SHIP-2 and PTEN Are Expressed and Active in Vascular Smooth Muscle Cell Nuclei, but Only SHIP-2 Is Associated with Nuclear Speckles*

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Recently, the control of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3)-dependent signaling by phosphatases has emerged, but there is a shortage of information on intranuclear PtdIns(3,4,5)P3 phosphatases. Therefore, we investigated the dephosphorylation of [32P]PtdIns(3,4,5)P3 specifically labeled on the D-3 position of the inositol ring in membrane-free nuclei isolated from pig aorta vascular smooth muscle cells (VSMCs). In vitro PtdIns(3,4,5)P3 phosphatases assays revealed the production of both [32P]PtdIns(3,4)P2 and inorganic phosphate, demonstrating the presence of PtdIns(3,4,5)P3 5- and 3-phosphatase activities inside the VSMC nucleus, respectively. Both activities presented the same potency in cellular lysates, whereas the nuclear PtdIns(3,4,5)P3 5-phosphatase activity appeared to be the most efficient. Immunoblot experiments showed for the first time the expression of the 5-phosphatase SHIP-2 (src homology 2-domain-containing inositol phosphatase) as well as the 3-phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) in VSMC nuclei. In addition, immunoprecipitations from nuclear fractions indicated a [32P]PtdIns(3,4,5)P3 dephosphorylation by both SHIP-2 and PTEN. Moreover, confocal microscopy analyses demonstrated that SHIP-2 but not PTEN colocalized with a speckle-specific component, the SC35 splicing factor. These results suggest that SHIP-2 may be the primary enzyme for metabolizing PtdIns(3,4,5)P3 into PtdIns(3,4)P2 within the nucleus, thus producing another second messenger, whereas PTEN could down-regulate nuclear phosphoinositide 3-kinase signaling. Finally, intranuclear PtdIns(3,4,5)P3 phosphatases might be involved in the control of VSMC proliferation and the pathogenesis of vascular proliferative disorders.

Vascular smooth muscle cell (VSMC) migration and proliferation are key factors in the development of atherosclerosis,

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¶ The abbreviations used are: VSMC, vascular smooth muscle cell; PI3K, phosphoinositide 3-kinase; PI, phosphoinositide; PtdIns(4,5)P2, phosphatidylinositol-4,5-bisphosphate; PtdIns(3,4)P2, phosphatidylinositol-3,4-bisphosphate; PtdIns(3,4,5)P3, phosphatidylinositol-3,4,5-trisphosphate; PtdIns(4)P, phosphatidylinositol-4-monophosphate; PtdIns(3,4)P1, phosphatidylinositol-3,4-bisphosphate; PtdIns(3,4,5)P1, phosphatidylinositol-3,4,5-trisphosphate; PtdIns(3,4,5)P2, phosphatidylinositol-3,4,5-trisphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; SHIP, src homology 2 domain-containing inositol phosphatase; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; HPLC, high performance liquid chromatography; CREB, cyclic AMP-responsive element binding protein.

PKB, protein kinase B; PKC, protein kinase C; PTEN, phosphatase and tensin homolog deleted on chromosome 10; SHIP, src homology 2 domain containing inositol phosphatase; FTIC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; HPLC, high performance liquid chromatography; CREB, cyclic AMP-responsive element binding protein.
Thus, the production of PtdIns(3,4,5)P3 from PtdIns(4,5)P2 by SHIP proteins may convert one signal to another, whereas PTEN could mediate inactivation of both PtdIns(3,4,5)P3 and PtdIns(3,4,5)P3 signals.

Most research on PI3K-dependent transduction pathways focused on the plasma membrane, but 3-Pls and their biosynthetic machinery are also present in the nucleus, suggesting that they could be major players in nuclear signaling. Indeed, recent highlighted expression of an active class IA PI3K in the nucleus of different cell types, such as neurons, hepatocytes, and osteosarcoma cells (19, 20), and epitope-tagged PI3K translocation to the nucleus after serum stimulation in HepG2 cells (21). We also characterized an endogenous G protein-activated PI3K from membrane-free VSMC nuclei (22). This PI3K-related enzyme specifically generated PtdIns(3,4,5)P3 from a pre-existing nuclear pool of PtdIns(4,5)P2. Interestingly, Boronenkov et al. (23) demonstrated that PtdIns(4,5)P2 is spatially located in speckles, which are nuclear structures involved in pre-mRNA processing in mammalian cells. To date, the mechanisms controlling the level of nuclear PtdIns(3,4,5)P3 are not understood, and there is a lack of knowledge about nuclear PtdIns(3,4,5)P3-phosphatases. Immunohistochemical studies showed that PTEN is expressed in both the cytoplasm and the nucleus (24–26). However, nuclear PTEN activity was not addressed, and SHIP proteins have not yet been identified in the nucleus. Therefore, we investigated 3-PI phosphatase activity in membrane-free nuclei isolated from pig aorta VSMCs. Our data provide the first evidence for the presence of two active 3-PI phosphatases inside the cell nucleus. Moreover, the activities of these 5- and 3-phosphatases correlate with the expression of active SHIP-2 and PTEN in the nuclear compartment, respectively, and SHIP-2 seems to be the primary enzyme responsible for hydrolyzing PtdIns(3,4,5)P3 in VSMC nuclei. Finally, our results reveal that SHIP-2, but not PTEN, concentrates in nuclear speckles, suggesting that SHIP-2 may be involved in pre-mRNA processing.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—All culture reagents were obtained from Invitrogen. [γ-32P]ATP (300 Ci/mmol) was purchased from Perkin Elmer Corp. Antibodies specific for PTEN (sc-9747 and sc-9145), SHIP-1 (sc-1964), SHIP-2 (sc-14502 and sc-14504), horseradish peroxidase-conjugated anti-goat antibody, and blocking peptide (sc-14502P) were from Santa Cruz Biotechnology, Inc. Horseradish peroxidase-conjugated anti-rabbit antibody, and blocking peptide (sc-14502P) were from Upstate Biotechnology, Inc. Horseradish peroxidase-conjugated anti-mouse antibody, and blocking peptide (sc-14502P) were from Dako. Antibody against the p85α subunit of PI3K (06-195) was from Upstate Biotechnology, Inc. Monoclonal anti-nucleolin p62 antibody was from BD Transduction Laboratories. An ECL system was purchased from Amersham Biosciences. Phosphatidylinositol-4-monophosphate (PtdIns(4)P), phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2), phosphatidylinositol, and phosphatidylserine were obtained from Avanti Polar Lipids, Inc. All other reagents were obtained from Sigma.

Cell Culture and Isolation of VSMC Nuclei—VSMCs were prepared from the thoracic aortas of pigs 6 weeks of age using the explant technique and cultured as described previously (27). For all experiments, VSMCs were used from the second to the fifth passage. VSMCs were arrested in the G0-G1 phase by complete serum starvation and then induced to progress through the cell cycle by adding 10% fetal calf serum as described previously (3).

Highly purified VSMC nuclei, stripped of their nuclear envelopes, were obtained as described previously (22). Briefly, growing VSMCs or VSMCs progressing through the cell cycle were washed twice with ice-cold calcium- and magnesium-free phosphate-buffered saline and then with a hypotonic buffer. VSMCs were then lysed at 4°C in the hypotonic buffer containing 1% Nonidet P-40 and 1% deoxycholic acid at 4°C and were sheared by three passages through a 25-gauge needle. The cell lysate was layered over a 0.3 M/2 M sucrose discontinuous gradient and was centrifuged at 500 × g for 15 min at 4°C. Membrane-enriched nuclei were recovered at the interface 0.3 M/2 M sucrose, washed once with the phosphate assay buffer, and disrupted by sonication (10 kHz for 3 s) using an ultrasonic cell disrupter (Brandson Sonifier 250). An average of 0.5 × 10^6 nuclei were obtained from 1 × 10^6 cells (1 × 10^6 cells and 1 × 10^6 nuclei contained 300 and 30 μg of proteins, respectively).

Fluorescence and Immunoprecipitation—Proteins from whole cell or purified nuclei were separated by SDS-PAGE using 10% polyacrylamide gels, transferred onto nitrocellulose membrane (Schleicher and Schuell), and immunoblotted as described previously (28). Membranes were incubated overnight at 4°C with the appropriate primary antibody, polyclonal (sc-9145) or monoclonal (sc-7974) anti-PTEN (1:1200 and 1:100, respectively), anti-SHIP-1 (sc-14502, 1:200), or anti-SHIP-2 (sc-14502, 1:200), overnight at 4°C with the corresponding secondary antibody: FITC-conjugated anti-goat (1:20), or anti-mouse (1:200), TRITC-conjugated anti-rabbit (1:200) or anti-mouse, TRITC-conjugated anti-mouse (1:200), or anti-mouse (1:200), FITC-conjugated anti-goat (1:20) or anti-mouse (1:200), TRITC-conjugated anti-rabbit (1:200) or TRITC-conjugated anti-mouse (1:200). Finally, cells were mounted in Vectashield (Vector Laboratories, Inc.) and examined by confocal scanning laser immunofluorescence microscopy using a LSM510 ZEISS microscope.

Active 3-Phosphoinositide Phosphatases in VSMC Nuclei

3-Phosphoinositide Phosphatase Assays—[γ-32P]PtdIns(3,4)P2 and [γ-32P]PtdIns(3,4,5)P3 labeled at the D-3 position of the inositol ring were prepared. First, class IA PI3K was immunoprecipitated from 10^7 growing VSMCs with anti-p85α antibody as described previously (29). Second, protein A/G agarose-conjugated anti-SHIP antibodies, followed by addition of a recombinant sheep, aggarose-conjugated protein A/G for 1 h at room temperature. After washing, cells were stained 1 h at room temperature with the corresponding secondary antibody: FITC-conjugated anti-goat (1:20) or anti-mouse (1:200), TRITC-conjugated anti-rabbit (1:200) or TRITC-conjugated anti-mouse (1:200). Finally, cells were mounted in Vectashield (Vector Laboratories, Inc.) and examined by confocal scanning laser immunofluorescence microscopy using a LSM510 ZEISS microscope.

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RESULTS

VSMC Nuclei Contain Phosphoinositide 5- and 3-Phosphatase Activities—As we described previously (22), our isolation procedure yields nuclei of high purity. In nuclear preparations, lactate dehydrogenase and 5’-nucleotidase activities, recognized as markers for cytoplasm and plasma membranes, respectively, represented <0.3% of the activity in the total homogenate. Immunoblot analysis revealed that nuclear fractions were devoid of tubulin, a cytoskeleton marker, but were highly enriched with nucleoporin p62, a protein of the nuclear pore complex. Electron microscopy analysis showed that the lysis procedure maintained the nucleolar structure and removed the nuclear envelope to leave the lamina and nuclear pore remnants.

We reported previously that PtdIns(3,4,5)P3 is the only 3-PI synthesized from endogenous precursors within the VSMC nuclei stripped of their nuclear envelopes. However, the mechanisms regulating the level of nuclear PtdIns(3,4,5)P3, especially its degradation pathway(s), remains unclear. Therefore, we first investigated whether PtdIns(3,4,5)P3 could be dephosphorylated by nuclear enzymes in growing VSMCs. Nuclear and cellular extracts were incubated 30 min with purified [32P]PtdIns(3,4,5)P3 specifically labeled on the D-3 position of the inositol ring. Then, the dephosphorylated radioactive products were analyzed by HPLC. HPLC profiles of both membrane-depleted nuclei (Fig. 1, middle panel) and whole-cell lysates (Fig. 1, lower panel) revealed a peak of [32P]PtdIns(3,4)P2 (4,306 ± 919 dpm/1.5 × 107 nuclei, n = 3; 16,911 ± 693 dpm/107 cells, n = 3) and a peak of 32P-labeled inorganic phosphate (1,249 ± 295 dpm/1.5 × 107 nuclei, n = 3; 11,621 ± 924 dpm/107 cells, n = 3), demonstrating the existence of PtdIns(3,4,5)P3 5- and 3-phosphatase activities in VSMCs, respectively. [32P]PtdIns(3,4,5)P3 alone was analyzed as control (Fig. 1, upper panel). Interestingly, the 5-phosphatase activity was higher than the 3-phosphatase activity in the nuclear compartment, whereas these activities presented a similar potency in the total cellular homogenate. We next carried out time-course studies to compare the activities of the two PtdIns(3,4,5)P3 phosphatases in the nuclear compartment and whole-cell lysate and to determine which phosphatase preferentially uses PtdIns(3,4,5)P3 as substrate in the nucleus (Fig. 2). Nuclear extract incubation with [32P]PtdIns(3,4,5)P3 up to 60 min increased synthesis of both [32P]PtdIns(3,4)P2 and 32P (Fig. 2, upper panel). Moreover, we observed a higher level of [32P]PtdIns(3,4,5)P3 synthesis compared with [32P]PtdIns(3,4)P2 along the kinetic points. In contrast, [32P]PtdIns(3,4,5)P3 incubation with cell lysate resulted in a rapid accumulation of [32P]PtdIns(3,4)P2 sustained until 60 min and showed a constant 32P increase (Fig. 2, lower panel). Indeed, nuclei and cell lysates showed interesting differences in their relative 32P:[32P]PtdIns(3,4)P2 ratio. This ratio was constant in nuclei (between 0.21 and 0.24), whereas it continuously increased from 0.44 to 1.33 in VSMC homogenates. Moreover, nuclei exhibited a lower 32P:[32P]PtdIns(3,4)P2 ratio, which was about 6-fold less than the one observed for total VSMCs at 60 min. These data suggest that PtdIns(3,4,5)P3 is hydrolyzed by both 5- or 3-phosphatases in VSMCs but is preferentially hydrolyzed by a 5-phosphatase in the nuclear compartment. To test whether PtdIns(3,4,5)P3 could be dephosphorylated by a nuclear 3-phosphatase, purified [32P]PtdIns(3,4)P2 labeled on the D-3 position was used as substrate. As shown in Fig. 3, incubation of nuclei with [32P]PtdIns(3,4)P2 induced a significant production of 32P (2,888 ± 737 dpm/1.5 × 107 nuclei, n = 3) characteristic of a PtdIns 3-phosphatase activity. Altogether these results indicate the presence of active PtdIns(3,4,5)P3 5- and 3-phosphatases in VSMCs, but in nuclei, the PtdIns(3,4,5)P3 5-phosphatase could be the primary enzyme responsible for hydrolyzing PtdIns(3,4,5)P3.

SHIP-2 and PTEN Are Expressed and Active within VSMC Nuclei—The above data revealed the existence of both 5- and 3-phosphatase activities in VSMC nuclei. Previous studies showed that SHIP proteins and PTEN dephosphorylate the D-5 and the D-3 positions of 3-PI, respectively, and PTEN was localized in the nucleus (24–26). Therefore, we next sought to determine whether these phosphatases are expressed in VSMC nuclei. By using immunoblot experiments, SHIP-1, which is mainly expressed in hematopoietic cells, was undetectable in VSMCs (data not shown). In contrast, the more ubiquitous SHIP-2 was present in VSMCs (Fig. 4A). Indeed, we detected a 155 kDa protein in both the total cell homogenate (Fig. 4A, lane 1) and the nuclear fraction (Fig. 4A, lane 2). Relative to the total lysate, 3.6 ± 0.5% (n = 4) of the cellular SHIP-2 protein was quantified within VSMC nuclei. A positive control with human anaplastic lymphoma cells (FEPD cells) also showed a single band at 155 kDa (Fig. 4A, lane 3). Moreover, the SHIP-2 detection was specific because no signal was observed when the primary antibody was pre-incubated with a 5-fold excess of...
blocking peptide (Fig. 4A, lane 4–6). A duplicate blot probed with an antibody to nucleoporin p62 revealed that nuclei used in these experiments were highly enriched with this nuclear pore protein (× 9.3 ± 0.2; n = 4) and confirmed the purity of our nuclear fractions (Fig. 4B). Thus, these results demonstrate for the first time the expression of the 5-phosphatase SHIP-2 within membrane-depleted nuclei.

We next checked the expression of the 3-phosphatase PTEN in VSMCs (Fig. 5). We used a monoclonal anti-PTEN antibody and loaded the same amount of proteins from cell homogenates (Fig. 5A, lane 1) or from purified nuclei (Fig. 5A, lane 2). Western blot analysis showed an immunoreactive protein at 57 kDa characteristic of PTEN in both preparations. Moreover, cellular and nuclear PTEN staining quantification revealed that 17.5 ± 1.1% (n = 4) of PTEN protein was present in the nucleus. To ascertain PTEN expression in VSMC nuclei, we also performed immunoprecipitation experiments from nuclear fractions. When PTEN was immunoprecipitated with a polyclonal antibody and probed with a monoclonal antibody, a single band at the expected molecular weight was detected (Fig. 5B, lane 1). The same result was obtained by using a monoclonal antibody for anti-PTEN immunoprecipitation and a polyclonal antibody in immunoblotting (Fig. 5B, lane 2). Therefore, these data strongly suggest that SHIP-2 and PTEN are also present within the VSMC nucleus.

To investigate whether SHIP-2 and PTEN could be the nuclear PtdIns(3,4,5)P₃ 5- and 3-phosphatase activities, respectively, we immunoprecipitated each enzyme from nuclear extracts. The data shown are representative of three independent experiments.
Fig. 5. Intranuclear PTEN expression in VSMCs. Proteins were fractionated on 7.5% SDS-PAGE, transferred, and assayed by Western blot. A, proteins from 2 × 10^6 VSMCs (lane 1) and 2 × 10^7 purified nuclei (lane 2) were fractionated on 7.5% SDS-PAGE, transferred, and probed with monoclonal anti-PTEN antibody. B, PTEN was immunoprecipitated from 10^7 nuclei with either specific polyclonal (lane 1) or monoclonal (lane 2) antibody and then immunoblotted with monoclonal and polyclonal anti-PTEN antibody, respectively. The data shown are representative of three independent experiments.

Fig. 6. Nuclear SHIP-2 and PTEN activities in VSMCs. Protein from 1.5 × 10^6 membrane-free nuclei were immunoprecipitated with either anti-SHIP-2 antibody (middle panel) or monoclonal anti-PTEN antibody (lower panel), and PtdIns(3,4,5)P_3 activity was assayed as described in Fig. 1. Purified [32P]PtdIns(3,4,5)P_3 was incubated 30 min at 37 °C with the immunoprecipitates. Then radioactive lipids were extracted and analyzed by HPLC. As control, a HPLC profile of purified [32P]PtdIns(3,4,5)P_3 is shown in the upper panel. The data shown are representative of three different experiments. PtdIns(3,4,5)P_3, PtdIns(3,4)P_2, Pi, inorganic phosphate.

Active 3-Phosphoinositide Phosphatases in VSMC Nuclei

The nuclear localization of PTEN but not SHIP-2 is cell cycle-dependent—Because SHIP-2 and PTEN have been implicated in cell growth, we investigated whether the targeting of SHIP-2 and/or PTEN to the nucleus was dependent upon the VSMC cell cycle. Quiescent VSMCs were stimulated by fetal calf serum for different times, and the expression of SHIP-2 and PTEN were studied in nuclei isolated from VSMCs at different points of the cell cycle. Flow cytometry analysis (Fig. 7A) indicated that nuclei at 14 h after stimulation were predominantly in the G_1 phase, nuclei at 18 h were at the transition between the G_1 and S phases, and the majority of the nuclei at 24 h were in S phase. Western blotting analyses showed that nuclear SHIP-2 expression did not significantly change during the cell cycle (Fig. 7B). On the other hand, nuclear PTEN levels increased when quiescent VSMCs entered the cell cycle (∼1.6), reached a maximum (∼2.5) at the 24 h time point as the majority of VSMCs were in S phase, and then decreased as the cells begin to enter in the G_2 phase (Fig. 7C).

SHIP-2 but not PTEN colocalizes with nuclear speckles—To further characterize the intranuclear location of SHIP-2 and PTEN, we performed double immunofluorescence labeling (FITC and TRITC) on growing VSMCs using confocal scanning laser microscopy (Fig. 8). In agreement with immunoblot studies, both proteins were present in the nuclei (Fig. 8A), and their labeling was independent of the fixation method used (data not shown). No green or red labeling was observed when the primary antibodies were omitted (data not shown). PTEN was clearly expressed in nuclei, whereas SHIP-2 was present at low levels compared with cytosol. In addition, a SHIP-2 and PTEN merge revealed a distinct subnuclear expression pattern, suggesting a compartmentalization of the PtdIns(3,4,5)P_3 phosphatases inside the nucleus. Because both phosphatases also showed a punctuated nuclear staining reminiscent of nuclear speckles, cells were then labeled with an antibody directed against a speckle-specific component, the SC35 splicing factor (31), and with either anti-SHIP-2 or anti-PTEN antibodies. Interestingly, SHIP-2 colocalized with the SC35 protein, as indicated by yellow in Fig. 8B. In contrast, PTEN does not seem associated with SC35 and localized out of the nuclear speckles (Fig. 8C). Therefore, SHIP-2 but not PTEN colocalizes with nuclear speckles in VSMCs. The distinct nuclear localization of SHIP-2 and PTEN was also detected in a variety of cell lines tested, such as Caco-2 and HeLa cells (data not shown).

Nuclear speckles are functionally connected to mRNA metabolism, and their morphology correlates with transcriptional activity. As observed by Boronenkov et al. (23), the treatment of VSMCs with the transcriptional inhibitor α-amanitin caused reorganization of the speckle component SC35 into fewer and larger structures (Fig. 9). Moreover, double labeling of treated VSMCs demonstrated that SHIP-2 redistributed identically with SC35 in response to the inhibition of transcription (Fig. 9).
The same results were obtained with 5,6-dichlorobenzimidazole riboside treatment (50 μg/ml; 4 h) (data not shown). These observations suggest a physical association of SHIP-2 with some components of nuclear speckles.

DISCUSSION

In the present study, we have provided the first evidence that the second messenger PtdIns(3,4,5)P₃ is hydrolyzed by both 5- and 3-phosphatases within VSMC nuclei. Furthermore, we showed that the 5-phosphatase SHIP-2 and the 3-phosphatase PTEN are expressed and active in the nucleus, and that SHIP-2 but not PTEN is associated with nuclear speckles.

The production of 3-PI by PI3Ks is now well documented in the nuclei of different cell types (19, 20). Our findings reinforce the notion of a nuclear 3-PI cycle, apart from that occurring in the plasma membrane. Therefore, the combined action of the phosphatase may turn-off 3-PI signaling pathways by decreasing the level of intranuclear PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂. Currently, the functions of intranuclear PtdIns(3,4)P₃ and PtdIns(3,4,5)P₃ are not understood, but it is likely that they may act as intranuclear mediators and may induce or maintain the activation of downstream targets such as the serine/threonine kinases PKCζ or PKB. Both protein kinases were identified in nuclei of numerous cells, among which were VSMCs (22). Indeed, PtdIns(3,4,5)P₃ generated in the nucleus seems to act as the driving force for the nuclear translocation of PKCζ in nerve growth factor-stimulated PC12 cells (32) and in rat hepatocytes treated with C2-ceramides (33). Therefore, nuclear PtdIns(3,4,5)P₃ might control the nucleocytoplasmic shuttling of PKCζ, and PtdIns(3,4,5)P₃ phosphatases modulate PKCζ access to its nuclear targets such as the nucleolin, a major nuclear protein involved in several aspects of ribosome biogenesis (34). It is now evident
that PKB is activated by PtdIns(3,4,5)P3 and PtdIns(3,4)P2-dependent phosphorylations (threonine-308 and serine-473) in the cytoplasm (35) and then migrates to the nucleus (36). However, the role of nuclear PKB still remains unclear, but nuclear PtdIns(3,4)P2 and PtdIns(3,4,5)P3 could sustain its activation in the nucleus. The 5-phosphatase activity may switch PtdIns(3,4,5)P3-dependent signals to PtdIns(3,4)P2 and consequently regulate nuclear PKB targets. In contrast, the 3-phosphatase activity should attenuate these signals and down-regulate nuclear targets of PKB, such as the forkhead family transcription factors, FKHR, FKHL1 and AFX, that control the expression of pro- and anti-apoptotic genes (37, 38).

Our results suggest that the regulation of the nuclear PtdIns(3,4,5)P3 cycle might be different between the nucleus and the plasma membrane. We observed that nuclear 5-phosphatase activity is more efficient than the 3-phosphatase activity to metabolize PtdIns(3,4,5)P3. In contrast, these activities presented the same potency in total cell homogenates. These differences could be explained by the down-regulation of PTEN in the VSMC nucleus. In fact, the prevailing view is that the phosphorylation of the cluster Ser/Thr located at the PTEN phosphorylation sites of PTEN (42), and the in vivo and in vitro phosphorylation of both serine by the casein kinase 2 inhibited PTEN activity toward PtdIns(3,4,5)P3 (40). Our preliminary data seem to indicate that PTEN phosphorylation is higher in the nucleus than in the cytoplasm, and as shown in other cell types, casein kinase 2 localizes to the nucleus in VSMCs. Therefore, it is possible that PTEN lipid phosphatase activity is weaker in the nucleus than in cytoplasm. However, we cannot exclude the up-regulation of nuclear SHIP-2 in our conditions. Unfortunately, SHIP-2 activation is poorly documented, and its regulation mechanisms are unknown. It is also possible that other unidentified PtdIns(3,4,5)P3 5- or 3-phosphatases are present in the nucleus of VSMCs.

Interestingly, immunocytochemistry experiments showed a different subnuclear localization of the 3-PI phosphatases: SHIP-2, but not PTEN, concentrates in nuclear speckles. Moreover, SHIP-2 might directly interact with speckles because the phosphatase reorganized identically with speckles when transcribed activity was inhibited. These immunohistochemical results are very intriguing, because class II PI3K is unable to phosphorylate PtdIns(4,5)P2 whereas active SHIP-2 dephosphorylates PtdIns(3,4,5)P3. Nevertheless, class IA PI3Ks, which display a preference in vivo for PtdIns(3,4,5)P3, were present in the nucleus (45, 46) and might transiently localize to speckles and produce PtdIns(3,4,5)P3. Concerning class IB PI3K, no subnuclear localization of PI3K in VSMCs could be done because all tested antibodies were unable to reveal PI3Kγ by immunocytochemical studies (22). The remarkable subnuclear localization of SHIP-2 suggests that 3-PIs could be critical regulators of transcription. This hypothesis is supported by the observations that PtdIns(3,4,5)P3 and PtdIns(4,5)P3 can reverse the inhibition of transcription caused by histone H1 (47). Moreover, active PKB phosphorylates cyclic AMP-responsive element binding protein (CREB) and promotes target gene expression through the reactivation of the CREB-binding protein/p300, and Von Mickelze et al. (48) recently demonstrated a relocation of CREB-binding protein/p300 into speckles after transcriptional inhibition.

It is also interesting to note that PI3K inhibitors, as well as PTEN or SHIP-2 overexpression, down-regulated the cell cycle kinase inhibitor p27kip and blocked cell cycle progression in the G1 phase (3, 17, 18). Concerning PTEN, it was believed that only the lipid phosphatase activity of PTEN mediated growth suppression. However, a recent report showed that PTEN inhibits cell cycle progression through the cooperation of its protein phosphatase, which down-regulates cyclin D1 and its lipid phosphatase activity, which up-regulates p27kip (49). Hence, important questions for future studies include the investigation of protein activity of nuclear PTEN. Moreover, subcellular partitioning of PTEN might also play some role in the progression of different tumor types. There is now some evidence that PTEN is localized mainly in the nucleus in non-neoplastic cells, but its expression was found to be highly decreased in the nucleus and became predominantly cytoplasmic in neoplastic cells (24, 26, 50). Moreover, nuclear PTEN levels seem to vary differently in the cell cycle of cancerous and non-cancerous cells. We report that the highest level of nuclear PTEN was associated with the greatest proportion of VSMCs in S phase. On the other hand, Ginn-Pease and Eng (51) showed that in MCF7 breast cancer cells overexpressing PTEN, a decreased PTEN level was associated with an increased proportion of cells in S phase. These observations could suggest a deregulation of nuclear PTEN targeting with tumorigenesis.

Thus, nuclear 3-PI metabolism via SHIP-2 or PTEN should have functionally distinct outcomes with implications for these enzymes as targets in novel therapies of proliferative diseases, such as atherosclerosis and cancer.

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