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Review

Nuclear phosphoinositide specific phospholipase C (PI-PLC)-ß1: a central intermediary in nuclear lipid-dependent signal transduction

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Summary. Several studies have demonstrated the existence of an autonomous intranuclear phosphoinositide cycle that involves the activation of nuclear PI-PLC and the generation of diacylglycerol (DG) within the nucleus. Although several distinct isozymes of PI-PLC have been detected in the nucleus, the isoform that has been most consistently highlighted as being nuclear is PI-PLC-ß1. Nuclear PI-PLC-ß1 has been linked with either cell proliferation or differentiation. Remarkably, the activation mechanism of nuclear PI-PLC-ß1 has been shown to be different from its plasma membrane counterpart, being dependent on phosphorylation effected by p44/42 mitogen activated protein (MAP) kinase. In this review, we report the most up-dated findings about nuclear PI-PLC-ß1, such as the localization in nuclear speckles, the activity changes during the cell cycle phases, and the possible involvement in the progression of myelodisplastic syndrome to acute myeloid leukemia.

Key words: Nucleus, Phosphoinositides, Proliferation, Differentiation, Myelodisplastic syndromes

Introduction

PI-PLC plays a pivotal role in the transmembrane signal transduction pathways by catalyzing the hydrolysis of phosphoinositide 4,5 bisphosphate (PtdIns 4,5 P_2) to yield the intracellular second messengers diacylglycerol (DG) and inositol 1,4,5 trisphosphate (Ins 1,4,5 P_3) in response to the interaction of various ligands with cell surface receptors in most eukaryotic cells (Rebecchi and Pentyala, 2000).

Ins 1,4,5 P_3 induces the release of Ca^{2+} from the internal stores, and DG activates protein kinase C (PKC). These two processes have important consequences for many cellular activities including cell to cell communication, secretion, proliferation, and differentiation (Rhee, 2001). PI-PLC family comprises twelve isoforms that can be subdivided into five types (β, γ, δ, ε, ζ) based on their structural differences. The activation of PI-PLCs located in the plasma membrane, in response to extracellular agonists, has been extensively investigated and many aspects of their regulation are now understood (Rhee, 2001). However, evidence has accumulated suggesting the presence of PI-PLCs in the cell nucleus (Martelli et al., 2003, 2004). The isoform which has most consistently been found to be associated with the nucleus is PI-PLC-ß1. PI-PLC-ß1 was the inositide-specific phospholipase originally identified at the nuclear level (Martelli et al., 1992). It is now established that this PI-PLC isoform plays an important role in the control of cell proliferation, because it mediates the mitogenic effect of insulin-like growth factor 1 (IGF-1) in Swiss 3T3 cells (Manzoli et al., 1997) and regulates cell cycle progression in mouse erythroleukemia (MEL) cells (Faenza et al., 2000). In IGF-1-stimulated Swiss 3T3, nuclear PI-PLC-ß1 is responsible for the rise in the DG mass which occurs in the nucleus. Such an increase in the DG levels attracts to the nucleus the α isoform of PKC (Neri et al., 1998). Moreover, it has been proposed that PI-PLC-ß1 is critically involved in resumption of meiosis in mouse oocyte (Avazeri et al., 2000, 2003).

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In this article, we shall first briefly summarize our current general knowledge about PI-PLC-ß1 and then review, in more detail, studies that have shed light on the regulation and function of nuclear PI-PLC-ß1.

PI-PLC-ß1

The sequences of the eukaryotic PI-PLCs contain a string of modular domains organized around a catalytic α/ß barrel formed from the characteristic X- and Y-box regions. They include a pleckstrin homology (PH) domain, EF-hand motifs, and a single C2 domain that immediately follows the Y-box region (Fig. 1). Moreover, ß-subtypes PI-PLC have at their carboxy-terminal end extensions of ~400 amino acid residues (P/G domain) that contain sequences important to membrane binding, nuclear localization, and their activation by G protein subunits (Rhee, 2001).

As to PI-PLC-ß1, its PH domain serves as membrane tether, while the function of EF-hand motifs still waits for elucidation. The C2 domain of PI-PLC-ß1 binds specifically to GTP-charged Gaq, its physiological activator. Furthermore, the C2 domain of ß isozymes appears to operate in concert with the carboxy-terminal extension to effectively engage these proteins to the membrane. PI-PLC-ß1 exists as alternatively spliced variants -ß1a (150-kDa) and -ß1b (140-kDa). The -ß1b variant replaces 75 carboxy-terminal residues of the original PI-PLC-ß1 cDNA with a unique 32-amino acid sequence (Bahk et al., 1998).

Nuclear PI-PLC-ß1

In 1992, our laboratory for the first time showed by immunocytochemical and immunochemical analysis the presence of PI-PLC-ß1 in the nucleus of quiescent Swiss 3T3 fibroblasts, while PI-PLC-γ1 was confined to the cytoplasm (Martelli et al., 1992). Most importantly, it was also demonstrated that in response to a mitogenic stimulation with IGF-1, the activity of nuclear PI-PLC-ß1 increased from two to three folds. The intranuclear presence of PI-PLC-ß1 has subsequently been confirmed by other investigators who employed different cell lines and tissues. In Table 1 we summarize the cell types and tissues where PI-PLC-ß1 has been detected in the nucleus.

However, the relative distribution of this isozyme, between the cytoplasm and the nuclear fraction, is highly controversial, because it has been reported to range from being completely nuclear (e.g. Martelli et al., 1992; Marmiroli et al., 1994), to being as little as 2% of total cellular PI-PLC-ß1 (Divecha et al., 1993). The discrepancy might depend on the existence of the two splice variants of PI-PLC-ß1 (referred to as a and b, see above). It might be that in some cell types (e.g. Swiss 3T3, Saos-2) PI-PLC-ß1b is much more abundantly expressed than PI-PLC-ß1a, whereas in others (rat hepatocytes) the opposite is true. This fact may provide an explanation for the controversial results obtained by immunoblotting analysis in which a monoclonal antibody that recognizes both the subtypes was employed.

Subnuclear localization of PI-PLC-ß1

Molecular structure analysis has revealed that, among PI-PLC isozymes, the four members of the ß family are unique, in that they contain a high proportion of basic residues located at their carboxyl-terminal domain. Kim et al. (1996) demonstrated that this region was critical for allowing nuclear localization of these isozymes. In particular, substitution with isoleucine of the cluster of lysine residues 1056, 1063, and 1070 (a

Table 1. Cell types in which PI-PLC-ß1 has been localized to the nucleus.

CELL TYPE	REFERENCES
Swiss 3T3 mouse fibroblasts	Martelli et al., 1992; Maraldi et al., 1995; Liu et al., 1996; Billi et al., 1997; Xu et al. 2001a; 2001b; Manzoli et al., 1997;
Rat Hepatocytes	Divecha et al., 1993;Bertagnolo et al.,1995; Crljen et al., 2004
Mouse erythroleukemia cells	Martelli et al., 1994; Divecha et al., 1995; Faenza et al., 2000
Saos-2 human osteosarcoma cells	Marmiroli et al., 1994
C6Bu-1 rat glioma cells	Bahk et al., 1998
Chinese hamster lung cell line CCL39	Fee et al., 1994
C2C12 rat myoblasts	Faenza et al., 2003
HL60 human leukemia cells	Neri et al., 2002; Lukinovic-Skudar et al., 2005
Human natural killer lymphocytes	Vitale et al., 2001;Ponti et al., 2002
Mouse oocyte	Avazeri et al., 2000; 2003

Fig. 1. Structural domains of PI-PLC-ß1. Please note that the cartoon is only intended to indicate the order of appearance of the various domains as it does not accurately reflect the precise length of each domain.

cDNA mutant referred to as M2b) almost completely abolished nuclear localization of PI-PLC-ß1. There are other features which might explain why PI-PLC-ß1b is more abundantly expressed in the nucleus. The last 32 amino acids of PI-PLC-ß1b located at the carboxy-terminus are different to those of PI-PLC-ß1a (see Fig. 2). These amino acids form a α-helix/proline/basic residue motif which might act as an additional nuclear localization signal. Moreover, PI-PLC-ß1a has in its unique carboxy-terminus a typical nuclear export signal (LXLXXLXXV) which may result in this variant being less concentrated in the nucleus, in analogy with PI-PLC-δ1 (Yamaga et al., 1999) (Fig. 2).

Immunoelectron microscopy investigations have shown that PI-PLC-ß1 localizes at the interchromatin granule clusters (Zini et al., 1993, Maraldi et al., 1995; Tabellini et al., 2003). These structures correspond to nuclear speckles which appear at the fluorescence microscope level as irregular, punctuate structures which vary in shape and size (Lamond and Spector, 2003).

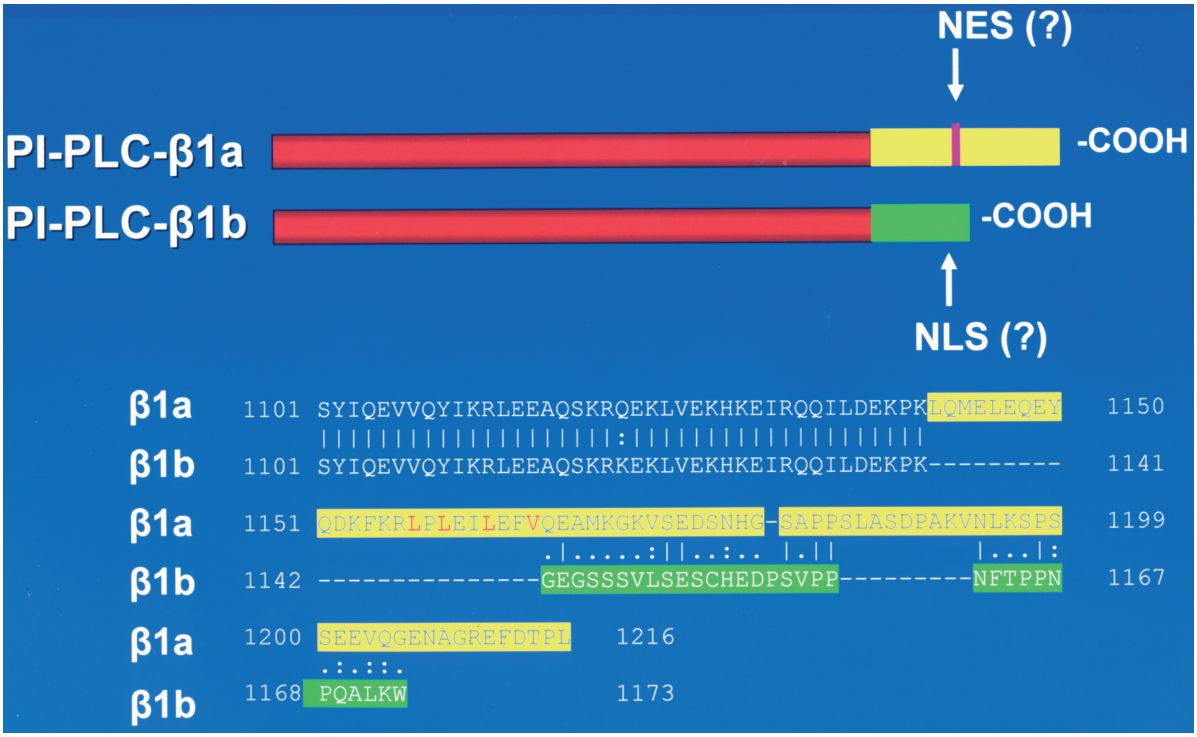

Fig. 2. Sequence alignment of PI-PLC-ß1 splicing variant carboxy-terminus. The stretches of amino acids unique to PI-PLC-ß1a (yellow) and PI-PLC-ß1b (green) are shown. The residues which constitute the putative NES of PI-PLC-ß1a are indicated in magenta.

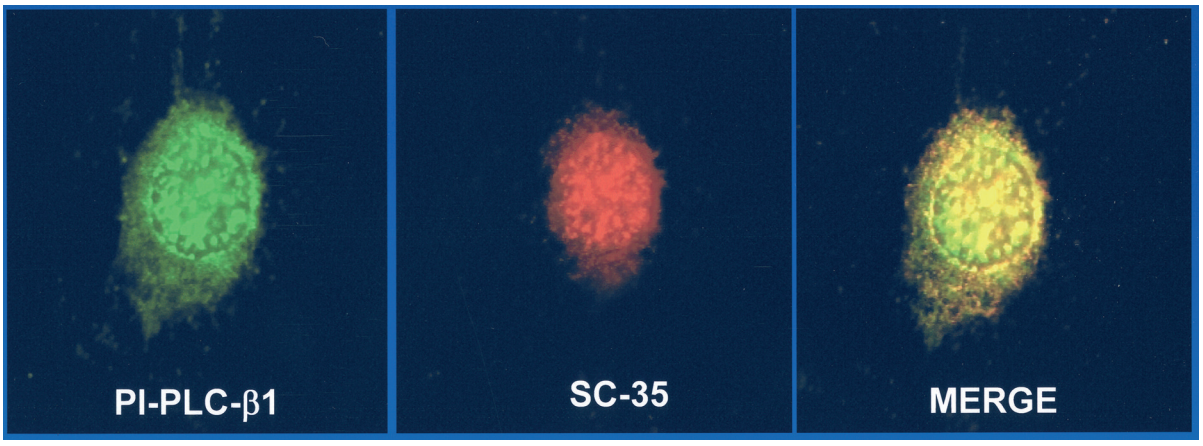

Fig. 3. PI-PLC-ß1 localizes to nuclear speckles in mouse C2C12 myoblasts. Cells growing on cover slips were fixed, permeabilized and double immuno-stained for PI-PLC-ß1 (mouse monoclonal) and SC-35 (goat polyclonal). Secondary antibodies were a fluorescein isothiocyanate-conjugated anti-mouse IgG (green) and a Cy3-conjugated anti-goat IgG (red). The merged image shows almost complete colocalization of the two signals.

Speckles are subnuclear structures that are enriched in pre-messenger RNA splicing factors as well as protein kinases and phosphatases. Double immunofluorescence labelling shows that PI-PLC-ß1 co-localizes with the speckle marker SC-35 (Fig. 3). It is noteworthy that speckles contain other elements of nuclear phosphoinositide metabolism such as PtdIns 4,5 P_2, phosphatidylinositol phosphate kinases, phosphoinositide 3-kinase (PI3K) C2α, SHIP-2 (which is a phosphatase targeting 3-phosphorylated inositol lipids, see Déléris et al., 2003), and diacylglycerol kinase (DGK) -θ (Martelli et al., 2004). Moreover, speckles contain profilin I, a PtdIns 4,5 P_2-binding protein (Skare et al., 2003), as well as a snRNP-associated form of actin (Nakayasu and Ueda, 1984). Interestingly, PtdIns 4,5 P2 regulates actin-binding proteins (Zhao et al., 1998). The function of all of these constituents of the inositide metabolism is unclear in relationship with nuclear speckles, although evidence suggests that PtdIns 4,5 P2 might be important for pre-mRNA splicing (Osborne et al., 2001).

Furthermore, it has been shown that most of nuclear PI-PLC-ß1 is associated with the nuclear matrix (Zini et al., 1995a), a nuclease and salt resistant framework which is thought to be of fundamental importance for determining nuclear structure and function (Martelli et al., 2002).

Regulation of nuclear PI-PLC-ß1 activity

At the plasma membrane, activation of PI-PLC-ß isoforms mainly occurs through the α subunit of herotrimeric G-proteins. The α subunits (α_q, α_{11}, α_{14}, α_{16}) of all of the four members of the Gq family of α subunits are capable of activating PI-PLC-ß family isozymes. The most powerful activators of PI-PLC-ß1 are α_q and α_{11} subunits. However, PI-PLC-ß1 could also be activated by the ßγ heterodimer, albeit weakly (Rhee, 2001).

Activation of nuclear PI-PLC-ß1 by G proteins seemed unlikely, given that nobody has ever demonstrated the presence within the nucleus of GTP-binding proteins of the Gq class. Accordingly, we always failed to stimulate nuclear PI-PLC-b1 activity with either GTP-γ-S or AlF4 (Martelli et al., 1996). Thus, we sought to determine whether or not other mechanisms could control the activity of nuclear PI-PLC-ß1. Phosphorylation is a widely used mechanism for reversibly regulating protein structure and function. Conformational or electrostatic changes promoted by phosphorylation modulate the enzymatic activity and macromolecular interactions of a plethora of cellular proteins. Our laboratory for the first time demonstrated that, in response to IGF-1 stimulation of quiescent Swiss 3T3 cells, activation of nuclear PI-PLC-ß1 was paralleled by its hyperphosphorylation. Interestingly, if activation of the p42/44 mitogen-activated protein (MAP) kinase signalling pathway was prevented by the selective pharmacological inhibitor PD98059, the hyperphosphorylation of PI-PLC-ß1 was no longer detectable, suggesting an involvement of MAP kinase in this phenomenon (Martelli et al., 1999). The increase in phoshorylation was paralleled by up-regulation of nuclear PI-PLC-ß1 activity.

Subsequently, our group reported similar findings obtained with either insulin-treated NIH 3T3 cells (Martelli et al., 2000) or interleukin-2-exposed human natural killer (NK) cells (Vitale et al., 2001). It is important to recall here that in insulin-treated NIH 3T3 fibroblasts, we observed a hyperphosphorylation of PI-PLC-ß1b, i.e. the form which is more abundantly expressed within the nucleus (Bahk et al., 1998). A hyperphosphorylation on serine residues of nuclear PI-PLC-ß1b which paralleled its activation, has been recently reported by another group which employed as experimental models regenerating rat liver (Crljen et al., 2004) or HL60 human acute leukemia cell line (Lukinovic-Skudar et al., 2005). In regenerating rat liver, serine hyperphosphorylation of nuclear PI-PLC-ß1b was detected 6 h after operation coinciding with increased enzymatic activity. A later peak of nuclear PI-PLC-ß1b was detected 20 h after hepatectomy and in this case PI-PLC-ß1b was not hyperphosphorylated but rather was tightly bound to the nuclear matrix (Crljen et al., 2004). It is interesting that we recently reported that in PC12 cells treated with nerve growth factor the activity of nuclear DGK-θ increased in parallel with its increased association with the nuclear matrix (Tabellini et al., 2004). Therefore, the association with the matrix may be responsible for the increase in the activity of both DGK-θ and PI-PLC-ß1b. Other enzymes, such as DNA primase and polymerase-α, have been reported to become more active upon their binding to the nuclear matrix (Tubo and Berezney, 1987).

In nocodazole-synchronized HL60 cells activation of nuclear PI-PLC-ß1b was detected at either 1 h (G2/M phase) or 8.5 h (late G1 phase) after the block (Lukinovic-Skudar et al., 2005). An increase in the serine phosphorylation of nuclear PI-PLC-ß1b was detected at the same times and could be blocked, together with enzyme activity, by PD98059. Most likely, this nuclear PI-PLC activity corresponds to the PI-PLC activity detected by others at the G2/M phase transition in aphidicolin-synchronized HL60 cells (Sun et al., 1997). It has been demonstrated that DG produced by this PI-PLC was responsible for activating nuclear PKC-ß2, whose activity is required for mitosis (Sun et al., 1997).

However, the conclusive demonstration that p42/44 MAP kinase was responsible for the activation of nuclear PI-PLC-ß1 came from an elegant series of experiments, performed with IGF-1-treated Swiss 3T3 cells, showing that: a) IGF-1 induced a rapid (within 2 min) nuclear translocation of active p42/44 MAP kinase; b) nuclear PI-PLC-ß1 and activated p42/44 MAP kinase could be coimmunoprecipitated; c) recombinant PI-PLC-ß1 could be efficiently phosphorylated in vitro by activated p42/44 MAP kinase, but not by protein kinase

A (PKA); d) the p42/44 MAP kinase phosphorylation site of PI-PLC-ß1 was mapped at serine 982, which lies within a PSSP motif located in the carboxy-terminus of PI-PLC-ß1; e) phosphopeptide analysis showed a similarity between in vivo 32P-labeled PI-PLC-ß1 and PI-PLC-ß1 phosphorylated in vitro by recombinant p42/44 MAP kinase; f) if Swiss 3T3 cells overexpressed a mutant PI-PLC-ß1 in which serine 982 was replaced by glycine, nuclear phospholipase activity was not activated by IGF-1, and the mitogenic effect of the growth factor was markedly attenuated. Taken together, these findings strongly suggested that p42/44 MAP kinase phosphorylated nuclear PI-PLC-ß1 at serine 982, and such a phosphorylation played a critical role in the activation of the phospholipase and was also crucial to the mitogenic action of IGF-1 (Xu et al., 2001a). These findings appear very intriguing and completely unexpected, as they have never been reported for plasma membrane-associated PI-PLC-ß1. Since the p42/44 MAP kinase phosphorylation site is within the carboxyl terminus of PI-PLC-ß1 (which has been shown to be essential for its Gαq-mediated activation at the plasma membrane), it is conceivable that this phosphorylative event may affect the binding to this region of other, as-yet-unidentified, nuclear proteins that consequently stimulate PLC activity. Indeed, the MAP kinase-mediated phosphorylation does not increase per se the in vitro activity of purified PI-PLC-ß1.

Phosphorylation of PI-PLC-ß1 is not an entirely new regulatory mechanism, because at the plasma membrane it might be utilized to inhibit PI-PLC activity. PKA has been shown to directly phosphorylate in vitro PI-PLC-ß2 or -ß3, thereby inhibiting their activation by Gbγ subunits. Here, the putative phosphorylation site is serine 954, which determines an uncoupling of receptors that activate PI-PLC-ß3 through Gi/o while preserving the activation by receptors that utilize Gq. However, PKA can phosphorylate PI-PLC-ß3 also at serine 1105 and in this case the outcome is a partial block of the Gq-dependent activation (Rhee, 2001). Moreover, PI-PLC-ß isoforms are also substrates for PKC. It was initially demonstrated that treatment of a variety of cells with phorbol esters (which are powerful PKC activators) resulted in the inhibition of receptor-coupled PI-PLC-ß activity. Moreover, PKC phosphorylated in vitro bovine PI-PLC-ß at serine 887, but without any concomitant effect on phospholipase activity, so that the physiological relevance of this phosphorylative event remained unclear (Rhee, 2001). Subsequently, it has been shown that PKC-α and -ε (but not PKC-ζ) phosphorylated in vitro PI-PLC-ß1, whereas PI-PLC-ß3 was poorly phosphorylated by PKC-α (Rhee, 2001). In this case, PKC phosphorylation of PI-PLC-ß1 resulted in significant activity inhibition, but the Gαq stimulation was unaffected while Gßγ subunits blocked the PKC-α-mediated phosphorylation of PI-PLC-ß1 and antagonized its inhibition.

Translocation of PKC-α to the nucleus is one of the earliest events that occur in IGF-1-treated Swiss 3T3 cells (Neri et al., 1998). DG produced by nuclear PI-PLC-ß1 serves as a chemoattractant for PKC-α. The functions of PKC-α, once translocated in the nucleus, are unclear, but recent evidence indicates it may be involved in a negative feedback regulation of PI-PLC-ß1 activity (Xu et al., 2001b). Indeed, treatment of Swiss 3T3 cells with Go6976, a selective inhibitor of PKC-α, caused a sustained elevation of IGF-1-dependent PI-PLC-ß1 activity. Two-dimensional phosphopeptide mapping and site-directed mutagenesis demonstrated that PKC-α phosphorylated nuclear PI-PLC-ß1 at serine 887. Moreover, overexpression of either a PI-PLC-ß1 mutant in which the PKC phosphorylation site serine 887 was replaced by alanine, or a dominant-negative PKC-α, resulted in a sustained activation of nuclear PI-PLC-ß1 in response to IGF-1 stimulation. All in all, these findings indicated that a negative feedback regulation of nuclear PI-PLC-ß1 by PKC-α was a critical step in the termination of the IGF-I evoked signals that activated inositol lipid cycle within the nucleus. However, an explanation of how phosphorylation of nuclear PI-PLC-ß1 at serine 887 can modulate its activity remains to be defined. As reported above, there is no evidence that a G protein is involved in the regulation of nuclear PI-PLC-ß1 activity, while in vitro phosphorylation of purified PI-PLC-ß1 by PKC-α seems to affect the interaction of the phospholipase with the Gßγ subunits. It might also be that in this case PI-PLC-ß1 phosphorylation leads to changes in the interaction with other regulatory nuclear protein(s) that remain to be identified.

Functions and targets of PI-PLC-ß1

1. Nuclear PI-PLC-ß1 and cell proliferation

Nuclear PI-PLCß1 plays an important role as a mediator of the mitogenic stimulus exerted by IGF-1 on Swiss 3T3 mouse fibroblasts, because inhibition of PI-PLC-ß1 expression by antisense RNA renders these cells far less responsive to IGF-1, but not to platelet-derived growth factor (Manzoli et al., 1997). A similar conclusion could be reached in a study in which nuclear PI-PLC-ß1 activity increase elicited by IGF-1 was blocked by a selective pharmacological inhibitor (Neri et al., 1998). In this case, there was no increase in intranuclear DG mass, PKC-α did not migrate to the nucleus, and Swiss 3T3 mouse fibroblasts did not enter S phase in response to IGF-1. Activation of nuclear PI-PLC-ß1 by IGF-1 has also been reported in HL60 human leukemia cells (Neri et al., 2002), but in this case the relationship with cell proliferation was not investigated.

How might PKC-α affect proliferation rates of Swiss 3T3 cells? Very recent findings have pointed out that, in NIH 3T3 mouse fibroblasts treated with powerful tumor promoter 12-myristate 13-acetate (PMA), PKC-α and PKC-ε activate the cyclin D1 and cyclin E promoters and thus markedly elevate the levels of both cyclin D1 and E. This results in higher proliferation rates. Up-

regulation of cyclin D1 expression is mainly mediated through the AP-1 transcription factor enhancer element present in the cyclin D1 promoter (Soh and Weinstein, 2003). We do not know whether or not it is nuclear PKC-α which is directly involved in this kind of regulation, but this is a possibility that should be investigated. In fact, PMA is known for inducing nuclear migration of PKC-α (Schmalz et al., 1996, 1998) and an increase in nuclear PKC-α and AP-1 transcriptional activity has been reported in B16 mouse melanoma cells in response to retinoic acid (Gruber et al., 1995).

It has also been shown that the overexpression of nuclear PI-PLC-ß1 commits mouse erythroleukemia (MEL) cells to proliferate even in the absence of serum and that this correlates with the activation of the cyclin D3/cdk4 system. As a consequence of this enforced expression, retinoblastoma protein is phosphorylated and E2F-1 transcription factor is activated as well (Faenza et al., 2000). It is presumed that the overexpression of PI-PLC-ß1 provides a tonic level of nuclear enzyme activity which approximates to that obtained in untransfected Swiss 3T3 mouse fibroblasts stimulated with IGF-1. However, at present it is unclear how increased expression of nuclear PI-PLC-ß1 could up-regulate cyclin D3 and cdk4. We might speculate that it is somehow linked with the emerging role played by inositol polyphosphates in controlling gene expression (Martelli et al., 2004).

2. Nuclear PI-PLC-ß1 and cell differentiation

There is additional evidence for a PI-PLC-ß1 role in nuclear signaling. In the case of the MEL cell mentioned above, DMSO-dependent erythroid differentiation is accompanied by a decrease in nuclear PI-PLC-ß1 enzymatic activity and protein as well as DG mass (Martelli et al., 1994; Divecha et al., 1995; Zini et al. 1995b). These findings might be in relationship with the increased in vitro phosphorylation of PtdIns 4,5 P_2 detectable in isolated nuclei of MEL cells (Cocco et al., 1987). Indeed, an increase in nuclear PtdIns 4 P mass (Martelli et al., 1995) due to a decreased PI-PLC-ß1 activity could result in enhanced PtdIns 4,5 P_2 synthesis. Conversely, differentiation is attenuated by maintaining high nuclear PI-PLC-ß1 levels via transfection of a PI-PLC-ß1 cDNA construct, whereas a mutant that lacks the nuclear localization sequence (NLS) has no effect (Matteucci et al., 1998). It is interesting that in cells overexpressing nuclear PI-PLC-ß1 there was a reduced amount of p45/NF-E2, a transcription factor that regulates ß-globin gene expression (Faenza et al., 2002). This observation might help explain why MEL cells with increased intranuclear amounts of PI-PLC-ß1 failed to differentiate in response to DMSO.

The findings with erythroid differentiation in MEL cells, however, contrast with the pattern of expression of PI-PLC-ß1 seen in other differentiating systems. For example, differentiation of C2C12 rat myoblasts in response to mitogen withdrawal and insulin stimulation is characterized by a marked increase in nuclear PI-PLC-ß1 (Faenza et al., 2003). In this case, the timing of PI-PLC-ß1 synthesis and its accumulation in the nucleus precedes that of the late muscle marker Troponin T by 24 h. Moreover, the expression of a transfected PI-PLC-ß1 mutant lacking the NLS acted as a dominant negative for nuclear translocation of PI-PLC-b1 and suppressed the differentiation of C2C12 myoblasts into multinucleate myotubes. These results suggest that nuclear PI-PLC-ß1 is a key player in myoblast differentiation by functioning as a positive regulator in this process.

The opposite results obtained with MEL cells and C2C12 myoblasts beg the question of whether there is a common mechanism in place. One possible explanation that encompasses all of the above systems is provided by the observation that some PKC isoforms (a target of DG produced by nuclear PI-PLC-ß1), depending on the timing of activation in the G1 phase, either positively or negatively regulates the cell cycle. For example, in vascular endothelial cells, the treatment of cells with phorbol ester in late G1 phase inhibits DNA synthesis, whereas it induces DNA synthesis in early G1 phase (Zhou et al., 1993, 1994). Further studies have shown that the late G1 effects are specific for the PKC-α isoform and are due to a direct suppression of E2F activation (Nakaigawa et al., 1996). These findings raise the possibility that nuclear PKC-α activation can have quite opposite outcomes at two independent points in cell cycle progression and further provide an explanation for the apparently contradictory association of nuclear PI-PLC-ß1 within different differentiation models.

Another mechanism specific for the differentiation of C2C12 myoblasts proposes that myogenic factors regulate not only tissue-specific gene expression but also the exit from the cell cycle. At the onset of differentiation, MyoD up-regulates cyclin D3 which then sequesters unphosphorylated retinoblastoma protein leading to irreversible exit of differentiating myoblasts from the cell cycle (Cenciarelli et al., 1999). In this connection, it is very interesting that our unpublished data reveal that down-regulation by siRNA of nuclear PI-PLC-ß1 during insulin-induced myogenic differentiation of C2C12 cells results in lowered expression of cyclin D3. Whether this mechanism has parallels with tissue-specific factors in other differentiation models remains to be seen, but our findings indicate that cyclin D3 expression is a specific target of nuclear PI-PLC-ß1 signaling in at least two unrelated experimental systems.

3. Nuclear PI-PLC-ß1 and meiosis resumption

A novel role for nuclear PI-PLC-ß1 has recently emerged. During the resumption of meiosis in the mouse oocyte, the enzyme translocates to the nucleus, apparently to perichromatin and interchromatin granules, and this is followed by a later shift to the nucleoplasm, as demonstrated by immuno-electron microscopy

analysis (Avazeri et al., 2000, 2003). Importantly, microinjection into the nucleus of an antibody to PI-PLCß1 blocked the germinal vesicle breakdown, that is part of meiosis. Also in this experimental system, nuclear migration of PI-PLC-ß1 has been related to DG generation and attraction to the nuclear compartment of DG-sensitive PKC isoforms -α, -ß1 and -ß2 (Avazeri et al., 2004).

PI-PLC-ß1 and disease

Our laboratory has recently shown the possible involvement of PI-PLC-ß1 in myelodisplastic syndrome (MDS). MDS constitutes a group of hematological disorders characterized by peripheral blood cytopenia secondary to bone marrow dysfunction and occurs predominantly in adult patients (usually >60 years of age). It evolves in acute myeloid leukaemia (AML) in about 30% of the cases after variable intervals from diagnosis (Steensma and Tefferi, 2003). The clinical transition is demonstrated by the clonal proliferation of the hematopoietic precursor that generates leukemic blasts unable to differentiate. It is considered that the evolution to AML is associated with additional genetic changes acquired by MDS patients. Moreover, AML evolving from MDS is much less responsive to chemotherapeutic agents than is *de novo* AML (Steensma and Tefferi, 2003). Approximately half of MDS patients have a detectable chromosome abnormality, usually a total or partial deletion of chromosome 5 or 7 and/or trisomy 8, whereas translocations and amplifications are not very frequent. Allelic loss has been found in chromosome 6q, 7p, 10p, 11q, 14q and 20q, and, even if there is no specific relationship between most of the rearrangements and the clinical outcome, MDS patients with abnormal karyotype are usually thought to be at a higher risk of developing AML than MDS patients having normal karyotype. Nevertheless, the management of MDS patients showing normal karyotype by means of classic cytogenitic techniques is still a problem. It has recently been observed that the clinical follow-up of these patients is not sufficient since some of them have surprisingly worse and poorer clinical outcomes than expected (Steensma and Tefferi, 2003).

Our group had previously mapped the gene encoding PI-PLC-ß1 to the short arm of chromosome 20 (Peruzzi et al., 2000).

In a group of AML patients with an undefined karyotype due to the presence of complex chromosome rearrangements and indecipherable markers, SKY analysis disclosed rearrangements of chromosome 20 consisting in total or partial gains or losses in five individuals. Using a specific probe for the PI-PLC-ß1 gene, FISH analysis disclosed the loss of one allele of the gene in all the patients examined (Lo Vasco et al., 2004). Rearrangements of the short arm of chromosome 20 have been detected in a number of patients with solid tumors but rarely in hematological disorders (Peruzzi et al., 2000). In all five patients the 20p rearrangement was associated with the deletion of PI-PLC-ß1 gene. Nevertheless the association with other chromosome aberrations hampered the definition of the role played by the 20p abnormalities in both the origin and the evolution of the disease (Lo Vasco et al., 2004).

More interesting are the data about patients affected by MDS or AML and having normal high resolution GTG banding karyotype. It has been found that the AML patients with PI-PLC-ß1 gene monoallelic deletion died in a time frame ranging from 1 to 12 months and all the MDS patients with the deletion died in a time frame ranging from 1 to 6 months after developing AML. The total painting for chromosome 20 resulted normal in all of the MDS and AML patients (Lo Vasco et al., 2004). To establish the amplitude of the deletion and the possible involvement of genes other than PI-PLC-ß1 within the 20p12 region, we used a probe for another gene localized in the same band, PI-PLC-ß4 gene (being the distance between the two genes as long as less than 0.1 Mb). FISH analysis revealed that all patients bearing the monoallelic deletion of PI-PLC-ß1 were normal as far as PI-PLC-ß4 gene was concerned, suggesting that the absence of one allele of PI-PLC-ß1 gene could be due an interstitial deletion as wide as less than 0.1 Mb, (Lo Vasco et al., 2004). Immunocytochemical analysis by means of anti PI-PLC-ß1 antibody, on all the AML and MDS patients that resulted normal at FISH analysis, showed normal staining of the nucleus. In contrast, all the AML and MDS patients bearing the monoallelic deletion of PI-PLC-ß1 gene show reduced immunostaining intensity when compared to controls using the same time of exposure.

It is worthwhile mentioning here that the clinical evolution and the progression of the disease of the MDS patients with monoallelic PI-PLC-ß1 gene deletion, has been worse than expected. Therefore, the genetic anomaly affecting a key signalling PI-PLC seems to be critical for pathophysiology of MDS and these data give the first clue that PI-PLC-ß1 might be involved in the progression of the disease. However, we do not know how deletion of one allele of the PI-PLC-ß1 gene might affect the evolution of MDS.

Concluding remarks

The availability of various new tools for phospholipid research has resulted, over the last five years, in several important discoveries in the field of nuclear PI-PLC-ß1. In particular, the mechanism of activation of PI-PLC-ß1 has been partly clarified and it seems to be peculiar to the nucleus.

One of the major challenges now is to identify other proteins nuclear PI-PLC-ß1 interact with, because this information will certainly be of great help in understanding the exact and multifaceted functions of this enzyme.

We feel that a better knowledge in the field of nuclear phosphoinositide metabolism may lead to new

and targeted therapeutic intervention, most likely in neoplastic disease, as indicated by our data about evolution of MDS and monoallelic deletion of PI-PLC-ß1. Indeed, given that the regulation of the nuclear inositol lipid cycle is different in many instances from the plasma membrane cycle, it should be possible to design drugs with the aim to specifically interfere at various steps of nuclear phosphoinositide-dependent signaling pathways.

The results reviewed here have set new stages for nuclear inositol lipid metabolism; the task at hand is to decipher its physiological relevance, to dissect the complexities of the various nuclear signaling networks and, ultimately, to elucidate the downstream targets. These and other future investigations will certainly enhance our knowledge and will highlight the multiple emerging roles played by phosphoinositides in the extremely complex nuclear microenvironment.

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