Distinctive regulation of v-Src-associated phosphatidylinositol 3-kinase during PC12 cell differentiation

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In chicken embryo fibroblasts, the binding of v-Src to PtdIns 3-kinase requires Src homology domains, SH3, SH2 and the SH1 or kinase domain, which induces the cytoskeletal disruption associated with fibroblast transformation. In the rat phaeochromocytoma PC12 cell line, v-Src has a different effect on the cytoskeleton, inducing neurite extension rather than cytoskeletal disruption. Here we show that v-Src-induced neurite outgrowth is suppressed by the selective PtdIns 3-kinase inhibitor LY294002, suggesting that this effect of v-Src in PC12 cells also requires the activity of the lipid kinase. However, in contrast with chicken embryo fibroblasts, the association of PtdIns 3-kinase with v-Src in PC12 cells is delayed until several hours after activating the v-Src tyrosine kinase. Furthermore the v-Src-associated p85 regulatory subunit of PtdIns 3-kinase is not phosphorylated on

tyrosine in PC12 cells and associates only weakly with isolated v-Src homology domains (SH3/SH2) in a Src kinase-independent manner. However, p85 and v-Src both associate with an unidentified protein (of molecular mass approx. 68 kDa; termed p68), which becomes tyrosine phosphorylated concomitantly with the association of both p85 and PtdIns 3-kinase with v-Src in PC12 cells. Thus we conclude that the mode of regulation of v-Src-associated PtdIns 3-kinase is cell-context-dependent and that p68 might act as an adaptor protein to mediate the association of p85 and v-Src in PC12 cells. The different regulation of PtdIns 3-kinase in PC12 and in chicken embryo fibroblasts in response to v-Src activity might reflect the different cytoskeletal rearrangements induced by this oncoprotein in the two cell types.

INTRODUCTION

v-Src, the protein tyrosine kinase encoded by the transforming gene of Rous sarcoma virus, induces cytoskeletal disassembly leading to cell transformation in chicken embryo fibroblasts (CEF) (reviewed in [1]). In the rat phaeochromocytoma cell line PC12 [2], however, the expression of v-Src results in neurite outgrowth [3,4], indicative of differentiation into sympathetic neuron-like cells [5]. It remains unclear how v-Src can induce quite different biological effects in these two cell types.

v-Src requires association with PtdIns 3-kinase to transform CEF [6,7]. Because the lipid products of PtdIns 3-kinase are now implicated in cytoskeletal rearrangements [8,9], v-Src-associated PtdIns 3-kinase is a likely mediator of the actin cytoskeleton disruption and focal adhesion disassembly that are responsible for the rounded morphology characteristic of transformed fibroblasts [10,11]. Our previous work demonstrated that, in CEF, the v-Src SH2 domain can bind the p85 regulatory subunit of PtdIns 3-kinase in a phosphotyrosine-dependent manner and that combined v-Src SH3 and SH2 domains, when present in the same tandem arrangement as in the entire v-Src molecule, co-operate in binding tyrosine-phosphorylated p85 [12]. Furthermore the activation of v-Src-associated PtdIns 3-kinase in CEF does not occur as a result of direct binding to the v-Src SH3/SH2 domains but requires the tyrosine kinase activity of v-Src. Indeed, the association of PtdIns 3-kinase with v-Src in CEF occurs with kinetics that are indistinguishable from the kinetics of activation of the tyrosine kinase of temperature-sensitive (ts) v-Src [12]. Thus our results indicate that v-Src stimulates the association

and activation of PtdIns 3-kinase by tyrosine phosphorylation of p85 in CEF [12].

The observation that wortmannin inhibits nerve growth factor (NGF)-stimulated neurite extension in PC12 cells [13] suggests a requirement for PtdIns 3-kinase in this process. This has recently been supported by the use of a dominant inhibitory p85 molecule to demonstrate that NGF-stimulated neurite extension and neurite maintenance in these cells requires the activity of PtdIns 3-kinase [14]. These results, together with the known binding of PtdIns 3-kinase to v-Src on its activation in fibroblasts, suggest that v-Src-associated PtdIns 3-kinase might also be involved in generating the cytoskeletal changes responsible for the outgrowth of neurites in PC12 cells expressing the oncoprotein.

We therefore investigated the regulation and role of PtdIns 3-kinase during v-Src-induced neurite extension in PC12 cells that stably express ts v-src LA29 [15]. We show that v-Src-induced neurite outgrowth in PC12 cells is inhibited by the PtdIns 3-kinase inhibitor LY294002 and demonstrate that the mode of regulation of PtdIns 3-kinase is different from that operating in CEF, perhaps reflecting the different biological consequences of v-Src activity in the two cell types.

MATERIALS AND METHODS

Cell culture and generation of PC12 cells stably expressing ts v-src

PC12 cells were routinely cultured at 39.5 °C in plastic tissue-culture flasks coated with type I rat tail collagen (Collaborative Biomedical Products, Bedford, MA, U.S.A.). Growth medium

Abbreviations used: CEF, chicken embryo fibroblasts; GST, glutathione S-transferase; NGF, nerve growth factor; SH, Src homology; ts, temperature-sensitive.

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Figure 1 Expression of ts v-Src in PC12 cells induces neurite extension at permissive temperature

(a) v-Src expression, detected by immunoblotting, is shown in PC12/F29 sub-clone (lane 4) and is compared with expression in parental PC12 cells (lane 3), CEF/R29 (lane 2) and CEF control cells (lane 1). Protein lysate (5 μ g) was loaded for each. (b) Morphology of PC12 parental cells detected by phase-contrast microscopy. (c, d) Morphology of PC12/F29 at restrictive (39.5 °C; c) and shifted to permissive (35 °C; d) temperature for 36 h. Magnification $\times 132$.

was Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) heat-inactivated fetal calf serum (Advanced Protein Products, Brierley Hill, Birmingham, U.K.), 5 % (v/v) heat-inactivated horse serum (Sigma, Poole, Dorset, U.K.), 0.375 % sodium bicarbonate, 1 mM sodium pyruvate and 2 mM glutamine.

PC12 cells were transfected with an fpGV retroviral expression vector [16] carrying the neomycin resistance marker and the ts v-src LA29 gene by using DOTAP transfection reagent (Boehringer Mannheim, Lewes, E. Sussex, U.K.). The G418 concentration

used for selection was 600 ng/ml. Cells were treated with 40 μ M LY294002 (from a 5 mM stock solution in ethanol) or with an equivalent volume of ethanol as control. Cells were re-fed with medium containing fresh drug every 12 h.

Immunoprecipitation

Subconfluent cells were lysed in modified PLC lysis buffer [50 mM Tris/HCl (pH 7)/1 % (v/v) Triton X-100/10 % (v/v) glycerol/1.5 mM $MgCl_2$ /1 mM EGTA/150 mM NaCl/1.8 μ g/ml aprotinin/0.1 mM sodium orthovanadate/0.5 mM NaF/10 mM β -glycerophosphate/10 mM sodium pyrophosphate/1.25 mM PMSF]. The lysates were clarified by centrifugation and protein concentrations were determined with the bicinchoninic acid protein assay kit (Pierce, Chester, Cheshire, U.K.). The clarified lysates were adjusted for protein concentration and aliquots were incubated overnight at 4 °C with mouse monoclonal anti-(v-Src) (EC10) or anti-(p85- α) antibody (Upstate Biotechnology, Lake Placid, NY, U.S.A.), or with normal mouse serum immobilized on Protein A-Sepharose beads coated with rabbit anti-mouse serum (all from Sigma). The immunoprecipitates were pelleted, washed and used for PtdIns 3-kinase assays or Western blotting analysis.

Glutathione S-transferase (GST) fusion protein binding

GST fusion proteins were prepared as described in [12]. Subconfluent cells were lysed in modified PLC lysis buffer and aliquots of lysate containing equal amounts of cellular protein were incubated with glutathione-agarose-bound GST-SH3, GST-SH2 or GST-SH3/S2 fusion proteins for 2 h at 4 °C. The complexes were then pelleted, washed and used for PtdIns 3-kinase assays or Western blotting analysis.

PtdIns 3-kinase assays

PtdIns 3-kinase was measured in anti-v-Src immunoprecipitates by using a 3:1 sonicated mixture of phosphatidylinositol and phosphatidylserine by the method described in [12].

v-Src kinase assays *in vitro*

Anti-v-Src immunoprecipitates, washed once with modified PLC lysis buffer and three times with PBS supplemented with 1 % (v/v) NP-40, were incubated for 5 min at 30 °C with 5 μ Ci of [γ - 32 P]ATP (3000 Ci/mmol; Amersham, Little Chalfont, Bucks, U.K.) in kinase buffer [100 mM Pipes (pH 6.8)/2 mM $MnCl_2$ /10 μ M sodium orthovanadate]. The reaction was stopped by the addition of 2 $\times$ sample buffer [0.08 M Tris/HCl (pH 6.8)/10 % (v/v) glycerol/5 % (w/v) SDS/0.5 % (w/v) Bromophenol Blue/4 % (v/v) 2-mercaptoethanol]. The products were resolved on SDS/polyacrylamide [7.5 % (w/v)] gels and transferred to a nitrocellulose membrane by semi-dry blotting, after which an autoradiograph was taken on Fuji RX medical X-ray film (Fuji, London, U.K.).

Immunoblotting

Proteins separated by SDS/PAGE [7.5 % (w/v) gel] were transferred to nitrocellulose by semi-dry blotting. After blocking overnight with 3 % (w/v) BSA (Sigma) in PBS supplemented with 0.2 % (v/v) Tween-20, the membranes were incubated for 1 h with mouse monoclonal anti-phosphotyrosine (PY20) (Upstate Biotechnology), anti-p85 or anti-v-Src (EC10) primary antibody followed by a 1 h incubation with polyclonal sheep

anti-mouse horseradish peroxidase-linked secondary antibody (Amersham). Detection was by enhanced chemiluminescence (Amersham). For re-probing with a different primary antibody, bound antibody was stripped off the membrane by incubation with 0.2 M glycine solution, pH 2.5, supplemented with 1% (w/v) SDS, at room temperature for 1 h. Stripping efficiency was confirmed by autoradiography.

RESULTS

v-Src-induced neurite outgrowth in PC12 cells is inhibited by LY294002

PC12/F29 cell clones were obtained by G418 selection of PC12 cells transfected with fpGV encoding ts *v-src* LA29. v-Src expression varied between clones but was consistently lower than that observed in CEF expressing ts *v-src* LA29 (CEF/R29 cells) (Figure 1a). The PC12/F29 clone expressing the highest concentrations of v-Src was chosen for further study (Figure 1a). PC12/F29 cells displayed recognizable neurite extension after 24–48 h of growth at the permissive temperature (35 °C) (Figure 1c). At the restrictive temperature (39.5 °C), PC12/F29 cells were morphologically similar to parental PC12 cells (Figure 1b), although short neurite extensions were evident in some cells, presumably as a consequence of some residual v-Src activity at 39.5 °C. Treatment of PC12/F29 cells with the selective PtdIns 3-kinase inhibitor, LY294002, suppressed v-Src-induced neurite extension (Figure 2c). Thus, as shown by others [3,4], the activity of v-Src in our PC12/F29 sub-clones induces their differentiation. Furthermore the biological activity of v-Src in PC12 cells requires PtdIns 3-kinase, as it does also during fibroblast transformation.

Association of PtdIns 3-kinase with v-Src is delayed in PC12 cells

To determine whether the regulation of v-Src-associated PtdIns 3-kinase in PC12/F29 cells was similar to the regulation observed in fibroblasts [12], we first examined the kinetics of binding of PtdIns 3-kinase to v-Src. In contrast with the rapidly induced association of PtdIns 3-kinase with v-Src during the first 60 min after a shift to the permissive temperature in CEF, there was no stimulated binding in PC12/F29 cells during this period (Figure 3a). To ensure that the v-Src tyrosine kinase was rapidly reactivated in PC12/F29 cells, we performed immune-complex kinase assays and demonstrated that v-Src autophosphorylation was evident as early as 10 min after the shift to permissive temperature (Figure 3b). Thus v-Src reactivates in PC12 cells with a kinetics that is indistinguishable from that in CEF [17]. We next examined a period between 0 and 12 h after activation of v-Src and observed an increase in v-Src-associated PtdIns 3-kinase between 2 and 4 h, which reached a maximum after 6 h at the permissive temperature (Figure 3c). Thus, despite a very similar and rapid reactivation of the tyrosine kinase activity in CEF and PC12/F29 cells, the kinetics of association of PtdIns 3-kinase with v-Src was different in the two cell types, being substantially delayed in the PC12 cells.

Ability of the p85 and PtdIns 3-kinase from PC12 cells to associate with the v-Src homology domains is independent of kinase activity

As the primary mode of binding of v-Src to PtdIns 3-kinase is by the interaction of the p85 regulatory subunit with the Src SH3 domain [18] but also involves the SH2 domain after activation of

Figure 2 Suppression of PC12 cell differentiation by LY294002

(a, b) Morphology of PC12/F29 cells at restrictive temperature (a) and shifted to permissive temperature (b) for 30 h respectively in the presence of 40 μ M ethanol solvent control. (c) Morphology of PC12/F29 cells shifted to permissive temperature in the presence of 40 μ M LY294002 made as $\times 100$ stock in ethanol. Magnification $\times 195$.

Figure 3 v-Src stimulates binding of active PtdIns 3-kinase with different kinetics in fibroblasts and PC12 cells

(a) Anti-v-Src immunoprecipitates from lysates of PC12/F29 or CEF/R29 cells, which had been shifted to the permissive temperature for various times between 0 and 60 min, were assayed for associated PtdIns 3-kinase activity. The averaged results of three independent experiments are represented as fold activation compared with cells at restrictive temperature. (b) Reactivation of the v-Src kinase is measured by autophosphorylation of v-Src immunoprecipitates *in vitro*. (c) PtdIns 3-kinase activity associated with v-Src from PC12/F29 cells that had been shifted to the permissive temperature for various times between 0 and 12 h.

the v-Src kinase in CEF [12], we examined the binding of PC12 p85 to the v-Src SH3 or SH2 domains, alone or in combination, and expressed as GST-fusion proteins. We found that binding of p85 from PC12/F29 cell lysates to the isolated SH3 or SH2 domains was barely detectable at either restrictive or permissive

Figure 4 Binding of the p85 regulatory subunit of PtdIns 3-kinase to the SH3 and SH2 domains

(a) Lysates of PC12/F29 (or CEF/R29) cells, which had been grown at restrictive temperature (39.5 °C) or shifted to permissive temperature (35 °C) for 8 h were incubated with GST alone (–), GST-SH3 (SH3), GST-SH2 (SH2) or GST-SH3/SH2 (SH3/SH2) fusion proteins. Bound proteins were separated by SDS/PAGE and p85 was detected by immunoblotting. (b) Total cellular p85 in PC12 and PC12/F29 cells at 39.5 °C and 35 °C was measured by immunoblotting. (c) Comparison of total cellular p85 in PC12 and CEF.

temperature; however, consistent with our results in fibroblasts [12], the binding of p85 to the combined SH2/SH3 domains was greater than the binding to either domain alone (Figure 4a). Furthermore the amount of p85 bound was independent of temperature, indicating that v-Src activity in PC12 cells did not stimulate p85 binding to the combined SH domains of v-Src (Figure 4a). We confirmed that the amount of cellular p85 was similar in PC12 cells and the PC12/F29 derivative cell line at both temperatures (Figure 4b). We also confirmed that the single p85 species from PC12 cells was present at an level equivalent to that of the two p85 species from CEF (Figure 4c). The nature of the two species detected in anti-p85 immunoblots of lysates from CEF is not known, although both are forms of p85- α because the antibody used in these experiments was specific for the α isoform of the p85 protein. One possibility is that the two species are differently phosphorylated. Multiple phosphorylated forms of p85 are known to exist because treatment of bovine brain p85 with serine/threonine phosphatase decreases the number of forms of the protein [19]. In our experiments we did not detect the p55 form of the regulatory subunit of PtdIns 3-kinase in either CEF or PC12 cells (results not shown).

Thus, in contrast with the v-Src kinase-dependent binding of p85 from CEF to the SH3/SH2 domains (Figure 4a) [12], p85 from PC12/F29 cells does not bind the SH domains in a phosphorylation-dependent manner. Consistent with this, we did not detect temperature-dependent tyrosine phosphorylation of p85 precipitated by the GST-SH domain fusion (results not shown). Furthermore PtdIns 3-kinase activity from PC12/F29

Table 1 PtdIns 3-kinase activity associated with GST-SH domains

PtdIns 3-kinase from PC12 and PC12/F29 cell lysates that associated with GST-SH domain fusion proteins was measured as described in the Materials and methods section. The activity is given as fold increase relative to GST controls. The results are given as means $\pm$ S.D. for three replicate experiments.

Cell line	Temperature ($^{\circ}$ C)	GST fusion protein	PtdIns 3-kinase activity (normalized to GST control)
PC12/F29	39.5	SH3/SH2	20.1 $\pm$ 3.6
PC12/F29	35	SH3/SH2	17.6 $\pm$ 2.5
PC12	39.5	SH3/SH2	16.6 $\pm$ 3.4

cells that associated with the v-Src SH3/SH2 domains was similar at both temperatures, and indeed similar to parental PC12 cells (Table 1), indicating that PtdIns 3-kinase activity binding to the SH domains in PC12 cells is also not influenced by the tyrosine kinase activity of v-Src. This is again in contrast with the v-Src homology domain-associated PtdIns 3-kinase activity in CEF/R29, which is substantially elevated as a consequence of v-Src-induced tyrosine phosphorylation of p85 in these cells [12]. Thus we conclude that, in addition to delayed kinetics, the mode of PtdIns 3-kinase's binding to v-Src is also different in CEF and PC12 cells, with association in the latter case not being mediated by the binding of tyrosine-phosphorylated p85 to the SH3/SH2 domains after activation of the v-Src kinase.

p85 associates with v-Src and a 68 kDa phosphotyrosine-containing protein in PC12 cells

The results presented above demonstrate that the v-Src SH3/SH2 domains are insufficient to mediate the kinase-dependent binding of PtdIns 3-kinase from PC12/F29 cells to v-Src (the latter being shown in Figure 3c). We therefore examined whether p85 and v-Src associated with other tyrosine-phosphorylated proteins in PC12/F29 cells by probing anti-p85 immunoprecipitates with anti-phosphotyrosine antibodies. In contrast with CEF [12], p85 immunoprecipitated from PC12/F29 cells was not detectably phosphorylated on tyrosine in PC12 cells, even after the activation of v-Src (Figure 5a). The presence of p85 and its gel migration position were confirmed by re-probing the immunoblot (Figure 5a) with anti-p85 serum (Figure 5b). However, we detected a p85-associated tyrosine-phosphorylated protein with an estimated molecular mass of 68 kDa (Figure 5a), and a 60 kDa species that was confirmed as v-Src by re-probing with a Src kinase-specific antibody. Furthermore, quantification by optical densitometry showed that tyrosine phosphorylation of the 68 kDa protein increased steadily after activation of v-Src by a shift to 35 $^{\circ}$ C, reaching a maximum at approx. 6 h, at which time v-Src-associated PtdIns 3-kinase activity was also elevated (Figure 3c). In addition, the amount of tyrosine-phosphorylated v-Src associated with p85 also increased after a shift to the permissive temperature (Figures 5a and 5d), with kinetics that paralleled the association of the tyrosine-phosphorylated 68 kDa species (termed p68) (Figures 5a and 5c). Tyrosine-phosphorylated forms of Src kinase or p68 were not detected in anti-p85 immunoprecipitates from parental PC12 cells (Figures 5a, 5c and 5d).

To distinguish between the possibilities that p85 was present in distinct complexes with v-Src and with p68 (shown in Figure 6a) or was simultaneously complexed with both (shown in Figure 6b), we tested whether p68 was bound to p85 independently of v-Src. v-Src was therefore removed from PC12/F29 cell lysate by

Figure 5 Association of a 68 kDa tyrosine-phosphorylated protein with p85 and v-Src in PC12/F29 cells

(a) Phosphotyrosine-containing proteins associated with p85 were detected by immunoprecipitating p85 from cell lysates of PC12/F29 cells (grown at restrictive temperature or shifted to permissive temperature for various times between 0 and 8 h) and immunoblotting the associated proteins with anti-phosphotyrosine as probe. IgG from the precipitating antibody, an unidentified species (?), which was also present in PC12 lysates, and the gel migration positions of v-Src, p85 and a 68 kDa species (p68) are shown. (b) Detection of p85 in the lysates used in (a) by immunoblotting with anti-p85 as probe. (c) Densitometric quantification of tyrosine-phosphorylated p68 associated with p85 after shift of PC12/F29 cells to permissive temperature for various times. (d) Densitometric quantification of tyrosine-phosphorylated v-Src associated with p85 after shift of PC12/F29 cells to permissive temperature for various times.

immunodepletion, and anti-p85 immunoprecipitates of depleted lysate were probed with anti-phosphotyrosine. Despite the immunoprecipitation of a similar amount of p85 from PC12 and PC12/F29 cell lysates (Figure 6c), association with v-Src and p68 was again evident only in v-Src-expressing PC12/F29 cells (Figure 6d). However, when lysates were first depleted of v-Src, no tyrosine-phosphorylated p68 was present in anti-p85 immunoprecipitates (Figure 6d), indicating that p85 and v-Src associated with the same pool of tyrosine-phosphorylated p68 protein. To confirm that tyrosine-phosphorylated p68 had been removed from the lysate during v-Src depletion, we detected tyrosine-phosphorylated p68 in the anti-v-Src immunoprecipitates (Figure 6e). Thus p85, tyrosine-phosphorylated p68 and v-Src form a trimolecular complex, presumably in association with the p110 catalytic subunit of PtdIns 3-kinase, raising the possibility that

Figure 6 p85, v-Src and p58 are present in a trimolecular complex

(a) Models depicting the possible bimolecular interactions between p85 (in association with the catalytic PtdIns 3-kinase subunit p110) and tyrosine-phosphorylated p68 or v-Src. (b) Model depicting trimolecular complex. (c) Demonstration that equivalent amounts of p85 are immunoprecipitated from PC12 and PC12/F29 as detected by immunoblotting of precipitates with anti-p85 as probe. (d) Phosphotyrosine-containing p85-associated proteins immunoprecipitated from PC12 and PC12/F29 cell lysates (grown at the permissive temperature of 35 °C) by anti-p85 antibodies, before and after depletion of cell lysates of v-Src; v-Src and p68 are shown. (e) Phosphotyrosine-containing proteins immunoprecipitated from lysates of PC12 and PC12/F29 by anti-v-Src antibodies during immunodepletion in (d) above; v-Src and associated tyrosine-phosphorylated p68 are shown.

p68 is an adaptor protein mediating the association of PtdIns 3-kinase with v-Src in PC12 cells (Figure 6b).

DISCUSSION

Inhibition of NGF-induced neurite outgrowth in PC12 cells by wortmannin or by the expression of a dominant inhibitory mutant form of p85 demonstrated that PtdIns 3-kinase activity is required for PC12 cell differentiation [13,14]. c-Src, the cellular homologue of the v-Src oncoprotein, is present at the plasma membrane of neurite growth cones, specifically at the sites of

attachment to the substratum [20] in PC12 cells treated with NGF, and is required for the NGF-induced neuronal phenotype [21]. This, together with the finding that it is not NGF-receptor-associated PtdIns 3-kinase that is required for neurite extension of PC12 cells [22], suggests that activated c-Src might have a role in NGF-induced, PtdIns 3-kinase-dependent neurite extension.

Here we provide evidence that neurite outgrowth induced by the activation of a ts v-Src protein also requires the activity of PtdIns 3-kinase, because treatment with the compound LY294002 suppressed the v-Src-induced biological activity. In the case of stimulation by NGF, wortmannin did not block the NGF-induced extension of neurites when added transiently at the time of treatment with NGF, but was effective when added 5–10 h after NGF [13], suggesting that PtdIns 3-kinase is required for a late phase in NGF-stimulated PC12 cell differentiation that coincides with neurite extension [13]. In ts v-Src-expressing PC12/F29 cells, temperature-induced PtdIns 3-kinase associates with v-Src only after several hours of growth at the permissive temperature, consistent with PtdIns 3-kinase activation's being a relatively late event during PC12 cell differentiation. The much more rapid association of PtdIns 3-kinase with ts v-Src during transformation of CEF [12] might reflect the earlier appearance of recognizable cytoskeletal remodelling after a shift to the permissive temperature in fibroblasts.

Our results demonstrate that the regulation of v-Src-associated PtdIns 3-kinase is different in ts v-Src-expressing fibroblasts and PC12 cells. In addition to the delayed kinetics mentioned above, there is no temperature-dependent tyrosine phosphorylation of p85 in PC12/F29 cells. Moreover, the association of p85 or PtdIns 3-kinase from PC12 cells with the SH3/SH2 domains of v-Src is independent of temperature, despite a clear kinase-dependent association with immunoprecipitated v-Src. This apparent discrepancy can be explained by the observed association of p85 with both v-Src and a tyrosine-phosphorylated species, p68, in ts v-Src-expressing PC12 cells. Furthermore the association of tyrosine-phosphorylated p68 with p85 increases after the activation of v-Src, the timing of this increase being correlated with increased binding of tyrosine-phosphorylated v-Src to p85 and with increased v-Src-associated PtdIns 3-kinase activity. These findings are consistent with a role for p68 as an adaptor molecule that mediates the association between v-Src and the p85 subunit of PtdIns 3-kinase in PC12 cells and raises the possibility that p68 might function in a similar manner to polyoma middle T antigen, which acts as a docking protein for c-Src and PtdIns 3-kinase after phosphorylation at a specific tyrosine residue [23,24]. Thus our observations might imply the existence of a functional homologue of middle T in eukaryotic cells. Consistent with this idea, we found that v-Src and p85 were present in a trimolecular complex with tyrosine-phosphorylated p68.

The identity of p68 and its role in PC12 cell differentiation are unknown. However, we note that in a comparative study of NGF-induced compared with v-Src-induced PC12 cell differentiation, specific tyrosine phosphorylation of a 66 kDa protein, among others, was observed in PC12 cells in which v-Src was active [4]. Antibodies reactive with potential PtdIns 3-kinase and Src substrates or binding partners in the 60–70 kDa range, specifically paxillin [25], p62/p68^{SAM} [26–29] and the 62 kDa isoform of Shc [30], did not react with p68 in immunoblots. Thus a functional analysis of the role of p68, identified here as a potential mediator of v-Src-induced, PtdIns 3-kinase-dependent PC12 cell differentiation, awaits its purification and cloning.

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