[Frontiers in Bioscience 13, 2452-2463, January 1, 2008]

# Nuclear phospholipase C beta1 and cellular differentiation

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## 1. ABSTRACT

Phosphoinositides (PI) are the most extensively studied lipids involved in cell signaling pathways. The bulk of PI is found in membranes where they are substrates for enzymes, such as kinases, phosphatases and phospholipases, which respond to the activation by cell-surface receptors. The outcome of the majority of signaling pathways involving lipid second messengers results in nuclear responses finally driving the cell into differentiation, proliferation or apoptosis. Some of these pathways are well established, such as that of PI-specific phospholipase C (PI-PLC), which cleaves phosphatidylinositol-4,5-bisphosphate ($PIP_2$) into the two second messengers diacylglycerol (DAG) and inositol-1,4,5-trisphosphate ($IP_3$). Two independent cycles of PI are present inside the cell. One is localized at the plasma membrane, while the most recently discovered PI cycle is found inside the nuclear compartment. The regulation of the nuclear PI pool is totally independent from the plasma membrane counterpart, suggesting that the nucleus constitutes a functionally distinct compartment of inositol lipids metabolism. In this report we will focus on the signal transduction-related metabolism of nuclear PI and review the most convincing evidence that the PI cycle is involved in differentiation programs in several cell systems.

## 2. INTRODUCTION

The first demonstration of the existence of phospholipids in chromosomes comes from the 1958 work of La Cour and colleagues (1). In the 1970s, Manzoli and colleagues showed that addition of phospholipids to purified nuclei could influence *in vitro* transcription (2). In the early 1980s, the same group demonstrated that negatively charged lipids led to chromatin decondensation, while positive charged lipids had the opposite effect. In 1987, the first demonstration came from a work by Cocco et al., that a nuclear PI metabolism exists and it is regulated during cell differentiation (3). Since then, progress has been made on the regulation of nuclear PI, as well as their role in cellular functions, such as growth and differentiation. Nevertheless, much still needs to be understood about the function, regulation and physical properties of this nuclear component. For example, while it is clear that these PIs are not part of the nuclear envelope but they reside within the nuclear compartment, the physicochemical form of nuclear lipids still remains totally obscure (4).

We know today that lipid signaling molecules are essential components of that extremely complicated, multistep process that allows one extracellular signal to be transduced inside the cell, to the nucleus. In the nuclear

compartment, lipid second messengers elicit reactions that regulate gene transcription, DNA replication or repair, and DNA cleavage, eventually resulting in cellular differentiation, proliferation, apoptosis, or many other cell functions. Inositol-containing phospholipids are the most intensively studied lipid second messengers. Although most of the research on signal transduction pathways based on PIs has been devoted to phenomena that take place at the cell periphery and plasma membrane, it has become clear that the nuclear PI cycle is regulated in a totally independent manner from that at the plasma membrane level. This suggests that nuclear inositol lipids themselves can modulate nuclear processes, as important as transcription and pre-mRNA splicing, growth, proliferation, cell cycle regulation and differentiation.

Phosphatidylinositol(4,5)bisphosphate ($PIP_2$) is one central lipid molecule in the PI cycle. It is the substrate of enzymes involved in signal transduction, such as phosphatidylinositol-specific phospholipase C (PI-PLC) and phosphoinositide 3'-OH kinase (PI3K), thus producing the second messengers diacylglycerol (DAG), inositol trisphosphate ($IP_3$) and phosphatidylinositol(3,4,5)trisphosphate ($PIP_3$). $PIP_2$ has also been shown to be directly involved in chromatin remodeling, by binding to proteins such as histone H1 and the Brahma-related gene associated factor (BAF) complex (5, 6). This double function of $PIP_2$ in the nucleus, both as substrate for second messenger generation and as chromatin-remodeling element, adds further emphasis to the importance of the enzymes responsible for nuclear $PIP_2$ metabolism.

In this review we will confine our focus to the nuclear PI-PLC, and only briefly consider nuclear PI3K and PIP kinases. In particular, we will review the most updated literature on PI-PLC beta1, but it should be kept in mind that also PI-PLC gamma1 and PI-PLC delta1 isoforms are present in the nucleus and function in a cell cycle-dependent manner. In particular, PI-PLC gamma1 is activated in the cytosol by receptor tyrosine kinases and translocates to the nucleus, where it acts as a guanine nucleotide exchange factor (GEF) for the nuclear GTPase PI3K enhancer (PIKE), which subsequently activates PI3K.

## 3. NUCLEAR PHOSPHOINOSITIDES AND PI-PLC

### 3.1. The cycle of nuclear phosphoinositides

The nuclear PI signaling centers around the synthesis and metabolism of $PIP_2$, which can directly modulate the activity of effector proteins and is substrate of signaling generating enzymes. Following activation of nuclear PI-PLC, $PIP_2$ is hydrolyzed into the two products DAG and $IP_3$, which exert second messenger roles within the nucleus. In Swiss 3T3 cells treated with insulin-like growth factor-1 (IGF-1), a nuclear DAG increase has been related to protein kinase C (PKC)-alpha migration to the nucleus (7). With the blockade of PI-PLC activity, and subsequent lack of nuclear DAG increase, PKC-alpha fails to translocate to the nucleus (8). Also PKC-betaII migrates to the nucleus in response to a nuclear increase in DAG mass, in HL60 (9) and U937 human leukemia cells (10). These observations suggest that nuclear DAG is the attracting force that drives some PKC isozymes to the nucleus. Different PKC isoforms have their distinct localization inside the nucleus. While PKC-alpha has been seen in the nucleus interior except for nucleoli, the –betaII isoform localizes at the nuclear membrane (11), PKC-delta is only found in the nucleoli and PKC-epsilon is localized in the pore complex and nuclear envelope (12). PKC isozymes are involved in the regulation of DNA replication, RNA synthesis and processing, gene expression, nucleo-cytoplasmic transport and chromatin structure (for a recent review on nuclear PKC, see ref. (13)). Increased levels of nuclear PKC have also been related to mechanisms which regulate the differentiation of cells, mainly of hematolymphopoietic lineage (reviewed in ref. (14)), although there is a lack of functional studies to explain how PKC actually influences cell differentiation. We need to remind that PI is not the only nuclear source of DAG. Nuclear DAG can also be produced from phosphatidylcholine (PC) by phospholipase D and PA-phosphohydrolase. DAG derived from PI is polyunsaturated, while the PC-derived DAG is monounsaturated and saturated. Some investigators have suggested that only polyunsaturated DAG is the real second messenger responsible for PKC activation, while the monounsaturated and saturated DAG has no second messenger function (reviewed in (15, 16)).

$IP_3$ is the hydrophilic second messenger produced by PI-PLC-mediated hydrolysis of $PIP_2$, and may have a role in nuclear calcium ($Ca^{2+}$) regulation. In the cytosol, $IP_3$ binds to $IP_3$-receptors that release $Ca^{2+}$ from the intracellular storage, while the source of nuclear $Ca^{2+}$ is still controversial (17, 18). It is known that $Ca^{2+}$ pumps are present in the outer nuclear membrane, and there is evidence for the presence of functional $IP_3$ receptors that span the inner nuclear membrane with their binding domains facing the nucleoplasm. However, a formal link between $IP_3$ produced within the nucleus and nuclear $Ca^{2+}$ is still missing. Yet, locally produced $IP_3$ also has another important role in nuclear functions: it is the precursor of a different series of second messengers, the highly phosphorylated inositols, which have been recently implicated in chromatin remodeling and mRNA export (19-22).

$PIP_2$ is also substrate of another important signaling generating enzyme, the PI3K (for a review, see ref. (23)). The phosphorylation of $PIP_2$ by PI3K produces $PIP_3$, but this enzyme can also generate PI(3)P and $PI(3,4)P_2$. Members of the PI3K family are considered as oncogenes, because they control cell cycle progression, differentiation, survival, invasion and metastasis, and oncogenesis. Several biological effects of this enzyme are mediated through the downstream target Akt, a serine/threonine protein kinase. Both PI3K and Akt have been found in the nucleus (23). $PIP_3$ is then converted back to $PIP_2$ by the action of phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which can also produce PI(4)P from $PI(3,4)P_2$. In a significant number of cancers, PTEN is mutated or inactivated, resulting in the constitutive activation of the PI3K signaling pathway (24).

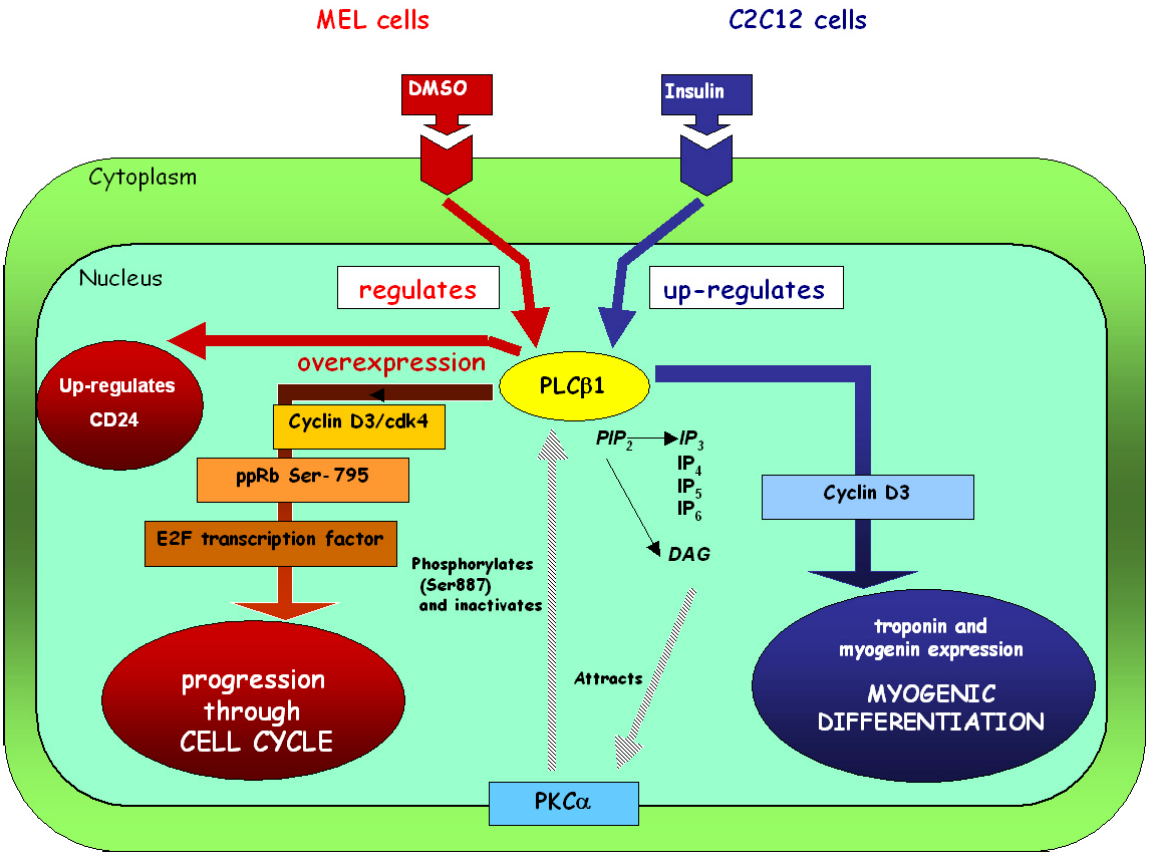


**Figure1**. PI-PLC beta1 is at the center of two differentiative signaling pathways. In **MEL cells** (red pathway), PI-PLC beta1 causes stimulation of cyclinD3/cdk4 system, pRb phosphorylation and E2F activation, with the final endpoint of cell cycle progression. PI-PLC beta1 signaling is responsible for the overexpression of CD24, an early surface marker for lymphopoiesis. In **C2C12 cells** (blue pathway), nuclear PI-PLC beta1 is involved in the differentiative pathway that is elicited by insulin and causes myogenic differentiation.

Nuclear DAG is phosphorylated by a highly regulated enzyme, DAG kinase (DGK), to yield another second messenger, phosphatidic acid (PA). This implies that DGK has a double function within the nucleus: to down-regulate the DAG-dependent signaling, and to produce PA. Among the nine mammalian DGK isozymes described until today, six of them have been detected in nuclei of different cell types (for a review, see ref. (25)). DAG and PA are also important intermediates in the biosynthesis of lipids.

An additional group of enzymes which are important in nuclear PI cycle, is the variety of kinases that phosphorylate PI in the 3', 4' and 5' positions of the inositol ring. These phosphate residues can be utilized by phosphatases to complete the cycle and increase the number of different PI phosphates isomers. Three classes of PI phosphate kinases (PIPK) have been described, the types I, II and III. Type I and II both generate $PIP_2$ although by utilizing different substrates. PIPKI-alpha and PIPKII-beta have been detected in the nucleus, and controversy still remains about the nuclear localization of PIPKII-alpha (26, 27). Also PI4K has a nuclear localization; in particular, it has been found associated to the nuclear matrix (28).

## 4. ROLE AND REGULATION OF NUCLEAR PIS AND PI-PLC

### 4.1. $PIP_2$ and chromatin remodeling

In 1982, Manzoli and colleagues showed that negatively charged lipids lead to chromatin decondensation, while positively charged lipids have the opposite effect (29). Since then, several observations confirmed the idea that phospholipids are responsible for chromatin structure and remodeling (30). $PIP_2$ binds to the C-terminal domain of histone H1. One mechanism proposes that $PIP_2$ binding releases H1 from DNA and reverses the inhibition of RNA polymerase II acted by histone H1 (6). Depletion and phosphorylation of histone H1 unfolds chromatin fibers and, in turn, increases transcriptional activity.

A different mechanism that explains how $PIP_2$ can modulate chromatin remodeling, involves BAF (5). The BAF complex is mainly soluble in resting T-lymphocytes, where nuclei are small and compact with dense heterochromatin. In response to T-lymphocyte stimulation, the nucleus increases with the appearance of

chromatin and activation of T-specific genes. At the same time, the BAF complex becomes associated with an insoluble nuclear fraction, corresponding with the nuclear matrix. Interestingly, BAF insolubilization can be mimicked by incubating resting T-cells nuclei with exogenously added $PIP_2$. However, the evidence that nuclear $PIP_2$ increases with T-cell activation is still missing. The BAF complex contains a SWI/SNF2 like ATPase core, BRG-1, and 12 more subunits (31). Two subunits are beta-actin and BAF53, an actin-related protein. Association of the BAF complex to the nuclear matrix requires the presence of all three subunits beta-actin, BAF53 and BRG-1 (21). BRG-1 has two actin-binding domains, one of them contains a lysine-rich region which is required for BRG-1 function and binds to $PIP_2$ (32). The idea is that $PIP_2$ disrupts the interaction of BRG-1 with actin resulting in the exposure of a site on actin which binds to the nuclear matrix. This mechanism would be analogous to $PIP_2$-mediated capping of actin, which stimulates actin polymerization (33). Interestingly, the retinoblastoma protein, which recuits the BAF comples to regulate gene expression (34), interacts and activates type I PIPK (35). Increased nuclear $PIP_2$, in turn, can lead to a stronger association of the BAF complex with the nuclear matrix. Jones and Divecha have proposed that the BAF complex interacts with and recruits type I PIPK to control localized $PIP_2$ synthesis (36).

Several chromatin regulating proteins contain a plant homeodomain (PHD) finger. Considering that a subset of PHD finger domains interacts with PIs, it has been proposed that modulating the amount of PIs in the nucleus may lead to regulating different PHD containing proteins. One example of such PHD-containing proteins is ASH2, which interacts with SKTL, a predicted Drosophila type I PIPK. ASH2 has bee shown to localize to the nucleus by immunohistochemistry, while SKTL has a nuclear localization signal (NLS). Both SKTL and ASH2 accumulate on polytene chromosomes. Since histone H1 hyperphosphorylation within euchromatin dramatically increases on *sktl* and *ash2* mutant polytene chromosome, it has been suggested that $PIP_2$ might play a role in maintaining transcriptionally active chromatin via histone H1 phosphorylation. During the assembly of nucleosomes, histone acetylation regulates histone H1 binding and chromatin condensation (37, 38). H1 displacement from the chromosome is necessary for H3 acetylation and transcription. SKTL/ASH2 chromatin remodeling complexes would bind to chromatin, where $PIP_2$ can be produced and can bind to H1. This interaction would lead to H1 displacement and prevent H1 hyperphosphorylation, which in turn leads to chromatin decondensation, histone acetylation and activation of transcription (39).

### 4.2. PI-PLC

PI-PLCs are soluble multidomain proteins ranging in molecular masses from 85 to 150 kDa (for a comprehensive review see ref. (40)). Four –beta, two –gamma and four –delta isoforms, with numerous splice variants, have been described in mammals. The sequences of eukaryotic PI-PLC contain a string of modular domains organized around a catalytic alpha/beta-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. Additional regulatory motifs are present in the –beta and –gamma subtypes.

In the crystallographic structure of PI-PLC-delta1, the catalytic domain is formed from the X and Y regions, of 147 and 118 residues respectively. While the requirement for inositol is absolute, various features of the polar headgroup affect substrate preference. Although the prokaryotic forms of PI-PLC prefer PI and PI-glycans, the eukaryotic enzymes have an order of preference that is generally $PIP_2$ > PI(4)P > PI. Neither form is capable of hydrolyzing the 3-phosphorylated PIs. A single calcium ion is bound to the active site. Although other domains in PI-PLC have the potential to bind calcium, the single catalytic calcium ion seems to be the only essential metal. Surrounding the active site is a ridge of hydrophobic residues, which could insert into the membrane surface in a process required for full enzymatic activity. Unlike the –beta and –delta subtypes, the X/Y-spanning polypeptide in PI-PLC-gamma is extensive, consisting of multiple adaptor domains. The PI-PLC-gamma isoforms contain two Src homology (SH)2, and SH3 and a single PH domain that engage both protein and lipid binding partners.

Most eukaryotic PI-PLCs contain a single PH domain of about 130 amino acids. Because PH domains lack any obvious catalytic properties and are found in proteins associated with the membranes, it was suggested that these domains function as adaptors or tethers, linking their host proteins to the membrane surface. Principal binding partners are PIs and the beta/gamma-subunits of heterotrimeric G proteins. Although the PH domains of the beta-isoforms may also serve as membrane tethers, they are not PI specific. PI-PLC beta1and –beta2 bind strongly to membranes regardless of the presence of these lipids. Moreover, PI-PLC beta1, PI-PLC beta2 and –beta3 fail to bind $IP_3$ or other inositol polyphosphate or polyphosphoinositide analogs.

PI-PLC isoforms have up to four EF-hand motifs, each consisting of a helix-loop-helix structure. The first two EF-hands present in mammalian, possess residues that appear capable of binding calcium or magnesium ions, however there is no evidence other that they actually bind metal ions. Although the EF-hand region may have an important regulatory function, it has yet to be identified.

C2 motifs, of about 120 residues, have been identified in numerous proteins other that PI-PLC, many of which function in lipid-signaling pathways. In the –delta1 isoform, three loops form the binding sites for up to three calcium ion. Disruption of these calcium sites fails to affect the calcium-dependent hydrolysis of $PIP_2$ in detergent micelles and PC bilayers. Both –beta and –gamma subtypes also contain C2 domain motifs, yet the key residues involved in calcium ligation are not conserved. Although these domains may have been retained as an integral part pf the PI-PLC catalytic core, they could also function in recognition of other regulatory lipids and proteins. The

later possibility is consistent with the findings that the PI-PLC beta1 C2 domain binds specifically to GTP-charged $alpha_q$, its physiological activator in the cytosol.

A single C2 domain and short peptide cap the C-terminal ends of –gamma and –delta isozymes, while –beta subtypes have extensions of about 400 amino acid residues that contain sequences important for membrane binding, nuclear localization, and their activation by G protein subunits. Deletion of this entire C-terminal region from PI-PLC beta1does not destroy catalytic activity but abolishes activation by $Galpha_q$ and related proteins in vitro and in living cells. Deletion of the C-terminus of PI-PLC beta1or PI-PLC beta1, also inhibits binding to PA, association of PI-PLC beta1to the particulate fraction of the cell. Most importantly for the purpose of this review, removal of the C-terminal region from the PI-PLC beta1 inhibits its transfer to the nucleus (40).

### 4.3. PI-PLC beta1 is present inside the nucleus, in nuclear speckles

Four PI-PLC beta isotypes and additional splice variants have been identified in mammals (for a comprehensive review see ref.(40)). These isoforms are regulated by heterotrimeric GTP-binding proteins, and have a high GTPase stimulating (GAP) activity. Mammalian PI-PLC betas are differentially distributed in tissues, with the PI-PLC beta1 being most widely expressed, especially in specific regions of the brain. PI-PLC beta1 exists as alternatively spliced variants beta1a and beta1b, which differ in their carboxy-terminal residues (41). In the cytoplasm, PI-PLC betas function as effector enzymes for receptors belonging to the rhodopsin superfamily of transmembrane proteins that contain seven transmembrane spanning segments. They are activated by a variety of stimuli and require special combinations of Galpha and Gbeta/gamma subunits to couple to the effector.

PI-PLC beta1 is the predominant isoform which is found inside the nucleus of several cell lines (42, 43), and the C-terminal is essential for the nuclear localization (44). The first demonstration came from observations on Swiss 3T3 mouse fibroblasts. These cells were brought to a quiescent state and then stimulated with the mitogen insulin-like growth factor-1 (IGF-1). When their membrane-stripped nuclei were analyzed, activation of PI-PLC was evidenced by a decrease in nuclear PI(4)P and $PIP_2$ mass and a concomitant increase of DAG levels, with a translocation of PKC to the nucleus. No changes in PI(4)P, $PIP_2$ and DAG amount was seen in total cells homogenate and in nuclei in which the nuclear envelope was still present. On the contrary, bombesin stimulated PI-PLC activity at the plasma cell level and not in the nucleus (7). These observations suggested a PI-PLC activation specifically inside the nucleus, and that the nuclear activation of PI-PLC was totally independent from the PI metabolism at the plasma membrane level. The following year, our group demonstrated that the IGF-1-responsive PLC activity in the nucleus of Swiss 3T3 cells was PI-PLC beta1 (42).

The evidence that the nucleus contains PIs and PI cycle enzymes, leads to the next question: where are they located in the nucleus? We know that the PIs and enzymes involved in nuclear signaling are not located at the nuclear envelope, and are separate from defined membrane structures, but may be in a protein-lipid compartment. Using immunohistochemical approaches, at least half of the nuclear $PIP_2$ localizes at electron-dense intranuclear particles (45). Interestingly not only $PIP_2$, but also PIPKIalpha and PIPKIIbeta are localized in the same site (26, 45). These intranuclear foci are separate from the nuclear envelope or invaginations of the envelope that span in the nucleus. Colocalization studies with pre-mRNA splicing factors revealed that these particles are interchomatin granule clusters involved in mRNA processing, also known as “nuclear speckles”. They are thought to be storage sites for splicing factors, they contain small nuclear ribonucleoproteins (snRNP) and a hyperphosphorylated form of RNA polymerase II (46-48), and probably play a role in coupling transcription to mRNA splicing (49, 50). Nuclear speckles change morphology during transcription; they become smaller and more numerous when transcriptional activity is increased, while they appear larger and fewer with inhibition of transcription (51, 52). $PIP_2$ colocalizes with the splicing factor SC35, hyperphosphorylated RNA polymerase II, snRNAs, snRNP and with Sm protein, a snRNP component used as marker for nuclear speckle. Interestingly, $PIP_2$ colocalization appears during interphase and not during mitosis, suggesting that $PIP_2$ containing complexes undergo dynamic changes during cell cycle. Also, immunodepletion of $PIP_2$ and its interacting proteins from nuclear extracts of HeLa cells results in an inhibition of mRNA splicing *in vitro* (45).

In addition to PIPKs and $PIP_2$, also PI-PLC beta1 has been reported to localize to nuclear speckles, together with DGK theta, PI-PLC delta4, PI3K C2alpha, PTEN and SHIP2 (28, 53, 54). Our group demonstrated an association between PI-PLC beta1 and both DGK zeta and PIPKalpha, in immunoprecipitation experiments with a PI-PLC beta1 specific antibody. With immuno-electron microscopy we also showed that DGK theta, PI-PLC beta1 and $PIP_2$ are associated to electron-dense particles within the nucleus, that correspond to nuclear speckles as revealed by using the antibody against SC-35 (55).

These findings suggest that nuclear speckles are important sites of nuclear PI signaling, and strengthen the idea that nuclear PIs play a role in mRNA processing. One possible mechanisms is that $PIP_2$ is involved in regulating these processes by interacting with the enzymatic core of the splicesome or cytoscheletal proteins such as protein 4.1, which localizes with nuclear speckles and splicing factors (56). Another possibility, is that $PIP_2$ acts only as substrate for PI-PLC beta1, producing second messengers which, in turn, regulate nuclear events. As mentioned in the next paragraph, one known role of DAG is to act as nuclear attractor for PKC-alpha, or as substrate for DGK. $IP_3$, instead, can be further phosphorylated to generate several inositol phosphates which, in yeast, regulate transcription and RNA export (19, 20).

### 4.4. Nuclear PI-PLC beta1 has a role in mitogenesis

Our laboratory showed that the role of PI-PLC beta1 is essential in the IGF-1 mitogenic signaling pathway, because downregulation of this enzyme by antisense RNA causes an insensitivity of Swiss 3T3 cells to IGF-1, but not, for example, to platelet-derived growth factor (57). This was confirmed by a separate study, where the IGF-1-dependent nuclear PI-PLC beta1 activity increase was blocked by a selective pharmacological inhibitor. In this case, there was no increase in nuclear DAG mass, PKC-alpha failed to translocate to the nucleus and Swiss 3T3 cells did not enter the S phase (8).

The PI-PLC beta1-dependent increase of nuclear DAG is then turned off by the activation of nuclear DGK, which converts DAG to PA. Exposure of Swiss 3T3 cells to IGF-1 also causes stimulation of nuclear DGK activity. Inhibition of DGK by pharmacological inhibitors results in elevated DAG mass and a retention of PKC-alpha in the nuclear compartment for a longer period of time. As a final outcome of the treatment with the DGK inhibitors, IGF-1 mitogenic stimulation is markedly enhanced (58). One plausible interpretation is that DAG levels in the nucleus attract PKC-alpha to the nucleus, which is essential in the mitogenic signaling elicited by IGF-1. More recent studies have shown that in NIH3T3 mouse fibroblasts which were treated with the tumor promoter 12-myristate 13-acetate (PMA), PKC-alpha and PKC-epsilon activate the cyclin D1 and cyclin E promoters, resulting in increase of the two cyclins and, in turn, higher proliferation rates (59). This offers a possible mechanism by which PKC-alpha could affect the proliferation rate of these cells. In fact, PMA is known for inducing nuclear migration of PKC-alpha (60, 61), and increase of nuclear PKC-alpha and AP-1 transcriptional activity has been reported in B16 mouse melanoma cells in response to retinoic acid (62).

### 4.5. Regulation of PI-PLC beta1

As described above, PI-PLC betas have a C-terminal domain which is important for Galpha protein binding, as well as membrane binding and nuclear localization. There is no doubt that in the cytoplasm both $Galpha_q$/$alpha_{11}$ and Gbeta/gamma subunits activate PI-PLC beta1. Gbeta/gamma, differently from the Galpha subunit, interacts with N-terminal PH domain of the PI-PLC. But Galpha proteins probably fail to regulate the nuclear PI-PLC beta1 signaling because, although some reports show that $Galpha_i$ can translocate to the nucleus (63), there is no evidence that $Galpha_q$/$alpha_{11}$ is present in this compartment. This is also supported by the observation that neither GTP-gamma-S nor $AlF_4^-$ stimulate PI-PLC beta1 in nuclei form MEL cells (64).

Among the PI-PLC betas, the PI-PLC beta1 isoform is the only one to have a p42/44 mitogen-activated protein kinase (MAPK) phosphorylation site in the C-terminal tail. Thus, one candidate for PI-PLC beta1 regulation is MAPK. In fact, if MAPK nuclear translocation is inhibited in Swiss 3T3 cells, the IGF-1-induced hyperphosphorylation of PI-PLC beta1 is abolished (65). This is confirmed in insulin-treated NIH 3T3 cells (66) and IL-2 treated human primary Natural Killer cultures (67), where inhibition of MAPK with PD098059 results in a lack of activation of nuclear PI-PLC beta1. IGF-1 stimulation of quiescent Swiss 3T3 cells causes p42/44 MAPK activation and translocation to the nucleus, where it phosphorylates PI-PLC beta1 in Ser 982, in the C-terminal domain (68). This phosphorylation, though, is not sufficient to activate PI-PLC beta1. One hypothesis is that the phosphorylation might cause a recruitment of additional regulatory elements which enhance PI-PLC beta1 activity. A Ser 982 Gly mutation of PI-PLC beta1 acts as a dominant negative for IGF-1 induced mitogenesis (68). By contrast, a PI-PLC beta1 Ser 982 Gly mutant which also lacks the nuclear localization sequence has no effect in the response to IGF-1. Hence, this may be an activation mechanism which is distinct from that at the plasma membrane level, and peculiar to the actions of the nuclear PI cycle.

The transient activation of nuclear PI-PLC beta1 can be terminated via PKC-alpha translocation to the nucleus (69). Phosphopeptide mapping and site directed mutagenesis have indicated that PKC-alpha phosphorylates PI-PLC beta1 at Ser 887. Overexpression in Swiss 3T3 cells of a Ser 887 Ala mutant or a dominant negative PKC-alpha mutant evoked a sustained activation of nuclear PI-PLC beta1 in response to IGF-1 stimulation (69). It still remains to be explained how this phosphorylation actually acts to inactivate PI-PLC beta1 activity. In different studies, it has been shown that *in vitro* phosphorylation of recombinant PI-PLC beta1 by activated PKC-alpha fails to alter the PLC activity, while it appears to affect the PI-PLC beta1 interaction to Gbeta/gamma (70, 71). This implies a colocalization of both up- and down-regulatory mechanisms involving Ser 887 and suggests a role for Gbeta/gamma subunits in the process. Another hypothesis can be drawn, that PKC-alpha mediated phosphorylation of PI-PLC beta1 causes a change in the interaction with other nuclear proteins, yet to be identified.

## 5. ROLE OF NUCLEAR PI-PLC BETA1 IN CELLULAR DIFFERENTIATION

### 5.1. PI-PLC beta1 in mouse erythroleukemia

In 1987, our laboratory showed that nuclear PI metabolism changes during dimethyl sulfoxide (DMSO)-induced erythroid differentiation of a mouse erytholeukemia cell line (MEL) (3). Subsequently, we demonstrated that the DMSO-induced differentiation of these cells is accompanied by a progressive decrease of replicative activity and accumulation of nuclear $PIP_2$ (72). The increased *in vitro* synthesis of $PIP_2$ in nuclei of differentiating MEL cells led to the novel idea that a decrease of nuclear PI-PLC activity may occur during erythroid differentiation, and that the nuclear PI-PLC activity could be the target for anti-leukemia drugs. Tiazofurin, an antitumor drug capable of inducing differentiation of K562 and MEL cells, was then shown to inhibit a nuclear PI-PLC activity with a concomitant accumulation of nuclear $PIP_2$ mass (73). This was supported by the findings that DMSO-induced differentiation is associated with a decrease of PI-PLC beta in the nucleus of MEL cells. Also PI-PLC gamma is

present in these nuclei, but the expression of this isoform remains unchanged during differentiation (74). This observation was confirmed by a different group, who also showed that the specific isoform involved in DMSO-induced differentiation is PI-PLC beta1 (43). At the same time, we demonstrated that also the tiazofurin-induced decrease of nuclear PI-PLC activity was due to a decrease of the PI-PLC beta1 isoform (75). These observations inspired a series of investigations, aimed to establish the function of PI-PLC beta1 in erythroid differentiation.

First of all, the nuclear localization of PI-PLC beta1 was shown to be crucial for the differentiation of MEL cells. Indeed, when wild type PI-PLC beta1 is overexpressed in MEL cells, the DMSO-induced differentiation is inhibited and the expression of beta-globin is almost completely abolished, together with the activity of the p45/NF-2E transcription factor. Instead, when a mutant lacking the ability to localize at the nucleus is expressed, the cells differentiate and the expression of beta-globin, a marker for erythroid differentiation, increases in response to DMSO (76). A later report confirmed that the specifically nuclear overexpression of PI-PLC beta1a and PI-PLC beta1b significantly reduces the expression of p45/NF-E2 transcription factor, which is an enhancer binding protein for the expression of the beta-globin gene. Again, the expression of the mutant lacking the ability to localize at the nucleus did not affect the level of the hematopoietic factor, as compared to wild type cells. In order to establish whether p45/NF-2E is a specific target for nuclear PI-PLC beta1, the behaviour of GATA-1, -2 and -3 was examined in DMSO-induced MEL cells. GATA transcription factors are important regulators of gene expression in hematopioetic lineages. The results showed that GATA-1 and -3 are present in these cells and are induced my DMSO, but totally unaffected by changes of PI-PLC beta1 expression. This led to the conclusion that PI-PLC beta1 specifically acts on p45/NF-E2 (77).

To determine whether nuclear PI-PLC beta1 is involved in cell cycle progression, cyclin D3, cyclin E, cdk2 and cdk4 protein expression was determined in serum starved and serum-stimulated MEL cells. In hematopoietic cells lines, upregulation of cyclin D2 and D3 prevents granulocyte differentiation (78), and both cyclins, in complex with cdk4 or cdk6 and cyclinE-cdk2 phosphorylate pRb, a well known tumor suppressor. pRb is important in gating S phase entry through its ability to repress genes activated by theE2F family of transcription factors. The results showed that the nuclear overexpression of PI-PLC beta1is directly responsible for the expression and activation of cyclin D3-cdk4 complex, which is known to stimulate progression through G1. Cyclin E, which instead is responsible for the G1-S transition of the cell cycle, is not affected by PI-PLC beta. Also pRb and E2F, downstream targets of cyclin D3-cdk4 complex pathway, are activated by PI-PLC beta1 (79).

Another clue for a role of PI-PLC beta1 signaling during differentiation comes from recently published data showing that nuclear PI-PLC beta1 upregulates the expression of CD24 in MEL cells. CD24 is an antigen involved in differentiation and hematopoiesis, it is considered as a critical molecule in the metastasizing ability of solid tumors and is overexpressed in a number of leukemias. When PI-PLC beta1 expression is abolished with the use of small interfering RNA, CD24 expression is down-regulated. The regulation of PI-PLC beta1 on CD24 is mediated at the transcriptional level at least in part, since PI-PLC beta1 affects the promoter activity of CD24. Moreover, the upregulation of CD24 is higher during erythroid differentiation of MEL cells (80).

A different target of nuclear PI-PLC beta1 in MEL cells has been identified with a proteomic approach. The protein profiles of wild-type MEL cells and nuclear PI-PLC beta1-overexpressing MEL cells were compared. The protein SRp20 appeared to be differentially expressed in the two systems, being down-regulated in cells with PI-PLC beta1 overexpression. Immunoprecipitation and colocalization analysis then followed, to show that PI-PLC beta1 interacts with SRp20 at the nuclear level (81). SR proteins are nuclear phosphoproteins (82) found in nuclear speckles. Some SR proteins, like SRp20 are not confined at the nucleus, but shuttle continuously between nucleus and cytoplasm, probably exerting a role in the communication between the two compartments (83). Also, it has been shown that SRp20 is a novel downstream target of insulin signaling in hematopoietic cells, and that the insulin-induced reduction of SRp20 effectively enhances an insulin-dependent accumulation of cyclin D3 (84).

### 5.2. PI-PLC beta1 in muscle cells differentiation

The C2C12 myogenic cell line has been widely used to study muscle cell differentiation, and the involvement of PI-PLC beta1 in this process. Muscle development is characterized by a few well defined steps. The first step involves the determination of which cell will give rise to the myoblast, then the proliferating myoblasts withdraw from the cell cycle, to align and fuse to form multinucleate myotubes. Before the onset of muscle differentiation, proliferating myoblasts express the two myogenic transcription factors MyoD and Myf5. Once activated, MyoD and Myf5 induce the withdrawal of myoblasts from the cell cycle and expression of myogenin and transcription factors from the MEF2 family. These two groups of protein cooperate for the activation of muscle structural genes (85). Skeletal muscle contraction, instead, is regulated by Troponin and Tropomyosin. The Troponin complex is constituted of a $Ca^{2+}$-binding subunit, Troponin C, Troponin I and Tronin T. The latter subunit is responsible for binding to Tropomyosin and is used as a marker of differentiation (86).

C2C12 cells contain at least 3 PI-PLC beta isoforms: PI-PLC beta1, -beta3 and -beta4. PI-PLC beta1 is mainly located at the nucleus, while -beta3 is both in the nucleus and in the cytoplasm, and -beta4 is only found at the cytoplasm. During insulin-induced myoblast differentiation, PI-PLC beta1 is greatly increased at the nuclear level, while the expression of -beta3 remains unchanged (87).

A more detailed look at the involvement of PI-PLC beta1 in muscle differentiation, evidenced that both PI-PLC beta1a and PI-PLC beta1b isoforms are increased

in the nucleus during myogenesis in C2C12 myoblasts. The nuclear increase occurs during the first 24 hours of differentiation, which corresponds to very early stages of differentiation, when the myogenic marker Troponin T is not yet expressed. The nuclear localization of PI-PLC beta1 is essential for induction of differentiation, as seen by overexpressing a PI-PLC beta1 mutant which loses nuclear localization. When this mutant is expressed, it stays in the cytoplasm and acts as a dominant negative for nuclear localization of PI-PLC beta1 (76). As a result of the lack of PI-PLC beta1 nuclear localization, insulin-induced myogenesis is inhibited, as assessed by myogenin expression (88).

An important function of nuclear PI-PLC beta1 activation during early myoblast differentiation has been highlighted in a recent study, which evidenced that PI-PLC beta1 regulates cyclin D3 expression by acting on its promoter (89). Even though the expression of most cyclins is down-regulated during cell cycle arrest, a notable exception is cyclin D3. In fact, cyclin D3 expression plays a critical role in the Myo-D-mediated arrest of cell cycle which precedes myoblast differentiation. At the onset of differentiation, MyoD activates cyclin D3, which then sequesters unphosphorylated retinoblastoma protein (pRb), leading to the irreversible withdrawal of differentiating myoblasts from the cell cycle (90). Consistently with a role of cyclin D3 in muscle differentiation, it has been shown that cyclin D3 5'noncoding region contains several E-boxes with binding sites for myogenic transcription factors which activate muscle-specific structural genes (91). In C2C12 cells, PI-PLC beta1 and PI-PLC gamma1 expression is low in undifferentiated state, and increases as myoblasts elongate and fuse. They reside in different subcellular compartments, with PI-PLC beta1 mostly in the nucleus and -gamma1 in the cytoplasm. Both PI-PLC enzymes are required for myogenin expression and activation of cyclin D3 promoter during the differentiation of myoblasts to myotubes, although affecting different regions of the cyclin D3 gene. This indicates that both isoforms are crucial regulators of the mouse cyclin D3 gene, and that insulin-induced PI-PLC signaling activates at least two different lipid-dependent signaling pathways.

The relationship between function and location of PI-PLC beta1 and PI-PLC gamma1 seems to be supported by data showing the importance of the nuclear organization to achieve muscle differentiation. Nuclear PI-PLC beta1 could be involved in nuclear lamina reorganization by acting on cyclin D3. In fact, it has been demonstrated that changes in internal lamin A/C organization in muscle cells are influenced by cyclin D3 with the involvement of pRb (92).

Nuclear PI-PLC beta1 activation during early skeletal muscle differentiation could also be important because it causes $IP_3$ increase, which mediates $Ca^{2+}$ release. It has been demonstrated that the basal level of nuclear $IP_3$ increases significantly in dystrophic muscle when compared to normal cells (93). Therefore, PI-PLC beta1 could have a role in muscle hypertrophy taking place in human muscular dystrophy.

Co-localization studies have evidenced that in C2C12 cells, nuclear PI-PLC beta1 is bound to DGK-zeta, in nuclear speckles. Like PI-PLC beta1, also nuclear DGK-zeta increases during myoblast differentiation, and impairment of DGK-zeta upregulation markedly inhibits differentiation (94). The physical binding of these two enzymes could have a functional meaning. In fact, PI-PLC beta1 produces DAG, which is the substrate for DGK. DGK produces PA which, in turn, has been shown to activate PI-PLC beta1 by binding to its C-terminal domain (95). It should be noted that DGK-zeta interacts with, and is activated by the Rb family proteins pRb, p107 and p130 (96).

Interestingly, it has been proposed that the different subcellular expression of PI-PLC beta isoforms during the establishment of myotube differentiation is related to a spatial-temporal signaling event, involved in myogenic differentiation. Once again, the subcellular localization appears to be a key element for the diverse signaling activity of PI-PLC betas (87).

## 6. PERSPECTIVE

The existence of a PI cycle inside the nucleus is well established. It constitutes an autonomous lipid-dependent signaling system, independently regulated from its plasma membrane counterpart and it modulates cell cycle progression and differentiation. Among all the PI-PLC isoforms, we have focused to the PI-PLC beta1 because it is the most extensively investigated in the nuclear compartment. PI-PLC beta1 is a key player in the regulation of nuclear inositol lipid signaling, and, as discussed above, its function could also be involved in nuclear structure because it hydrolyses $PIP_2$, a well accepted regulator of chromatin remodeling.

The findings on the role of nuclear PI-PLC beta1 in erythroid differentiation in MEL cells, are somehow in contrast with its role in muscle differentiation of C2C12 rat myoblasts. In both cases, nuclear localization is crucial for the function of PI-PLC beta1. Yet, while in MEL cells DMSO-dependent erythroid differentiation is accompanied by a decrease of PI-PLC beta1, C2C12 myoblasts have a marked increase of nuclear PI-PLC beta1 in response to mitogen withdrawal and insulin stimulation. Of course, every cell type represents a potentially unique biological system, and what looks like discrepancies may simply represent the basis of biological divergence of cells functions. Nevertheless, this contrast leads to the question of whether there is a common mechanism of action of nuclear PI-PLC beta1 in the differentiation of different cell types.

One possible explanation is provided by the observation that some PKC isoforms, which are target of PI-PLC-derived DAG, either positively or negatively regulate the cell cycle, depending on the timing of activation during the G1 phase. For instance, in vascular endothelial cells, the treatment of cells with phorbol esters in late G1 phase inhibits DNA synthesis, while it induces DNA synthesis in early G1 phase (97, 98). Further studies

have shown that the late G1 effects are specific for the PKC-alpha isoform and are due to a direct suppression of E2F activation (99). These observations pose the possibility that nuclear PKC-alpha activation can have opposite outcomes if activated at two independent points in the cell cycle progression, and provide an explanation for the apparently contradictory association of nuclear PI-PLC beta1 in different differentiation models.

Another common element of PI-PLC beta1 function in the differentiation of MEL cells and C2C12 cells is the activation of cyclin D3. Nuclear PI-PLC beta1 activates cyclin D3 in both systems. This cyclin, then, has an opposite effect in the two cell types, promoting the differentiation of myoblasts to myotubes in the case of C2C12 cells, and stimulating the progression through the G1 phase of the cell cycle in the case of MEL cells(100).

One less direct, but very intriguing, link between PI-PLC beta1 and differentiation comes from the finding that one allele of this enzyme is deleted in a subset of high risk multiple displacement syndrome (MDS) patients. These patients have a normal karyotype, but show an unpredictable aggressive disease course and develop acute myeloid leukemia in a shorter time frame, in comparison to MDS patients whose alleles for PI-PLC beta1 are both intact (101, 102).

## 7. ACKNOWLEDGEMENTS

This work was supported by AIRC, Italian Ministry of University and Research FIRB and Cofin grants and CARISBO Foundation**.**

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**Key Words:** Phospholipase C, nucleus, Differentiation; Transcription Factor, Review

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