Nuclear phosphatidylinositol 3,4,5-trisphosphate, phosphatidylinositol 3-kinase, Akt, and PTEN: emerging key regulators of anti-apoptotic signaling and carcinogenesis

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Inositol lipid-derived second messengers have long been known to have an important regulatory role in cell physiology. Phosphatidylinositol 3-kinase (PI3K) synthesizes the second messenger 3,4,5'-phosphatidylinositol trisphosphate (PtdIns 3,4,5P3) which controls a multitude of cell functions. Down-stream of PI3K/PtdIns 3,4,5P_3 is the serine/threonine protein kinase Akt (protein kinase B, PKB). Since the PI3K/PtdIns 3,4,5P_3 /Akt pathway stimulates cell proliferation and suppresses apoptosis, it has been implicated in carcinogenesis. The lipid phosphatase PTEN is a negative regulator of this signaling network. Until recently, it was thought that this signal transduction cascade would promote its anti-apoptotic effects when activated in the cytoplasm. Several lines of evidence gathered over the past 20 years, have highlighted the existence of an autonomous nuclear inositol lipid cycle, strongly suggesting that lipids are important components of signaling pathways operating at the nuclear level. PI3K, PtdIns(3,4,5)P_3, Akt, and PTEN have been identified within the nucleus and recent findings suggest that they are involved in cell survival also by operating in this organelle, through a block of caspase-activated DNase and inhibition of chromatin condensation. Here, we shall summarize the most updated and intriguing findings about nuclear PI3K/PtdIns(3,4,5)P_3/Akt/PTEN in relationship with carcinogenesis and suppression of apoptosis.

Key words: PtdIns(3,4,5)P_3; PI3K; Akt; nucleus; apoptosis; cancer; PTEN.

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Transferring of signals from the plasma membrane to the cell nucleus is an extremely complex multistep process which strongly depends, among other molecules, on PtdIns lipid signaling molecules (Di Paolo and De Camilli, 2006). The repertoire of cellular processes known to be directly or indirectly regulated by this class of lipids has now dramatically expanded. Inositol phospholipids are concentrated at the cytosolic surface of membranes where they are substrates for phospholipases, kinases, and phosphatases. Among lipid kinases, PI3K has emerged as a key regulator of multiple signaling cascades, being involved in the control of many critical cell responses (Engelman *et al.,* 2006). PI3K synthesizes four species of non-canonical, 3'-phosphorylated inositides: PtdIns(3)P, PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PtdIns (3,4,5)P_3. Several lines of evidence indicate that members of PI3K family can also be considered as oncogenes, because they control cell cycle progression, differentiation, survival, invasion and metastasis, and angiogenesis (Cully *et al.,* 2006). Many biological effects of PI3K are mediated through the activation of the downstream target Akt, a 57-kDa serine/threonine protein kinase, which belongs to the family of the AGC protein kinases (Hanada *et al.,* 2004). Most of the studies performed on PtdIns-dependent signal transduction pathways have focused on events that occur at the plasma membrane and in the cytoplasm. However, phosphoinositides and their biosynthetic machinery are also localized in the nucleus (Irvine 2004; Martelli *et al.,* 2005a; Manzoli *et al.,* 2005). Remarkably, nuclear inositol lipid cycle is largely independent from that of the plasma membrane, suggesting that the nucleus constitutes a functionally distinct compartment for PtdIns metabolism. PtdIns(3,4,5)P_3,

PI3K, and Akt have been reported to be present in the nucleus (Martelli *et al.,* 2006a). In this review article, we shall update our knowledge of the roles played by these molecules in the nucleus in relationship with carcinogenesis and anti-apoptotic signaling. However, we shall firstly review some general data about 3′-phosphorylated inositides, PI3K, and Akt.

3'-phosphorylated inositol lipids and PI3K

Resting mammalian cells contain significant levels of PtdIns(3)P, but hardly any of the other 3'-phosphoinositides. While the overall levels of PtdIns(3)P do almost not increase upon cell stimulation with agonists, the levels of the other 3'-phosphoinositides can rise dramatically (Vanhaesebroeck *et al.,* 2001). Since these lipids are not the target of any known phospholipases, they are metabolized by phosphatases that act on the inositol ring. PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a 3′-phosphorylated inositol lipid-phosphatase which has received much attention recently, because of its role as a tumor suppressor gene (Sansal and Sellers, 2004). PTEN converts PtdIns(3,4)P_2 to PtdIns(4)P, and PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2. In a significant number of human cancers, PTEN is mutated and/or inactivated so that the PI3K signaling pathway is constitutively activated as a result of the high PtdIns(3,4,5)P_3 levels (Chow and Baker, 2006). Two other phosphatases, SHIP-1 and SHIP-2 (for Src Homology domain-containing Inositol Phosphatases), are capable of removing the 5-phosphate from PtdIns(3,4,5)P_3 to yield PtdIns (3,4,)P_2 (Backers *et al.,* 2003), but their role in down-regulating PI3K-dependent signals is not well understood, taking also into account that PtdIns (3,4,)P_2 shares several functions with PtdIns(3,4,5)P_3 (in addition to unique signalling properties) and may prolong the duration of PtdIns(3,4,5)P_3 signaling. There are multiple isoforms of PI3K in mammalian cells, and these are subdivided into three classes, referred to as I, II, and III (Vanhaesebroeck *et al.,* 2001). Our review will focus on class IA PI3Ks which are the most investigated because they are generally coupled to extracellular stimuli. They display a preference *in vivo* for PtdIns(4,5)P_2 as a substrate. Class IA PI3Ks are heterodimeric enzymes composed of a p110 catalytic subunit (α, β, and δ) and an adaptor/regulatory subunit. There are at least five adaptor proteins that are generated by expression and alternative splicing of three different genes (referred to as Pik3r1, Pik3r2, and Pik3r3). The regulatory subunits function as adaptors and act to localize PI3K to the plasma membrane by the interaction of their SH2 (Src homology) domains with phosphotyrosine residues in activated receptors. They also serve to stabilize p110 and to limit its activity.

Akt

At present, three members of the Akt family have been identified and are referred to as Akt1, Akt2, and Akt3. Although they are products of different genes, they are highly related exhibiting more than 80% sequence homology (Hanada *et al.,* 2004; Brazil *et al.,* 2004). In response to a variety of stimuli (hormones, growth factors, cytokines), inactive (cytosolic) Akt is recruited to the plasma membrane by the products of PI3K, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. Then, Akt is phosphorylated at threonine 308 by a phophoinositide-dependent kinase 1 (PDK1), whose activity strictly depends on 3'-phosphorylated inositol lipids, (Mora *et al.,* 2004) and at serine 473 by a still undefined kinase. This double phosphorylation activates Akt (Brazil *et al.,* 2004). A plethora of Akt substrates have been identified and these include, among the others, BAD, Raf1, members of the FoxO family of transcriptions factors, Iκ-B kinase, procaspase-9, GSK-3-α/β, mTOR, cyclin D1, $p27^{KIP1}$, $p21^{CIP1}$ (Brazil *et al.,* 2004). The large variety of proteins that are phosphorylated by Akt explains why this kinase has rapidly emerged as a key mediator of cell proliferation, differentiation, and survival. Moreover, increasing evidence indicates that Akt plays an important role in tumorigenesis and resistance to chemotherapeutic drugs (Fresno Vara *et al.,* 2004; Martelli *et al.,* 2005b; Martelli *et al.,* 2006b).

Nuclear 3'-phosphorylated inositol lipids and class I_A PI3Ks

The presence of these inositol lipids in the nuclear compartment has been demonstrated by means of different techniques (radioisotope labeling, immunocytochemistry, quantitative immunogold electron microscopy) (Deleris *et al.,* 2006; Martelli *et al.,* 2006a; Lindsay *et al.,* 2006) in a variety of cell types, including PC12 rat pheochromocytoma, Saos-2 human osteosarcoma, rat hepatocytes, HepG2 human hepatocarcinoma, and HL60 human

promyelocytic leukemia (reviewed in Neri *et al.,* 2002). While control of cytoplasmic class IA PI3K is quite well defined, regulation of its nuclear counterpart has been unclear. A major breakthrough has been achieved in PC12 cells stimulated with NGF. By means of a yeast two-hybrid approach, Ye et al. (2000) identified the protein PIKE (Phospho-Inositide 3-Kinase Enhancer) as a novel physiological regulator of nuclear class I_A PI3K. PIKE is a nuclear GTPase characterized by a PX domain and three proline-rich domains, which typically bind to SH3 domains of target proteins. Retroviral infection of PC12 cells showed that NGF-induced nuclear PI3K activity was blocked by a dominant-negative form of PIKE, and that PI3K activation by PIKE was GTP-dependent and required the presence of both p85 and p110 subunit. Subsequently, the same group identified nuclear phosphoinositide-specific phospholipase C (PI-PLC) γ1 as the guanine nucleotide exchange factor (GEF) for PIKE (Ye *et al.,* 2002). Indeed, the SH3 domain of PI-PLCγ1 directly bound the third proline-rich domain (amino acids 353-362) of PIKE and this interaction stimulated GDP dissociation, markedly enhanced GTP binding to PIKE, and was required for nuclear PI3K activation. This finding might partly explain the previous puzzling observation that the mitogenic activity of PI-PLCγ1 does not actually require it to be catalytically active, but does indeed require the SH3 domain to be present (Bae *et al.,* 1998). In addition, the same authors have suggested that down-regulation of nuclear PI3K activity could result from the interaction between PIKE and the protein 4.1N (Ye *et al.,* 2000). Indeed, in NGF-treated PC12 cells, they observed protein 4.1N translocation to the nucleus with a slower time course than for PI3K translocation and PIKE activation. The binding of the protein 4.1N to PIKE inhibited PIKE GTPase activity and prevented association between PIKE and PI3K, resulting in nuclear PI3K activity decrease. The initially identified PIKE isoform is now referred to as PIKE-S (for Shorter), because two more PIKE isoforms have been subsequently identified, PIKE-L (Longer) and PIKE-A, which specifically binds to active Akt. While PIKE-S is exclusively localized in the nucleus, PIKE-L occurs both in the nucleus and cytoplasm. However, its function in the nucleus has not been clarified yet (Ye, 2005).

Nuclear Akt

It is now clear that phosphorylated (active) Akt is present within the nucleus. Indeed, some of its substrates are resident within this organelle, such as the FoxO family of transcription factors (Arden and Biggs, 2002) or the transcriptional coactivator p300 (Pekarsky *et al.,* 2000). Either Akt1 or Akt2 have been reported to migrate into the nucleus in response to a variety of stimuli including serum, activation of B-lymphocytes, hypoglicemic coma, mitogenic stimulation with polypeptide growth factors such as insulin-like growth factor-1 (IGF-1), differentiating treatment of PC12 cells with NGF, or exposure of HL60 and NB4 cells to retinoids (Neri *et al.,* 2002; Matkovic *et al.,* 2006). The nuclear localization signal (NLS) motif of Akt has not been identified so far, nevertheless the oncogene Tcl1 may be involved in Akt nuclear localization (Pekarsky *et al.,* 2000). Whether Akt may be phosphorylated and activated within the nucleus, is controversial. There are reports showing that Akt did not require phosphorylation for entering the nucleus (e.g. Saji *et al.,* 2005). Even though PDK1 has been identified in the nucleus (Kikani *et al.,* 2005), several lines of evidence suggest that Akt migrates to the nucleus after having been phosphorylated at the plasma membrane and that nuclear PDK1 does not target Akt. Rather, it seems that PDK1 nuclear translocation may be a mechanism to sequestrate it from activation of cytosolic signaling pathways (Lim *et al.,* 2003). Indeed, a recent report has demonstrated that in NGF-stimulated PC12 cells Akt phosphorylation is essential for nuclear translocation and retention (Xuan Nguyen *et al.,* 2006). There exists quite an ample body of literature on the localization of active Akt in the nucleus of neoplastic cells. The presence of nuclear phosphorylated Akt has been reported in lung, breast, prostate, and thyroid cancers, as well as in acute myeloid leukemia blasts (Lee *et al.,* 2002; Nicholson *et al.,* 2003; Van de Sande *et al.,* 2005; Vasko *et al.,* 2004; Brandts *et al.,* 2005; Montironi *et al.,* 2005). It is intriguing that in the prostate, the extent of Akt nuclear localization increases during the progression from normal tissue to low grade prostatic intraepithelial neoplasia (PIN), high grade PIN, and tumor (Van de Sande *et al.,* 2005). Furthermore, in prostatic carcinomas the extent of Akt nuclear localization correlates with the Gleason score, which is the most powerful predictor of tumor progression after prostatectomy

(Montironi *et al.,* 2005). All three Akt isoforms display a classic leucine rich, leptomycin-sensitive nuclear export sequence (NES). Stable overexpression of Akt1 with a non-functional NES, resulted in persistent nuclear localization of Akt1 and enhanced cell migration *in vitro* of $Akt1^{-/-}$ fibroblasts (Saji *et al.,* 2005). This finding may further support the hypothesis that Akt nuclear localization is somehow involved in some aspects of carcinogenesis and/or tumor progression.

Nuclear PTEN

There are several reports which have addressed the issue of nuclear PTEN. Four, non-traditional, putative NLS motifs have been identified in PTEN. Mutations in each of the four NLS-like region of PTEN did not alter entry into the nucleus. However, when mutations were combined, it was found that nuclear localization of PTEN was affected, thereby indicating that nuclear import requires two NLS-like motifs acting in concert. Double NLS mutants did not interact with the major vault protein (MVP), a previously hypothesized nuclear-cytoplasmic transport protein (Chung *et al.,* 2005). Consistently with this hypothesis, down-regulation of MVP decreased the nuclear localization of PTEN (Minaguchi *et al.,* 2006). Recently, however, it has been suggested that PTEN enters the nucleus by a Ran GTPase-dependent mechanism (Gil *et al.,* 2006). In contrast, others, have claimed that PTEN enters the nucleus mainly by diffusion (Liu *et al.,* 2005). Whichever the case, there is general consensus over the fact that a decrease in nuclear PTEN characterizes several types of human neoplasia, including thyroid carcinoma and melanoma (Gimm *et al.,* 2000; Whiteman *et al.,* 2002). Interestingly, in MCF-7 breast cancer cells, intranuclear PTEN levels correlate with the cell cycle, with the highest levels being observed at/or before G0-G1. Therefore, it has been suggested that nuclear PTEN could help coordinate cell cycle arrest (Ginn-Pease and Eng, 2002). This could be achieved through a down-regulation of cyclin D1 and involved a specific down-modulation of MAP kinase by nuclear localized PTEN (Chung *et al.,* 2006). Interestingly, NGF-mediated differentiation of PC12 cells (which associates with reduced cell proliferation) is characterized by increased levels of nuclear PTEN (Lachyankar *et al.,* 2000). Furthermore, nuclear PTEN alone is capable of suppressing anchorage-independent growth of U251 MG cells without inhibiting Akt activity. Growth suppression induced by nuclear PTEN is dependent on possessing a functional lipid phosphatase domain (Liu *et al.,* 2005). Therefore, it seems plausible that this effect of PTEN is related to a decrease in intranuclear 3'-phosphorylated inositol lipid mass, and not to its protein phosphatase activity. Nevertheless, others have shown that intranuclear PtdIns(3,4,5)P_3 levels are insensitive to PTEN expression in the nucleus (Lindsay *et al.,* 2006). Catalytically active nuclear PTEN enhanced cell apoptotic responses (Gil *et al.,* 2006) and this effect could be in relationship with the observation that nuclear PTEN forms a complex with p300 and plays a role in maintenance of high p53 acetylation in response to DNA damage thus regulating the p53 levels (Li *et al.,* 2006). As for Akt, an interesting correlation between PTEN nuclear localization and cell proliferation/differentiation and transformation has begun to take shape. Indeed, PTEN usually localizes to the nucleus of primary normal cells. For example, thyroid follicular cells, normal melanocytes, and pancreatic islet cells express PTEN prodominantly in the nucleus, whereas thyroid carcinomas, melanomas, and endocrine pancreatic tumors show a dramatic reduction in PTEN nuclear staining (Gimm *et al.,* 2000; Whitman *et al.,* 2002; Perren *et al.,* 2000). Interestingly, in follicular thyroid tumors, the intranuclear PTEN levels are inversely correlated to the localization of Akt: while nuclear PTEN diminishes during the progression from normal tissue to adenoma to carcinoma, the amount of phosphorylated Akt within the nucleus increases (Vasko *et al.,* 2004). Nevertheless, it remains to be established whether this findings could be related to a PtdIns(3,4,5)P_3-dependent phosphorylation of Akt which takes place inside the nucleus.

Involvement of 3'-phosphorylated inositol lipid metabolism and Akt in NGF-dependent anti-apoptotic signaling of PC12 cells

PI3K/Akt pathway is by far the most important signaling network for cell survival. Traditionally, anti-apoptotic signaling by PI3K/Akt has been thought to take place at the plasma membrane level and in the cytoplasm (Franke *et al.,* 2003). However, recent findings point to the likelihood that nuclear PI3K plays an essential role in promoting cell survival also through nuclear PtdIns (3,4,5)P_3 synthesis (Ye, 2006). PI3K migrates to the PC12 cell

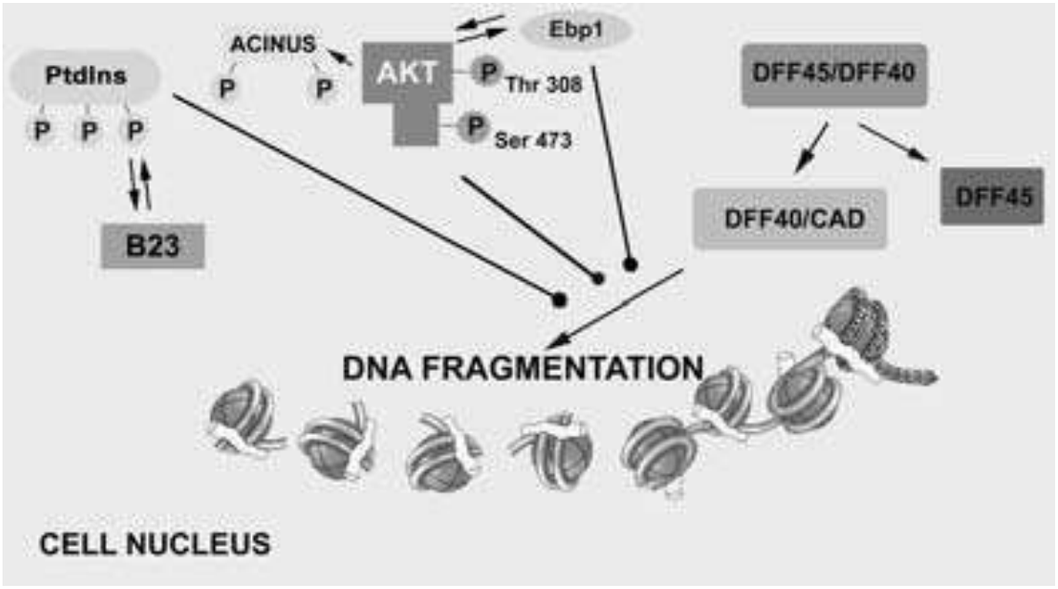

Figure 1. Schematic diagram showing the relationship between PtdIns (3)P, activated Akt and DNA fragmentation inside the nucleus. The pathways depicted hint at the anti-apoptotic role of this signalling cascade.

nucleus in response to NGF (Neri *et al.,* 1999). Taking advantage of a cell-free system, it has been shown that nuclei isolated from NGF-treated PC12 cells were resistant to DNA fragmentation factor/caspase activated DNase (DFF40/CAD) - dependent DNA cleavage initiated *in vitro* by activated cell-free apoptotic solution, consisting of HEK293 cell cytosol supplemented with purified active caspase-3 (Ahn *et al.,* 2004). Nuclei from constitutively active PI3K adenovirus-infected cells displayed the same resistance as those treated with NGF, whereas PI3K pharmacological inhibitors, immunodepletion of PI3K from nuclear extracts with anti-p110 antibody, and dominant negative PI3K or PIKE abolished it. PtdIns (3,4,5)P_3 alone, but not PtdIns (3,4)P_2, PtdIns (4,5)P_2 or PtdIns (3)P, mimicked the anti-apoptotic effect of NGF. The involvement of nuclear PtdIns (3,4,5)P_3 in the protecting role of NGF was also substantiated by an experiment in which isolated nuclei were preincubated with PTEN and then analyzed for DNA fragmentation. It was found that PTEN pre-treatment abolished the protective effect of NGF, even though it was not demonstrated that PTEN actually decreased the amount of nuclear PtdIns (3,4,5)P_3 (Ahn *et al.,* 2004). Since NGF treatment stimulates migration of phosphorylated Akt to the nucleus of PC12 cells (Borgatti *et al.,* 2003), the role of nuclear Akt in the anti-apoptotic action of NGF was also examined. It turned out that nuclei isolated from cells overexpressing wild type or constitutively active Akt were resistant to internucleosomal DNA cleavage, whereas those from dominant-negative Akt-infected cells showed DNA cleavage in spite of NGF treatment, thus demonstrating that nuclear Akt is required for NGF-mediated anti-apoptotic signaling (Figure 1). Nevertheless, in the absence of NGF treatment, all the nuclei displayed DNA degradation, suggesting that Akt activation alone is not sufficient to inhibit DNA cleavage (Ahn *et al.,* 2004). The same group identified protein B23/nucleophosmin as a receptor for nuclear PtdIns (3,4,5)P_3. Indeed, depletion of B23 from nuclear extracts or B23 knockdown abolished NGF-dependent protective effect in PC12 cells, whereas overexpression of B23 prevented apoptosis (Ahn *et al.,* 2005). Protein B23 directly interacts with and inhibits active CAD in a PtdIns (3,4,5)P_3-dependent fashion. As to anti-apoptotic action of nuclear Akt, it has been recently shown that Akt phosphorylates acinus on Ser 422 and 573, resulting in its resistance to caspase-dependent cleavage and inhibition of acinus mediated chromatin condensation (Hu *et al.,* 2005). Acinus, which induces apoptotic chromatin condensation after cleavage by caspase-3 without inducing DNA fragmentation is essential for apoptotic chromatin condensation *in vitro* and *in vivo* (Sahara *et al.,* 1999). Furthermore, nuclear Akt prevents DNA fragmentation by CAD through its association with protein kinase C-phosphorylated p48 isoform of nucleolar protein Ebp1 (Figure 1) (Ahn *et al.,* 2006).

Conclusions

As is clear from this overview, nuclear PI3K, PtdIns(3,4,5)P_3, Akt, and PTEN may be involved in key cellular processes, including carcinogenesis and apoptosis protection. While our knowledge of how this signaling cascade could result in neoplastic transformation is virtually non-existent, we understand more about its involvement in blocking apoptosis. A challenge for the future will be to better elucidate the anti-apoptotic functions of nuclear PI3K/ PtdIns (3,4,5)P_3/Akt/PTEN signaling. For example, we do not know whether or not this system is operative only in neural cells (Ye, 2005) or also in other cell types, including hepatocytes and cardiomyocytes, as preliminary evidence would suggest (Martelli *et al.,* 2006a). A central question is whether this pathway is also activated by other neurotrophins which protects neural cells from apoptosis, such as IGF-1. Identification of additional targets and/or interacting partners within the nucleus will be of outmost importance for a better comprehension of the roles played by this signal transduction system. Furthermore, it should not be forgotten that nuclear PI3K seems to be critically involved in processes other than tumorigenesis and apoptosis, such as myeloid cell differentiation (Bertagnolo *et al.,* 2004). However, further elucidation of this complex and peculiar nuclear signaling pathway is expected to provide new potential targets for pharmacological interventions in major human diseases, including cancer and degenerative disorders in which inappropriate apoptosis is thought to play a fundamental role, such as heart failure, Parkinson's disease, and amyotrophic lateral sclerosis.

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