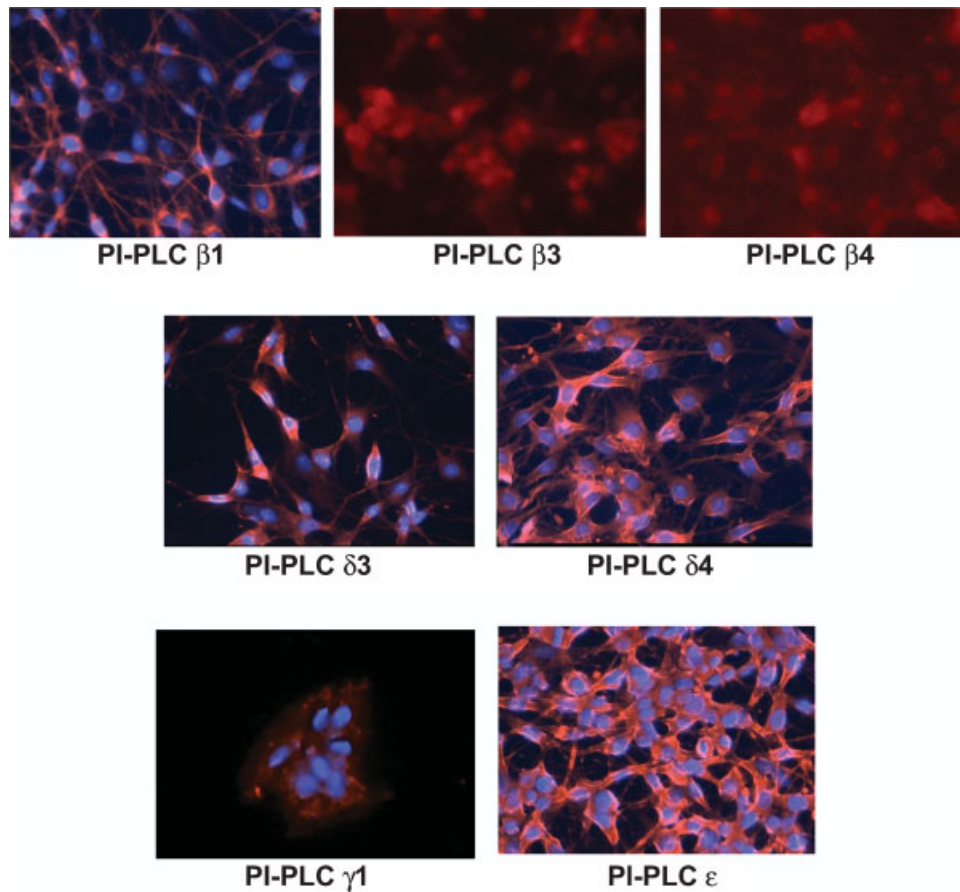
Fig. 3. Immuncytochemical distribution of specific immunoreactivity for various PI-PLC isoforms in an established rat astrocytoma (C6) cell line (40×). After staining with anti-PI-PLC isoform-specific antibodies, cells were counterstained with DAPI in order to label nuclei.

Table I). This finding is substantially in line with the well-known involvement in other tissues of PI-PLC enzymes (especially PI-PLC β1) in the progression of the cell cycle [Faenza et al., 2000] and their supposed involvement in

TABLE III. Immunocytochemical Distribution of PI-PLC Isoforms in a Cultured Rat Astrocytoma (C6) Cell Line

PI-PLC isoenzymes	Nucleus	Cytoplasm
β1	−	++
β3	++	+
β4	++	+
δ3	+/−	++
δ4	+/−	++
γ1	−	+
ε	+/−	++

++, strong signal; +, weak signal; +/−, very weak signal; −, no signal.

malignant transformation [Lo Vasco et al., 2004].

In the present investigation we used immunocytochemistry to study the cellular localization of PI-PLC isoforms we had identified previously by means of RT-PCR. As a matter of fact, in the complex cascade of events involving PI-PLC signaling, the control of cell proliferation may be influenced not only by a differential expression of specific isoforms (depending on cell type), but also by a specific intracellular localization of the enzyme activity [Santi et al., 2003]. Actually, in line with literature data, we found a marked nuclear localization of PI-PLC β3 isoform in both primary and neoplastic astrocytic cells, even though in C6 cells this isoform is less nuclear as compared with primary cultured astrocytes and is also slightly cytoplasmatic (Figs. 2 and 3). This nuclear localization (for β3 and β4

isoforms) has been described widely in various cell types and could be related to the well-known involvement of the PI-PLC β family in cell cycle progression [Cocco et al., 2003].

As far as the cytoplasmic localization may concern, we detected a heavy specific immunoreactivity for isoforms PI-PLC δ4 and ε in both astrocytes and C6 cells (Figs. 2 and 3). These similar pattern of distribution suggest that both models may be useful for future experimental studies. However, the expression of PI-PLC δ3 (Fig. 1) deserves some comment because level of PI-PLC δ3, which is poorly expressed in normal tissues [Pawelczyk, 1999] but is highly expressed in C6 astrocytoma cells (Figs. 1B and 3) and other neoplastic tissues [Pawelczyk and Matecki, 1998], may be interpreted as a neoplastic upregulation of this enzyme.

In conclusion, the present experiments suggest that various PI-PLC isoforms may play important roles in glial cells. This would be of great interest as glia plays a fundamental role in homeostasis and trophism of the nervous system under normal and pathological conditions [Ambrosini and Aloisi, 2004]. Namely there is the likelihood that some PI-PLC isoforms could be involved, in glial cells, in the maintenance of normal phenotype while other could be related to the transformed one. Indeed PI-PLC β3 isozyme as been shown to increase in promyelocytic cell line HL60 following induction of differentiation and this could fit with higher level of this isozyme in primary glial cells as compared to astrocytoma ones. In addition PI-PLC γ and δ isoforms have been described to be involved in a continuous shuttling between cytoplasm and nucleus during cell cycle, hinting at the role in cell cycle progression [for review see Cocco et al., 2001b]. All in all the present work depicts a detailed scenario of PI-PLCs expression in primary cultured astrocytes and astrocytoma cells, paving the way for further investigations on neoplastic transformation of glial cells.

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