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Intranuclear domains involved in inositol lipid signal transduction*

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Once experimentally established that isolated membrane-free nuclei were capable of synthesizing polyphosphoinositides (Cocco *et al.*, 1987) and that some agonists induced changes of inositide metabolism at the nuclear but not at the cytoplasmic level (Martelli *et al.*, 1992), the question arised of the precise localization of this signaling system within the nucleus (Irvine and Divecha, 1992). In fact, it appeared of fundamental interest to determine whether the inositides and the related enzymes are restricted to the nuclear envelope membranes, or localized at DNA-containing structures. In the first case, the system could represent an extension of the transduction system located at the cell membrane, in the second, it could be autonomous and involved in the regulation of genomic functions.

Experimental responses to the localization problem have been accumulated in the last years (for comprehensive reviews, see D'Santos *et al.*, 1998; Neri *et al.*, 1999; Maraldi *et al.*, 1999). This study is particularly concerned with the possible involvement of nuclear inositides in the modulation of essential nuclear functions, such as chromatin remodeling and transcript splicing.

MULTIPLE ROLES OF POLYPHOSPHOINOSITIDES

Redefinition of the roles of polyphosphoinositides in cell biology could also account for the localization of these molecules at multiple cell sites. The variety of phosphorylated inositides do not act solely as precursors of second messengers, but play additional roles. PIP_3, originated by the phosphorylation of PIP_2 at the 3' position by PI 3-kinase, is not hydrolyzed by phospholipases but acts itself as a signal, capable of activating target proteins through interaction with PH and SH2 domains. PIP_2 can directly interact with protein modules (Toker, 1998). Several nuclear proteins present a PI-binding motif and, as some actin-associated proteins affecting the cytoskeleton assembly, could modulate nuclear activities. In this contest, it appears crucial to identify the nuclear sites of interaction between PIP_2 and the related enzymes (kinases and phospholipases). In fact, since the global level of PIP_2 does not vary dramatically in response to agonists, it is conceivable that variations occur locally through compartmentalization processes. PIP_2-binding proteins, therefore, might play crucial roles in signaling regulation, allowing lipid substrate-enzyme interactions to occur at specific sites. The lipid-derived second messengers and/or the inositide-protein interactions could, in turn, modulate functions localized at the same sites, in a sort of channeled pathway controlled by the inositide signaling. The platform for these multiple interactions is conceivably constituted by structural proteins containing PH domains.

NUCLEAR SITES OF PHOSPHOLIPID LOCALIZATION

Lipids were considered for a long time as structural constituents of cell membrane without spe-

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cific functions. However, even before the demonstration of the signaling role of the inositides (Berridge and Irvine, 1984), phospholipids were identified as minor chromatin components affecting DNA stability, gene expression and release of ribonucleoproteins (Manzoli *et al.*, 1982; 1985). Autoradiographic and cytochemical evidence were provided on the localization of nuclear phospholipids at the interchromatin granules in the interchromatin domain (Maraldi *et al.*, 1984; 1987), in association with nuclear matrix proteins (Maraldi *et al.*, 1992; 1993). The involvement of nuclear phospholipids in DNA replication control was deduced by the observed decrease of their amount just in the cell undergoing S phase. This suggested that an increased phospholipase activity could release lipid-derived second messengers capable of affecting DNA polymerase activity (Maraldi *et al.*, 1993; 1994). Although based on methods that did not allow the identification of the phospholipid species, these findings identified the nuclear sites at which, subsequently, the inositides and the related signaling enzymes have been localized.

The functional importance of the inositides as signaling molecules partly obscured the role of other phospholipids, that has been re-evaluated only in recent times (D'Santos *et al.*, 1998). Among phosphoinositides, the linchpin is PIP_2, as source of second messengers, precursor of PIP_3, and signaling molecule on its own right. Moreover, since reliable antibodies are available only against PIP_2, its localization within the nucleus has been deeply investigated. The results obtained by different groups using complementary techniques agree in localizing PIP_2 at two main sites: chromatin-containing domains and interchromatin granules. These findings are accounted by the presence of PI-binding motif in a variety of nuclear proteins, among which some histones, polymerases and matrix constituents (Yu *et al.*, 1998). The presence of a soluble pool of PIP_2, associated to the chromatin, and of an insoluble pool, associated to the inner nuclear matrix, has been demonstrated by fractionation techniques and ultrastructural immunocytochemistry. Interestingly, the nuclear matrix-associated insoluble pool is insensitive to detergents and is extractable only by phospholipase hydrolysis (Maraldi *et al.*, 1994). Its localization, by confocal microscopy, occurs at speckles (Boronenkov *et al.*, 1998), and at clusters of interchromatin granules by electron microscopy immunogold techniques (Mazzotti *et al.*, 1995). Quantitative variations of the nuclear PIP_2 associated to the chromatin were observed in response to agonists affecting nuclear phospholipase activity, such as IGF-I in 3T3 rat fibroblasts (Maraldi *et al.*, 1995), and IL-1 in Saos-2 human osteosarcoma cells (Zini *et al.*, 1996). In both cases, the increased hydrolysis of nuclear PIP_2 resulted in the activation of transcription factors affecting cell cycle and differentiating pathways.

In conclusion, phosphoinositides can interact with chromatin and nuclear matrix proteins constituting a platform which could tether other proteins through interactions with PH and SH2 domains. Owing to their functional roles, the localization of lipid kinases, phospholipases, and second messenger-activated protein kinases was widely investigated.

LIPID KINASES

The presence of inositides in the nucleus *per se* could not account for the existence of a signaling system independent from that at the cell membrane. However, the presence in the nucleus of the enzymes involved in inositide metabolism represents a formidable piece of evidence supporting this possibility. The inositol ring is phosphorylated at different position by a family of kinases; three of them were identified in the nucleus, namely PI 4-kinase, PI(5)P4-kinase and PI(4)P5-kinase. These enzymes were demonstrated to be nuclear matrix-associated (Payrastre *et al.*, 1992), and localized at speckles in association with SC-35 splicing factor (Boronenkov *et al.*, 1998). Inositides can be phosphorylated also at the 3 position by PI 3-kinase. D3-phosphoinositides act as signals that modulate nuclear functions through Akt/PKB, JNK and some PKC isoforms. Also PI 3-kinase has been identified associated to the nuclear matrix (Zini *et al.*, 1996). The inositol kinases can reach the nucleus upon agonist-induced activation at the membrane receptor, as demonstrated for p85 PI 3-kinase subunit translocation induced by IL-1 receptor occupancy in Saos-2 cells (Maraldi *et al.*, 1997; Marmiroli *et al.*, 1998; Bavelloni *et al.*, 1999). Moreover, once translocated to the nucleus, a lipid kinase could be compartmentalized by a substrate-driven mecha-

nism at specific sites (Anderson *et al.*, 1999). A cross-talk between divergent inositide phosphorylation pathways mediated by PIP-kinase and PI 3-kinase might occur at the level of substrate availability as demonstrated at the cytoskeletal level (Lu *et al.*, 1998). In any case, actin-regulatory proteins carrying PIP_2 binding sites, such as nuclear CapG, in association with nuclear matrix actin (Zhao *et al.*, 1998), could represent structural elements that promote the assembly of the inositol substrates and the related enzymes.

In these instances, the inositides act directly on nuclear targets; more frequently these last are activated by second messengers derived from the hydrolysis of polyphosphoinositides by specific nuclear phospholipases. The identification of some members of the PI-PLC family in the nucleus and their co-localization with the inositide substrates represented a fundamental step in the characterization of the signaling system operating at the nuclear level.

PI-PHOSPHOLIPASES

Since the first studies on 3T3 cells responding to IGF-I, the rise of a phosphodiesterase acticvity exclusively at the nuclear level was demonstrated to depend on the PI-PLC-β_1, while cytoplasmic isoforms were not affected (Martelli *et al.*, 1992). All four PLC-β isoforms, which are differently expressed at the nuclear level (Cocco *et al.*, 1999), are considered to interact with negatively charged nuclear inositides through long C-terminal segments of positively charged amino acids not present in other PLC isoforms (Kim *et al.*, 1996). Nevertheless, also other PLC isoforms, namely γ_1 and δ_4, were reported to be involved in nuclear signaling events in specific cell types (Zini *et al.*, 1994 and 1995; Liu *et al.*, 1996). In this case, interactions with nuclear phosphoinositides can occur through PH domains and can account for a substrate-specific compartmentalization.

The involvement of PLCs in signaling responses at the nuclear level is sustained by several experimental findings. In fact, their amount and activity can be up- or down-regulated by agonists affecting either cell proliferation or differentiation (Maraldi *et al.*, 1995). The interactions of PLCs with nuclear components are particularly stable, being maintained after the extraction of soluble components (Zini *et al.*, 1993). Finally, the sites of their localization correspond to specific domains involved in pre-mRNA processing, where other elements of the signaling system, such as the inositides and the targets of lipid-derived second messengers, were also identified (Maraldi *et al.*, 1993 and 1999).

PLCs are constitutively expressed, or actively translocated to the nucleus. At least in the case of PLCβ_1 over-expression, the enzyme was found within the nucleus also in the absence of stimuli (Marmiroli *et al.*, 1996). PLC-β_1 presents a consensus nuclear targeting sequence (Suh et al,. 1988), but it can also interact with negatively charged molecules (Kim *et al.*, 1996). PLC-β_1 presents a MAPK-consensus sequence regulated by phosphorylation, and the activation of nuclear PLC-β_1 requires both PI 3-kinase activity on PKB/Akt and the integrity of the cytoskeleton (Martelli *et al.*, 1999). Translocation of PI 3-kinase appears to be an early event in the activation of nuclear PLC-β_1 in response to IL-1 in Saos-2 cells (Marmiroli *et al.*, 1994; 1998). The activation of other nuclear PLC isoforms is less defined and could involve Ca^{++} in the case of the δ isoform, and nuclear receptor tyrosine kinases in the case of the γ isoform (Csermely *et al.*, 1995).

NUCLEAR TARGETS

The downstream part of the inositide signaling pathway in the nucleus involves lipid-derived second messengers and their target enzymes, as well as direct lipid-protein interactions. The hydrolytic products of PIP_2 are differently involved; in fact, DAG represents a direct activator of PKC, while the role of IP_3 in the regulation of Ca^{++} concentration within the nucleus is indirect and not fully elucidated. DAG represents a lynchpin of the transduction cascade, being originated by the inositides and by PC (D'Santos *et al.*, 1998). The two pools of DAG are differently involved in cell responses and could be localized at specific regions of the nucleus owing to the specificity of PIP_2-PLC and PC-PLD interactions (Petitt *et al.*, 1997). In any case, PKC is activated mainly by PIP_2-derived DAG in short-lasting responses (D'Santos *et al.*, 1999). Interestingly, PKC translocation to the nucleus follows the activation of PLC-β_1, but precedes the synthesis of PI necessary to trigger S phase (Murray

and Fields, 1998). PKC is a cytosolic enzyme which, upon stimulation, translocates at different cell sites, interacting with membrane-associated receptors, cytoskeletal proteins, and nuclear components. Since PKC does not present nuclear localization signals, either DAG or isoform-specific binding proteins can convey the enzyme to the nucleus (Irvine and Divecha, 1992; Imoto *et al.*, 1994). Once translocated to the nucleus, PKC has been found to be co-localized with PIP_2 (Zini *et al.*, 1994) and PLC-β_1 (Maraldi *et al.*, 1999), at the level of clusters of interchromatin granules. PKC has been demonstrated to phosphorylate lamin B *in vivo* thus inducing nuclear lamina disassembly (Baudrier *et al.*, 1992). Other nuclear substrates, among which DNA and RNA polymerases and transcription factors were reported to be phosphorylated by PKC *in vitro* (Imoto *et al.*, 1994).

Another nuclear target of the inositide sygnaling is PKB/Akt. This lipid-dependent protein kinase could affect the activity of PLC-β_1 (Meier *et al.*, 1997; Andjelkovic *et al.*, 1997). The peculiarity of PKB is its dependence on PI 3-kinase, which is translocated to the nucleus and localized at nuclear matrix-associated interchromatin granules (Zini *et al.*, 1996).

Protein kinases are not the unique targets of inositide signaling. In fact, polyphosphoinositides interact with proteins involved in chromatin remodeling, thus affecting its template availability, which is a prerequisite of transcription factor binding. Chromatin remodeling complexes, such as BAF, are capable of modifying nucleosome structure in order to allow the binding of transcription factors *in vivo*. The BAF complex, once activated by inositides, Ca^{++}, and PKC, tightly binds to the nuclear matrix and causes massive chromatin de-condensation and nuclear volume enlargement. The responsive element of BAF is the PIP_2-binding actin-regulatory protein CapG, which can compete with PI 3-kinase for its PIP_2 substrate. Therefore, chromatin remodeling complex can be modulated in a multiple way by PLCs and PI 3-kinase (Zhao *et al.*, 1998).

NUCLEAR DOMAINS INVOLVED IN INOSITIDE SIGNALING

The identification of elements of inositide signaling at nuclear sites has been achieved by immunocytochemical techniques at confocal and electron microscopy level (Maraldi *et al.*, 1999). Each method presents advantages and drawbacks; however, the results obtained are generally consistent and complementary. The main advantage of fluorescent-labeled probes at confocal microscope is the possibility of examining the whole cell maintaining its three-dimensional organization, while gold-labeled probes at electron microscope ensure very high resolution and quantitative evaluations. The availability of GFP-tagged probe technique, overcomes the limits of traditional cytochemistry based on fixed specimens, and allows one the use of *in vivo* localization assay. The results till now obtained with this technique confirm those previously obtained with conventional immunocytochemistry (Bavelloni *et al.*, 1999).

The use of *in situ* nuclear matrix preparations allowed one to identify the localization sites of the insoluble versus soluble pools of some elements of the signaling system. The PIP_2 soluble pool is quite diffused within the nucleus; in fact it is localized at both heterochromatin and interchromatin domains, in agreement with the presence of a large number of nuclear proteins presenting PI-binding motifs. As a consequence of the interactions between polyphosphoinositides and nuclear proteins such as histones, polymerases and BAF complexes, PIP_2 localization sites have been detected on the heterochromatin, mainly at its periphery, on some nucleolar components, and on ribonucleoproteins in the interchromatin domains. The insoluble pool, on the other hand, is restricted to interchromatin granules. Other elements of the signaling system were detected to co-localize with PIP_2 at the same sites (Maraldi *et al.*, 1999). The molecular events occurring at these districts, that is transcription of hnRNA, and pre-mRNA processing, have been widely investigated. Nascent transcripts correspond to perichromatin fibrils (Fakan, 1994), while interchromatin granule clusters, detectable as fluorescent speckles at confocal microscope, contain many of the splicing factors (Mattern *et al.*, 1999). Splicing can occur both co- and post-transcriptionally, that is at the perichromatin fibers as well as at the interchromatin granules (Smith *et al.*, 1999). Activation of splicing factors involves phosphorylation, that modulates their shuttling from transcriptional to post-transcriptional sites (Misteli and Spector, 1997). Therefore, the presence of a signaling system at these sites, co-localized with the protein kinases involved in the phosphorylation of splicing

factors, strengths the possibility of a functional involvement of inositides into crucial steps of nuclear metabolism.

CONCLUSIONS

The control mechanisms on signaling based on polyphosphoinositides are multiple, and the compartmentalization at different cell sites seems to be a crucial one. The peculiarity of the nuclear organization, based on functional domains instead of membrane-delimited organelles, requires massive re-organization of its constituents (matrix, chromatin, ribonucleoproteins) in correspondence with the phases of the cell cycle and in response to differentiation stimuli (Maraldi *et al.*, 1998). The nuclear autonomous signaling system, characterized by the multiple roles of the inositides, acting not only as source of second messengers, but also as signals through interactions with protein modules, appears to ensure the requested flexibility to allow the genome to be differently modulated.

The localization of the components of the signaling system at specific nuclear domains, based on immunocytochemistry at confocal and electron microscope, represents a key moment for the interpretation of the mechanisms modulated by the signaling pathway. The next steps in this direction will involve the use of GFP-tagged probes and the isolation of subnuclear fractions capable of responding to agonists. Once this goal will be achieved, a new nuclear domain, the transduceosome, should be fully characterized.

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