CMLS, Cell. Mol. Life Sci. 57 (2000) 379–393
1420-682X/00/030379-15 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Nuclear inositides: inconsistent consistencies

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Abstract. It is now clear that phosphoinositides, which play a major role in the regulation of a variety of cellular processes in the cytoplasm, are found within the nucleus. Their role in this subcellular compartment is still contentious; however, data has suggested that nuclear inositides generate substrates, such as PtdIns(4,5)P2, utilised by a number of nuclear signalling pathways: for example, nuclear phospholipase C and the PtdIns 3-kinase cascade. There is also evidence that PtdIns(4,5)P2 may play a role in the localisation and regulation of a number of nuclear proteins such as the BAF complex, which is involved in the regulation of chromatin structure. Although the presence of nuclear inositides has been demonstrated in a number of different cell types, suggesting that it is ubiquitous, there are many inconsistencies within the literature concerning the locations and isotypes of enzymes that are involved in their regulation and in the potential second messengers which are generated by them. This review aims to highlight some of these inconsistencies in order to focus on areas that need further characterisation.

Key words. Diacylglycerol; phospholipase C; phospholipase D; phosphoinositides; nucleus; signal transduction.

Introduction

We and others have recently produced reviews that have outlined the majority of studies pertaining to inositol lipids in the nucleus. This review, however, will concentrate on a number of the main concepts and attempt to outline some of the consistencies, inconsistencies and the gaps that need to be filled. The reader is therefore referred to these other reviews for a full literature survey [1–3]. Potential nuclear targets of protein kinase C (PKC) will also not be considered; however, the reader is referred to other excellent reviews [4–8].

Inositol lipids

Inositol lipids are made up of an inositol head group that is linked, via a phosphate diester bond, to a 1,2-diacylglycerol (DAG) moiety. The DAG acts to maintain this lipid as part of the membrane, whereas the inositol head group forms a potential interface between components in the cytosol and the membrane. PtdIns can be phosphorylated in a number of positions, by a number of different kinases to yield at least seven potential second messengers (see fig. 1) which act in two ways. Some of these can be cleaved by a specific phospholipase to yield both a membrane-bound and a soluble cytosolic second messenger. This has been shown to be the case for the hydrolysis of PtdIns(4,5)P2 by PIC to generate DAG, which acts to stimulate PKC [9], and Ins(1,4,5)P3, which regulates calcium release from internal stores [10, 11]. In this case PKC acts as a sensor which integrates these two signals, leading to the phosphorylation of key cellular components [12]. The mechanism by which DAG activates PKC is thought to involve binding to the membrane, followed by a conformational change which leads to an opening of the active site, and then activation of the enzyme. Thus this lipid acts not only to localise, but also plays a key role in its activation. This idea now appears to be a recurring phenomenon, as a number of phosphorylated forms of inositol lipids have also been shown to bind, and therefore specifically localise, key signalling enzymes. One of the first examples of this paradigm is the activation of protein kinase B (PKB). This protein kinase contains a pleckstrin homology (PH) domain, examples of which have been shown to be important in the interaction with

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proteins, such as the $\beta\gamma$ subunits of heterotrimeric G proteins, or with lipidic components of the membrane [13–19]. Interestingly, the PH domain shows a high affinity for both PtdIns(3,4,5)P3 and PtdIns(3,4)P2 [20–23] and is able to inhibit the activity of the enzyme. The enzyme is recruited to the membrane through the receptor-mediated increase in mass of the PtdIns(3,4,5)P3 and PtdIns(3,4)P2 [24–26], via the interaction of these lipids with the PH domain. In what can be thought of as a similar mechanism as the activation of PKC by DAG, the PH domain-mediated inhibition of the kinase activity is overcome, probably through a conformational change in the protein's structure. This opens the catalytic loop and allows the phosphorylation of key residues, leading to its activation [21, 23, 27]. Interestingly, the phosphorylation of these residues is carried out by an upstream kinase that also contains a PH domain sensitive to PtdIns(3,4,5)P3 [28–30]. Thus the production of this lipid leads not only to the recruitment of PKB but also its upstream kinase PDK-1 [31]. Removal of the PH domain or fusion of the enzyme to a domain which targets the chimera to the membrane leads to the constitutive phosphorylation of these same residues and thus activation of the enzyme [23]. Physiologically, this targeting mechanism is important, as demonstrated by a viral oncogene which creates a fusion protein that is constitutively targeted to the membrane.

Another domain, called the FYVE domain (so called after the first four proteins that were suggested to contain this domain), has been shown to specifically bind to PtdIns(3)P, though whether this regulates the activities of the enzymes that bind to it or is purely a localisation phenomenon remains to be discovered [32–36]. It should be mentioned here that there is increasing evidence that PtdOH, which can be generated by the action of a phospholipase D (PLD) or by a phospholipase C (PLC) followed by phosphorylation of the DAG by a DAG kinase, is also an important second messenger, and it is likely that it acts in the above manner, leading to the binding and relocation of a target protein [37–42]. Although no specific domains have been identified that interact with this lipid, a number of downstream kinases have been suggested to be targets of PtdOH production [43–46].

That inositol lipids are able to target proteins and lead to their activation is now an established role for these lipids, and it is likely that the different lipids will utilise different binding domains. There are now seven different phosphorylated forms of PtdIns which are present in cells, and together with the fact that they are likely to be located in various intracellular compartments, this would provide a network of target sites able to activate and localise proteins important in the regulation of a number of key pathways within the cell.

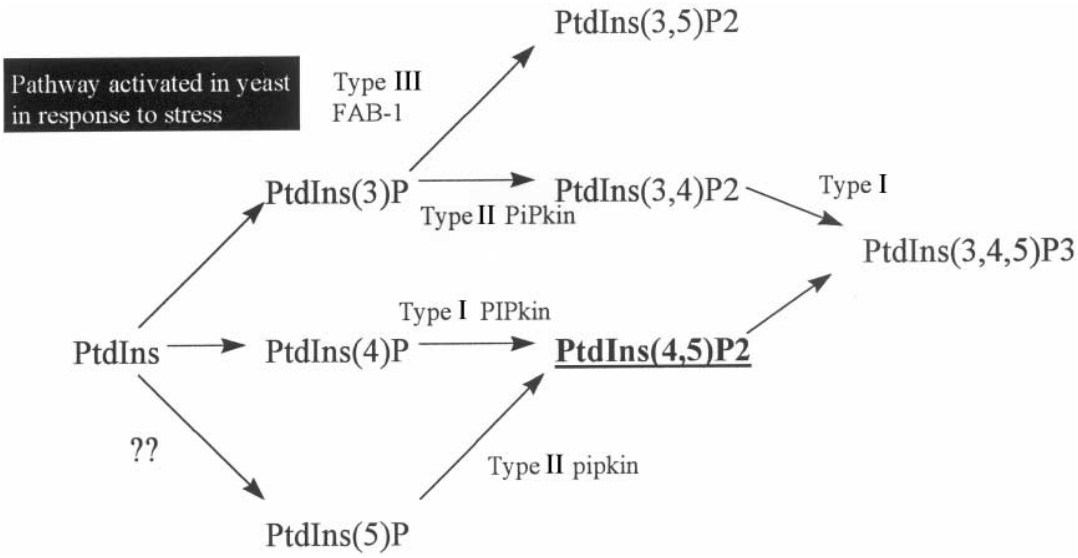

Figure 1. PtdIns is converted into seven new higher-phosphorylated forms, each with the potential to act as a second messenger. In the case of PtdIns to PtdIns(5)P, no enzyme activity has been found. It is possible that the generation of PtdIns(5)P could come from the 4-dephosphorylation of PtdIns(4,5)P2. The diagram also illustrates that it is possible to generate all of the above lipids with a combination of a type I and type II pipkinase, if PtdIns(3)P is already present. The generation of PtdIns(3,5)P2 appears to be the specific function of the type III pipkinase, the yeast homologue being FAB1. There is evidence that the type I pipkinase will also phosphorylate PtdIns(3)P to PtdIns(3,5)P2; however, a mammalian homologue of FAB1 has now been found.

Although the role of these lipids in the cytosolic compartment of a cell is well established, their role in the nucleus has generated a certain amount of controversy. However, the availability of complementary DNAs (cDNAs) and antibody probes to the enzymes that modify these lipids [47–49] and their unequivocal nuclear localisation should finally put the issue of contamination to rest, and open up new investigation into putative nuclear targets.

Nuclear phosphatidylinositol lipids and the enzymes that synthesise and degrade them

This section will deal with the evidence for the presence of inositides, and the enzymes that make them within the nucleus. For a full review of the literature the reader is referred to other more comprehensive reviews [1, 50]. Smith and Wells [51–53] were the first to demonstrate that when purified intact rat liver nuclei were incubated with 32P-ATP, PtdOH, PtdIns(4)P and PtdIns(4,5)P2 were rapidly labelled. The interpretation of this is that the enzymes that phosphorylate DAG, PtdIns and PtdIns(4)P, together with their substrates, are present in nuclei. As this experiment uses endogenous substrates, it also suggests, importantly, that the enzymes and the lipids that they phosphorylate are present in the same location. This type of experiment has been carried out using nuclei from various tissues and cell types with results that are essentially the same as the original data from Smith and Wells [54–61]. A number of studies have taken these experiments further, in order to define whether this nuclear pathway is a target of regulation by growth factors and cellular processes [55, 57, 58, 62–67]. Nuclei isolated from control cells, or from those differentiated down an erythroid pathway, showed differences in their labelling patterns after incubation with 32P-ATP. Specifically, the amount of label incorporated into PtdIns(4,5)P2 was substantially increased after differentiation [55, 60]. This may have been due to changes in the inositol lipid-modifying enzymes (either kinases/phosphatases or phospholipases) or in the levels of their substrates, or both. This experiment, however, led to the hypothesis that this nuclear inositide cycle could be regulated distinctly from that of the plasma membrane. Further, when quiescent Swiss 3T3 cells were stimulated with IGF-1, changes in the nuclear inositides were seen, with no such changes in the whole cells [57]. These changes were shown to reflect mass levels of the various inositides, and the simplest interpretation of the data was that insulin-like growth factor 1 (IGF-1) led to an increase in the activity of a nuclear PIC, which hydrolysed either PtdIns(4)P or PtdIns(4,5)P2, leading to enhanced production of nuclear DAG [68, 69]. This increase in nuclear DAG occurred concomitantly with translocation of PKC to the nucleus, a physiological downstream target of this lipid [69–72]. Indeed, IGF-1 was shown to enhance the activity of a nuclear phosphoinositidase C (PIC) whilst having no effect on its cytosolic counterpart [73, 74]. Studies, either on differentiation of murine erythroleukemia (MEL) cells [63, 75, 76] or proliferation, induced after partial hepatectomy [64, 77, 78], and their effects on the nuclear inositide cycle, have led to the suggestion that regulation of a nuclear PIC may be a key event during proliferation and terminal differentiation. Thus a nuclear PIC is able to regulate the mass levels of nuclear DAG, which in turn regulates progression through the cell cycle, perhaps through regulation of PKC activity. Extension of this hypothesis would suggest that improper control of the levels of nuclear DAG should lead to problems with progression through the cell cycle. Indeed, studies carried out by Topham et al. [79, 80] demonstrated that DAG kinase ζ, which is partially nuclear-localised, is phosphorylated after stimulation of PKC, by epidermal growth factor (EGF) or phorbol ester (TPA), and this leads to its efflux from the nucleus (fig. 2). This efflux appears to correlate with an increase in the mass of nuclear DAG. The PKC phosphorylation site was mapped to a putative nuclear localisation sequence, which, when phosphorylated, prevents the enzyme from entering the nucleus. In support of the above hypothesis, overexpresion of this enzyme led to a decrease in the nuclear DAG and a doubling in the cell cycle time, with cells becoming blocked in G1. Interestingly, these effects were dependent on both the DAG kinase activity and its nuclear localisation (see fig. 2).

Things are never what they seem

Although the above studies would suggest that the picture in the nucleus is simple, in that PtdIns(4,5)P2 can be synthesised through the sequential phosphorylation of PtdIns to PtdIns(4)P and then to PtdIns(4,5)P2, which in turn is utilised by a nuclear PIC to generate DAG. The emerging data suggest that life is never as simple as this. A number of studies would suggest that the generation and utilisation of PtdIns(4,5)P2 within the nucleus is as complicated as that in the plasma membrane, suggesting that this lipid has multiple roles in the nucleus.

PIPkins: These are the enzymes that are able to phosphorylate PtdInsP to PtdInsP2. The nomenclature used here is deliberately nonspecific, as it is unsure as yet what the specificity of the different kinases is in vivo. Since the cloning of the first PIPkin, it has become obvious that there are at least three subfamilies which share a common catalytic domain, but differ in respect

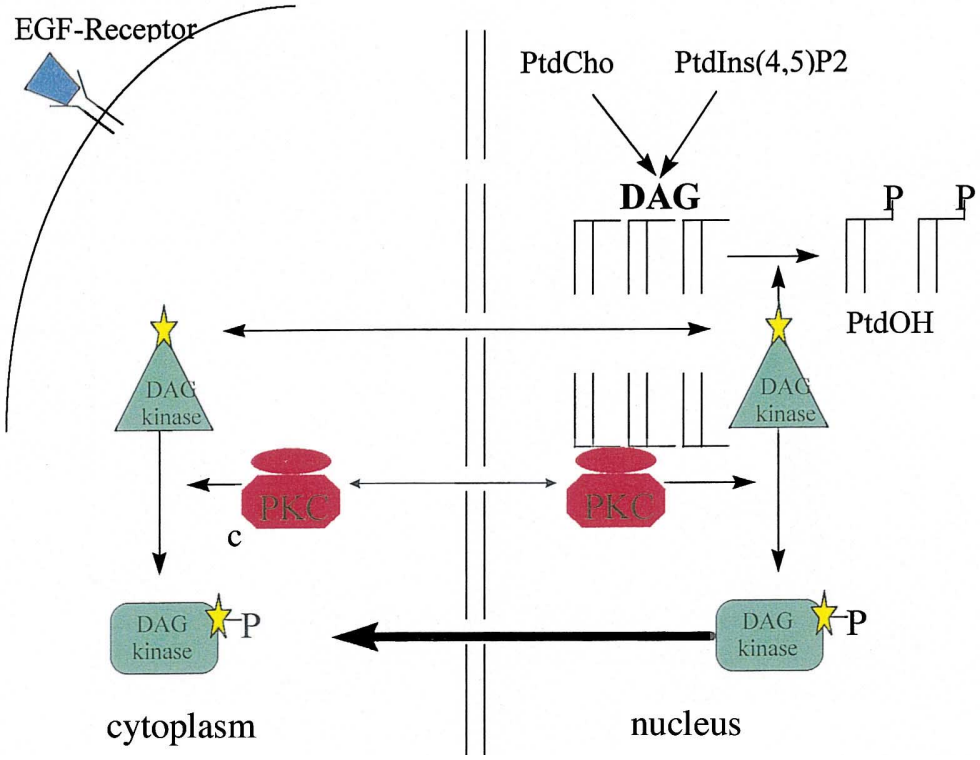

Figure 2. Stimulation of PKC either by EGF or by TPA leads to enhanced nuclear PKC activity, which leads to the phosphorylation of DAG kinase ζ. This phosphorylation prevents the translocation DAGζ to the nucleus with a subsequent loss of the enzyme. This in turn leads to an accumulation of DAG within the nucleus which could further lead to a stimulation of PKC. How the DAG is made is not defined, although it could potentially come from either the hydrolysis of lipids such as PtdCho or PtdIns(4,5)P2, or through the de novo synthesis pathway. Whether PKC is nuclear or is translocated to the nucleus is also not defined, although there is evidence for translocation induced by EGF treatment of Swiss 3T3 cells [72]. Overexpression of the DGKζ leads to a cell cycle arrest which is dependent on the activity of the enzyme and its ability to be nuclear-localised. This suggests that nuclear DAG is required for cell cycle progression or that the product PtdOH can lead to this inhibition. The second is unlikely, as in some cells it has been suggested that PtdOH is a mitogenic factor [172].

to which substrate they phosphorylate [47, 81–89]. The type III proteins, typified by the FAB 1 protein from yeast, phosphorylate only PtdIns(3)P on the 5 position to generate PtdIns(3,5)P2 [90]. There is no evidence as yet that this lipid, or the enzyme that makes it, is present in the nucleus, and therefore it will not be discussed here (however, for interest see [90, 91]). The type I PIPkins, of which there are at least three isoforms, phosphorylate PtdIns(4)P on the 5 position to generate PtdIns(4,5)P2. However, this enzyme has an odd specificity in that in the test tube it will also phosphorylate PtdIns(3)P on both the 4 and the 5 position, to produce PtdIns(3,4,5)P3 [84, 92, 93]. The enzyme is activated by PtdOH in vitro, and this has been suggested to be an in vivo mechanism, perhaps through PLD, for its activation [84, 86–88, 94]. It is still not known which of the three isoforms is responsible for the phosphorylation of PtdIns(4)P to PtdIns(4,5)P2 that is used by a receptor-activated PIC. These isoforms are also able to bind to small molecular weight G proteins from the rho family [95–98] and have been suggested to be important in the regulation of PtdIns(4,5)P2 required for a number of intracellular signalling pathways, such as vesicle transport and cytoskeletal dynamics [38, 99–103].

The type II family, which also has at least three isoforms [83, 85, 89], was initially identified as a PtdIns(4)P 5-kinase and has been redesignated as a kinase that preferentially phosphorylates the 4 position of PtdInsP and whose substrate in vivo is not known, but presumed to be PtdIns(5)P [104]. The enzyme will also phosphorylate PtdIns(3)P to generate PtdIns(3,4)P2, although it has been suggested that kinetically it prefers PtdIns(5)P as a substrate [81]. Very little is known about the regulation of this enzyme in vivo, although the crystal structure of the type IIβ has been determined [105]. Two of the isoforms appear to be regulated by phosphorylation on serine and threonine residues in response to growth

factors [89, 106], and there are reports that the β-isoform is able to bind to activated EGF and tumour necrosis factor (TNF) [107] receptors.

The redesignation of this enzyme leads to a number of new problems in the understanding of the pathways of synthesis of PtdIns(4,5)P2 in the nucleus. Immunological analysis using antibodies to the type II enzyme demonstrates that a protein is present in nuclei of a number of different cell types [62, 108]. Moreover, overexpression of green fluorescent protein (GFP)-type II PIPkin β or type IIα shows that both of these enzymes localise to the cytosolic and nuclear compartment (our unpublished data and [108]). Further immunological analysis has suggested that the type II enzyme colocalises with messenger RNA (mRNA) splicing complexes in specific locations within the nucleus [108]. These data demonstrate unequivocally that PIPkins are present in the nucleus and are not present solely through contamination from other compartments during nuclei isolation. What are these PIPkins doing there? After in vitro labelling of isolated nuclei using 32P-ATP, we analysed which position of the inositol head group the radiolabel had been transferred to. This demonstrated that the majority of radiolabel was present in the 5 position. This suggests that the major route of PtdIns(4,5)P2 production in the nuclei is through a type I PIPkinase. Indeed, purification of PIPkins from pig liver nuclei demonstrated the presence of a type I enzyme that was only able to phosphorylate PtdIns(4)P but not PtdIns(5)P. The question then is, What is the type II enzyme doing in the nucleus? Does this enzyme generate PtdIns(4,5)P2 for utilisation by a nuclear PIC or does it serve some other purpose such as PtdIns(4,5)P2 generation involved in other processes? Also, for the type II enzyme to be active requires that its substrate should be present. There are no data on the presence of either PtdIns(3)P or PtdIns(5)P in the nucleus. As stated earlier, although kinetically PtdIns(5)P is a better substrate than PtdIns(3)P, the absolute nuclear concentrations of these lipids, or their localisation, compared with the type II enzymes, are not known, and therefore it is impossible to define the real in vivo substrate and the product it would make. The presence, however, of both a type II and a type I PIPkinase would suggest that the regulation of PtdIns(4,5)P2 levels within the nucleus is as complicated as that in the cytosol, with subnuclear pools of PtdIns(4,5)P2 being differentially utilised.

Nuclear DAG

Stimulation with growth factors or proliferation (induced by partial hepatectomy) leads to an increase in the levels of nuclear DAG, which is correlated with an increase in nuclear PIC activity [64]. In contrast to this, when MEL cells were terminally differentiated, leading to cell cycle arrest, a decrease in the level of nuclear DAG was observed which correlated with downregulation of nuclear PIC activity [1, 60, 63, 75, 109]. More specifically, differentiation led to a decrease in the transcription of the gene encoding PLCβ1 [75]. We and others have demonstrated that this was potentially the nuclear PIC activity, as this was the only immunologically detectable isoform of PIC in rat liver nuclei [62, 63, 73, 110, 111]. Thus the increase/decrease in nuclear DAG during different treatments has been ascribed to the changes in this isoform of PIC. Using a beautiful technique set up by Kennerly, we tried to establish the identity of the precursor lipid for the nuclear DAG, with the assumption that it would be derived from hydrolysis of a PtdIns lipid. The technique is well suited to analysis of nuclear lipids, as it does not depend on isolation of mass quantities of lipid but is based on the analysis of PtdOH after separation according to the number of double bonds they contain, i.e. their degree of saturation. PtdIns is predominantly polyunsaturated, whereas the PtdCho is predominantly saturated or monounsaturated. PtdIns and PtdCho were hydrolysed using specific PLCs, and their respective DAGs were phosphorylated with a nonspecific DAG kinase to generate labelled PtdOH. These were then separated by argentation chromatography, which distinguishes molecular species by their degree of saturation. These patterns of unsaturation were then compared with the overall nuclear DAG pool after phosphorylation to PtdOH by the same DAG kinase. The result demonstrated that the majority of nuclear DAG could not be derived from the hydrolysis of PtdIns and must either come from the de novo pathway or be derived from the hydrolysis of other lipids such as PtdCho [76]. Upon differentiation of these cells, which was shown to lead to a decrease in nuclear PIC activity, the decrease in nuclear DAG occurred in species that are already present in control nuclei and therefore unlikely to be derived from PICβ1-mediated cleavage of PtdIns lipids. This suggests either that the decrease in nuclear DAG is unrelated to the downregulation of PLCb1, or that there is a feedforward mechanism such that the small amount of PtdIns-generated DAG leads to the activation of another PLC that hydrolyses other lipids, such as PtdCho, leading to enhanced DAG production [76]. The answer is not yet clear. However, previous data in 11c9 cells also suggested that thrombin stimulation led to an increase in nuclear DAG mass, which was most likely derived from PtdCho hydrolysis [112, 113]. We have carried out this analysis in a number of different cell types (including Swiss 3T3 cells) and always find that the majority of DAG resembles PtdCho rather than PtdIns. How nuclear DAG is generated and whether nuclei contain a PtdCho-specific PLC is sug-

gested, but not demonstrated. This also raises the interesting question of which species of DAG are involved in the regulation of cell cycle progression. In the study carried out by Topham et al. [80], overexpression of nuclear DAG kinase, leading to the decrease in nuclear DAG content, led to cell cycle block. Which types of DAG are required for cell cycle progression is not known, but it is important to ascertain this as it defines which type of PLC is regulated during the cell cycle.

What of the role of PtdIns-derived lipids and PIC activation in the nucleus? Formally it has not been demonstrated that the PICs that have been shown to be present in the nucleus are able to hydrolyse their endogenous substrates. There is no doubt that these enzymes are present in the nuclear compartment [their activity can be demonstrated using exogenous substrates, and they have been immunologically identified both by Western blotting and by immunoelectron microscopy (for a review, see [1])]; however, it is not clear which if any of the endogenous substrates they can utilise. A study carried out by Sun et al. [114] suggested that intact, isolated nuclei were able to generate DAG when incubated at 30 °C. No analysis of this DAG was shown, but the use of inhibitors suggested it was via a nuclear PIC. In MEL cells we were able to demonstrate a similar increase in nuclear DAG on incubation of isolated intact nuclei; however, subsequent analysis by argentation chromatography showed that this DAG was predominantly mono- or disaturated, again suggesting that it is derived from sources other that PtdIns hydrolysis [76]. So is there a PIC-mediated DAG production, and which of the PtdIns lipids are turned over by this enzyme? Surprisingly, the answer came by analysis of the PtdOH after in vitro labelling in the presence of 32P-ATP. Argentation analysis of the PtdOH showed that it was predominantly polyunsaturated and was similar in composition to the PtdIns-derived lipids. Thus it appears that the DAG can be derived from PIC-mediated hydrolysis, but it is fed directly to a DAG kinase and is phosphorylated to PtdOH [76]. This raises a number of important questions. Which of the PtdIns lipids is hydrolysed in vivo? This point is important, as it determines which head group is released. In light of the contentious issue surrounding the presence of the Ins(1,4,5)P3 receptor on the inner nuclear membrane and the specific regulation of nuclear calcium, only hydrolysis of PtdIns(4,5)P2 would generate this head group. It is also not clear whether the role of the PIC-mediated hydrolysis is to generate new second messengers such as DAG, Ins(1,4,5)P3 and PtdOH, or to attenuate the signalling capacity of PtdIns(4,5)P2. Which PIC is acting within the nucleus? There is evidence for the presence of a number of different PIC isoforms present within the nucleus [1], and this may reflect the various cell types that have been studied, or it may reflect the need for different PICs in the regulation of multiple nuclear pools of PtdIns(4,5)P2.

Nuclear PICs

A number of candidate PICs have been shown to be present within the nucleus, and a review of this evidence has been presented elsewhere. However, a number of studies have highlighted the importance of PICβ1. PICβ1 consists of two isoforms, PICβ1a and PICβ1b, derived from differential splicing of one gene. They are identical except for the C-terminal 42 amino acids. PICβ1a is also 43 amino acids longer. Both transcripts appear to be expressed in a variety of tissues, and both are always expressed together, although their relative levels differ, the reason for which is not known. Immunocytochemical analysis, using isoform-specific antibodies, suggests that the PICβ1b is mainly present in the nucleus, whereas the PLCb1a is mainly present in the cytosol [115, 116]. This data is in direct contradiction to data from Martelli et al. [73], which suggested that PLCb1 was an exclusively nuclear-localised enzyme. Although the antibody utilised in their study would have recognised both isoforms, one would still have expected at least an equal distribution of the two isoforms within the cytoplasm and the nucleus. Immunoblotting studies on rat liver nuclei suggested that although PICβ1 was present in the nucleus, it only represented 2–5% of the total immunoreactivity [62]. We also found this to be the case in Swiss 3T3 cells and in murine erythroleukemia cells. In our hands, overexpression of PICβ1a in either Swiss 3T3, porcine endothelial cells or in MEL cells localises the enzyme to the cytosol/membrane with very little staining in the nucleus. However, studies have emphasised the importance of PICβ1a in nuclei. Data obtained in MEL cells suggested a mass decrease in the levels of nuclear DAG after differentiation which was correlated with a decrease in the levels of PICβ1 associated with the nucleus [63, 75, 111, 117, 118]. This has led to the hypothesis that downregulation of this enzyme leads to a decrease in nuclear DAG and that somehow this is important in the decision for differentiation. A recent study would suggest that this may be the case, as overexpression of PICβ1a from a cytomegalovirus immediate-early (CMV) promoter (and therefore not susceptible to downregulation during differentiation) can block the differentiation process, but only if the enzyme can benuclear localised [119]. This data would suggest that PICβ1 does have a key role to play in differentiation, although the relationship between nuclear DAG and PICβ1 expression is unclear. However, data using MEL cells also suggests that a PIC activity that is able to hydrolyse endogenous PtdIns lipids is not downregulated during differentiation even though

PICβ1 was shown to suggest the presence of multiple nuclear PIC activities. Further evidence for the importance of PICβ1 has come from antisense-PICβ1 knockouts in Swiss 3t3 cells which inhibited IGF-1-mediated proliferation, suggesting an important role for this enzyme in governing cell proliferation [120] (it should be noted here that mice that are homozygous for a PICβ1 deletion still develop normally [121]). Nuclear PICβ1 has also been implicated in a role for meiotic reinitiation in the mouse oocyte [122–124], as this was inhibited by nuclear injection of anti-PICβ1 antibodies.

Potentially, one of the most exciting developments was the cloning of a novel isoform of the δ isoforms of PIC [78, 125]. PICδ4 was cloned using polymerase chain reaction (PCR) from a regenerative rat liver cDNA library [78]. The authors showed that this enzyme is transcriptionally regulated, such that the protein is only present during S-phase of the cell cycle, but more important, it appears to be localised to the nucleus [78] (it should be mentioned here that this clone was also isolated by another group who have not postulated a nuclear-specific role for this enzyme [125]). There are, however, four splice variants of this enzyme that appear to be generated from one gene [125, 126]. Three of these are active; however, ALT111 has a deletion which overlaps with part of the X catalytic domain and renders this variant inactive. ALT 111 has been suggested to be a regulatory molecule, which is able in vivo to inhibit the activity of endogenous PICs [126]. Whether all of these isoforms are present in the nucleus and whether ALT 111 has a dominant-negative role there is unknown. An understanding of the regulation of this enzyme and its intranuclear localisation with respect to the PICβ1 isoform will be important for a full picture of the various roles of nuclear PtdIns(4,5)P2.

The presence of multiple isoforms of enzymes that make and degrade PtdIns(4,5)P2 would suggest that PtdIns(4,5)P2 and phospholipases in the nucleus are important in regulating a number of key nuclear events, some of which are highlighted below.

Nuclear PtdIns(4,5)P2

Potentially PtdIns(4,5)P2 could represent a signalling molecule within the nucleus. Its levels could be regulated either by its synthesis and/or by its hydrolysis. The demonstration that both the type I and the type II PIPkins are present in nuclei would suggest that the levels of this lipid are regulated in a number of different ways. PtdIns(4,5)P2 within the nuclear matrix is probably organised such that its hydrophobic tail is bound to some protein, whereas its head group is able to sit out into the nucleosol. This would make it ideal for use as a targeting agent, as a recent study has suggested [127]. Stimulation of immature T cells leads to their movement from G0 into G1 of the cell cycle. This traversal is accompanied with a decondensation of chromatin and an increase in nuclear size (these two events are thought to be important in the regulation of transcription and for DNA synthesis to occur). One of the nuclear complexes thought to be important is the BAF complex (fig. 3). This is a large complex of at least 13 proteins which, on binding to DNA and the nuclear matrix, is thought to be important in its remodelling. On stimulation of the T cells, there is an increase in the amount of Brahma-related gene 1 associated factor (BAF) complex that becomes associated with the nuclear matrix. Interestingly, the ability of this complex to associate with these nuclei is PtdIns(4,5)P2-dependent [127]. However, no data have yet demonstrated an increase in the amount of PtdIns(4,5)P2 during this process of maturation or that this binding is specific to PtdIns(4,5)P2 [over that obtained with PtdIns(4)P, PtdIns(3,5)P2 or PtdIns(3,4)P2]. It is, however, possible that stimulation leads to the regulation of a PIPkinase, which leads to an increase in the nuclear PtdIns(4,5)P2 levels, and that this is important in the translocation and regulation of the BAF complex. Previous data have suggested that the addition of lipids, such as PtdIns or PtdCho, could lead to profound effects on the transcription, in vitro, of a number of genes through their effects on chromatin structure [128–134].

PtdIns(4,5)P2 can also bind specifically to histones H1 and H3, and this binding is able to inhibit the histone-mediated repression of RNA-polymerase 1, leading to enhanced in vitro transcription [135]. It has also been suggested to be important in the regulation of RNA efflux by its interaction and activation of a nuclear envelope-associated ATPase [136]. More recent data have suggested that the type II PIPkinases may play a role in the regulation of messenger RNA (mRNA) splicing, as immunofluorescence showed a colocalisation of this enzyme with RNA splicing complexes within the nucleus [108]. These authors were also able to show a partial colocalisation with nuclear PtdIns(4,5)P2 using antibodies specific for this lipid. Thus PtdIns(4,5)P2 may play a role in chromatin remodelling during different nuclear processes, such as transcription, DNA synthesis and during the condensation/decondensation of chromatin during mitosis.

However, what is really required is the definition of specific nuclear targets which interact with PtdIns(4,5)P2. These should show a high specificity over PtdIns(4)P or PtdIns(5)P and also show isomer specificity. Elucidation of some of these targets will be important for future studies.

Nuclear calcium

The issue over specifically regulating nuclear calcium, without the need to increase cytosolic calcium, would give the cell more specific control over calcium-regulated nuclear processes. There is no doubt about the importance of nuclear calcium in the regulation of gene transcription [137–139] and a number of other key intranuclear events (such as apoptosis [140, 141]); however, the issue of whether intranuclear calcium is regulated independently of cytosolic calcium is contentious, and arguments for and against will be reviewed in other articles in this issue and have also been presented elsewhere [142–147]. However, there is good evidence that Ins(1,4,5)P3 receptors, which are able to regulate calcium release from the lumen of the endoplasmic reticulum, are present in the inner nuclear membrane [148], and that application of Ins(1,4,5)P3 can lead to the efflux of calcium from the lumen of the nuclear envelope into the nucleosol [149, 150]. Whether Ins(1,4,5)P3 can release calcium from an intranuclear store has been studied mainly using microinjection. These studies have unequivocally demonstrated that intranuclear injection of Ins(1,4,5)P3 can cause release of calcium into the nucleosol, even when heparin is present in the cytosol to block the Ins(1,4,5)P3-mediated efflux of calcium from the endoplasmic reticulum [122–124, 151–153]. Moreover, this nuclear efflux is blocked if heparin is injected into the nucleus. The experiment carried out the other way is not so convincing. This is the release of nuclear calcium when Ins(1,4,5)P3 is injected into the cytosol with heparin. In some cases this blocks nuclear calcium release, whilst in other studies there is no effect. In a study with HeLa cells, histamine caused both a nuclear and cytosolic calcium release, with the nuclear release not being blocked by heparin when present in the cytosol [151]. The implication is either that histamine is able to cause hydrolysis of PtdIns(4,5)P2 within the nucleus, or that Ins(1,4,5)P3 generated at the plasma membrane is able to diffuse into the nucleus and lead to calcium release. Thus it can be seen that the question of the diffusability of Ins(1,4,5)P3 is an important issue. The contradictions seen in these studies may reflect the speed with which the Ins(1,4,5)P3 is metabolised. However, due to the nature of the experiments (microinjection into whole cells), this is impossible to assess. The question of whether Ins(1,4,5)P3 can be generated on the inside of the nucleus in response to growth factors, and whether this could release calcium, is not known. In the most well characterised situation of nuclear inositide regulation (IGF-1stimulation of Swiss 3T3 cells), PtdIns(4,5)P2 levels within the nucleus were shown to decrease, whereas no change was seen in the whole cell levels of this lipid. In contrast to this, bombesin led to hydrolysis of PtdIns(4,5)P2 at the plasma membrane without any effect on the nuclear PtdIns(4,5)P2. Measurement of calcium changes in these cells demonstrated that, as expected, bombesin caused an increase in cytoplasmic calcium; however, no nuclear (or cytosolic) calcium changes were observed with IGF-1 treatment. This experiment has not been carried out in other systems where the location of the hydrolysis of PtdIns(4,5)P2 has been characterised. The data from this single experiment would suggest either that the Ins(1,4,5)P3 that is generated is rapidly metabolised, such that it is unable to mobilise calcium within the nucleus, or that the decrease in the mass of PtdIns(4,5)P2 is not due to receptor-activated nuclear PIC hydrolysis. The decrease measured may be due to the hydrolysis of PtdIns(4)P, its precursor, which by mass flow could lead to a perceived decrease in the mass of PtdIns(4,5)P2. In support of this, a nuclear PIC activity has been characterised that, in vitro, will hydrolyse PtdIns, PtdIns(4)P and PtdIns(4,5)P2, but shows a higher specificity towards PtdIns(4)P. This is interesting in the light of previous data that suggested that DNA polymerase α was specifically activated by Ins(1,4)P2, the water-soluble head group of the hydrolysis of PtdIns(4)P (see fig. 3); however, this data has not been confirmed. Biochemically, we have not been able to measure changes in the mass of Ins(1,4,5)P3, nor have we been able to show an increase in labelled Ins(1,4,5)P3 coincidentally with the increase in PtdOH, during the time course of in vitro labelling of MEL cell nuclei. However, these data should be contrasted with those of Avazeri et al. [124], who have been able to implicate nuclear PICβ1 in nuclear calcium oscillations.

Other roles for PtdIns(4,5)P2 include its phosphorylation to PtdIns(3,4,5)P3 by a PtdIns 3-kinase [24, 154, 155]. Previous data suggested that this enzyme was not present in nuclei purified from either rat liver or from MEL cells. However, recent data suggest that rat liver nuclei are able to make PtdIns(3,4,5)P3 in a wortmannin-dependent manner [156], suggesting the involvement of a classical PtdIns 3-kinase. Further data have suggested that in Saos 2 cells, a PtdIns 3-kinase translocates to the nucleus in an interleukin (IL)1-dependent manner [157, 158]. What this enzyme does there is unknown. However, there are reports that a downstream activator, PKB, also translocates to the nucleus after stimulation by growth factors [22, 159]. Thus the concomitant translocation of PtdIns 3-kinase would lead to enhanced production of PtdIns(3,4,5)P3 within the nucleus, required for the activation of PKB, which can phosphorylate and inhibit the fork head family of transcription factors, which are important in apoptosis [160, 161]. In relation to this, proteins from the centaurin family, which are also PtdIns(3,4,5)-P3-binding proteins, have also been suggested to be present in the nucleus, although their function in vivo is

unknown [162]. Further work needs to be carried out to verify these findings and to assess whether the nuclear PKB is active and phosphorylates these substrates and whether there is an IL-1 increase in the levels PtdIns(3,4,5)P3 in the nucleus. The development of new probes for these lipids, either antibodies or specific PH domains, may be a way to carry this out.

Conclusions

The demonstration that inositide-modifying enzymes are present in the nucleus was carried out over 15 years ago and has been confirmed by many studies in different laboratories. The data have been met with a certain amount of scepticism, and rightly so, as the isolation of any intracellular organelle is always likely to suffer from different degrees of contamination with other organelles. However, with the cloning of inositide-signalling enzymes and the development of new immunological probes, a number of these enzymes have been shown to be present in the nucleus using techniques that do not require isolation of the organelle, such as immunofluorescence. Using this technique, both the type I and the type II PIPkinases, at least two DAG kinases and a number of PICs have been shown to reside in the nucleus. With the development of specific PH domain probes, coupled to the GFP, it is now possible to look at single-cell nuclear PtdIns(4,5)P2 levels and to study its intracellular localisation using methods that do not require prior fixation. It is therefore worth mentioning previous work carried out,

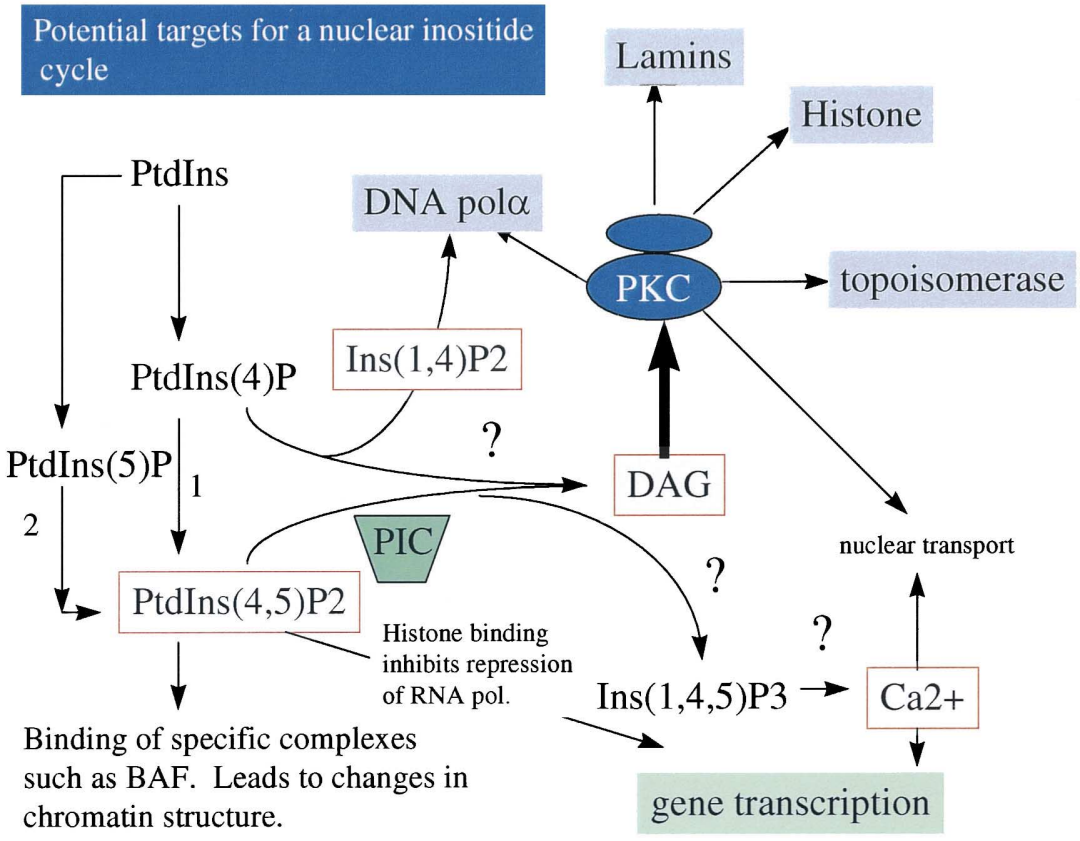

Figure 3. PtdIns(4,5)P2 is generated in two ways, either by the 5-phosphorylation of PtdIns(4)P by a type I PIPkin or by the 4-phosphorylation of PtdIns(5)P by a type II PIPkin. In this diagram the pools of PtdIns(4,5)P2 are the same; however, in the nucleus this may not be the case. The PtdIns(4,5)P2 may then be used to target proteins or complexes such as the BAF complex to specific areas of the nucleus. PtdIns(4,5)P2 or PtdIns(4)P may be used as a substrate for a PIC activity, both yielding DAG, which may activate nuclear PKC (a number of studies have demonstrated that isoforms of PKC are either present or are translocated in response to growth factors, e.g. [72, 113, 173–176]) directly or may lead to the generation of DAG from other sources such as PtdCho, which can activate PKC. There are a number of nuclear targets that have been shown to be phosphorylated by PKC, and in the case of DNA α polymerase [177] and topoisomerase 1 and 11 [178–180], this also leads to its activation. Evidence has also been presented for the phosphorylation of lamins by PKC [181, 182]. Whether this is important for dissolution during mitosis is still contentious. Activated PKC has also been suggested to be important in the specific phosphorylation of transcription factors [183]. Cleavage of PtdIns(4,5)P2 by PIC would also yield Ins(1,4,5)P3, which may lead to the specific regulation of calcium within the nucleus. Question marks represent pathways which are unclear.

mainly in the group of Nadir Maraldi, who have looked at the localisation of PtdIns(4,5)P2 with antibodies generated specifically against this lipid [163–166]. These authors have shown that PtdIns(4,5)P2 is specifically localised to areas of the nuclear matrix, and that unlike the membrane-bound PtdIns(4,5)P2, they are not extracted from these structures using detergents [118, 158, 167–171]. These authors have over the years also carried out a number of useful control experiments (such as phospholipase C digestion after fixation to demonstrate specificity of the antibody) to verify these results. The use of the new PH domain probes may confirm what these authors have maintained for a long time and may enhance the information by allowing real time visualisation of the changes in nuclear PtdIns(4,5)P2. There is no doubt now that these enzymes and the lipids that they modify are present in the nucleus (see fig. 3); however, what their role there is, is still not known. Is PtdIns(4,5)P2 present as a substrate for a nuclear PIC activity and/or does it have a role of its own, perhaps in targeting proteins to specific domains of the nucleus? The presence of both the type I and the type II enzymes in the nucleus would suggest specific localisations and distinct roles for the PtdIns(4,5)P2 that they are able to generate. The issue of nuclear calcium is still a contentious issue; however, the presence of both a PIC and receptors for Ins(1,4,5)P3 would argue for a calcium regulatory role in the nucleus. How is the nuclear DAG pool regulated? Likely there are at least two pools of DAG that are regulated through the action of two distinct phospholipases, and it is possible that these pathways interact and signal to each other. Which phospholipases are present and which lipids they hydrolyse are still not known. What is missing the most is a nuclear-specific inositide-signalling enzyme. So far the immunological data would suggest that most of the enzymes share their time between the two compartments, and this makes it difficult to solely target the nuclear pathway. The best candidate for such a nuclear-specific enzyme at the moment is PICδ4, and understanding its regulation and function will be crucial to this field. That these enzymes shuttle between the cytoplasm and the nucleus should send out a warning to the sceptics amongst us. Most whole cell preparations are contaminated with nuclei!

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