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Phosphoinositide 3-kinase is associated to the nucleus of HL-60 cells and is involved in their ATRA-induced granulocytic differentiation*

S. Capitani, M. Marchisio, L.M. Neri, F. Brugnoli, A. Gonelli, and V. Bertagnolo

Department of Morphology and Embryology, Human Anatomy Section, Via Fassato di Mortara 66, 44100 Ferrara, Italy.

Phosphoinositide 3-kinases (PI 3-kinases), a class of enzymes implicated in growth factor-induced mitogenic response and many other intracellular signalling pathways, act by phosphorylation at the D-3'-OH position of the myo-inositol ring of phosphoinositides (reviewed in Kapeller and Cantley, 1994; Toker and Cantley, 1997; Vanhaesebroeck et al, 1997; Domin and Waterfield, 1997). The products of these reactions, 3-phosphoinositides, are not substrates of any known phospholipase C, are not components of the canonical phosphoinositide turnover and are now regarded as important second messengers, whose downstream effects are implicated in many processes including regulation of the cell cycle, cellular transformation, vesicle transport and reorganisation of the cytoskeleton (Domin and Waterfield, 1997).

The class IA PI 3-kinases, which are regulated by growth-factor receptor tyrosine kinases, are tightly coupled heterodimers constituted of a regulatory 85 (or 55) kDa and a catalytic 110 kDa protein. Five types of p85-like regulatory subunits exist: p85α, p85β, p55 (termed γ or $p55^{PIK}$) and two alternative splices of p85α (p55α and p50α) (Domin and Waterfield, 1997; Fruman *et al.*, 1996). All five interact with p110 kDa catalytic subunits of which three distinct isoforms have been identified: α, β and δ. Sequence and structural analysis of the regulatory subunit shows that it is a multidomain protein, without intrinsic catalytic activity, which mediates association of the enzyme with tyrosine-phosphorylated motifs of upstream signalling molecules or with specific proline-rich regions of a number of cellular proteins. Target motifs can be found in the cytoplasmic domains of receptor tyrosine kinases or in adapter molecules that serve as their primary enzymatic targets.

Increased levels of 3-phosphorylated inositides have been detected during diverse cellular processes, including cell growth, and maturation (Soltoff *et al.*, 1992; Varticovski *et al.*, 1994; Ai *et al.*, 1995; Domin and Waterfield, 1997). The recent explosion in interest in the role of PI 3-kinases in cellular processes has been facilitated by the availability of a range of reagents which have greatly simplified experimental investigations in this area. The most widely used of these are two specific and cell-permeable inhibitors of PI 3-kinase, namely wortmannin (Ui *et al.*, 1995) and LY294002 (Vlahos *et al.*, 1994).

PI 3-kinase is present in HL-60 promyelocitic cell line (Volinia *et al.*, 1995), a well established model to study granulocytic differentiation when treated with all-*trans* retinoic acid (ATRA), that is known to act through the RAR/RXR nuclear receptors, members of the thyroid/steroid receptors superfamily of transcription factors (Chambon, 1994). Data obtained in our laboratory have demonstrated that the ATRA-induced granulocytic differentiation of HL-60 cell line is accompa-

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nied by a specific pattern of expression and activity of enzymes, like phospholipase C and protein kinase C, related to the phosphoinositide signalling pathways (Zauli *et al.*, 1996; Bertagnolo *et al.*, 1997). In order to determine whether PI 3-kinase plays a role in HL-60 cell differentiation as well, we have monitored the amount and the activity of the enzyme during the differentiation process triggered by ATRA.

Immunochemical analysis, performed on different cell fractions obtained from HL-60 induced to granulocytic differentiation after treatment with 1μM ATRA, demonstrated the presence of the enzyme also in the nuclear compartment, in which it significantly increased during the differentiation process. The subnuclear distribution of PI 3-kinase was further explored, and the enzyme, that showed a salt- and detergent-resistance, appeared to be tightly bound to the nuclear matrices. In addition, all the examined nuclear fractions obtained from cells treated with ATRA showed increased levels of PI 3-kinase (Marchisio *et al.*, 1998). The prevalence of the enzyme in the cytoplasm together with a discrete distribution inside the nucleus of HL-60 cells was confirmed by morphological analysis by *in situ* immunocytochemistry combined with confocal microscopy. In particular, confocal analysis of isolated nuclei and nuclear matrices showed that the anti p85 immunofluorescence, which painted a fine fibrogranular meshwork in the inner nuclear domains, was markedly increased after HL-60 cell differentiation (Marchisio *et al.*, 1998). This suggest that the distribution of the enzyme and the sites of its activity may be spatially regulated by the nuclear matrix, which it is also known to govern other key nuclear functions, like DNA replication, repair and transcription.

The analysis of PI 3-kinase activity, evaluated by *in vitro* assays, and performed on the different cell fractions, demonstrated that also purified nuclei were able to produce 3-phosphorylated lipids, and that, when exogenous substrates were supplied, the *in vitro* synthesis of PtdIns(3,4,5)P_3 was resumed by all nuclear fractions and reached higher levels in nuclei and nuclear matrices purified from ATRA-treated cells.

Since it has been shown that the fungal product wortmannin inhibits agonist stimulated PI 3-kinase activity at nanomolar concentrations, this molecule was employed to verify the role of this enzyme in granulocytic differentiation of HL-60 cells. In particular, we demonstrated that the contemporary ATRA and wortmannin administration significantly reduced tendency of the cells to mature morphologically, so that after 96 hrs of treatment with the two drugs, the differentiation was observable only in less than 20% of the cells. Accordingly, combined treatment with ATRA and wortmannin largely reduced, in both cells and nuclei, PI 3-kinase activity, as judged by the recovery of PtdIns(3,4,5)P_3 (Bertagnolo *et al.*, 1999).

It is known that growth factor receptors with intrinsic tyrosine kinase activity, upon activation, autophosphorylate their cytoplasmic moiety on several tyrosine residues, which form distinct binding sites for SH2 domain-containing proteins. These activated receptors can directly recruit a number of enzymes involved in various signal transduction pathways, including components of the lipid-dependent signalling pathways (Reedijk *et al.*, 1992; Klinghoffer *et al.*, 1996; Clark *et al.*, 1998). Other agonists, which do not possess an intrinsic tyrosine kinase activity, are still able to activate non receptor protein tyrosine kinases, inducing the tyrosine phosphorylation of several substrates. A number of phosphotyrosine-containing proteins, defined as adapter proteins, are recognised to play an important role in linking the signal transduction emanating from both activated receptors or non receptor PTK to lipid-dependent enzymes, such as PI 3-kinase and PLC-γ1.

Since ATRA has a nuclear receptor that belongs to the transcription factor family (Chambon, 1994) it is likely that the observed increase in nuclear PI 3-K derives from a transcriptional event regulated by ATRA. However, activation of PI 3-kinase in this system probably requires a mechanism different from those involving receptor or non receptor tyrosine kinase, which rapidly activate the enzyme (Varticovski *et al.*, 1994; Cantley *et al.*, 1991).

To identify and characterise the mechanism involved in the recruitment and activation of PI 3-kinase during granulocytic differentiation induced by ATRA, phosphorylated proteins that coimmunprecipitated with PI 3-kinase were analysed in HL-60 treated with 1μM ATRA, which induced terminal granulocytic differentiation of most cells. Two phosphoproteins showing an association with

the p85 regulatory subunit after ATRA treatment, in both cells and nuclei, were identified as PLCγ1 and Vav, respectively (Bertagnolo *et al.*, 1998).

The protooncogene Vav is expressed exclusively in hematopoietic cells and contains an array of structural motifs including a guanine nucleotide exchange domain (GEF) for the Rho/Rac/CDC42 family of small GTPases, a pleckstrin homology domain and two src homology 3 domains (SH3) that flank on SH2 domain (Collins *et al.*, 1997). By means of these different motifs, Vav is capable of physically associating with a number of proteins, both in the cytoplasm and in the nucleus, playing a role in several distinct cellular functions, like cell proliferation and maturation, cytoskeletal reorganisation, regulation of gene expression and apoptosis (Clevenger *et al.*, 1995; Fischer *et al.*, 1998; Bachmann *et al.*, 1999; Raab *et al.*, 1997; Kong *et al.*, 1998).

The binding of the Vav SH2 domain to phosphorylated PTKs is thought to serve for the recruitment of activated kinases, which in turn can phosphorylate Vav (Collins *et al.*, 1997). Human Vav protein contains 31 tyrosine residues with unknown functional significance, except for Tyr174, which is phosphorylated by Lck (Collins *et al.*, 1997). *In vitro*, tyrosine phosphorylated Vav binds to a host of SH2 domains, including those present in PLC γ1 and in the p85 regulatory subunit of PI 3-K.

In a recent work it has been reported that both PtdIns (4,5)P2 and PtdIns (3,4,5)P3 interact with Vav, modulating the ability of Lck to phosphorylate and activate Vav in response to mitogens. It has been proposed that the PH domain of Vav, when complexed to PtdIns (4,5)P2, inhibit Vav GEF activity. On the other hand, phosphorylation of Vav by Lck was enhanced in the presence 3-phosphorylated lipids, suggesting that activation of PI 3-kinase could serve to eliminate a Vav inhibitor and simultaneously produce activators of Vav activity (Han *et al.*, 1998).

In our cell model Vav is present in both cells and nuclei and increased its amount and its level of tyrosine phosphorylation during ATRA-induced differentiation. In particular, our data demonstrated a preferential nuclear localisation of tyrosine-phosphorylated Vav during the course of ATRA-induced granulocytic differentiation of HL-60 cells (Bertagnolo *et al.*, 1998).

Since phosphorylated Vav is known to bind SH2-containing proteins and, according to our previous data, HL-60 granulocytic differentiation is accompanied by a selective nuclear increase in PLC-γ1 and PI 3-kinase, both containing SH2 domains, the interaction between these proteins was analysed. None of the enzymes coimmunoprecipitated with Vav in untreated cells and nuclei, while a significant amount of both enzymes associated with Vav after granulocytic differentiation. The existence of protein-protein association between Vav, PLC-γ1 and p85 was confirmed by cross immunoprecipitations with anti-PLC-γ1 antibody and by the finding of a PI 3-kinase activity in anti-Vav immunoprecipitates after ATRA treatment (Bertagnolo *et al.*, 1998).

The association of non-phosphorylated p85 with the tyrosine phosphorylated Vav suggests that this binding might be mediated through the SH2 domains of p85. To elucidate whether the two SH2 domains of p85 were responsible for its interaction with Vav, cell and nuclear lysates were adsorbed on GST-fusion N-terminal and C-terminal p85-SH2 proteins. Our data demonstrated that, in control cells, ATRA treatment increased the Vav binding to both N- and C-terminal SH2s, and most of Vav copurified with C-terminal p85 SH2. On the contrary, in isolated nuclei, it was the SH2-N-terminal that increased its association with tyrosine phosphorylated proteins in differentiated conditions.

The data reported above demonstrated, for the first time, the presence of Vav/PLC-γ1/PI 3-K proteins including complexes in the nucleus of differentiating HL-60 cells. Since Vav is the only of the Vav/PLC-γ1/PI 3-K proteins to possess a nuclear localisation sequence, it is possible that Vav is directly involved in the nuclear transportation of PLC-γ and PI 3-kinase.

The downstream targets of PI 3-K products include some PKC isoforms and cytoskeletal proteins (Domin and Waterfield, 1997), whose role in the differentiation process has to be fully elucidated, and whose recruitment might be directly mediated by PIP_3 itself. These observations suggest that the nuclear increase in PI 3-kinase activity during HL-60 treatment with ATRA may activate specific PKC isoforms, which are required for full granulocytic differentiation of this cell model.

Several possibilities can be envisioned to explain the progressive accumulation of Vav adapter protein in the nucleus of HL-60 cell line induced to differentiate by ATRA. Some studies have already reported the presence of Vav in the nuclear compartment (Hobert *et al.*, 1994; Clevenger *et al.*, 1995), and recent observations may support a role of Vav for the regulation of gene expression and other nuclear functions (Katzav *et al.*, 1995; Hobert *et al.*, 1996; Romero *et al.*, 1996). It has also been demonstrated that Vav can associate to several cytoskeletal proteins, including tubulin, zyxin and polymerised actin. Although the meaning of the Vav association to proteins of the cytoskeleton is not known, it is possible that Vav translocates into the nucleus via components of the cytoskeleton. Since we and other groups of investigators have demonstrated that the nuclear matrix contains all the inositol lipid substrates for PLC-γ1 and PI 3-K, the presence of Vav inside the nucleus might suggest an essential role of this protein in targeting PLC-γ1 and PI 3-K enzymes to their relevant nuclear substrates.

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