Minireview

Re-examination of the mechanisms regulating nuclear inositol lipid metabolism

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Abstract Although inositol lipids constitute only a very minor proportion of total cellular lipids, they have received immense attention by scientists since it was discovered that they play key roles in a wide range of important cellular processes. In the late 1980s, it was suggested that these lipids are also present within the cell nucleus. Albeit the early reports about the intranuclear localization of phosphoinositides were met by skepticism and disbelief, compelling evidence has subsequently been accumulated convincingly showing that a phosphoinositide cycle is present at the nuclear level and may be activated in response to stimuli that do not activate the inositol lipid metabolism localized at the plasma membrane. Very recently, intriguing new data have highlighted that some of the mechanisms regulating nuclear inositol lipid metabolism differ in a substantial way from those operating at the cell periphery. Here, we provide an overview of recent findings regarding the regulation of both nuclear phosphatidylinositol 3-kinase and phosphoinositide-specific phospholipase C-β1.

Key words: Nucleus; Inositol lipid; Phosphatidylinositol 3-kinase; G protein; Phospholipase C-β1; Phosphorylation; Mitogen-activated protein kinase; Protein kinase C

1. Introduction

Inositol phospholipids represent a very minor fraction of cell phospholipids, yet they are involved in a number of key functions in several signaling pathways and in membrane traffic [1,2]. Indeed, it is hard to find a cell process in which inositol phospholipids have not been implicated. In the 'canonical' inositol lipid cycle, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) is hydrolyzed by means of a phosphoinositide-specific phospholipase C (PI-PLC) in response to a wide variety of stimuli, including growth factors, hormones, and neurotransmitters, that act on specific receptors localized at the plasma membrane. This reaction generates two second messengers of paramount importance, 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) [3,4]. The former may either activate DAG-dependent protein kinase C (PKC) isoforms or be converted to phosphatidic acid, which also has signaling functions; the latter liberates Ca^{2+} from intracellular stores [5]. More recently, the discovery of the D-3 phosphorylated inositides, such as phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3), and the enzyme which synthesizes them, i.e. phosphatidylinositol 3-kinase (PI3K), has considerably renewed the interest of cell and molecular biologists for phosphatidylinositol metabolism [6]. D-3 phosphorylated inositides are not substrates for phospholipases, but serve as second messengers themselves and regulate a plethora of cell functions.

Since the late 1980s, it was suggested that similar inositol lipid metabolism operates within the nucleus [7]. These early findings were met with disbelief, and rightly so, because it was generally thought to be the consequence of nuclear preparations being contaminated with residues of the plasma membrane. Indeed, the isolation of any intracellular organelle is always likely to suffer from different degrees of contamination with other organelles. However, in the following years, data coming from independent laboratories (reviewed in [8–12]) unequivocally demonstrated that the nucleus is endowed with its own inositide cycle. The recent widespread use of molecular biology techniques has greatly contributed to the demonstration that the results regarding nuclear inositide cycle are not a consequence of contamination. A characteristic feature of the inositol lipid cycle present in the nucleus is its operational distinctiveness from the cycle present at the plasma membrane. In other words, many agonists that stimulate the membrane cycle do not activate the nuclear cycle and vice versa. In other cases, if an agonist stimulates both cycles, it does so in a temporally distinct manner [8–12].

Since the nucleus contains inositol lipid cycle enzymes that are apparently the same as those involved at the plasma mem-

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Abbreviations: DAG, 1,2-diacylglycerol; IGF-I, insulin-like growth factor-I; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; MAP, mitogen-activated protein; MEL, mouse erythroleukemia; NGF, nerve growth factor; PH, pleckstrin homology; PKA, protein kinase A; PKC, protein kinase C; PI-PLC, phosphoinositide-specific phospholipase C; PIKE, phosphoinositide kinase enhancer; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PI3K, phosphatidylinositol 3-kinase; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate

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brane level, it was assumed that their regulation should be similar. However, recent studies have highlighted that the regulation of the nuclear polyphosphoinositide metabolism may be quite different from the plasma membrane cycle.

Here, we shall review the most recent findings regarding the regulation of nuclear PI3K and PI-PLC-β1, i.e. two key enzymes of the inositol lipid cycle. These data further strengthen the notion that the nuclear polyphosphoinositide metabolism is independent of the plasma membrane cycle.

2. Nuclear D-3 phosphorylated inositides and PI3K

The D-3 phosphorylated inositides found in mammalian cells are PtdIns(3)P, PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PtdIns(3,4,5)P_3. Resting mammalian cells contain significant levels of PtdIns(3)P, but hardly any of the other D-3 phosphorylated inositides. While the overall levels of PtdIns(3)P do almost not increase upon cell stimulation, the levels of the other D-3 phosphorylated inositides can rise sharply [13]. Even though these lipid molecules are not the target of any known phospholipases, they are metabolized by phosphatases that act on the inositol ring. PTEN is a 3′-phosphatase which has received a lot of attention recently, because it was previously identified as a tumor suppressor gene. PTEN converts PtdIns(3,4)P_2 to PtdIns(4)P, and PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2. In a significant number of human cancers, PTEN is mutated and inactivated so that PI3K signaling is constitutively activated as a result of the high PtdIns(3,4,5)P_3 levels [14].

There are multiple isoforms of PI3K in mammalian cells, and these are subdivided into three classes, referred to as I, II, and III [6,13]. The most studied are class I PI3Ks, because they are generally coupled to extracellular stimuli. They display a preference in vivo for PtdIns(4,5)P_2. Class IA PI3Ks consist of a p110 catalytic subunit (α, β, and δ) and a p85 regulatory adaptor subunit (α, β). In contrast, class IB PI3K or PI3Kγ is made of a p110γ catalytic subunit and a p101 regulatory subunit, unrelated to p85. p110γ can be activated in vitro by the βγ subunits of heterotrimeric G proteins [6].

A number of reports have highlighted the presence of PI3K in the nucleus of different cell types, including neurons, hepatocytes, human osteosarcoma and promyelocytic leukemia cells (reviewed in [10,12]). Both class IA (e.g. [15]) and class IB (e.g. [16]) PI3Ks have been detected in the nucleus or translocate there in response to various stimuli. Moreover, Bacqueville et al. [17] have shown the presence of a 117-kDa PI3K, immunologically related to p110γ, in the nucleus of pig aorta vascular smooth muscle cells.

Very recently, the presence of a class II PI3K, PI3K C2β, has been reported in the rat hepatocyte nucleus [18]. Interestingly, PI3K C2β activity increased at 20 h after partial hepatectomy, suggesting it plays a role in liver regeneration. Intranuclear PI3K is not restricted to mammalian cells, because this kinase has also been found in nuclei of plant cells, where it colocalizes with transcription sites, an indication that this enzyme might be important for mRNA synthesis [19]. Furthermore, the occurrence in the nucleus of D-3 phosphorylated inositol lipids has been reported by means of techniques ranging from in vivo labeling with [^{32}P]orthophosphate [20,21], to immunostaining with specific monoclonal antibodies [22], to the use of FYVE domain probes [23]. It is worth recalling here that the nucleus also contains other components of the PI3K signaling pathways, such as PKC-ζ [21,24], PKB/Akt [25], and PTEN [26]. However, the functional significance of these intranuclear pathways remains to be elucidated.

2.1. The regulation of nuclear PI3K

While control of cytoplasmic PI3K is quite well defined (e.g. [6]), regulation of nuclear PI3K has been obscure. In some cases, however, it does not seem to differ in a substantial way from what happens in the cytoplasm. Indeed, nuclear PI3Kγ is sensitive to pertussis toxin, an indication that nuclear G_i/G_0 proteins are likely to be involved in its activation [17], while PI3K C2β, which has been found in the nucleus, is conceivably activated through calpain-mediated proteolysis, as reported for platelets [18]. Indeed, in normal liver PI3K C2β bands at approximately 180 kDa, whereas 20 h after partial hepatectomy (when maximal PI3K was measured) a 18-kDa gel shift was observed [18].

A major breakthrough has recently been achieved regarding the control of class IA nuclear PI3K in PC12 cells stimulated with nerve growth factor (NGF). By means of yeast two-hybrid analysis it has been identified as a novel nuclear 753-amino acid GTPase, named PIKE (for phosphoinositide kinase enhancer), which binds to the p85 regulatory subunit of PI3K, resulting in activation of the catalytic subunit [27]. The amino acid sequence of PIKE displays in its carboxy-terminal portion a substantial homology to several G proteins, such as centaurin γ-a, Rab7, or R-Ras. PIKE also possesses a pleckstrin homology (PH) domain. By Northern blot analysis of rat tissues, PIKE was found to be mostly abundantly expressed in brain, and to a lesser extent also in lung, heart, and liver. When an epitope-tagged PIKE was overexpressed in HEK293 cells, it localized exclusively to the nucleus. NGF treatment of PC12 cells led to a five-fold increase in PIKE binding to GTP. Binding of PIKE to the p85 regulatory subunit of PI3K depends on both the amino terminal 261 amino acids and on the carboxy-terminal 40% of the GTPase, but not on its PH domain. PIKE also binds to the p110 catalytic subunit of PI3K, but in this case removal of the carboxy-terminal 40% of PIKE only modestly reduces binding (see Fig. 1). In cells transfected with p110 and PIKE, but lacking p85, no enhancement of PI3K activity was measured, demonstrating that p85 is required for the activation. At a first glance, this mechanism of activation of nuclear PI3K might appear similar to that reported for cytoplasmic PI3K, which can be stimulated by Ras (see [6] and references therein). However, in the case of the PI3K/Ras interaction, only the p110 catalytic subunit binds Ras, while PIKE binds both p110 and p85. Indeed, as emphasized above, p85 is absolutely required for PIKE-mediated activation of nuclear PI3K. Ye and coworkers [27] went further and demonstrated that protein 4.1N blocks the activation of PI3K by preventing PIKE association with PI3K. Protein 4.1N is a neuronal selective isoform of the erythrocyte membrane cytoskeleton protein 4.1R [28]. Protein 4.1N resides in the cytoplasm, and following challenging of PC12 cells with NGF, migrates to the nucleus over a period of hours, lagging behind PI3K translocation to the nucleus, which occurs within 30 min of stimulation. In contrast, it is currently thought that inhibition of plasma membrane class IA PI3K is mostly achieved through tyrosine dephosphorylation of growth factor receptor, which p85 binds to with its SH2 domains. In addition, a signal regulatory protein which negatively affects plasma

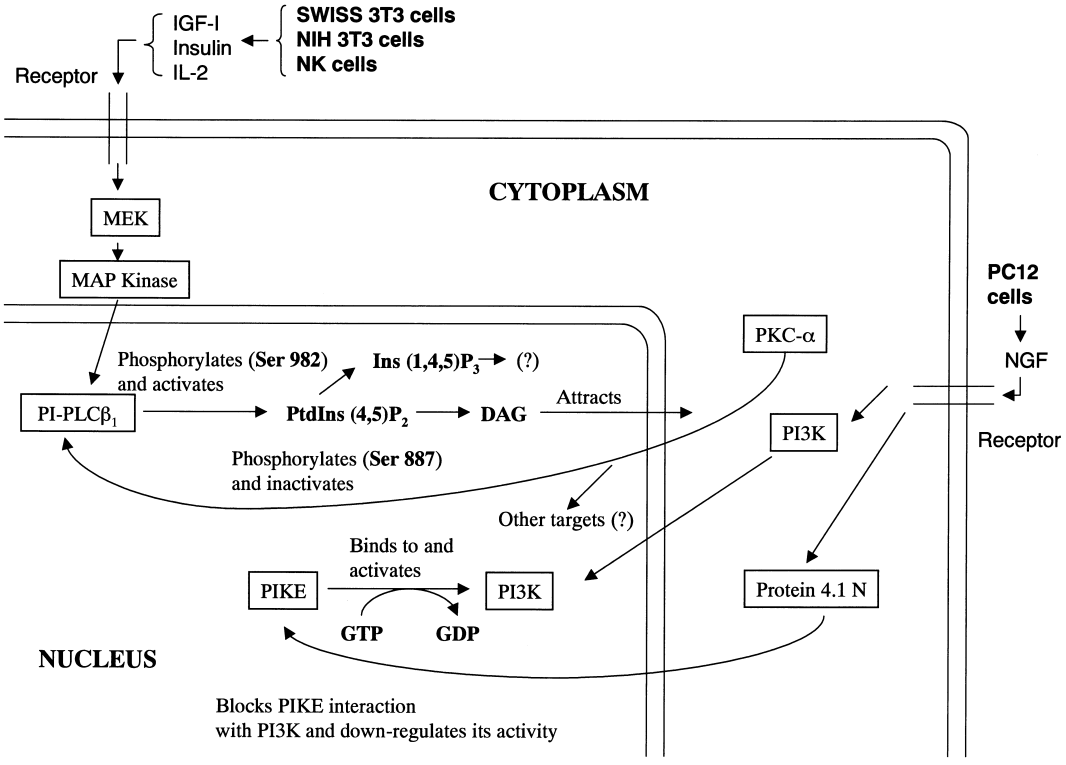

Fig. 1. Proposed models outlining the activation/inhibition of nuclear PI-PLC-β1 and PI3K. Various agonists (IGF-I, insulin, and interleukin-2 (IL-2)) activate the MAP kinase pathway. Activated MAP kinase migrates to the nucleus where it phosphorylates PI-PLC-β1 at serine 982, causing its activation. DAG produced by PtdIns(4,5)P_2 hydrolysis attracts to the nucleus PKC-α. PKC-α phosphorylates PI-PLC-β1 at serine 887, producing its inhibition. In PC12 cells, NGF treatment activates the nuclear GTPase PIKE, which binds to both p85 and p110 subunits of nuclear PI3K, thus activating it. Later on, protein 4.1N enters the nucleus and blocks PIKE interaction with PI3K, causing its inhibition.

membrane PI3K activity is SIRPα1. This protein was shown to reduce the association between phosphotyrosine phosphatase SHP2 and the p85 regulatory subunit of PI3K [29].

This mode of control of nuclear PI3K activity is extremely intriguing, but some questions remain unsettled. Firstly, it is not clear which protein activates the PIKE GTPase within the nucleus. Secondly, in many cell types, protein 4.1N is constitutively present in the nucleus, where it binds the abundant nuclear matrix protein NuMA (e.g. [30–32]). Moreover, 4.1N is concentrated in the speckless domains of the nucleus where it is involved in mRNA splicing [33,34]. However, in PC12 cells, 4.1N is scarcely expressed in the nucleus under basal conditions and translocates there in response to NGF [28]. Therefore, it remains to be established whether this kind of activation of nuclear class IA PI3K is universal or restricted to a single cell type. Thirdly, we and others [15,20] have reported a massive tyrosine phosphorylation of p85 recovered in the nucleus. Such a phosphorylation might somehow facilitate binding of PIKE to the regulatory subunit of PI3K, although its functional significance remains unclear. Thus, this issue needs additional investigation.

3. Nuclear PI-PLC-β1

PI-PLC-β isoforms function as receptors belonging to the rhodopsin superfamily of transmembrane proteins containing seven transmembrane spanning segments. PI-PLC-β1/β4 are activated by members of the α_q subfamily of the G proteins α subunit and, except for the β4 isoform, by G proteins βγ dimers (see [35] for an updated review on the issue). Moreover, in neutrophils, PI-PLC-β2 was found to be stimulated by the Rho GTPases CdC42Hs and Rac1 [36]. PI-PLC-β1 mainly hydrolyzes PtdIns(4,5)P_2, thus yielding DAG and Ins(1,4,5)P_3 [35].

PI-PLC-β1 was the inositide-specific phospholipase originally identified at the nuclear level [37,38]. It is now established that nuclear PI-PLC-β1 plays an important role in the control of cell proliferation, because it mediates the mitogenic effect of insulin-like growth factor-I (IGF-I) in Swiss 3T3 cells [39], or regulates cell cycle progression in mouse erythroleukemia (MEL) cells [40,41]. In IGF-I-stimulated Swiss 3T3, nuclear PI-PLC-β1 is responsible for the rise in the DAG mass which occurs in the nucleus. Such an increase in the DAG levels attracts to the nucleus the α isoform of PKC (see also later) [39]. Moreover, it has recently been proposed that PI-PLC-β1 is critically involved in resumption of meiosis in mouse oocyte [42].

3.1. The regulation of nuclear PI-PLC-β1

Activation of nuclear PI-PLC-β1 by G proteins seemed unlikely, given that nobody has ever demonstrated the presence within the nucleus of GTP-binding proteins of the G_q class. Nevertheless, in response to growth factor stimulation, the whole heterotrimeric GTP-binding protein G_i translocated to the nucleus [43], so that its βγ dimer might activate a nuclear PI-PLC-β isoform. Consistently, we always failed to stimulate nuclear PI-PLC-β1 activity with either GTP-γ-S or AlF_4 [44]. In contrast, GTP-γ-S stimulated polyphosphoinositide synthesis in isolated nuclei prepared from MEL cells, suggesting that inositol lipid phosphorylation in the nucleus

may be under the control of G proteins, in analogy with the plasma membrane [45].

Thus, we sought to determine whether or not other mechanisms could control the activity of nuclear PI-PLC-β1. 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 (e.g. [46]). Our laboratory demonstrated for the first time that in response to IGF-I stimulation of quiescent Swiss 3T3 cells, activation of nuclear PI-PLC-β1 was paralleled by its hyperphosphorylation. Interestingly, if nuclear translocation of p42/44 mitogen-activated protein (MAP) kinase was prevented by cytoskeletal depolymerization, the hyperphosphorylation of PI-PLC-β1 was no longer detectable, suggesting an involvement of MAP kinase in this phenomenon [47]. Similar results have subsequently been obtained with either insulin-treated NIH 3T3 cells [48] or interleukin-2-exposed human natural killer cells [49]. In these two reports, it was shown that activation of nuclear PI-PLC-β1 was prevented by treatment of cells with PD98059, a specific inhibitor of MAP kinase kinase, a further indication of the importance of the MAP kinase pathway. It is important to recall that in insulin-treated NIH 3T3 fibroblasts, we observed a hyperphosphorylation of PI-PLC-β1b, i.e. the form which is more abundantly expressed within the nucleus [39].

However, the conclusive demonstration that p42/44 MAP kinase was responsible for the activation of nuclear PI-PLC-β1 came only very recently from a series of experiments, performed with IGF-I-treated Swiss 3T3 cells, showing that: (a) nuclear PI-PLC-β1 and activated p42/44 MAP kinase could be coimmunoprecipitated; (b) recombinant PI-PLC-β1 could be efficiently phosphorylated in vitro by activated p42/44 MAP kinase, but not by protein kinase A (PKA); (c) the p42/44 MAP kinase phosphorylation site of PI-PLC-β1 was mapped at serine 982, which lies within a PSSP motif located in the carboxy-terminal tail of PI-PLC-β1; (d) phosphopeptide analysis showed a similarity between in vivo ^{32}P-labeled PI-PLC-β1 and PI-PLC-β1 phosphorylated in vitro by p42/44 MAP kinase; (e) if Swiss 3T3 cells overexpressed a mutant PI-PLC-β1 in which serine 982 was replaced by glycine, nuclear phospholipase activity was not activated by IGF-I, and the mitogenic effect of the growth factor was markedly attenuated. Taken together, these findings unequivocally suggested that p42/44 MAP kinase phosphorylates nuclear PI-PLC-β1 at serine 982, and such a phosphorylation plays a critical role in the activation of the phospholipase and is also crucial to the mitogenic action of IGF-I [50]. These findings appear very intriguing and completely unexpected, as they have never been reported for membrane-associated PI-PLC-β1. Since the p42/44 MAP kinase phosphorylation site is within the carboxyl terminus of PI-PLC-β1 (which has been shown to be essential for its $G\alpha_q$-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 phospholipase activity. Indeed, the MAP kinase-mediated phosphorylation does not increase the in vitro activity of purified PI-PLC-β1.

Phosphorylation of PI-PLC-β1 is not per se an entirely new regulatory mechanism, because at the plasma membrane it is utilized to inhibit PI-PLC activity. PKA has been shown to directly phosphorylate in vitro PI-PLC-β2 or -β3, thereby inhibiting their activation by Gβγ subunits. Here, the putative phosphorylation site is serine 954, which determines an uncoupling of receptors that activate PI-PLC-β3 through G_i/G_o while preserving the activation by receptors that utilize G_q. However, PKA can phosphorylate PI-PLC-β3 also at serine 1105, and in this case the outcome is a partial block of the G_q-dependent activation [41]. Moreover, PI-PLC-β isoforms are also substrates for PKC. It was initially demonstrated that treatment of a variety of cells with phorbol esters (that are powerful PKC activators) resulted in the inhibition of receptor-coupled PI-PLC-β activity. Moreover, PKC phosphorylated in vitro bovine PI-PLC-β at serine 887, but without any concomitant effect on phospholipase activity, so that the physiological relevance of this phosphorylative event remained unclear [51]. Subsequently, it has been shown that PKC-α and -ε (but not PKC-ζ) phosphorylated in vitro PI-PLC-β1, whereas PI-PLC-β3 was poorly phosphorylated by PKC-α [52]. In this case, PKC phosphorylation of PI-PLC-β1 resulted in significant activity inhibition, but the $G\alpha_q$ stimulation was unaffected, while Gβγ subunits blocked the PKC-α-mediated phosphorylation of PI-PLC-β1 and antagonized its inhibition.

Translocation of PKC-α to the nucleus is one of the earliest events that occur in IGF-I-treated Swiss 3T3 cells [53]. DAG produced by nuclear PI-PLC-β1 serves as a chemoattractant for PKC-α [37,54]. 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-β1 activity [55]. Indeed, treatment of Swiss 3T3 cells with Go6976, a selective inhibitor of PKC-α, caused a sustained elevation of IGF-I-dependent PI-PLC-β1 activity. Two-dimensional phosphopeptide mapping and site-directed mutagenesis demonstrated that PKC-α phosphorylated nuclear PI-PLC-β1 at serine 887. Moreover, overexpression of either a PI-PLC-β1 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-β1 in response to IGF-I stimulation. All in all, these findings indicated that a negative-feedback regulation of nuclear PI-PLC-β1 by PKC-α is 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-β1 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-β1 activity, while in vitro phosphorylation of purified PI-PLC-β1 by PKC-α seems to affect the interaction of the phospholipase with the Gβγ subunits. It might be that also in this case PI-PLC-β1 phosphorylation leads to changes in the its interaction with other regulatory nuclear protein(s) that remain to be identified.

4. Concluding remarks and future perspectives

Since the first observations that nuclei contain an inositol lipid cycle, considerable effort has been devoted to identifying the regulatory mechanisms of the enzymes involved in this metabolism. It now appears that inositol lipid metabolism within the nucleus is at least as complex as that within the cytoplasm. The progress made in defining the molecular

mechanisms of regulation of nuclear inositol lipid cycle has been painfully slow when compared with the spectacular progresses achieved for its cytoplasmic counterpart. The main reason why we know relatively little about these mechanisms it is that they are very difficult to study, as anyone who has tried to do so will testify. In spite of all the technical difficulties, since the pioneer days of nuclear inositol lipid signaling, a radical change has happened: we now have many new techniques and tools to study at the molecular level the events taking place within the nucleus. It is also true that our knowledge of similar pathways and processes outside the nucleus has tremendously advanced. Therefore, over the last few months, our comprehension of the molecular mechanisms underlying the upstream regulation of enzymes involved in nuclear inositol lipid cycle has substantially improved, as evidence reviewed here demonstrates. However, as underscored in this article, the events that regulate nuclear inositol lipid metabolism are not necessarily equal to those operating in the cytoplasm. This makes this field of investigation all the more exciting and intriguing. As emphasized by Robin Irvine in his recent review published in Science's stke (on line, September 5, 2000), other investigators should enter the fray and find out just what is going on within this microcosm of cellular signaling. The contribution of more laboratories would undoubtedly expedite the identification of additional regulatory mechanisms of nuclear inositide metabolism. Fundamental to such a challenge will be to understand how signaling by such a multitude of proteins is integrated inside the nucleus. In this regard, the yeast two-hybrid technology has already allowed the identification of a protein (PIKE) fundamental for the regulation of PI3K within the nucleus. The same technique, if applied for example to nuclear PI-PLC isoforms, might lead to the identification of the interacting partners important for their activation/inhibition. Post-translational modifications of proteins have already been demonstrated to play a fundamental role in the control of some aspects of nuclear inositol metabolism (see also [56]). Therefore, overexpression of other enzymes mutated in amino acid residues critical for the modifications to occur should improve our understanding of the exact role played by these changes, even though in some cases the use of protein overexpression might obscure many of the subtleties of lipid–protein interactions and their impact under natural conditions. This very same approach could be applied, for example, to definitively clarify the biological significance of tyrosine phosphorylation of the p85 regulatory subunit of nuclear PI3K, an issue whose importance is just beginning to be recognized [57].

Another important outstanding question is to define to what extent the complex interplay that exists between the different lipid-dependent signaling pathways (e.g. [58]) is operating also in the nucleus and how it is regulated.

A better knowledge of the regulation of nuclear inositol lipid metabolism appears desirable mostly because, if it will be determined that at least some molecular mechanisms are indeed peculiar to the nuclear compartment, this will then allow pharmaceutical intervention with clinical benefits to humans through the synthesis of new therapeutic agents having, as a specific target, the nuclear cycle while sparing the plasma membrane cycle.

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