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Nuclear Inositol Lipid Metabolism: More Than Just Second Messenger Generation?

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Abstract A distinct polyphosphoinositide cycle is present in the nucleus, and growing evidence suggests its importance in DNA replication, gene transcription, and apoptosis. Even though it was initially thought that nuclear inositol lipids would function as a source for second messengers, recent findings strongly indicate that lipids present in the nucleus also fulfil other roles. The scope of this review is to highlight the most intriguing advances made in the field over the last few years, such as the possibility that nuclear phosphatidylinositol (4,5) bisphosphate is involved in maintaining chromatin in a transcriptionally active conformation, the new emerging roles for intranuclear phosphatidylinositol (3,4,5) trisphosphate and phosphoinositide 3-kinase, and the evidence which suggests a tight relationship between a decreased level of nuclear phosphoinositide specific phospholipase C-β1 and the evolution of myelodisplastic syndrome into acute myeloid leukemia. J. Cell. Biochem. 96: 285–292, 2005.

Key words: nucleus; PtdIns(4,5)P_2; chromatin organization; phosphoinositide 3-kinase; apoptosis; phospholipase C; myelodisplastic syndrome

The presence in the nucleus of both polyphoshoinositides and the enzymes responsible for their metabolism has suggested that they may constitute a signaling system [Divecha et al., 2000; Irvine, 2003]. It is noteworthy that nuclear inositol lipid metabolism is independently regulated from its plasma membrane counterpart and is modulated in response to short-term growth factor signaling, cell cycle progression and during differentiation [Martelli et al., 2004]. Central to the regulation of nuclear inositol lipid signaling is PI-PLC. PI-PLC catalyzes PtdIns(4,5)P_2 hydrolysis to yield two fundamental second messenger: Ins(1,4,5)P_3 and DG. Several PI-PLC isoforms have been identified in the nucleus but the -β1 isozyme is the best characterized [Cocco et al., 2001]. In response to short term stimulation of

Abbreviations used: AML, acute myeloid leukemia; BAF, Brahma-related gene associated factor; CK2, casein kinase 2; DFF40/CAD, DNA fragmentation factor/caspase activated DNase; DG, diacylglycerol; DMSO, dimethylsulfoxide; FISH, fluorescent in situ hybridization; GEF, guanine nucleotide exchange factor; IGF-1, insulin-like growth factor-1; ING2, inhibitor of growth protein 2; Ins(1,4,5)P_3, inositol (1,4,5) trisphosphate; IRS-1, insulin receptor substrate-1; MAPK, mitogen-activated protein kinase; MDS, myelodisplastic syndrome; MEL, mouse erythroleukemia; NGF, nerve growth factor; PDK1, phosphoinositide-dependent kinase-1; NLS, nuclear localization signal; PHD, plant homeodomain; PKC, protein kinase C; PIKE, phosphoinositide 3 kinase enhancer; PI-PLC, phosphoinositide-specific phospholipase C; PIPkinase, phosphatidylinositol 4-phosphate-5-kinase; PI3K, phosphoinositide 3-kinase; PtdIns, phosphatidylinositol; PTEN, phosphatase and tensin homolog deleted on chromosome ten; SKTL, skittles; UBF1, upstream binding factor 1.

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quiescent Swiss 3T3 cells with IGF-1, nuclear PI-PLC-β1 is activated by p42/44 MAPK-dependent phosphorylation and subsequently down-regulated by another phosphorylation step effected by PKC-α, which is attracted to the nucleus by the DG produced by PI-PLC-β1 [Xu et al., 2001a,b]. Overexpression of PI-PLC-β1 in the nucleus is sufficient to drive 3T3 cells into S phase [Xu et al., 2001a]. In MEL cells, up-regulation of nuclear PI-PLC-β1 activity correlates with increased progression through the cell cycle [Faenza et al., 2000], whereas DMSO-induced differentiation causes down-regulation of nuclear PI-PLC-β1, an increase in nuclear phosphoinositide, and a decrease in nuclear DG mass [Divecha et al., 1995]. Moreover, a rise of nuclear PtdIns and PtdIns(4,5)P_2 mass takes place as synchronized NIH 3T3 cells progress from G_0 to G_1/S boundary [Stallings et al., 2005]. Therefore, in response to a variety of signals, activation/deactivation of PI-PLC-β1 and inositide metabolizing enzymes will generate different patterns of PtdIns, PtdIns(4,5)P_2, and DG within the nucleus [Martelli et al., 2003]. The D3-phosphorylated phosphoinositide PtdIns(3,4,5)P_3, which is the product of PI3K, is also present in the nucleus [Neri et al., 2002]. PtdIns(3,4,5)P_3 in the cytoplasm activates a variety of kinases, including Akt and PDK1, therefore influencing many cell responses [Prestwich, 2004]. However, the nuclear processes regulated by the PI3K signaling system have not yet been fully elucidated, even though it has been shown that nuclear PtdIns(3,4,5)P_3 is important for recruiting to the nucleus PKC-ζ [Neri et al., 1999]. A fundamental issue would be to understand whether the role of nuclear inositol lipids is restricted to the generation of second messengers or whether these lipids may function by themselves to regulate nuclear processes.

In this review, we shall highlight the most recent findings suggesting an involvement of PtdIns(4,5)P_2 in chromatin organization, as well as emerging data on new functions played by nuclear PI3K and PtdIns(3,4,5)P_3. Finally, we shall overview the evidence which links decreased levels of nuclear PI-PLC-β1 to the evolution of MDS in AML.

PtdIns (4,5)P_2 AND CHROMATIN ORGANIZATION

Previous findings dating back to the seventies showed that addition of phospholipids to purified nuclei could influence in vitro transcription. Indeed, negatively charged lipids led to chromatin decondensation, whereas positively charged lipids had the opposite effect [Manzoli et al., 1982]. Support to the hypothesis of a phosphoinositide-mediated control of transcription has come from an in vitro study which showed that in a *Drosophila* in vitro transcription system, PtdIns(4,5)P_2, when present at 10 μM, counteracted the histone H1-mediated repression of basal transcription by RNA polymerase II [Yu et al., 1998].

Moreover, a novel mechanism for the regulation of chromatin structure by inositol lipids came with the unexpected discovery that the interaction of the chromatin remodeling complex BAF could be regulated by the level of PtdIns(4,5)P_2 [Zhao et al., 1998]. Resting T-lymphocytes have small, compact nuclei with dense heterochromatin which, upon antigenic stimulation, increase in size with the appearance of euchromatin. These chromatin changes are conceivably required for the activation of T-cell specific genes. In response to T-lymphocyte stimulation with an antibody to the T-cell receptor, the BAF complex becomes associated with an insoluble nuclear fraction, conceivably corresponding to the nuclear matrix. Interestingly, BAF insolubilization could be mimicked by incubating resting T-cell nuclei with exogenously added PtdIns(4,5)P_2. However, even though these findings are consistent with a role for PtdIns(4,5)P_2 in regulating BAF complex localization, there is no evidence which indicates an increase in nuclear PtdIns(4,5)P_2 mass in response to T-cell activation. What are the molecular mechanisms underlying the interaction between PtdIns(4,5)P_2 and BAF? The BAF complex is composed of several proteins including actin, BAF53, and Brg1, which has ATPase activity. Brg1 has two domains which can interact with actin, one of which contains a lysine-rich stretch [Bourachot et al., 1999]. This lysine-rich domain is required for Brg1 function in vivo and importantly is capable of interacting with PtdIns(4,5)P_2 [Rando et al., 2002]. It might be that PtdIns(4,5)P_2 disrupts the interaction of Brg1 with actin resulting in the exposure of a site on actin which binds to the nuclear matrix. In other words, the mechanism would be analogous to PtdIns(4,5)P_2-mediated uncapping of actin, which stimulates actin polymerization [Schafer et al., 1996]. Interestingly, the retinoblastoma protein, which recruits BAF

complex to regulate gene expression [Olave et al., 2002], interacts with and activates type I PIPkinase, the enzyme which synthesizes nuclear PtdIns(4,5)P_2 [Divecha et al., 2002]. Therefore, it has been suggested that BAF chromatin remodeling complex may interact with and recruit type I PIPkinase to control localized PtdIns(4,5)P_2 synthesis [Jones and Divecha, 2004].

Further support to the hypothesis of a PtdIns(4,5)P_2-mediated control of chromatin organization has come from an investigation which shows that SKTL, a predicted *Drosophila* type I PIPkinase, interacts in vitro and in vivo with ASH2, a trithorax group protein [Cheng and Shearn, 2004]. The gene *sktl* encodes a protein that is 59% identical to human type I PIPkinase and 58% identical to mouse type I PIPkinase. Interestingly, SKTL possesses a NLS, while ASH2 is known to localize to nuclei by immunohistochemistry. Most significantly for this review, ASH2 contains a PHD finger, i.e., a putative double zinc finger involved in mediating protein–protein interactions and modifying chromatin structure. Furthermore, PHD fingers may act as domains capable of binding nuclear polyphosphoinositides [Gozani et al., 2003]. Both SKTL and ASH2 accumulate on polytene chromosomes. Since histone H1 hyperphosphorylation within euchromatin dramatically increased on *sktl* and *ash2* mutant polytene chromosomes, it has been suggested that PtdIns(4,5)P_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 [Ridsdale et al., 1990; van Holde and Zlatanova, 1996]. Displacement of histone H1 is required prior to target gene acetylation and transcription activation, because histone H1 inhibits histone H3 acetylation by hindering the access of histone acetyltransferases to histone H3 tail. It has been predicted that chromatin remodeling complexes would contain components that modify histone H1-chromatin interactions [Herrera et al., 2000]. Thus, it might be hypothesized that the PHD finger of ASH2 would bind PtdIns(4)P which could then be processed to PtdIns(4,5)P_2 by SKTL. When the ASH2-SKTL complex binds to chromatin, a source of PtdIns(4,5)P_2 could bind to and displace histone H1. The displacement of histone H1 would prevent its hyperphosphorylation and allow for chromatin decondensation, histone acetylation, and, eventually, activation of transcription [Cheng and Shearn, 2004].

NEW ROLES FOR NUCLEAR PI3K AND PtdIns (3,4,5)P_3

Class I PI3Ks were originally characterized as lipid kinases, although more than 10 years ago they were also shown to phosphorylate protein serine residues [Foukas and Shepherd, 2004]. So far, two proteins have been clearly identified as substrates for PI3K. One of them is the p85α regulatory subunit of PI3K itself, which is phosphorylated on Ser608 by the p110α catalytic subunit [Foukas et al., 2004]. This phosphorylation step down-regulates the lipid kinase activity and occurs in vivo in response to stimulation with growth factors, including insulin and platelet-derived growth factor. In contrast, p110β and p110δ autophosphorylate on Ser^{1070} and Ser^{1039}, respectively [Vanhaesebroeck et al., 1999; Czupalla et al., 2003]. Also in these cases, autophosphorylation results in a decreased lipid kinase activity. The other protein substrate for PI3K has been identified as IRS-1 which is serine phosphorylated in response to insulin or interferon challenging [Lam et al., 1994; Uddin et al., 1997].

However, a new potential protein substrate for PIK has been recognized in the nucleus. Recent evidence has shown that the UBF1, a regulator of RNA polymerase I activity which resides exclusively in the nucleolus, is phosphorylated in response to IGF-1 stimulation of mouse fibroblasts or myeloid cells [Drakas et al., 2004]. Following IGF-1 stimulation, IRS-1 migrates to nucleoli where it associates with UBF1 and the p110β subunit of PI3K. Considering that: (a) IRS-1 is a powerful activator of PI3K; (b) a highly purified PI3K was capable of phosphorylating UBF in vitro; (c) in vivo and in vitro phosphorylation of UBF1 was decreased by the PI3K inhibitor LY294002; it was concluded that PI3K is the most reasonable candidate for an IGF-mediated phoshorylation and activation of UBF1 [Drakas et al., 2004; Wu et al., 2005]. Although these findings appear very intriguing, we feel that, to reach this conclusion, additional experiments would be required. First, it would be necessary to demonstrate by immunocytochemistry that p110β migrates to nucleoli in response to IGF-1 challenging or it is resident there. Secondly, phosphopeptide map

analysis should be performed to show that in vivo phosphorylation pattern of UBF1 is equivalent to its in vitro phosphorylation by purified PI3K. Indeed, it is known that UBF1 can be phosphorylated also by p42/44 MAPK and CK2 [Voit et al., 1999; Stefanovsky et al., 2001]. Interestingly, CK2 is inhibited by LY294002 [Tolloczko et al., 2004] and there are reports showing that LY294002 could also block MAPK activation [Zhuang et al., 2004]. Furthermore, p42/44 MAPK is a classical downstream target of IGF-1 [e.g., Radcliff et al., 2005].

PI3K/Akt pathway is by far the most important signaling network for cell survival [Downward, 2004]. Traditionally, anti-apoptotic signaling by PI3K/Akt has been thought to take place at the plasma membrane level and in the cytoplasm [Franke et al., 2003]. However, recent findings point to the likelihood that also nuclear PI3K plays an essential role in promoting cell survival through nuclear PtdIns $(3,4,5)P_3$ and Akt. In PC12 cells, NGF treatment elicits powerful anti-apoptotic signaling cascades [Patapoutian and Reichardt, 2001]. In response to NGF, PI-PLCγ1 translocates to the nucleus where it acts as a physiological guanine-nucleotide-exchange factor for PIKE-S [Ye et al., 2002]. PIKE-S is a nuclear-specific GTPase that enhances PI3K activity and is regulated by protein 4.1N [Ye et al., 2000; Ye and Snyder, 2004]. Although PIKE was initially thought to reside only in the nucleus of nervous cells, it has been detected also in rat liver nuclei [Klein et al., 2004]. Therefore, PIKE might be a widespread regulator of nuclear PI3K activity. PI3K migrates to the PC12 cell nucleus in response to NGF [Neri et al., 1999]. Taking advantage of a cell-free system, it has been shown that nuclei isolated from NGF-treated PC12 cells were resistant to DFF40/CAD-dependent DNA fragmentation initiated in vitro by activated cell-free apoptotic solution, consisting of HEK293 cell cytosol supplemented with purified active caspase-3 [Ahn et al., 2004]. Nuclei from constitutively active PI3K adenovirus-infected cells displayed the same resistance as those treated by NGF, whereas PI3K pharmacological inhibitors, immunodepletion of PI3K from nuclear extracts with anti-p110 antibody, and dominant negative PI3K or PIKE abolished it. PtdIns $(3,4,5)P_3$ alone, but not PtdIns $(3,4)P_2$, PtdIns $(4,5)P_2$ or PtdIns (3)P, mimicked the anti-apoptotic effect of NGF. The involvement of nuclear PtdIns $(3,4,5)P_3$ in the protecting role of NGF was also substantiated by an experiment in which isolated nuclei were preincubated with PTEN (which dephosphorylates PtdIns $(3,4,5)P_3$ to PtdIns $(4,5)P_2$) and then analyzed for DNA fragmentation. It was found that PTEN pretreatment abolished the protective effect of NGF, even though it was not demonstrated that PTEN actually decreased the amount of PtdIns $(3,4,5)P_3$. In this connection, a good control would be constituted, in our opinion, by a mutated PTEN lacking the lipid phosphatase activity [Ramaswamy et al., 1999]. Since NGF treatment stimulates migration of phosphorylated Akt to the nucleus of PC12 cells [Borgatti et al., 2003], the role of nuclear Akt in the antiapoptotic action of NGF was also examined. It turned out that nuclei isolated from cells overexpressing wild type or constitutively active Akt were resistant to internucleosomal DNA cleavage, whereas those from dominant-negative Akt-infected cells showed DNA cleavage in spite of NGF treatment, demonstrating that nuclear Akt is required for NGF-mediated anti-apoptotic signaling (see Fig. 1). Nevertheless, in the absence of NGF treatment all the nuclei displayed DNA degradation, suggesting that Akt activation alone is not sufficient to inhibit DNA cleavage. The down-stream effectors of nuclear PtdIns $(3,4,5)P_3$ which prevent apoptosis have yet to be identified, however it should be emphasized that nuclear PtdIns(5)P modulates p53 activity and cell death through the interaction with its nuclear receptor ING2 [Gozani et al., 2003].

Furthermore, it should be emphasized that PIKE-mediated NGF-dependent cell survival also in intact cells, because apoptosis induced by staurosporine was much higher in PC12 cells in which PIKE has been knocked down by means of antisense oligonucleotides [Ahn et al., 2004].

NUCLEAR PI-PLC-β1 AND DISEASE

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-β1. Nuclear PI-PLC-β1 has been linked with either cell proliferation or differentiation [Cocco et al., 2001]. Our laboratory has recently shown the possible involvement of PI-PLC-β1 in MDS. MDS constitutes a heterogeneous group of hematological disorders characterized by peripheral blood cytopenia secondary to bone marrow dysfunction and

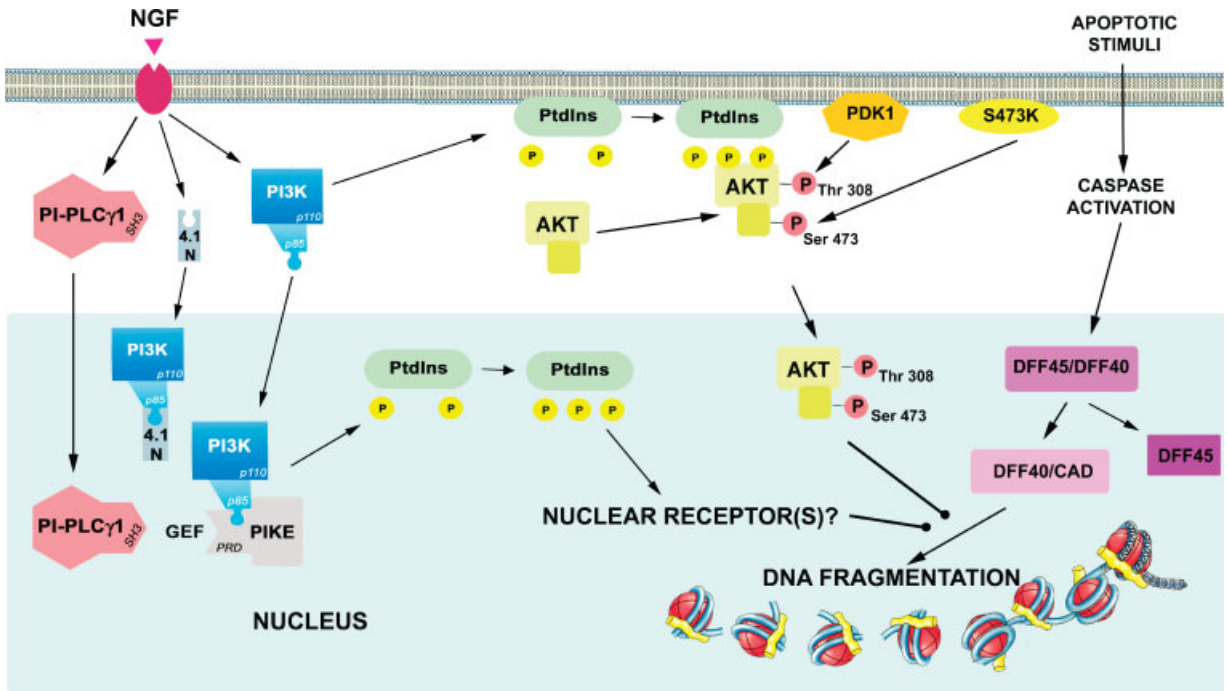

Fig. 1. Nerve growth factor (NGF) elicits nuclear PtdIns(3,4,5)P_3-dependent anti-apoptotic signaling in PC12 cells. In response to NGF treatment, there is intranuclear migration of a class IA PI3K (composed of a p110 catalytic subunit and a p85α regulatory subunit). Concomitantly, also PI-PLCγ1 translocates to the nucleus. The SH3 domain of PI-PLCγ1, acting as GEF, stimulate the activity of the nucleus-specific GTPase, PIKE. The target of the PI-PLCγ1 SH3 domain is one of the three proline-rich domains (PRD) of PIKE. PIKE then interacts with the p85α subunit of PI3K whose activity is stimulated. PI3K synthesizes intranuclear PtdIns(3,4,5)P_3 from PtdIns(4,5)P_2. Later on, protein 4.1N enters the nucleus to down-regulate PI3K activity. In non-apoptotic cells, DFF exists in the nucleus as a heterodimer, composed of a 45-kDa chaperone and inhibitor subunit (DFF45) (also called inhibitor of CAD (ICAD-L)) and a 40-kDa latent nuclease subunit (DFF40/CAD). Apoptotic activation of caspase-3 or -7 results in the cleavage of DFF45/ICAD and release of active DFF40/CAD nuclease [Widlak and Garrard, 2005]. PtdIns(3,4,5)P_3, through its interaction with yet unidentified nuclear receptor(s), blocks DFF40/CAD-dependent DNA fragmentation. To this end, phosphorylated (activated) nuclear Akt is also necessary but not sufficient. Akt recruiting at the plasma membrane requires PtdIns(3,4,5)P_3 synthesized by receptor-associated PI3K, while its phosphorylation is dependent on PDK1 and another, as yet unidentified, protein kinase (S473K).

occurs predominantly in adult patients (usually >60 years of age). It evolves in 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 [Hofmann et al., 2004]. 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 for developing AML than MDS patients having normal karyotype [Tchinda et al., 2003]. Nevertheless, the management of MDS patients showing normal karyotype by means of classic cytogenetic 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 outcome than expected [Steensma and Tefferi, 2003].

Our group had previously mapped the gene encoding PI-PLC-β1 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-β1 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-β1 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 appear the data about patients affected by MDS or AML and having normal high resolution GTG banding karyotype. We found that the AML patients with PI-PLC-β1 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-β1 within the 20p12 region, we used a probe for another gene localized in the same band, PI-PLC-β4 gene (being the distance between the two gene as long as less than 0.1 Mb). FISH analysis revealed that all patients bearing the monoallelic deletion of PI-PLC-β1 were normal as far as PI-PLC-β4 gene was concerned, suggesting that the absence of one allele of PI-PLC-β1 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-β1 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-β1 gene show reduced nuclear immunostaining intensity when compared to controls.

It is worthwhile mentioning here that the clinical evolution and the progression of the disease of the MDS patients with monoallelic PI-PLC-β1 gene deletion, has been worse than expected. Therefore, the genetic anomaly affecting a key signaling PI-PLC seems to be critical for pathophysiology of MDS and these data make the first clue that PI-PLC-β1 might be involved in the progression of the disease. However, we do not know how deletion of one allele of the PI-PLC-β1 gene with a consequent reduction of the amount of nuclear PI-PLC-β1 protein might affect the evolution of MDS. It might be that such a reduction negatively affects the differentiation program of MDS blasts. This would be somehow in contrast to our previous findings in MEL cells, because DMSO-induced erythroid differentiation is accompanied by a reduction of nuclear PI-PLC-β1 levels [Martelli et al., 1994]. Nevertheless, differentiation of C2C12 rat myoblasts in response to mitogen withdrawal and insulin stimulation is characterized by a marked increase in nuclear PI-PLC-β1 [Faenza et al., 2003]. Moreover, the expression of a transfected PI-PLC-β1 mutant lacking the NLS acted as a dominant negative for nuclear translocation of PI-PLC-β1 and suppressed the differentiation of C2C12 myoblasts into multinucleate myotubes. These results suggest that nuclear PI-PLC-β1 is a key player in myoblast differentiation by functioning as a positive regulator in this process. Thus, it might be that, depending on cell system and stimuli, nuclear PI-PLC-β1 has opposite effects on the outcome of the differentiation.

CONCLUDING REMARKS

Evidence reviewed here strongly suggests that the role of nuclear inositol metabolism goes beyond the generation of second messengers. The identification of nuclear inositide-binding proteins has established potential novel functions for these lipids within the nucleus, such as chromatin organization control and mRNA splicing [Osborne et al., 2001]. Signals that induce processes such as differentiation, proliferation, and apoptosis induce changes in inositol metabolism. We feel that a combination of compartmentalized and temporal changes in nuclear PtdIns $(4,5)P_2$ or PtdIns $(3,4,5)P_3$ might be detected by phosphoinositide-binding proteins to elicit different cellular responses, including changes to regulate gene expression, DNA replication or chromatin degradation. Moreover, the identification of a disease such as MDS whose malignant evolution seems to be tightly dependent on the amount of nuclear PI-PLC-β1

suggests that inositol lipids and their metabolizing enzymes might become interesting targets for the development of new therapeutic strategies for the treatment of cancer.

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