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Inositide-specific phospholipase C signalling in the nucleus*

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INTRODUCTION

The nucleus of eukaryotic cells contains all the information needed for cell proliferation and differentiation, however the initiation of these programmes are dependent on the signalling pathway elicited by different agonists. The existence of a nuclear phosphoinositide signalling stems from the early evidence that isolated nuclei posses the lipid kinases capable of phosphorylating phosphatidylinositol (PI) and phosphatidylinositol 4-phosphate (PIP). The synthesis of phosphatidylinositol 4,5-phosphate (PIP_2) was clearly increased only in the nuclear fraction from Friend cells terminally differentiated towards erythrocytes (Cocco *et al.*, 1987). On the contrary its amount along with that of PIP was decreased in nuclei of Swiss 3T3 cells stimulated to grow with insulin-like growth factor-I (IGF-I) (Manzoli *et al.*, 1989). Following these early observations we and others have demonstrated in several cell type the participation of the whole phosphoinositide cycle in the nucleus (Cocco *et al.*, 1994; Martelli *et al.*, 1992; Divecha *et al.*, 1991; Martelli *et al.*, 1994; Mazzoni *et al.*, 1992). Here we review the most recent achievements on this issue.

NUCLEAR PHOSPHOLIPASE C

The association of nuclear polyphosphoinositide hydrolysis and the progression of cell cycle has been suggested by the very first evidence that IGF-I, which acts via a classical tyrosine kinase receptor and does not involve inositol lipid cycle at the plasma membrane, activates the breakdown of separate pool of nuclear phospholipids to yield the second messenger diacylglycerol (for more details see Cocco *et al.*, 1994 and references therein) in a manner that is, at first sight, analogous to the well analysed plasma membrane counterpart (Berridge, 1993). Form separate laboratories evidence came out that in Swiss 3T3 cells (Martelli *et al.*, 1992) as well as in the rat liver (Divecha *et al.*, 1993a) a nuclear PLC signalling exists, characterised by a strict partitioning of PLC isoforms, since the γ isoform is confined to the cytoplasm and a β isoform (β_1) is specific for the nucleus. The activity of the nuclear isoform increases 2-3 fold within minutes of stimulation of Swiss 3T3 cells by IGF-I and this can be verified with measurement of the breakdown of polyphosphoinositides in the cell nucleus and by direct assay of PLC activity in nuclear extracts, whilst cytoplasmatic PLC activity assayed similarly does not alter on IGF-I stimulation (Divecha *et al.*, 1991; Martelli *et al.*, 1992). That the activation of PLC β_1 and that the hydrolysis of nuclear PI are a key step for the mitotic response has been suggested from the isolation of a 3T3 clone which binds IGF-I yet and fails both to activate nuclear PLC and to undergo cell division (Martelli *et al.*, 1991a).

Therefore the molecular scenario depicted by the activation of nuclear PLCβ_1 by IGF-I stimulation in Swiss 3T3 cells point out to an enhanced gener-

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ation of DAG (Divecha *et al.*, 1991) and this has been proposed as a likely mechanism for determining PKC translocation to the nucleus, which indeed occurs after IGF-1 treatment (Martelli *et al.*, 1991b). It is well known that the protein kinase C family comprises several isoforms differing in both enzymatic properties and cellular localisation (Buchner *et al.*, 1995). Since the canonical isoforms are dependent on DAG for their activation we decided to analyse whether or not these isoforms could translocate to the nucleus. For this aim we have used isozyme specify anti-PKC antibodies and we have shown that are expressed four PKC isozymes, i.e. α, βI, ε, ξ in Swiss 3T3 cells. After treatment with IGF-I only the α isoform translocates to the nucleus. Moreover confocal microscopy confirms the Western Blots and resolves the issue of the actual localisation of PKC in the nucleus after translocation since the *in situ* confocal sections through the equatorial plane of the cells indicate that an accumulation of PKC α within the nucleus is induced after stimulation of Swiss 3T3 cells by IGF-I (Neri *et al.*, 1994) as a consequence of increased nuclear PLC β_1 activity which induces an increase in the concentration of nuclear DAG (Martelli *et al.*, 1992; Divecha *et al.*, 1991). DAG likely represents an attractant for PKC α, although the exact mechanism for this remains still obscure. These data contribute to an understanding of how signals originating at the plasma membrane are transmitted to the nucleus. The steps leading to mitosis are characterised by an ordered sequence of events which links nuclear inositol lipid cycle to the translocation and stimulation of PKC α through the accumulation of nuclear DAG, a well known physiological activator of this isoform (Nishizuka, 1984). PKC could phosphorylate a number of nuclear proteins, besides nuclear lamins (Cocco *et al.*, 1994), involved in the early nuclear events leading to DNA replication. The role of PKCα in the nucleus has been recently highlighted by a report showing that nuclear DAG kinases is a substrate for PKCα (Topham *et al.*, 1998). In addition to IGF-I also Interleukin 1α stimulates nuclear PLC β_1 in human Osteosarcoma SaOS-2 cells (Marmiroli *et al.*, 1994) in a similar time frame indicating that also in this case the breakdown of nuclear PIP_2 represents one of the earliest events in the signalling evoked by the cytokine. More recently it has been confirmed that nuclear PLC β_1 is constitutively expressed in the nucleus and that its activation is related with the very early steps of nuclear signalling while a new PLC δ4 is expressed later on during the transition from G_1 to S phase (Liu *et al.*, 1996). The evidence of the existence of a nuclear inositol lipid cycle has been demonstrated by the large number of findings reported above. Albeit these results suggest a key role for PLC β_1 at the nucleus it was still lacking a direct link between nuclear PLC β_1 activation and IGF-I induced mitogenesis. For this reason we have set up two separate strategies in order to better clarify this issue. Since antisense technique offers the potential to block the expression of specific genes within the cell, we have tried to induce the silencing of the PLCβ_1 gene by overexpressing antisense mRNA. At the same time we have overexpressed the full length cDNA of PLC β_1 in the 5'-3' orientation. Swiss 3T3 cells were transfected with the antisense PLC β_1 cDNA and the measurement of PLC β_1 levels in these stably transfected cells indicated that the enzyme is absent and that this is accompanied by a dramatic reduction of the stimulation of DNA synthesis after IGF-1 stimulation (Manzoli *et al.*, 1997), which is known to induce activation of the nuclear PLC β_1 (Martelli *et al.*, 1992). On the contrary overexpression of full length PLC β_1 cDNA in the sense orientation increased dramatically the number of cell in S phase after IGF-I treatment (Manzoli *et al.*, 1997). Albeit further experiments are required to confirm the inhibition of DAG generation and of PKC translocation to the nucleus, these findings argue strongly for a key role for nuclear PLC β_1 in the very early steps of the signalling machinery responsible for the onset of DNA synthesis. That there is a direct link between PLC β_1 and DNA synthesis is also linked by the peculiar chromosome localisation of this PLC, since we have demonstrated the localisation of the PLC β_1 gene in the rat chromosome 3q35-36 (Calabrese *et al.*, 1995), which is a hot spot for genetic alterations in the rat since it is frequently rearranged in a number of tumours induced by chemical carcinogenesis (Debriec-Tychter *et al.*, 1991; Holeck *et al.*, 1989; Endo *et al.*, 1990). The nuclear localisation of PLC β_1 and the chromosomal localisation of its gene in a hot spot region intrigue about the relationship between normal and/or neoplastic growth and nuclear PLC signalling in the light of the suggest-

ed role of the autonomous nuclear signalling via inositol lipids in several all functions (Divecha *et al.*, 1993b; Maraldi *et al.*, 1994; Martelli *et al.*, 1996; York *et al.*, 1994a; York *et al.*, 1994b; Capitani *et al.*, 1990; De Vries *et al.*, 1995; Payrastre *et al.*, 1992). A new step of this story has been recently added by investigations aimed to ascertain whether other members of the PLCβ family could localise in the nucleus and which role could be exerted by each single isozyme upon mitogenic stimulation. Using NIH 3T3 cells it has been possible to obtain with two purification strategies, in the presence or in the absence of Nonidet P-40, both intact nuclei still maintaining the outer membrane and nuclei completely stripped of their envelope. In these nuclei we show that not only PLCβ$_1$ is present but also the other members of the family are detectable. The more abounding isoform is PLCβ$_1$ followed by PLCβ$_3$, PLCβ$_2$ and PLCβ$_4$ respectively. All the isoforms are enriched in nuclear preparations free from nuclear envelope and cytoplasmatic debris, indicating that the actual localisation of the PLCβ isozymes is in the inner nuclear compartment (Cocco *et al.*, 1999). Upon mitogenic stimulation the nuclear PLC activity increases to a higher extent in membrane stripped nuclei respect to intact nuclei, suggesting that the PLC located in the interior of the nucleus is involved in the phosphoinositide signalling events related to cell growth and the increase in PLC activity is due almost entirely to PLCβ$_1$ (Cocco *et al.*, in press). The stimulation of this PLC is paralleled by both its phosphorylation and translocation in the same time frame as MAP kinase to the nucleus and requires an intact cytoskeleton (Martelli *et al.*, 1999).

These findings strongly support the functional relevance during mitogenic stimulation of an autonomous inositol lipid cycle, which takes place at the nuclear interior, giving rise to a discrete topography which is directly related to the function of PLC signalling in that stimulation by a ligand, i.e. IGF-I, of a tyrosine kinase receptor activates only nuclear/nuclear matrix associated PLC but not the one associated to the plasma membrane. In this context it is interesting to report that at the nuclear envelope there is a separate phospholipase activity which utilises phosphatidylcholine as substrate following different types of stimulation (Baldassare *et al.*, 1997). The very beginning of the nuclear inositol lipid story has dealt with the changes occurring in the amount of PIP$_2$ in nuclei of Friend cells differentiated towards erythrocytes (Cocco *et al.*, 1987). Following this early report evidence has been obtained hinting at nuclear PLC β$_1$ as a central signalling enzyme during erythroid differentiation of Friend cells (Martelli *et al.*, 1994; Martelli *et al.*, 1995; Zini *et al.*, 1995; Divecha *et al.*, 1995). Moreover using the antileukaemic drug tiazofurin we have suggested nuclear PLC β$_1$ as possible target for chemiotherapeutic drugs capable of modulating differentiation of leukaemia cells (Manzoli *et al.*, 1995). Indeed tiazofurin, which is capable of inducing erythroid differentiation along with down-regulation of c-Ki-ras proto-oncogene (Olah *et al.*, 1988) and inhibiting an uncharacterised nuclear PLC activity in Friend cells (Billi *et al.*, 1993) affects expression of nuclear PLC β$_1$. Isolated nuclei from undifferentiated cells contain PLC β$_1$ which is markedly lowered in nuclei from DMSO or tiazofurin treated cells and this is accompanied by a down-regulation of PLC activity and a reduction of PLC β$_1$ mRNAs as evidenced by the Northern analysis (Martelli *et al.*, 1994; Manzoli *et al.*, 1995). Therefore the erythroid differentiation system *in vitro, i.e.* Friend erythroleukemia cells treated with different inducers, offered an opportunity of evaluating the relationship between the induction of haemoglobin synthesis and the activity of the enzymes of the nuclear inositol lipid cycle (Martelli *et al.*, 1995). The data obtained with this experiments suggest that the down-regulation, induced by DMSO or tiazofurin, of the nuclear PLC β$_1$ isoform is somehow related to with the establishment of erythroid differentiation and that this enzyme could be a possible target for anti-tumour drugs (Manzoli *et al.*, 1995).

More recently this issue has been deeper investigated and it has been shown that nuclear PLC β$_1$ is directly involved in switching the erythorleukemia cells programming for an undifferentiated to a differentiated state.

The availability of a mutant lacking the nuclear localisation signalling, which is determined by a cluster of lysine residues in the COOH- terminal tail (Kim *et al.*, 1996), gave us the possibility of analysing the different role of PLC β$_1$ depending on its subcellular localisation. Indeed when PLC β$_1$ is localised almost entirely in the nucleus, by overexpressing the wild-type cDNA containing the nuclear localisation signal, the differentiation towards erythrocytes, as determined by both the

expression of β-globin and the activation of the transcription factor NF-E2, essential for erythroid differentiation upon DMSO treatment (Andrews *et al.*, 1993), is abolished whilst Friend cells transfected with the PLC β_1 mutant lacking the nuclear localisation signal and overexpressing PLC β_1 only in the cytoplasm differentiate exactly as parental cells do (Matteucci *et al.*, 1998). This result assign to nuclear PLC β_1 a role as a negative regulator of erythroid differentiation and fits with the peculiar nuclear localisation of PLC β_1 in other tumour cells such as C6Bu-1 glioma cells (Bahk *et al.*, 1998).

It is worth mentioning that it has been recently reported that PIP_2 targets the BAF (Brm associated factor) complex to chromatin and nuclear matrix defining a direct interface between chromatin organisation and signal transduction (Zaho *et al.*, 1998). This chromatin remodelling factor acts similarly to transcription factors and its dependency on PIP_2 strengthens the contention that nuclear PLC activity is mainly involved in maintaining the optimal concentration of nuclear PIP_2.

All in all the data discussed above clearly demonstrate the existence of a PLC signalling in the nucleus and sustain the role of this pathway in the control of the early steps of the molecular machinery which regulates both proliferative and differentiative processes.

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