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The nuclear localization of 3'-phosphoinositide-dependent kinase-1 is dependent on its association with the protein tyrosine phosphatase SHP-1

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ABSTRACT

3'-Phosphoinositide-dependent protein kinase-1 (PDK1), the direct upstream kinase of Akt, can localize to the nucleus during specific signalling events. The mechanism used for its import into the nucleus, however, remains unresolved as it lacks a canonical nuclear localization signal (NLS). Expression of activated Src kinase in C6 glioblastoma cells promotes the association of tyrosylphosphorylated PDK1 with the NLS-containing tyrosine phosphatase SHP-1 as well as the nuclear localization of both proteins. A constitutive nucleo-cytoplasmic SHP-1:PDK1 shuttling complex is supported by several lines of evidence including (i) the distribution of both proteins to similar subcellular compartments following manipulation of the nuclear pore complex, (ii) the nuclear retention of SHP-1 upon overexpression of a PDK1 protein bearing a disrupted nuclear export signal (NES), and (iii) the exclusion of PDK1 from the nucleus upon overexpression of SHP-1 lacking the NLS or following siRNA-mediated knock-down of SHP-1. The latter case results in a perinuclear distribution of PDK1 that corresponds with the distribution of PIP3 (phosphatidylinositol 3,4,5-triphosphate), while a PDK1 protein bearing a mutated PH domain that abrogates PIP3-binding is excluded from the nucleus. Our data suggest that the SHP-1:PDK1 complex is recruited to the nuclear membrane by binding to perinuclear PIP3, whereupon SHP-1 (and its NLS) facilitates active import. Export from the nucleus relies on PDK1 (and its NES). The intact complex contributes to Src kinase-induced, Akt-sensitive podial formation in C6 cells.

1. Introduction

Phosphorylation regulates numerous facets of protein behaviour including localization, activity and intermolecular interactions. The phosphorylation status of the serine/threonine kinase PDK1 determines its effectiveness in contributing to the regulation of the phosphatidylinositol 3′-kinase (PI3K) signalling cascade. The pleckstrin homology (PH) domain of PDK1 binds to membrane-associated PI3K-generated lipid products, *e.g.* PIP3 (phosphatidylinositol 3,4,5-triphosphate), and once localized to the vicinity of the membrane PDK1 phosphorylates targets including Akt, p70S6K, p90RSK, serum and glucocorticoid-inducible kinase, and atypical isoforms of PKC [1].

Beyond its recognized localization and function in the cytoplasm, PDK1 also localizes to the nucleus. Constitutive nuclear PDK1 is not readily observed [2–5], yet nuclear PDK1 is detected following treatment of cells with leptomycin-B, an inhibitor of the nuclear export receptor CRM1, as well as by interfering with the CRM1-binding nuclear export signal (NES) in PDK1 itself [3,4]. In addition, nuclear PDK1 is clearly apparent upon stimulation with growth factors (*i.e.* insulin, IGF-1, NGF and PDGF) [3,4,6]. The insulin-induced accumulation of PDK1 in the nucleus is enhanced in embryonic fibroblasts devoid of PTEN (a phospholipid phosphatase) and is blocked by inhibition of the lipid kinase PI3K using wortmannin and LY294002, thus indicating that PDK1 nuclear import is regulated by the availability of PIP3 [3]. The detection of PIPs and key signalling components of the PI3K pathway, for example PI3K itself, Akt, PKCζ and p70S6K [7], in the nucleus certainly argues that PDK1 could target substrates in this compartment. A PDK1 protein bearing a loss-of-function NES mutation accumulates in the nucleus of MCF-7 cells, where it co-localizes with Akt [4]. This could explain the increase in phosphorylation, and concurrent repression of transcriptional activity, of FOXO3a, a forkhead transcription factor that, when phosphorylated, relocalizes to the cytoplasm where it is sequestered by 14-3-3 proteins. This nuclear PDK1/inactivation of FOXO3a could ultimately afford protection against pro-apoptotic stimuli [4,8]. In apparent contrast, the nuclear accumulation of a PDK1 protein lacking an NES clearly inhibits anchorage-independent growth of Comma-1D (mammary epithelial) cells and provides less protection against UV irradiation than does wildtype PDK1 in mouse mammary epithelial NMuMg cells [3]. Thus, nuclear PDK1 can facilitate cell type-dependent phenotypes.

These data clearly support the existence and functional relevance of nuclear PDK1, yet the actual mechanism used for PDK1 nuclear

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import is unknown. Phosphorylation of PDK1 has emerged as a possible contributing factor. Treatments as diverse as insulin, NGF, hydrogen peroxide, Src kinases (*e.g.* c-Src and c-Abl) and pervanadate (a pan-inhibitor of protein tyrosine phosphatases) can all increase the tyrosine phosphorylation of PDK1 [5,9–11]. Src kinase and pervanadate both induce the phosphorylation of PDK1(Tyr9), which facilitates additional phosphorylation, and ultimately PDK1 localization and activity [10]. Phosphorylation of PDK1(Ser396), adjacent to the NES, is important for nuclear import during IGF stimulation [4]. However, the non-phosphorylatable PDK1(Ser396Ala) variant can be detected in the nucleus following inhibition of nuclear export with leptomycin-B, while the double PDK1(Ser396Ala)/mNES variant is constitutively nuclear [4]. Clearly, several post-translational events influence PDK1 localization. If PDK1 nuclear import occurs *via* traditional nuclear pore complex-mediated transport, then the absence of a canonical nuclear localization signal (NLS) in PDK1 strongly suggests that its import into the nucleus must rely on a "piggy-back" type of transport, *e.g.* an obligatory association with an NLS-containing protein such as already demonstrated for the hepatitis B virus X protein (with IκBα: [12]) and the nuclear receptor binding factor-1, NRBF-1 (with PPARα: [13]).

Tyrosines targeted by Src kinase are flanked by sequences of amino acids that resemble motifs that are also targeted by the protein tyrosine phosphatase SHP-1 [14]. SHP-1 is known to negatively regulate PI3K/Akt signalling by dephosphorylating, for example, p85, the regulatory subunit of PI3K [15]. SHP-1, *via* its tandem SH2 domains, binds proteins expressing the consensus motif *p*YXX(V/I/L) (where *p*Y is *p*hospho-Tyrosine, *X* is any amino acid and V/I/L is Valine/Isoleucine/Leucine) [14,16]. Examination of the PDK1 deduced amino acid sequence reveals that Tyr9, Tyr373 and Tyr376, which are established Src kinase phosphorylation sites [10], also reside within *p*YXX(V/I/L) motifs. It is therefore not unreasonable to assume a direct regulatory role for SHP-1 in PDK1 phosphorylation and function. Although predominantly cytoplasmic, SHP-1 also contains a functional NLS [17] and can localize to the nucleus following stimulation with growth hormone in liver cells [18], with EGF in HEK293, COS-7, A431 and HT29 cells [17,19], and with IL-4 in NIH3T3 cells and IL-4 and IL-7 in PBLC-1 cells [20]. Of additional interest is the fact that SHP-1 does not express an NES and the mechanism underlying its export from the nucleus back to the cytoplasm is also unknown.

We hypothesized that SHP-1 associates with tyrosine-phosphorylated PDK1 and that this is a mutualistic association as it would permit the SHP-1:PDK1 complex to shuttle between the nucleus and the cytoplasm. Indeed, SHP-1 (and its NLS) would permit the nuclear import of PDK1 whereas PDK1 (and its NES) would permit the export of SHP-1 back to the cytoplasm. As specific tyrosines in PDK1 are potentially targets for both Src kinase and SHP-1, and tyrosine kinases have been implicated in process formation in C6 glioblastoma cells [21], we chose to test our hypothesis in Src kinase-expressing C6 cells.

2. Materials and methods

2.1. Reagents and antibodies

Protein concentrations were measured using the bicinchoninic acid-based assay (Pierce Biotechnology). Anti-SHP-1 was purchased from BD Biosciences. Anti-phosphotyrosine (4G10) was purchased from Upstate Biotechnology Inc. Antibodies recognizing Src kinase, GSK-3β and GSK-3β(pSer9), Akt [total, pSer473 and pThr308] and PDK1 were purchased from Cell Signalling Technology. Anti-β-actin, wortmannin and LY294002 (two PI3K inhibitors), and protease inhibitor cocktail were purchased from Sigma-Aldrich Co. Streptavidin-AlexaFluor 594 was purchased from Invitrogen. Secondary IgG-HRP conjugates were obtained from Cedarlane Laboratories Ltd.

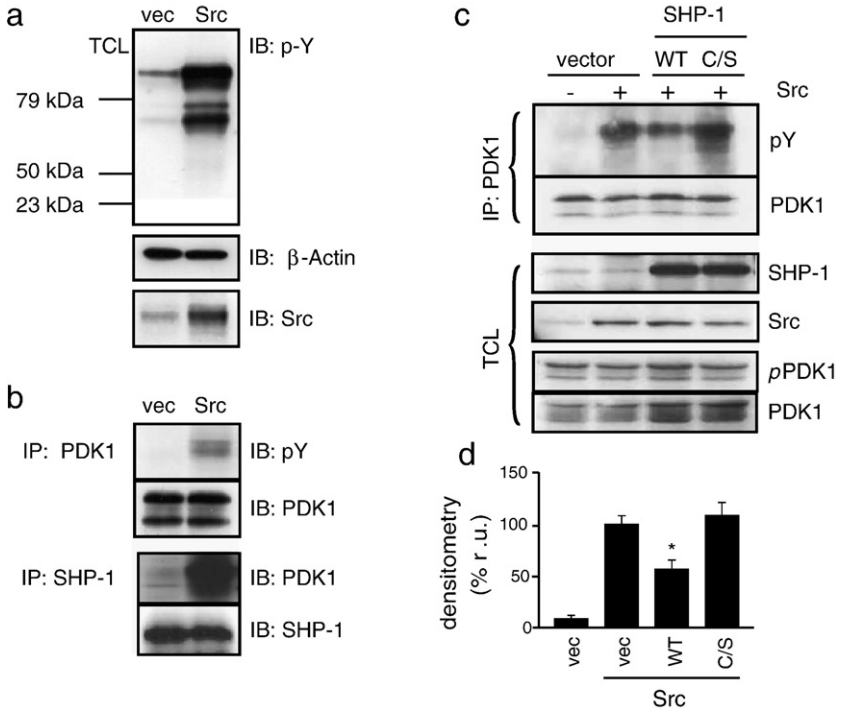

Fig. 1. Tyrosine-phosphorylated PDK1 associates with SHP-1. (a) Total cell lysates (TCL) from C6 cells expressing the constitutively active Src(Y527F) kinase (Src: *note*, vec: vector control) were examined for tyrosine phosphorylation (pY) status and for Src kinase expression. β-Actin was included as a loading control. (b) Endogenous PDK1 was immunoprecipitated from pre-cleared lysates and SDS-PAGE resolved proteins were probed with anti-pY and anti-PDK1. Pre-cleared protein from corresponding cultures was immunoprecipitated for endogenous SHP-1 and resolved proteins were probed with anti-PDK1 and anti-SHP-1. (c) Src kinase and either SHP-1 wildtype (WT) or the dominant-negative SHP-1(C455S) (C/S) protein were co-expressed in C6 cultures. The resolved anti-PDK1 immunoprecipitates were probed with anti-pY and anti-PDK1. TCL were probed for SHP-1, Src kinase and PDK1 (and phosphoSer241-PDK1: *p*PDK1). (d) The degree of tyrosine phosphorylation of endogenous PDK1 in (c) was quantitated using ImageJ ($n=3$–4). All Src-transfected groups were significantly different ($P<0.01$) from vec/no Src control. *$P<0.05$ versus Src/vec.

Protein-A/G-Sepharose and the enhanced chemiluminescence kit were obtained from GE Healthcare Bio-Sciences Inc.

2.2. Plasmids

The eGFP-PDK1 expression vector was obtained from Drs. S. Kim and J. Chung (Korea Advanced Institute of Science and Technology, Republic of Korea). The PDK1(R474A) mutation within the PH domain [22] and the double L380S/F383S mutation within the NES region [4] were generated using Quikchange (Stratagene). cDNA encoding SHP-1 (wildtype) and SHP-1(C455S) (catalytically inactive variant) were obtained from Dr. S.H. Shen (Biotechnology Research Institute, Montreal, Canada). N-terminal RFP-tagged SHP-1 was obtained by subcloning the SHP-1 cDNA into the pDsRed-monomer-C1 vector (Clontech Laboratories, Inc.). The NLS in SHP-1, found at the extreme C-terminal [17], was deleted [SHP1ΔNLS] by introducing a "TAG" (stop) codon at the beginning of the NLS. PH-mRFP1 was obtained from Dr. R.Y. Tsien (Howard Hughes Medical Institute, UCSD). c-Src (Y527F) (constitutively active) cDNA was PCR-amplified from the pBTM116-Src yeast vector [23] and subcloned into pcDNA3.1 (Invitrogen).

2.3. Cell culture and treatments

C6 (CCL-107) cells and HEK293 (CRL-1573) cells (ATCC: Rockville, MD) were cultured in DMEM (supplemented with 10% fetal bovine serum). ExGen500 (Fermentas Life Sciences) was used for gene transfection and experiments typically occurred 24 h post-transfection. Pharmacological manipulation of the nuclear pore complex was achieved using either Leptomycin-B (LMB, 50 nM, 3 h; Cedarlane Laboratories Ltd), or BAPTA-AM (100 μM, 30 min) and A23187 (5 μM, 10 min) (both from Sigma-Aldrich Co.).

2.4. Immunoprecipitation and Western blot analysis

Proteins were extracted into ice-cold lysis buffer: 20 mM Tris (pH 7.5), 1.0 mM EDTA, 1% Triton X-100, 10% Glycerol, 1% NP-40, 1 mM sodium orthovanadate, and protease inhibitor cocktail. Total cell lysate was cleared (5000 ×*g*, 10 min, 4 °C) for Western blot analysis (25–30 μg/lane) or pre-cleared with non-specific mouse or rabbit IgG for subsequent immunoprecipitation (~500 μg; 4 °C, overnight) with the appropriate antibody (1 μg/100 μg protein). Immune complexes were isolated with protein-A/G-Sepharose for 1 h (4 °C) and centrifugation at 10,000 ×*g* (3 × 15 min). Total cell lysates and immunoprecipitated proteins were resolved by SDS-PAGE and transferred to membranes that were probed overnight (4 °C) with the appropriate primary antibody. Protein detection relied on HRP-conjugated secondary antibodies and enhanced chemiluminescence.

2.5. Confocal fluorescence microscopy and indirect immunofluorescence microscopy

Cells cultured on chambered slides were treated and then fixed with 4% formaldehyde (30 min) and permeablized with 0.5% Triton-

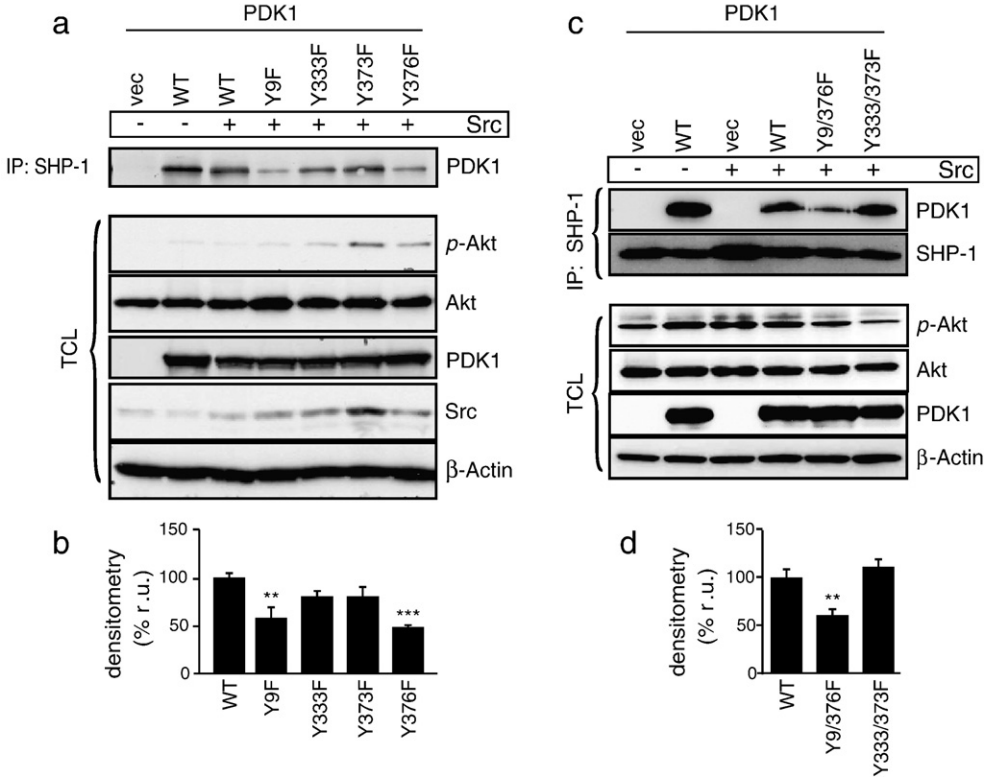

Fig. 2. Specific tyrosine residues in PDK1 mediate the association with SHP-1 in Src kinase-expressing C6 cells. (a) The PDK1 Y9F, Y333F, Y373F or Y376F substituted proteins were expressed in C6 cells co-expressing Src kinase (Src). Endogenous SHP-1 was immunoprecipitated and gel-resolved proteins were probed for PDK1. The expression of PDK1 and Src kinase was examined in corresponding total cell lysates (TCL) as was the phosphorylation status of Akt (*p*Akt). β-Actin was included as a loading control. (b) Densitometric analysis (ImageJ, $n=4$) of the co-immunoprecipitation of SHP-1 and the various PDK1 Y/F substituted proteins depicted in (a) are expressed as percentage of the co-immunoprecipitation of SHP-1 with wildtype (WT) PDK1 in the presence of Src (*i.e.* lane 3 on the gel in (a): 100.0% ± 9.05). (c) The PDK1 Y9/376F or Y333/373F double substituted proteins were co-expressed with Src kinase (Src). Endogenous SHP-1 was immunoprecipitated and resolved proteins were probed for PDK1 as well as for SHP-1. Corresponding cell extracts were examined for PDK-1 expression and for the phosphorylation status of Akt (*p*Akt). (d) Densitometry (ImageJ, $n=4$) was used to quantitate the degree of co-immunoprecipitation of SHP-1 with the PDK1 Y/F double substituted proteins depicted in (c). Values are expressed as percentage of the co-immunoprecipitation of SHP-1 with wildtype (WT) PDK1 in the presence of Src (*i.e.* lane 4 on the gel in (c): 100.0% ± 9.80). $^{**}P<0.01$ & $^{***}P<0.001$, versus WT/Src.

100/PBS for 15 min. Nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Co.) prior to addition of mounting medium, Pro-Long Gold antifade reagent (Invitrogen-Molecular Probes). For indirect immunofluorescence microscopy, cells were incubated with an anti-SHP-1 antibody at 4 °C overnight, then extensively washed and incubated with an AlexaFluor 594 secondary antibody with excitation/emission (ex/em) maxima of 590/617 nm. Direct fluorescence (using an Olympus FV300 laser scanning confocal microscope) relied on specific fluorophore tags, *i.e.* GFP-proteins were visualized using ex/em maxima of 488/507 nm, while RFP-proteins were visualized using ex/em maxima of 557/585 nm. ImageJ 1.32j (http://rsb.info.nih.gov/ij/) was used to determine the localization and relative quantity of proteins in the nucleus/cytoplasm along a bisecting line centered on the nucleus. ImageJ 1.32j was also used to measure podial outgrowth in relevant cultures.

2.6. Statistical analysis

Data are expressed as mean ± SEM. Significance (set at $P<0.05$) was assessed either by Student's *t*-test or by analysis of variance with *post-hoc* analysis relying on Bonferroni's Multiple Comparison Test (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. Tyrosine phosphorylated-PDK1 associates with SHP-1 and localizes to the nucleus

The overexpression of a constitutively active Src kinase enhanced tyrosine phosphorylation in C6 cell lysates (Fig. 1a) and increased the tyrosine phosphorylation of endogenous PDK1 (Fig. 1b). This also enhanced the co-immunoprecipitation of PDK1 with the tyrosine phosphatase SHP-1 (Fig. 1b). The tyrosine phosphorylation of endogenous PDK1 protein was diminished by co-expression of Src kinase and wildtype SHP-1, but not by co-expression of Src kinase and the dominant-negative SHP-1(C451S) protein [$F_{(3,14)}=33.48$, $P<0.0001$] (Fig. 1c,d).

Co-expression of Src kinase with specific PDK1 Y-to-F substituted proteins revealed that the PDK1(Y9F) and PDK1(Y376F) substitutions tended to interfere with the co-immunoprecipitation of PDK1 with endogenous SHP-1, whereas the PDK1(Y333F) and PDK1(Y373F) substitutions did not (Fig. 2a,b) [$F_{(4,19)}=8.726$, $P=0.0008$]. This suggests that the Y9 and Y376 residues in PDK1 may be binding targets for the SH2 domains in SHP-1. This was supported by the significant reduction in association between the PDK1(Y9/376F) double mutant and SHP-1, whereas the PDK1(Y333/373F) double mutant still clearly associated with SHP-1 (Fig. 2c,d) [$F_{(2,11)}=16.36$, $P=0.0010$].

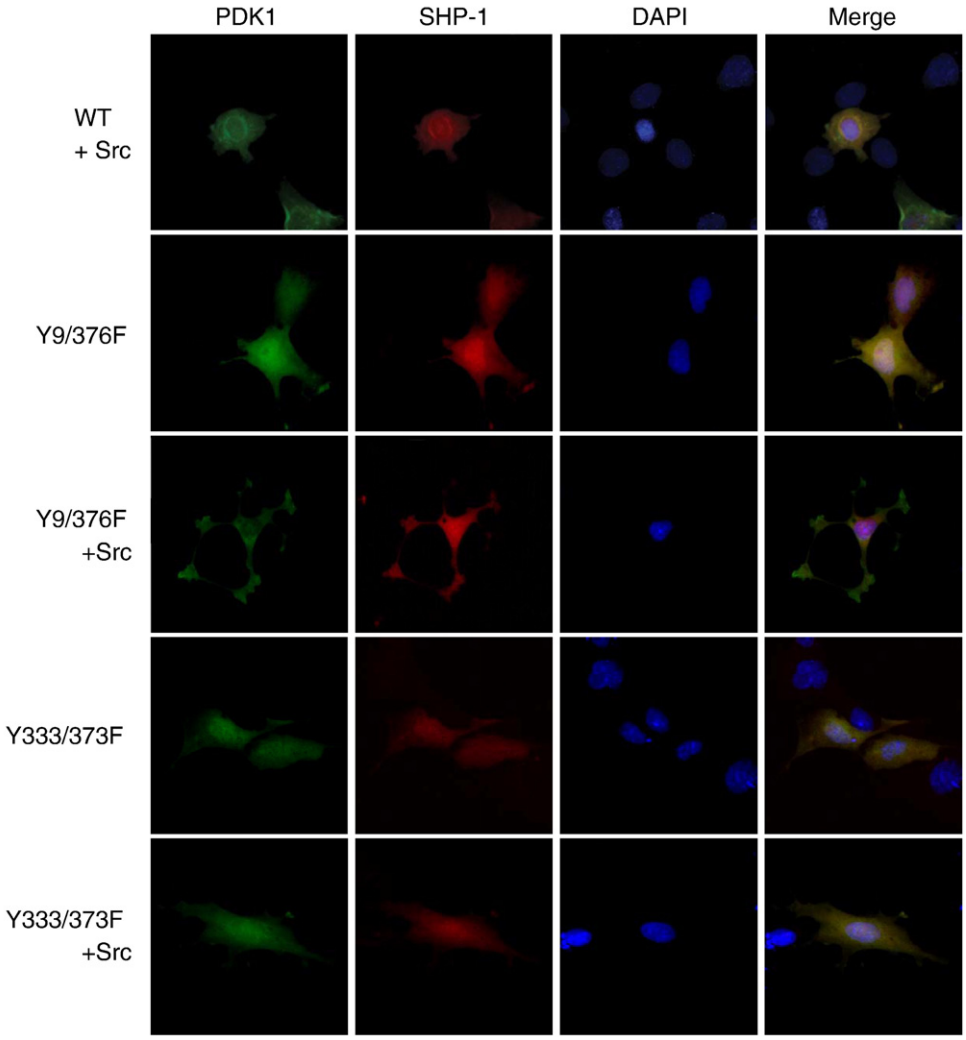

Fig. 3. Activated Src kinase promotes the nuclear accumulation of SHP-1 and PDK-1 in C6 cells. GFP-PDK1 [wildtype (WT) or either of the double Y9/376F and Y333/373F variants] were co-expressed with Src kinase (Src) in C6 cells. The distribution of GFP signal was examined by confocal microscopy. Endogenous SHP-1 was visualized by indirect immunofluorescence. Merged micrographs depict the subcellular localization of the two proteins. The nucleus is stained blue (DAPI fluorescence).

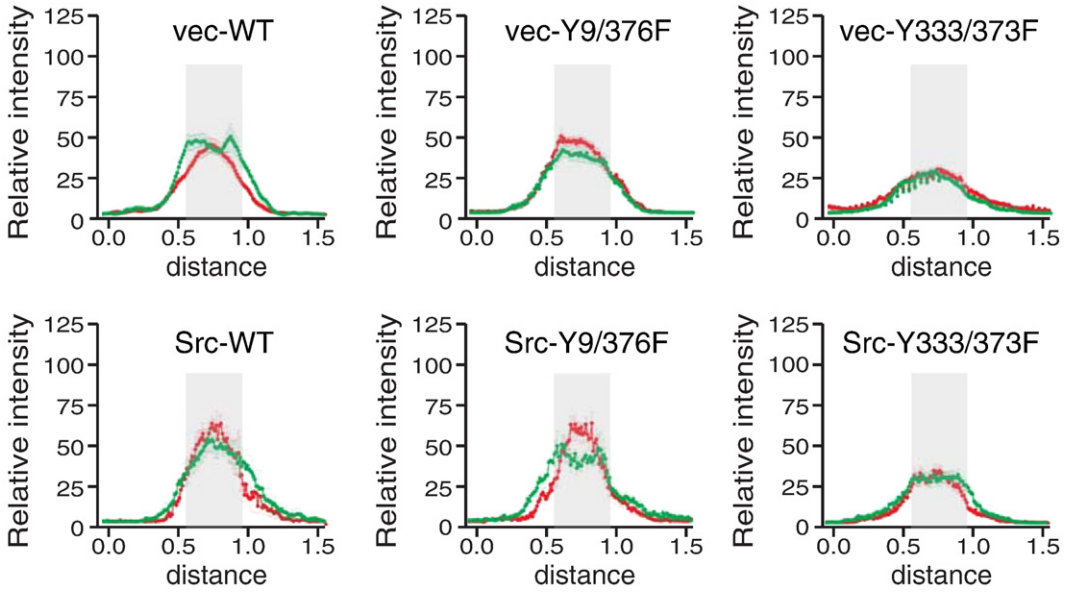

Fig. 4. The distribution of endogenous SHP-1 and of PDK-1 variants in Src kinase-expressing C6 cells. The distributions of the GFP-PDK1 [wildtype (WT) or either of the double Y9/376F and Y333/373F variants] and of endogenous SHP-1 presented in Fig. 3 were quantitated using Imagej 1.32j software (≥20 cells per sample, from two separate experiments) along a bisecting line centered on the nucleus. The distribution of GFP-PDK1 variants is depicted by the green lines, whereas the distribution of endogenous SHP-1 is depicted by the red lines. The *X*-axis label 'Distance' is the length of the bisecting line (Imagej) used in these, and all, experiments when determining the distribution(s) of PDK1 and SHP-1. The grey zones within the plots represent the average width of nuclei and are always centered along the bisecting line (*i.e.* at 0.75 along the 'Distance'). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Overexpressed PDK1 (wildtype) and endogenous SHP-1 co-localized in C6 cells overexpressing Src kinase (Figs. 3 & 4). Although SHP-1 also co-localized with the PDK1(Y9/376F) double mutant in C6 cells that do not express Src kinase (Fig. 3), these two proteins did not co-localize to the same degree in cultures co-expressing Src kinase (Fig. 3), thus reflecting the loss of co-immunoprecipitation between the two proteins we observed above (Fig. 2c,d). In contrast, the PDK1 (Y333/373F) double mutant clearly co-localized with SHP-1 (Fig. 3). These observations (quantitated using Imagej 1.32j, Fig. 4) confirm an integral role for the phosphorylation of specific tyrosine residues in

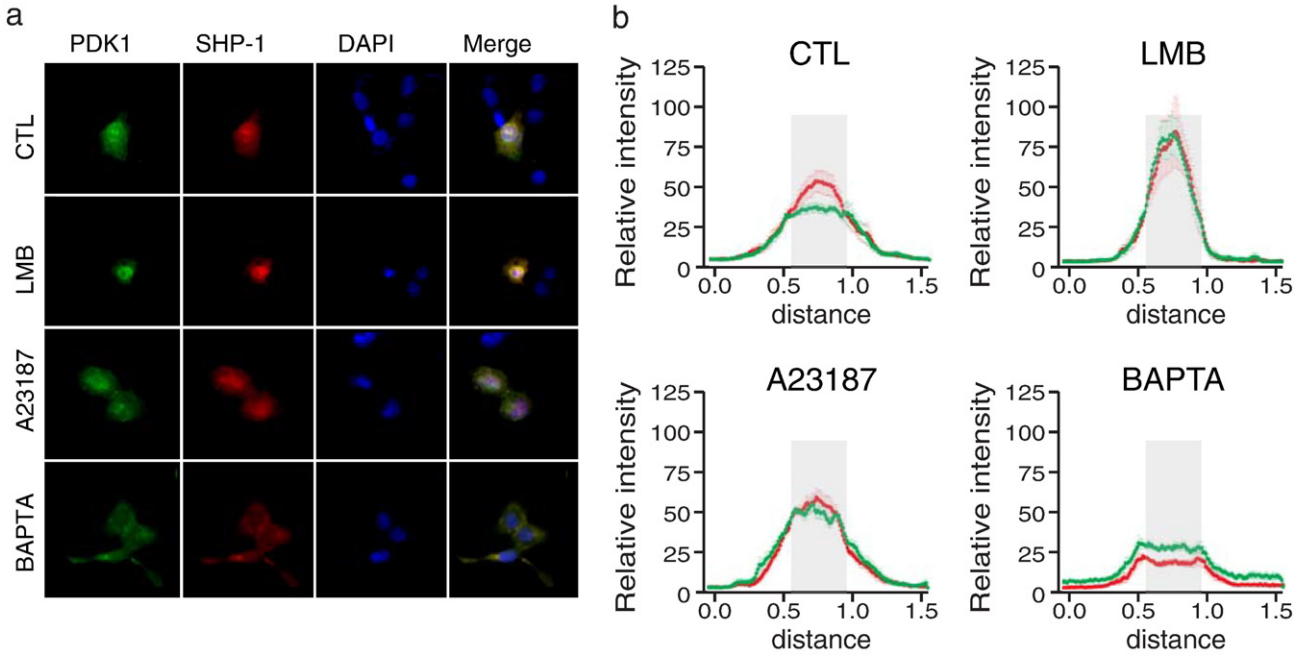

Fig. 5. The nuclear pore complex (NPC) mediates the nuclear localization of PDK1 and SHP-1. (a) C6 cells overexpressing GFP-PDK1 and RFP-SHP-1 were treated with leptomycin-B (LMB: inhibits NPC-mediated export), A23187 (activates NPC-mediated import) and BAPTA (inhibits NPC-mediated import). The distribution of fluorophore-tagged PDK and SHP-1 was determined by confocal microscopy and compared to that in unstimulated (control: CTL) cultures. DAPI staining was used to identify the nuclear compartment. (b) The distribution of GFP-PDK1 and RFP-SHP-1 was quantitated (using ImageJ) along a bisecting line centered on the nucleus. Plots represent the mean plot profile (±SEM) of $n \geq 12$ cells/culture condition from two–three separate experiments. The grey zones within the plots represent the average width of C6 cell nuclei. The distribution of GFP-PDK1 is depicted in green, while that of RFP-SHP-1 is depicted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

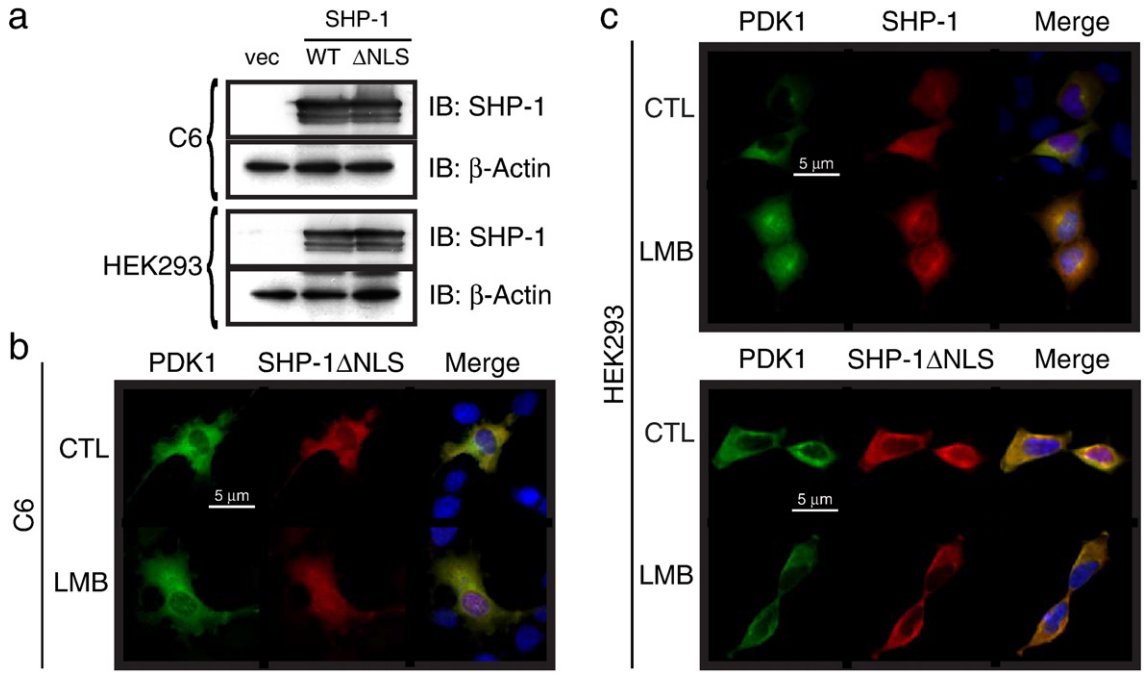

Fig. 6. The nuclear localization signal (NLS) in SHP-1 contributes to the nuclear localization of the SHP-1:PDK1 complex. (a) The expression of RFP-SHP-1 (wildtype: WT) and the RFP-SHP-1 NLS truncated protein (ΔNLS) in C6 cells and HEK293 cells was confirmed by Western blot analysis. The distribution of GFP-PDK1 in (b) C6 cells overexpressing RFP-SHP-1ΔNLS as well as in (c) HEK293 cells overexpressing either RFP-SHP-1 wildtype (SHP-1) or the NLS truncated protein (SHP-1ΔNLS) in the absence or presence of leptomycin-B (LMB: inhibits NPC-mediated export) was visualized using confocal microscopy. DAPI fluorescence defines the nucleus.

PDK1 for its association with SHP-1. While this is an important contribution to our understanding of PDK1 function, we were particularly interested by the fact that these fluorescence images also revealed that under certain conditions, *i.e.* in Src-expressing cells, the SHP-1 and PDK1 proteins tended to accumulate within the nucleus (Figs. 3 & 4, visualized using DAPI staining). The retention of SHP-1 in the nucleus of cultures co-expressing Src kinase and the PDK1(Y9/376F) double mutant (which binds less with SHP-1: Fig. 2c,d), not only suggests that SHP-1 might be facilitating the import of PDK1 to the nucleus, but it also suggests that the dissociation of SHP-1 from PDK1 could affect the export of SHP-1 from the nucleus. *In toto*, these data suggest the potential existence of a nucleo-cytoplasmic SHP-1:PDK1 shuttling complex. We chose to focus the remainder of this series of experiments on this important facet of the association between SHP-1 and PDK1.

3.2. The association between SHP-1 and PDK1 promotes a nucleo-cytoplasmic shuttling complex

To test whether SHP-1 and PDK1 do shuttle between the cytoplasm and nucleus, we treated naive C6 cells with leptomycin-B (LMB), a specific inhibitor of the nuclear export factor CRM1 and, by extension, of active nuclear export *via* the nuclear pore complex (NPC). This resulted in the nuclear accumulation of GFP-tagged PDK1 and RFP-tagged SHP-1 (Fig. 5a). Treatment with the calcium ionophore, A23187, which induces Ca^{2+}-calmodulin-dependent activation of the NPC and partial nuclear accumulation of SHP-1 in A431 cells [24], also results in nuclear accumulation of RFP-SHP-1 as well as GFP-PDK1 (Fig. 5a). In contrast, GFP-PDK1 and RFP-SHP-1 fluorescence remains predominantly cytoplasmic in cells treated with the Ca^{2+}-chelator BAPTA, which is known to abrogate nuclear import by inhibiting NPC formation and insertion into the nuclear envelope [25] (Fig. 5a). The distinct redistribution patterns of the fluorophore-tagged SHP-1 and PDK1 proteins with the various treatments were confirmed/quantitated using ImageJ 1.32j (Fig. 5b).

3.3. Both the nuclear localization signal (NLS) in SHP-1 and the nuclear export signal (NES) in PDK1 contribute to the nucleo-cytoplasmic shuttling of the SHP-1:PDK1 complex

SHP-1 is known to contain an active NLS [17]. Expression of SHP-1 in which the C-terminus NLS has been deleted [RFP-SHP-1ΔNLS] (Fig. 6a) results in the cytoplasmic accumulation of GFP-PDK1 in C6 cells (Fig. 6b). RFP-SHP-1ΔNLS is detected, albeit a much lesser extent than RFP-SHP-1 (*see* Fig. 5), in the nucleus indicating that the C-terminal NLS might not be the sole contributor to SHP-1 nuclear localization in C6 cells, as already suggested in studies using NIH-3T3 cells [20]. Yet Craggs and Kellie [17] demonstrated that the C-terminal

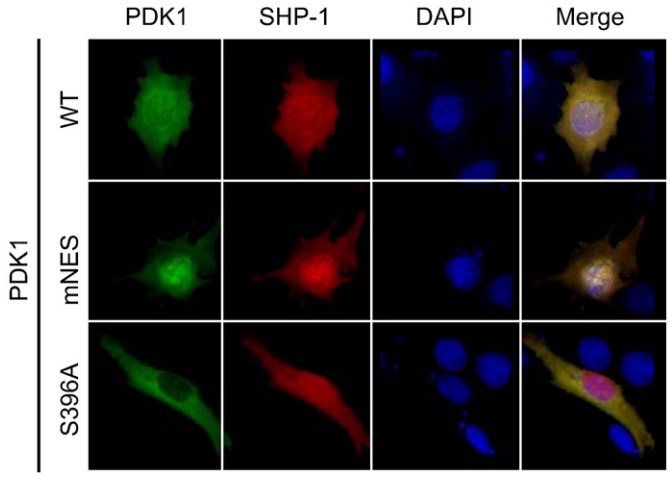

Fig. 7. PDK1 contributes to the distribution of the SHP-1:PDK1 complex. The distribution of RFP-SHP-1 was examined in C6 cells overexpressing either GFP-PDK1 wildtype (WT), GFP-PDK1(mNES) (bearing a mutation in the NES that impedes the NPC-mediated export of PDK1 from the nucleus) or GFP-PDK1(S396A) (a mutation that promotes the cytoplasmic retention of PDK1). Merged micrographs identify the subcellular localization of the two fluorophore-tagged proteins (nucleus is stained blue: DAPI fluorescence).

NLS is the sole regulator of SHP-1 nuclear localization in HEK293 cells. This is confirmed by the cytoplasmic accumulation of RFP-SHP-1ΔNLS (*cf.* RFP-SHP-1, wildtype) in HEK293A (Fig. 6c). GFP-PDK1 is also retained within the cytoplasm of these same cells. RFP-SHP-1ΔNLS and GFP-PDK1 are both excluded from the nucleus following treatment of HEK293A cells with LMB, clearly implicating the ability of SHP-1 (and its NLS) to translocate to the nucleus as an obligate factor in PDK1 nuclear localization (Fig. 6c).

GFP-PDK1(mNES) (whose nuclear export is impeded by a L379S/F383S mutation within the NES [4]) accumulates in the nucleus of C6 cells (Fig. 7). RFP-SHP-1 is also detected predominantly in the nucleus of these same cells. Expression of PDK1(S396A), which bears a Ser396Ala substitution that causes the cytoplasmic retention of the expressed protein in PTEN$^{-/-}$ and MCF-7 cells [4], is also detected almost exclusively in the cytoplasm of C6 cells (Fig. 7). A proportion of RFP-SHP-1 is distinctly nuclear in these unstimulated cells, suggesting that SHP-1 does not need to be complexed with PDK1 to enter the nucleus, yet it certainly appears to rely on PDK1 for export from the nucleus.

3.4. The PH domain in PDK1 plays a pivotal role in the localization and nuclear import of PDK1

PIP3 production and distribution was assessed indirectly using an RFP-tagged PH construct—the PH (pleckstrin homology) domain binds to PIP3s [26–28]. RFP-PH was homogeneously distributed in control C6 cells, whereas in cells expressing activated Src kinase it tended to concentrate in a distinct ring delineating the perinuclear region (Fig. 8a,b). GFP-PDK1 wildtype is also distributed throughout the cell in the absence of Src kinase and is clearly detected within the

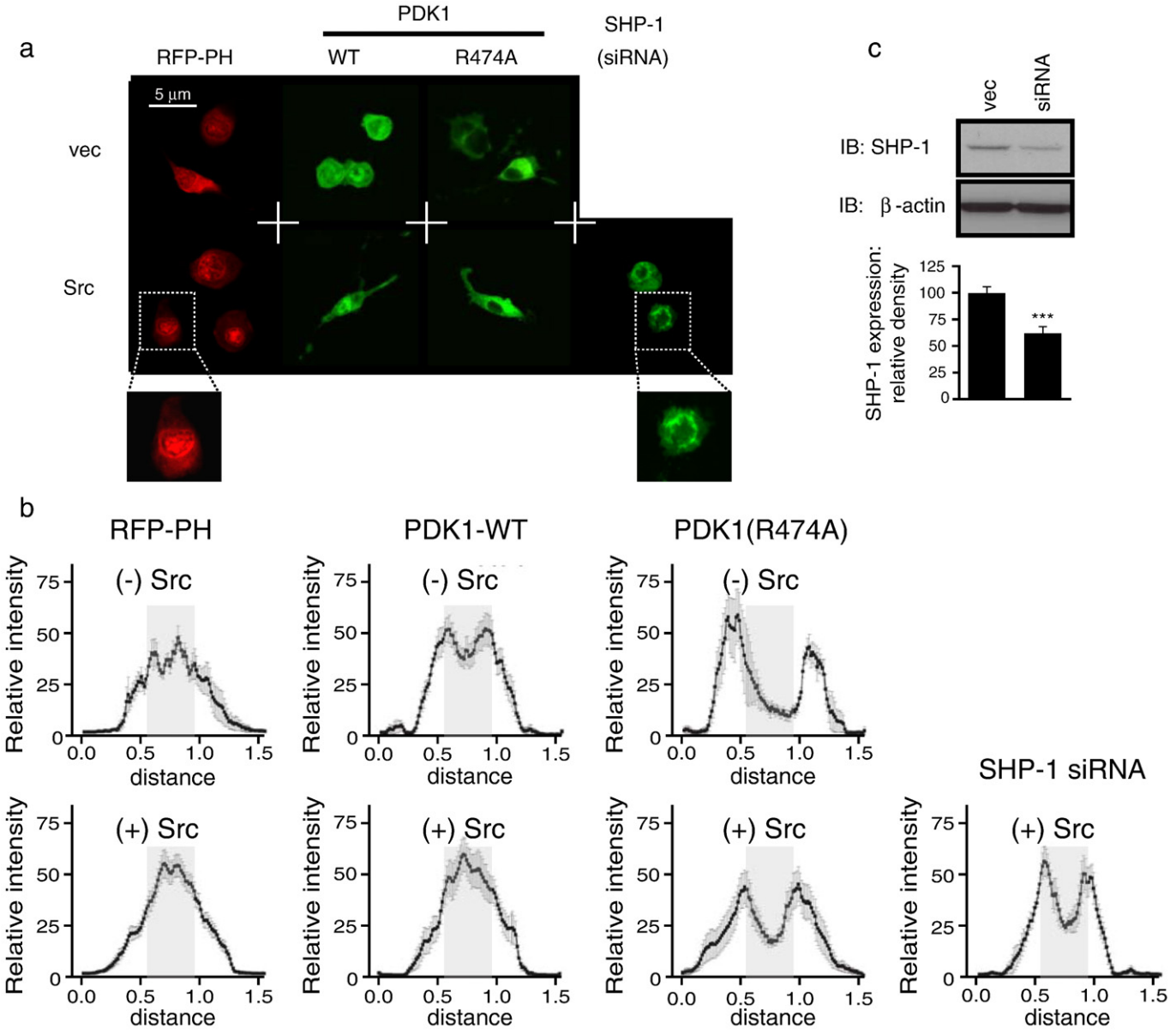

Fig. 8. The nuclear import of the SHP-1:PDK1 complex relies on sequential contributions by both PDK1 and SHP-1. (a) C6 cells co-expressing RFP-PH (to monitor the distribution of PIP3 production) and either activated Src kinase (Src) or the vector control (vec) was examined by confocal microscopy. (b) The distribution of RFP-PH in the absence [(−)Src, upper plot] or presence [(+)Src, lower plot] of Src was quantitated (using ImageJ and a bisecting line centered on the nucleus). Plots represent the mean plot profile (±st dev) of $n \geq 15$ cells/culture condition from two–three separate experiments. The grey zones within each plot represent the average width of nuclei along the bisecting line. (a,b) The distribution of GFP-PDK1 wildtype (WT) or GFP-PDK1(R474A) (a PH domain mutation that is incapable of binding to PIP3's) in C6 cells co-expressing activated Src(Y527F) kinase were also monitored and quantitated using ImageJ. (c) Targeted knock-down of endogenous SHP-1 using siRNA was confirmed by Western blot analysis ($t = 6.442$, $df = 8$, $P = 0.0002$) and was used to determine the contribution of SHP-1 to the distribution of GFP-PDK1 (a,b) in Src-expressing C6 cells.

nucleus of Src kinase-expressing cells (Fig. 8a,b). In contrast, the GFP-PDK1(R474A) protein (mutated within the PH domain so as to disrupt binding to PIP3 [22]) remains cytoplasmic even when co-expressed with Src kinase (Fig. 8a,b). The GFP-PDK1 wildtype is also excluded from the nucleus of Src kinase-expressing cells following siRNA-mediated knock-down of endogenous SHP-1 (Fig. 8a–c). In these cells, the detection of GFP-PDK1 also tends to localize to a perinuclear "ring". These combined data suggest that the PIP3-binding capacity of the PH domain of PDK1 is pivotal for the initial recruitment of the complex to the nucleus and that SHP-1 is subsequently necessary for import into the nucleus.

3.5. Src kinase-induced process formation in C6 cells is facilitated by the SHP-1:PDK1 complex

Tyrosine kinases are known to induce differentiation of C6 cells [21]. As such, we chose to examine the effect of the Src [tyrosine] kinase on C6 cell phenotype, using the formation of processes as a read-out. Cells were triple-transfected with activated Src kinase and SHP-1 (either wildtype or the ΔNLS truncated protein) and PDK1 (either wildtype or the mNES mutated protein). The formation and length of individual processes was visualized microscopically and quantitated using the ImageJ 1.32j software. Note that the intensities of the GFP and RFP signals in Fig. 9 were purposefully increased simply for ease of visualization of the extremely fine processes in these cultures.

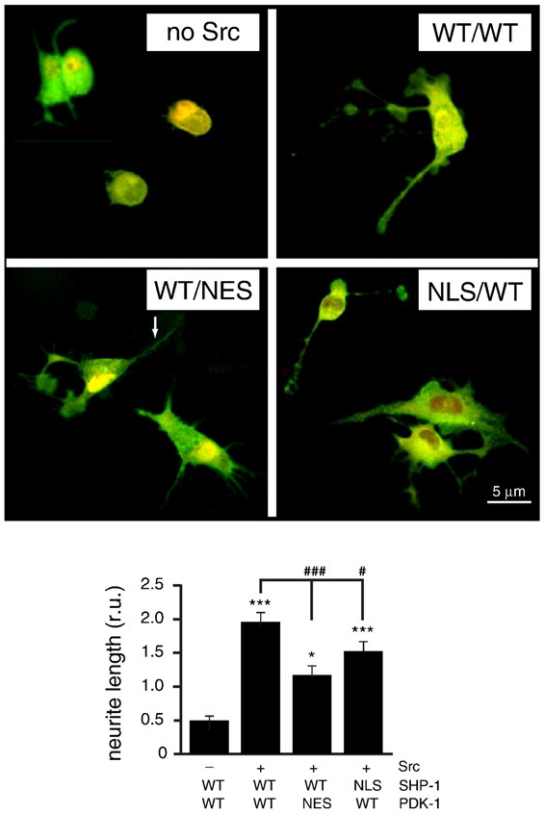

Fig. 9. Process formation in Src-expressing C6 cells is promoted by an intact SHP-1:PDK1 complex. (a) The appearance of processes in C6 cells was monitored in cultures overexpressing both wildtype RFP-SHP-1 and GFP-PDK1 in the absence (no Src) or presence (WT/WT) of Src kinase (Src). This was compared to cultures overexpressing (along with Src kinase) either RFP-SHP-1 wildtype and GFP-PDK1(NES) (WT/NES) or RFP-SHP-1ΔNLS and GFP-PDK1 wildtype (NLS/WT). (b) The lengths of individual processes were measured ($n = 20$–51 cells, from three separate experiments) using ImageJ. [note that fluorescence in these images were artificially enhanced for ease of visualizing the very fine processes]. $^{*}P<0.05$ & $^{***}P<0.001$, versus 'no Src' controls; $^{\#}P<0.05$ & $^{\#\#\#}P<0.001$, versus WT/WT (with Src).

Overexpression of both SHP-1 and PDK1 wildtype proteins enhanced Src kinase-induced process formation and this was significantly attenuated by overexpression of either the SHP-1ΔNLS or the PDK1(mNES) proteins [$F_{(3,137)} = 33.16$, $P<0.0001$] (Fig. 9). The expression of SHP-1ΔNLS or PDK1(mNES) also tended to induce a "ruffled" phenotype and/or the appearance of varicosities along the podial-like processes (Fig. 9). As expected, the cultures overexpressing either the wildtype proteins or the PDK1(mNES) had a distinct nuclear SHP-1/PDK1 signal (detected here as a "merged" yellow). GFP-PDK1 remained predominantly cytoplasmic, whereas there was a minor RFP signal in the nucleus of cultures expressing the RFP-SHP-1ΔNLS (as observed in Fig. 6) (Fig. 9).

3.6. Src kinase-induced process formation in cultures co-expressing GFP-PDK1 and RFP-SHP-1 reflects the inactivation of Akt

Differentiation is known to rely on the inactivation of Akt [29]. In keeping with this, Src kinase, which can induce differentiation, also inactivates Akt [30]. Treatment of naïve C6 cell cultures with the chemical PI3K/Akt inhibitor, LY294002, inhibits Akt phosphorylation (Fig. 10a), as expected. We then used LY294002 to test the contribution of Akt to Src kinase-induced process formation in C6 cells. However, treatment of C6 cultures co-expressing Src kinase, GFP-PDK1 and RFP-SHP-1 with LY294002 actually results in shorter processes [$F_{(2,139)} = 12.82$, $P<0.0001$] (Fig. 10b,c). This was unexpected as LY294002, and its ability to inhibit PI3K and Akt phosphorylation, should have enhanced the elongation of processes. Although this was initially perplexing, Western blot analysis subsequently revealed that in C6 cells co-expressing Src kinase/GFP-PDK1/RFP-SHP-1 and treated with LY294002, Akt is actually activated (confirmed by the phosphorylation of Akt as well as its downstream substrate, GSK-3β) (Fig. 10d). (it should be noted that, because of our concern over this observation, we repeated the experiment four times, using two separate lots of LY294002). Comparison with wortmannin, another compound routinely used to study the effects of PI3K/Akt inhibition on cell function, reveals that it does contribute to the inhibition of Akt phosphorylation (Fig. 10d), as expected. In contrast with LY294002, wortmannin does not inhibit the formation of processes in cultures co-expressing Src kinase/GFP-PDK1/RFP-SHP-1 (Fig. 10b,c). Interestingly, the phosphorylated pool of Akt in LY294002-treated cultures (Fig. 10d) appears to be localized predominantly at the plasma membrane (Fig. 10e). In contrast, the little residual phosphoAkt signal detected in wortmannin-treated cultures appears to be predominantly nuclear (Fig. 10e). While we do not have an immediate explanation for the fact that LY294002 and wortmannin exert opposite effects on Akt phosphorylation in our model (Fig. 10d,e), our combined data do confirm that both SHP-1 and PDK1 are required to enhance Src kinase-induced outgrowth of processes in C6 cells and that the inactivation of Akt signalling is positively correlated with this event.

4. Discussion

SHP-1 is known to negatively regulate PI3K/Akt signalling, at least in part, by targeting and dephosphorylating p85, the regulatory subunit of PI3K [15]. We now forward PDK1 as a novel target for SHP-1-mediated regulation of PI3K/Akt signalling. However, our data clearly indicate that the association between SHP-1 and PDK1 goes beyond the simple regulation of Akt signalling and that this association provides a mechanism for the import of PDK1 to the nucleus, *i.e. via* a "piggy-back" mechanism based on SHP-1 (by virtue of its NLS) [17] as an import vehicle.

Specific tyrosine residues in PDK1 contribute to its activation as well as to its ability to localize to the plasma membrane [10]. It is now well established that growth factors (*i.e.* insulin, NGF and IGF-1) that promote PDK1 tyrosine phosphorylation also promote PDK1 localization to the nucleus [3,4,6]. Yet it is surprising that the nuclear detection of PDK1 following stimulation with IFG-1, for example, is not

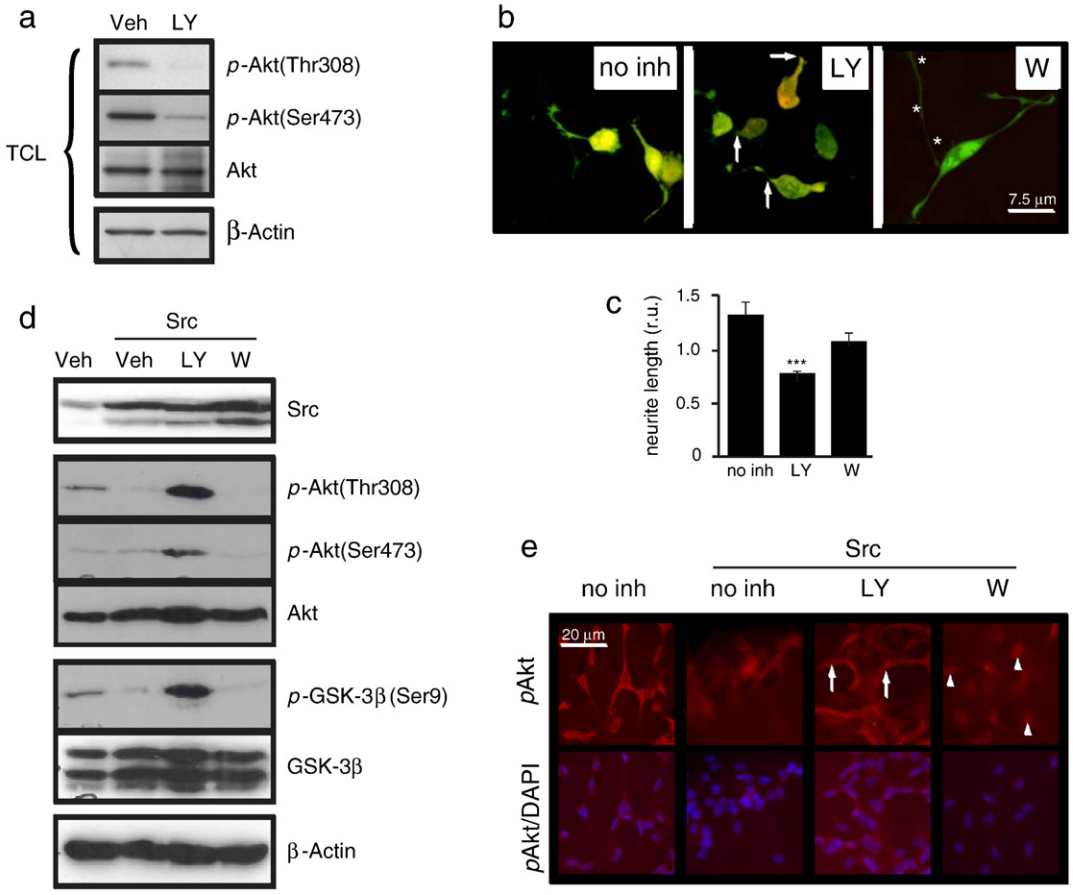

Fig. 10. Src kinase-induced process formation in C6 cells co-expressing SHP-1 and PDK1 coincides with the loss of Akt phosphorylation. (a) Non-transfected C6 cells were treated with the PI3K inhibitor LY294002 (LY, 25 μM) and the activation (phosphorylation) status of Akt was examined by Western blot. (b) C6 cell process formation was examined in cultures overexpressing Src kinase (Src), RFP-SHP-1 and GFP-PDK1. The cultures either did not receive any inhibitor (no inh), or were treated with either LY or another PI3K/Akt inhibitor wortmannin (W) (*n.b.* arrows indicate processes that otherwise might be difficult to see, whereas asterisks "*" indicate the elongated process from an out-of-field adjacent cell). (c) The effect of LY and W on process length was quantified using ImageJ ($n = 41$–56 cells, from three separate experiments). ***$P < 0.0001$ versus vehicle-treated (veh) controls. (d) The effect of treatment with LY and W on Akt phosphorylation was examined by Western blot. The phosphorylation of GSK-3β on Ser9 was included as it is an acknowledged target of activated Akt, while the expression of β-actin was included as a loading control. (e) The effect of treatment with LY and W on the phosphorylation of Akt on Thr308 (*p*Akt) was confirmed by indirect immunofluorescence. A series of images showing the distribution of DAPI are included to identify the nucleus of individual cells. Arrows indicate the detection of *p*Akt along the plasma membrane of LY-treated cells; arrowheads indicate the detection of *p*Akt in the nucleus of W-treated cells.

substantially different between non-transfected cells (*i.e.* looking at endogenous PDK1) or cells overexpressing PDK1 [4]. This suggests that a factor mediating PDK1 nuclear import may be saturated and, thus, rate-limiting. Overexpression of SHP-1, as done in the current study, overcomes this and thus supports SHP-1 as a "rate-limiting factor" in nuclear PDK1 localization. The association between SHP-1 and PDK1 is not unexpected given that SHP-1 is a general phosphatase of Src kinase substrates [31] and that PDK1 Y9, Y373 and Y376 are known targets of Src kinase [5,10]. Our data suggest that PDK1(Y9) and PDK1(Y376) are binding targets of SHP-1, presumably *via* its tandem SH2 domains, whereas the PDK1(Y333) and PDK1(Y373) residues are potentially catalytic targets. We continue to examine the role of these tyrosines in PDK1 function and cell phenotype.

The combined molecular weight of an SHP-1:PDK1 complex clearly exceeds the ~40 kDa threshold for passive diffusion into the nucleus. The complex undoubtedly requires energy-dependent active transport (*i.e.* importin/RanGTP-dependent) across the nuclear pore complex [32]. Our ability to alter the distribution of SHP-1 and PDK1 by chemically manipulating nuclear pore complex function clearly supports this notion. The reduction in nuclear detection of PDK1 in cells either overexpressing the SHP-1 protein devoid of an NLS [SHP-1ΔNLS] or in which endogenous SHP-1 has been knocked down clearly supports a pivotal role for SHP-1 in nuclear import of PDK1. Yet, the inability of the PDK1(R474A) PH domain-mutated protein to localize to the nucleus, even in Src kinase-expressing cells, suggests strongly that nuclear localization of the SHP-1:PDK1 complex requires an initial step that involves the recruitment of PDK1 (*via* its PH domain). This clearly implicates perinuclear PIPs in the process. This is not an unreasonable notion given that PDK1 is excluded from the nucleus following chemical inhibition of PI3K and is detected more readily in the nucleus of PTEN$^{-/-}$ embryonic fibroblasts (PTEN being the phosphatase that exerts part of its regulatory function by dephosphorylating PIP3's) [3]. Once in the proximity of the nuclear membrane, recognition of the NLS in SHP-1 by importins would permit transport across the nuclear pore complex. The fact that PH domains have micromolar affinities for PIPs [33] and that importin-α will bind NLS-containing cargo proteins with higher affinity, *i.e.* within the nanomolar range [34], supports the model of sequential recognition steps. This model would depend on an initial low-affinity mechanism for recruitment of PDK1 (*via* its PH domain) to perinuclear PIP3's, followed by a rapid high-affinity transfer, *via* SHP-1 (and its NLS), to the nuclear pore complex for the transport of both SHP-1 and PDK1 into the nucleus. SHP-1 itself has also been shown to bind to PIPs [35], but its inability to redirect the PDK1(R474A) PH domain-mutated protein to the nucleus certainly argues for PDK being the predominant recruiting factor to nuclear PIPs in the present model.

Src kinase targets PDK1 [5,10], yet it is also required for optimal activation of SHP-1 [36] and an adaptive feedback mechanism could exist given that SHP-1 is a negative regulator of Src kinase-targeted

substrates [14]. While nuclear SHP-1 clearly is required for NIH 3T3 cell proliferation [20] and appears to alter the tyrosylphosphorylation of several key transcription factors during myeloid cell differentiation [37], others [38] find that nuclear SHP-1 might exist within an inactive complex and does not associate with tyrosylphosphorylated proteins. In support of this, the loss of association between SHP-1 and the double-mutated PDK1(Y9/376F) variant (demonstrated herein using both co-immunoprecipitation and confocal microscopy) occurs within the soluble nuclear fraction (CFS and DDM, *unpublished data*), but we are as yet unclear as to why this should occur in this fraction and not in the cytoplasm. The redistribution of PDK1 to the nucleus, while clearly required for 3T3-L1 preadipocyte differentiation [39] and for proper activation of the nuclear PI3K machinery [1], might also provide a means for diminishing cytoplasmic PDK1 function, and, by extension, also inhibiting cytoplasmic Akt [2,3]. We [28] and others [21,40] clearly have associated C6 glial differentiation with inactivation of cytoplasmic Akt. Roymans et al. [21,40] implicated the PI3K cascade and tyrosine kinase activation in C6 glial cell process formation. We now demonstrate that the nuclear localization of the SHP-1:PDK1 complex promotes the extension of processes in Src kinase-expressing C6 cells and that nuclear PIP production contributes to this effect.

The interruption of the shuttling of the SHP-1:PDK1 complex between the nuclear and cytoplasmic compartments, *i.e.* with the SHP-1ΔNLS or with each of PDK1(mNES) or PDK1(S396A), tended to promote a "ruffled" phenotype rather than the elongations observed in cultures co-expressing the SHP-1 and PDK1 wildtype proteins. Interestingly, these PDK1 proteins can all affect Akt phosphorylation [3,41]. Neurite outgrowth and ruffle formation is dependent on distinct signalling cascades in PC12 cells [42]. Perhaps not surprising, preliminary investigations (CFS, DZ and DDM, *unpublished data*) indicate that the SHP-1:PDK1 complex also clearly contributes to NGF-induced differentiation of the preneuronal PC12 cell line, thus supporting reports of contributions by both SHP-1 [43,44] and a nuclear PI3K-mediated event [45] in this phenotypic change. As the appearance of podial-like elongations and ruffle formation in C6 cells appears to depend on the compartmentalization of SHP-1 and PDK1 to distinct subcellular [non-nuclear] compartments, we must assume that different signalling cascades could be influenced by the complex in a ligand-selective fashion. A previous study of ours suggested that C6 cell differentiation and anchoring of an active PI3K/p85 complex to the plasma membrane was partly sensitive to PDGF receptor, but not EGF receptor, stimulation and that the resultant loss of Akt phosphorylation occurred concurrently with a significant increase in nuclear PI3K activity [28]. The fact that Src kinase can activate PDK1 [5,10], all the while inhibiting Akt [30], also clearly supports the potential for uncoupling of the PI3K/PDK1/Akt pathway. The uncoupling of this pathway is not strictly associated with a differentiating phenotype. Indeed, we have observed an apoptotic phenotype following the induction of p85 and a concurrent dephosphorylation of Akt in human MCF-7 breast cancer cells in which SHP-1 has been induced (but c-Src kinase has been repressed) [46] and we have demonstrated that the antipsychotic haloperidol induces apoptosis and de-activates Akt [47,48] at a time when there is significant induction of PI3K p110 lipid kinase activity [47].

Finally, our present results clearly indicate that two commonly used PI3K inhibitors, LY249002 and wortmannin, do not exert identical effects in our Src kinase-expressing C6 cells. Although this initially surprised us, this observation is, in fact, not unique [49–51]. While we are currently unclear as to what mechanism(s) might account for this discrepancy, two conclusions can be immediately proposed. The first is a pragmatic one. The context in which either of these two inhibitors is used, particularly involving the study of Src kinase-mediated events, and the conclusions drawn from their use, must now be seriously considered. The second, and most germane to our current hypothesis, is that the respective effects of these two inhibitors on Akt phosphorylation in Src kinase-expressing cells clearly supports the loss of Akt activity as a primary signalling correlate in Src kinase/SHP-1/PDK1-induced C6 cell podial outgrowth.

The shuttling of PDK-1 between the cytoplasm and the nucleus has already been observed on several occasions in non-neuronal cells [2–5], yet the exact mechanism involved has remained unknown. Our data allow us to conclude that tyrosine-phosphorylated PDK-1 will associate with the protein tyrosine phosphate SHP-1 and that this complex facilitates PDK1 nuclear entry. In fact, the resulting complex is a mutualistic one as it also benefits SHP-1 by allowing it localize to different subcellular compartments along with PDK1. Our data also suggest that the shuttling of the SHP-1:PDK1 complex between the cytoplasm and the nucleus is apparently dependent on a specific sequence of events; initial recruitment of PDK1 to perinuclear PIPs, followed by SHP-1 (and its NLS)-mediated entry *via* the nuclear pore complex and PDK-1 (and its NES)-mediated nuclear export. This mutualistic "piggy-back" nucleo-cytoplasmic shuttling complex contributes to differentiating phenotypes in glial C6 [and preneuronal PC12] cells.

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