New frontiers of inositide-specific phospholipase C in nuclear signalling

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Strong evidence has been obtained during the last 16 years suggesting that phosphoinositides, which are involved in the regulation of a large variety of cellular processes in the cytoplasm and in the plasma membrane, are present within the nucleus. A number of advances has resulted in the discovery that nuclear phosphoinositides and their metabolizing enzymes are deeply involved in cell growth and differentiation. Remarkably, the nuclear inositide metabolism is regulated independently from that present elsewhere in the cell. Even though nuclear inositol lipids generate second messengers such as diacylglycerol and inositol 1,4,5-trisphosphate, it is becoming increasingly clear that in the nucleus polyphosphoinositides may act by themselves to influence functions such as pre-mRNA splicing and chromatin structure. This review aims at highlighting the most significant and up-dated findings about inositol lipid metabolism in the nucleus.

Key words: nucleus, inositide-specific phospholipase C, nuclear matrix, cell cycle, cell differentiation, regulation.

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The existence of a nuclear phosphatidylinositol (PtdIns) metabolism is now widely recognized (Martelli et al., 1999a) and hints at the nucleus as a functional compartment for PtdIns metabolism (Martelli et al., 2003). Indeed, it has been demonstrated that nuclei contain many of the enzymes involved in the classical PtdIns cycle, including kinases required for the synthesis of PtdIns(4,5)P2, inositide-specific phospholipase C (PI-PLC), and diacylglycerol kinase (DGK) (D'Santos et al., 1998; Martelli et al., 1999a; Cocco et al., 2001; Martelli et al., 2002a; Irvine, 2003). More importantly, specific changes in the nuclear PtdIns metabolism have been implicated in cell growth, differentiation, and neoplastic transformation (Divecha et al., 2000; Tamiya-Koizumi, 2002; Martelli et al., 2002b). The nucleus also contains 3-phosphorylated inositol lipids and the enzymes which synthesize them, i.e. phosphoinositide 3-kinases (PI3Ks) (Neri et al., 2002). The 3-phosphorylated lipids are not substrate for any know PI-PLC but act themselves as second messengers (Vanhaesebroeck et al., 2001).

In this review, we shall focus on nuclear PI-PLCs, namely PI-PLCβ1 and its involvement in cell proliferation and differentiation.

Nuclear polyphosphoinositide metabolism

Smith and Wells (1983) described the ability of isolated rat liver nuclear envelopes to synthesize *in vitro* phosphatidylinositol 4-phosphate (PtdIns(4)P), and phosphatidylinositol 4, 5-phosphate (PtdIns(4,5)P_2), even though the isolation of nuclei did not fulfill the rigorous criteria of purity, which allowed Cocco et al. (1987) to demonstrate, using detergent-treated murine erythroleukemia (MEL) cell nuclei, devoid of their envelope, that they were capable of synthesizing *in vitro* PtdIns(4)P and PtdIns(4,5)P_2, and nuclear PtdIns(4,5)P_2 synthesis became more pronounced if the cells were differentiated along the erythroid pathway by dimethyl sulfoxide (DMSO). To rule out cyto-

plasmic contamination of nuclear preparations the authors relied on electron microscopy analysis, measurement of enzymatic activities and/or immunoblotting analysis of markers for cytoplasmic constituents, such as glucose-6-phosphatase and β-tubulin, respectively. Indeed in the paper by Cocco et al. (1987) a strong clue for the existence of a separate nuclear inositide metabolism came from the observation that the striking changes in PtdIns(4,5)P_2 *in vitro* synthesis were only detectable in pure nuclear preparations and not in whole cell homogenates. In the following years, the availability of antibody probes to the enzymes of the inositol lipid cycle provided a stronger evidence that these molecules are localized also in the nucleus, by means of techniques that do not require organelle isolation, such as immunofluorescent staining. Nevertheless, it is clear that a nuclear fluorescent signal given by an antibody raised to a protein involved in phosphoinositide metabolism might also be interpreted as a consequence of either a cross-reaction and/or a fixation-generated artifact. The advent of green fluorescent protein (GFP) technology gave the opportunitys to study the enzymes involved in nuclear inositide metabolism in living cells (e.g. Bavelloni et al., 1999; Ciruela et al., 2000) overriding problems such as fixation, or inadequate antibody penetration. In addition, the identification of sequences which are essential to the nuclear localization of given enzymes and the possibility to express (in some instances as GFP hybrids) complementary DNA of the proteins of interest which are mutated, and have thus lost their capacity to localize in the nucleus, have been shown to represent other extremely valuable tools to unequivocally demonstrate intranuclear localization of phophoinositide metabolism-related enzymes, such as DGK, phosphatidylinositol 5-phosphate 4-kinase, and PI-PLCβ1 (Topham et al., 1998; Ciruela et al., 2000; Faenza et al., 2003).

Nuclear PI-PLCβ1, DAG and protein kinase C (PKC)α

The literature regarding nuclear PI-PLC is quite extensive (see Cocco et al., 2001) but the isoform that has been most consistently linked with signalling in the nucleus is PI-PLCβ1. The first hint that a PI-PLC activity might be activated in the nucleus came from experiments performed with quiescent Swiss 3T3 cells mitogenically stimulated with insulin-like growth factor-1(IGF-1). Within 2 min stimulation time, IGF-1 produced in membrane-stripped nuclei a decrease in in PtdIns(4)P and PtdIns(4,5)P_2 mass, a concomitant increase in DAG levels, and a translocation of protein kinase C (PKC) (Divecha et al.,1991). No changes in PtdIns(4)P, PtdIns(4,5)P_2, and DAG amount were detected in whole cell homogenates or in nuclei in which the envelope was still present. Bombesin, another powerful mitogen for these cells, stimulated inositide metabolism only at the plasma membrane level but not in the nucleus. These authors hypothesized about a possible role played by nuclear DAG to serve as chemoattractant for translocation of PKC to the nuclear compartment. In addition we demonstrated in nuclei of 3T3 mouse fibroblasts the presence of PI-PLCβ1 whose activity was up-regulated in response to IGF-1 stimulation (Martelli et al., 1992). In contrast, in these cells the -γ1 isoform of PI-PLC was confined to the cytoplasm. This kind of subcellular distribution of the two PI-PLC isoforms was subsequently confirmed in rat liver (Divecha et al., 1993). Nuclear PI-PLCβ1 plays an important role as a mediator of the mitogenic stimulus exerted by IGF-1 on Swiss 3T3 cells, because inhibition of PI-PLCβ1 expression by antisense RNA renders these cells far less responsive to IGF-1, but not to platelet-derived growth factor (Manzoli et al., 1997). If the function of nuclear DAG is to attract PKC isoforms within the nucleus, namely PKC-α (see Cocco et al., 2001 for more details) then it should conceivably exist a mechanism to turn off the signal. This role could be fulfilled by DGK, the enzyme which phosphorylates DAG yielding phosphatidic acid (PA) (Topham and Prescott, 1999). The fact that both isolated nuclear envelopes and nuclei produced *in vitro* radiolabeled PA, suggested the presence of DGK at the nuclear level (Smith and Wells, 1983; Cocco et al., 1987). Several independent laboratories have demonstrated the existence of DGK isoforms within the nucleus and have shown that this class of isozymes is indeed involved in controlling nuclear DAG mass after stimulation with a number of agonists (reviewed in Martelli et al., 2002a).

As to IGF-1-stimulated Swiss 3T3 cells, our laboratory has highlighted that exposure to this mitogen resulted in the stimulation of a nuclear DGK activity, but not of the same activity present in whole cell homogenate. An inverse relationship between nuclear DAG mass and DGK activity levels was shown by time course analysis. If 3T3 cells had been pre-incubated with two DGK pharmacological inhibitors, R59022 and R59949, the IGF-1-

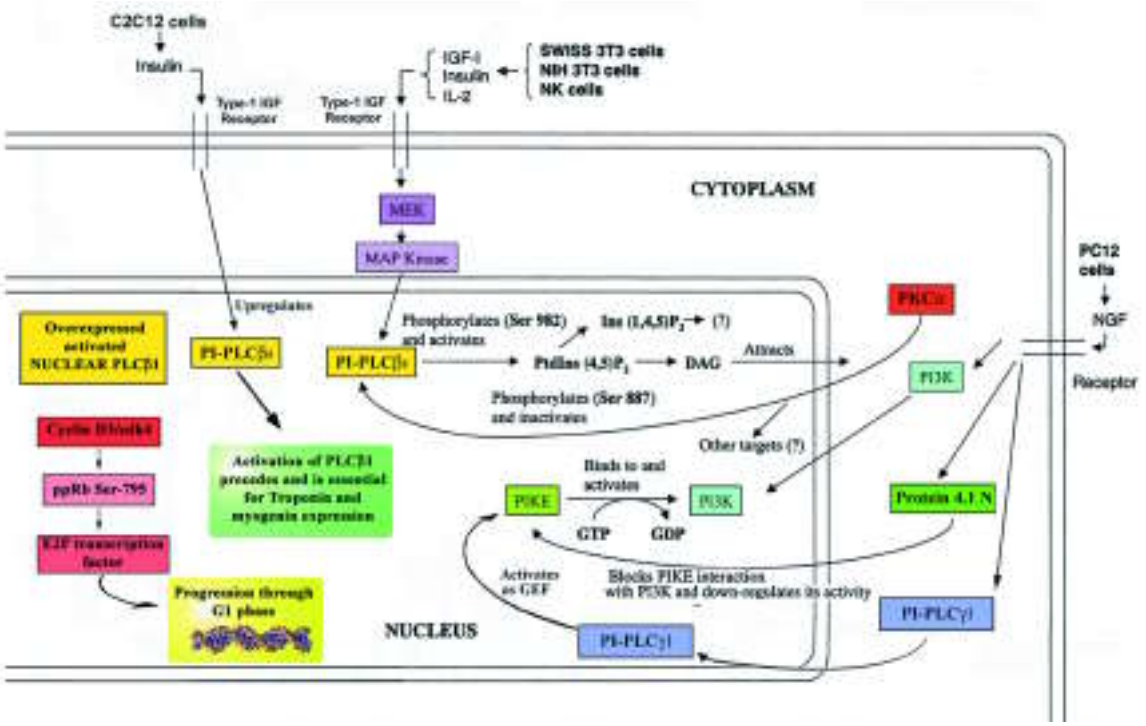

Figure 1. Schematic diagram summarizing the presence and signalling activity of nuclear PI-PLCs.

dependent rise in nuclear DGK activity was blocked and intranuclear levels of DAG stayed elevated for a longer period than in control cells. Also nuclear PKC-α activity remained higher in cells treated with the DGK inhibitors than in untreated cells. Furthermore, the two pharmacological inhibitors markedly potentiated the mitogenic effect of IGF-1. An inference based on these findings is that nuclear DGK plays a key role in regulating the levels of DAG present in the nucleus and that DAG and PKC-α are key molecules for the mitogenic effect which IGF-1 exerts on Swiss 3T3 cells (Martelli et al., 2000a).

Nuclear PI-PLCβ1 and its regulation

A key issue about nuclear PI-PLCβ1 is how it is regulated. The conventional view of PI-PLCβ1 activation comes from details of its action at the plasma membrane. It has been suggested that both Gαq/$_{11}$ and Gβγ_subunits can activate PI-PLCβ1. The region of PI-PLCβ1 that interacts with Gαq differs from that which interacts with Gβγ, the former binding to the extensive C-terminal tail characteristic of the PI-PLCβ isoforms while the latter has highest affinity for the N-terminal PH domain (Rebecchi and Pentyala, 2000; Rhee, 2001). Although there are reports that some subunits of heterotrimeric G-proteins, for example αi, can traslocate to the nucleus (Crouch and Simson, 1997), there is yet no evidence that αq/$_{11}$ is present within the nuclear compartment. Consistently, neither GTP-γ-S nor AlF4 stimulated nuclear PI-PLCβ1 *in vitro* activity (Martelli et al., 1996). A clue to a possible novel mechanism for activation of nuclear PI-PLCβ1 has come from the observation that it is hyper-phosphorylated in Swiss 3T3 nuclei in response to IGF-1 and that this is abolished by preventing the translocation of p42/44 mitogen-activated protein kinase (MAPK) to the nucleus (Martelli et al., 1999b). Supporting evidence was obtained with both insulin-treated NIH 3T3 cells (Martelli et al., 2000b) and IL-2 treated NK cells (Vitale et al., 2001) where activation of nuclear PI-PLCβ1 was blocked by PD098059, an inhibitor selective for the MAP kinase pathway. A more clear proof of a direct involvement of MAPK has come from the demonstration that, following IGF-1 stimulation of quiescent Swiss 3T3 cells, activated p42/44 MAPK translocates to the nucleus where it phosphorylates Ser 982 in the C-terminal tail of PI-PLCβ1 (Xu et al., 2001). This phosphorylation was inhibited by PD098059 and could be mimicked by recombinant PI-PLCβ1 protein and activated

MAPK *in vitro*. It is conjectured that phosphorylation *in vivo* might cause a recruitment of additional components which enhance PI-PLC_1 activity. The significance of Ser 982 phosphorylation to IGF-1 action, however, is seen in 3T3 cells stably transfected with PI-PLCβ1 harbouring a Ser 982 Gly mutation. This mutation acts in a dominant-negative manner on IGF-1 dependent mitosis (Xu et al., 2001) and this concurs with the findings of our previous report where PI-PLCβ1 was down regulated in 3T3 cells, resulting in loss of mitogenicity (Manzoli et al., 1997). By contrast, a PI-PLCβ1 Ser 982 Gly mutant which also lacks the nuclear localization sequence had no effect on the IGF-1 response (Xu et al., 2001). To our knowledge phosphorylation at this site is nucleus-specific and has not been reported for cytosolic PI-PLCβ1. This represents an activation mechanism which is distinct from that at the plasma membrane and peculiar to the actions of the nuclear phosphoinositide cycle. In addition, it is significant that within the PI-PLCβ family, the β1 isoform is the only one which possesses a MAPK phosphorylation site in its C-terminal tail.

Recent evidence suggests that PKC-α is involved in the regulation of nuclear PI-PLCβ1 in that PI-PLCβ1 is deactivated by PKC-α and that this is a critical step in attenuating the phospholipase activity that drives the nuclear inositol lipid cycle (reviewed in Cocco et al., 2002) .

Cell differentiation and nuclear PI-PLCβ1 signalling

The role of PI-PLCβ1 in nuclear signalling is strengthened by evidence obtained in differentiating systems. In case of MEL cell mentioned above, DMSO-dependent erythroid differentiation is accompanied by a decrease in nuclear PI-PLCβ1 enzymatic activity and protein as well as DAG mass (Martelli et al., 1994; Divecha et al., 1995). Conversely, MEL cells differentiation is attenuated by maintaining high nuclear PI-PLC β1 levels via transfection of a PI-PLC β1 cDNA construct, whereas a mutant that lacks the nuclear localization sequence (NLS) has no effect (Matteucci et al., 1998). It is interesting that in cell overexpressing nuclear PI-PLC β1 there was a reduced amount of p45/NF-E2, a transcription factor that regulates β-globin gene expression (Faenza et al., 2002). It has been shown that the overexpression of nuclear PI-PLC β1 commits MEL cells to progress into the G1 phase of the cell cycle even in the absence of serum and that this correlates with the activation of the cyclin D3/cdk4 system (Faenza et al., 2000). This supports the idea that differentiation requires withdrawal from the cell cycle and that the continued presence of PI-PLCβ1 in the nucleus maintains an undifferentiated, proliferative phenotype. However, at present is totally unclear how increased expression of nuclear PI-PLCβ1 could upregulate cyclin D3 and cdk4. In the physiological differentiation of C2C12 skeletal muscle cells in response to IGF-1 and insulin stimulation there is a marked increase in nuclear PI-PLCβ1 (Faenza et al., 2003). In this case, the timing of PI-PLCβ1 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-PLCβ1 mutant lacking the NLS suppressed the differentiation of C2C12 myoblasts into multinucleate myotubes. These results suggest that nuclear PI-PLCβ1 is also a player in myoblast differentiation by functioning as a positive regulator in this process, which is dependent on the activation of the type 1 IGF receptor, the same responsible for nuclear PI-PLCβ1 stimulation in quiescent 3T3 cells (see for a review Cocco et al., 2001). A possible link is that cyclin D3 is indeed a target of nuclear PI-PLCβ1 signaling since a mechanism specific for the differentiation of skeletal myoblasts implies that at the onset of differentiation, MyoD activates cyclin D3 which then sequesters unphosphorylated retinoblastoma protein leading to irreversible exit of differentiating myoblasts from the cell cycle (Cenciarelli et al., 1999).

PI-PLCs β 2,3,4, γ and δ in the nucleus

Molecular structure analysis has revealed that, among PI-PLC isozymes, the four members of the b family are unique, in that they contain a high proportion of basic residues located at their C-terminal domain. It has been demonstrated that this region is critical for allowing nuclear localization of these isozymes (Kim et al., 1996). Therefore, it came to no surprise that the -β2, -β3, and -β4 isozymes have been shown to be present in the nucleus of either HL60 or NIH 3T3 cells (Bertagnolo et al., 1997;Cocco et al., 1999). Since the amount of both PI-PLCβ2 and β3 isozymes increased in the nucleus following incubation of HL60 cells with either all-trans-retinoic acid (ATRA) or vitamin D3 (two differentiating agents, see later), it was concluded that these two members of the β family of PI-PLC

play some as yet unidentified important role at the nuclear level during differentiation of HL-60 cells (Bertagnolo et al., 1997).

Members of the -γ family of PI-PLC do not possess any known NLS. However, they have been reported to be present in the nucleus (e.g. Bertagnolo et al., 1995). In the nucleus of differentiated HL60 cells, PI-PLCγ1 forms an immunoprecipitable complex together with Vav (Bertagnolo et al., 1998). Since Vav possesses a nuclear localization signal, it might be that it facilitates intranuclear migration of PI-PLCγ1, conceivably through a piggy-back mechanism, as reported for other proteins such as IκBa (Turpin et al., 1999).

In this context the findings by Diakonova et al. (1997) appear interesting, given the demonstration that PI-PLCγ1 localized to the nucleus of highly transformed and proliferating cell lines but not to the nucleus of primary embryo skin or lung fibroblasts, where the enzyme was primarily cytoplasmic. An important suggestion from this study is that the differential subcellular localization of PI-PLCγ1 in normal or highly transformed cell lines could either be due to the degree of transformation of the cell type or be related with the S-phase of the cell cycle. The latter hypothesis seems more plausible because an increased amount of PI-PLCγ1 was detected in nuclei of 22 h regenerating rat liver (Neri et al., 1997).

PI-PLCδ1 has been reported to shuttle between the nucleus and the cytoplasm. Export from the nucleus requires a typical nuclear export sequence (NES), which was mapped at amino acid residues 164-177 of the EF-hand sequence. This leucine-rich functional NES is absent from PI-PLCδ4. Nuclear export of PI-PLCδ1 was sensitive to leptomycin B, a selective inhibitor of NES-dependent nuclear export (Yamaga et al., 1999). However, the functional significance of nuclear PI-PLCδ1 and of its export are at present undefined.

An 85-kDa PI-PLCδ4 isoform was found in nuclei from regenerating rat liver, serum-stimulated Swiss 3T3 cells, AH7974 rat ascites hepatoma cells and src-transformed 3Y1 cells, but not in nuclei from normal liver or quiescent fibroblasts (Liu et al., 1996). The nuclear δ4 isoform increases dramatically at the transition from the G_1 to the S phase, and remains at high levels to the end of the M phase (Liu et al., 1996). It has been claimed that this isoform is specific to the nucleus, although others have not confirmed this (Lee and Rhee, 1996).

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