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Neuronal differentiation signals are controlled by nerve growth factor receptor/Trk binding sites for SHC and PLCγ

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Differentiation and survival of neuronal cell types requires the action of neurotrophic polypeptides such as nerve growth factor (NGF). In the central and peripheral nervous system and the phaeochromocytoma cell model PC12, NGF exerts its effects through the activation of the signalling capacity of Trk, a receptor tyrosine kinase (RTK) which upon interaction with NGF becomes phosphorylated on tyrosines and thereby acquires the potential to interact with signal-transducing proteins such as phospholipase C-γ (PLCγ), phosphatidylinositol-3′-kinase (PI3′-K) and SHC. Mutagenesis of the specific binding sites for these src homology 2 (SH2) domain-containing substrates within the Trk cytoplasmic domain suggests a non-essential function of PI3′-K and reveals a major role for the signal controlled by the SHC binding site at tyrosine 490 and a co-operative function of the PLCγ-mediated pathway for neuronal differentiation of PC12 cells.

Key words: differentiation/nerve growth factor receptor/SHC/signal transduction/Trk

Introduction

The development and maintenance of cellular communication networks within the central and peripheral nervous system are regulated by neurotrophic factors, which through activation of specific cell surface receptors generate differentiation and survival signals in neuronal cell types. The prototypic neurotrophic polypeptide, nerve growth factor (NGF) (Cohen and Levi-Montalcini, 1956; Angeletti and Bradshaw, 1971; Scott *et al.*, 1983; Ullrich *et al.*, 1983), exerts its biological effects through the interaction with the receptor tyrosine kinase (RTK) Trk (Kaplan *et al.*, 1991; Klein *et al.*, 1991). Generation of cellular signals by receptors of this class involves as a first step the binding of src homology 2 (SH2) domain-containing cytoplasmic proteins to specific phosphotyrosine residues of the ligand-activated receptor (Koch *et al.*, 1991; Schlessinger and Ullrich, 1992; Pawson and Schlessinger, 1993), which in the β platelet-derived growth factor receptor (PDGF-R) are located within the juxtamembrane, kinase insertion and carboxy-terminal domains, and mediate the association of non-receptor tyrosine kinases Src, Fyn and Yes, the ras GTPase-activating protein (GAP), p85 of phosphatidylinositol-3′-kinase (PI3′-K), the phosphotyrosine phosphatase PTP1D and phospholipase C-γ (PLCγ) (Fantl *et al.*, 1992; Kashishian *et al.*, 1992; Rönnstrand *et al.*, 1992; Mori *et al.*, 1993; Valius *et al.*, 1993). For the NGF-receptor (NGF-R) Trk, we recently reported high-affinity binding of PLCγ, p85/PI3′-K and SHC to tyrosines 785, 751 and 490, respectively (Obermeier *et al.*, 1993a,b). While detailed information is available regarding the biochemical parameters that define the specificity of RTK–substrate interactions, little is known about the biological significance of the diverse signalling pathways utilized by individual RTK types and their functional relationships.

The rat phaeochromocytoma cell line PC12 represents a well-established model system for NGF-dependent neuronal differentiation (Tischler and Greene, 1975). Using this neuron-like tissue culture model, we investigated the roles of the NGF-R/Trk-associated primary signal transducers SHC, PI3′-K and PLCγ in neuronal differentiation. Individual and combined mutations of the corresponding phosphotyrosine binding sites within the Trk cytoplasmic domain were generated and shown to have differential effects on ligand-induced PC12 cell differentiation. Our experiments suggest a major role for SHC-mediated and a co-operative function for PLCγ-mediated signal transduction, whereas PI3′-K binding appeared to be dispensable for neurite formation.

Results

The PC12 rat phaeochromocytoma cell line is ideally suited for the investigation of molecular mechanisms underlying NGF-R/Trk-mediated neuronal differentiation signals resulting in the induction of neurite outgrowth. Because of the absence of PDGF receptors in PC12 cells (Heasley and Johnson, 1992), we chose an RTK chimaera approach (Riedel *et al.*, 1986) to investigate individual elements of the NGF-R/Trk signal under PDGF control within its normal cell environment, rather than in PC12-derived genetically altered mutant cell lines such as PC12-nnr5 (Green *et al.*, 1986), which may be altered in their signal processing features. By introducing tyrosine to phenylalanine (YF) mutations either singly at positions 785, 751 and 490 or in all possible combinations into PT-R (Figure 1), a chimaeric receptor consisting of the βPDGF-R extracellular domain and the transmembrane and intracellular domains of Trk, we analysed the significance of signalling pathways connected to SHC, PLCγ and p85/PI3′-K phosphotyrosine target sites for NGF-R/Trk-specific signal transduction, which ultimately leads to neuronal differentiation and is essential for the maintenance of the neuronal network. As gene transfer vehicles, we employed the Moloney murine leukemia virus-based retrovirus vector system pLEN (Adam *et al.*, 1991), which utilizes the neomycin resistance gene

as selection marker. PC12 cells were engineered by infection with the respective recombinant retroviruses to stably express the wild-type chimaera PT-R, tyrosine binding site-deficient PT-YF receptor mutants and, as a control, the kinase-negative PT-KM receptor, which carried a point mutation at position 538 in the ATP-binding site converting an essential lysine residue to methionine. Moreover, as a control for receptor overexpression, we employed PC12 cells infected with a wild-type Trk virus in our experiments. Constitutive differentiation of PT-virus-infected cells by PDGF present in culture sera was prevented by the use of 2.5% rather than 5% fetal calf serum (FCS) and horse serum (HS) from platelet-poor plasma.

Polyclonal cell lines obtained by recombinant virus infection and pooling of G418 selected clones were used for all experiments to eliminate the possibility of clonal variance. In order to determine the expression levels and autophosphorylation capacities of the chimaeric receptors, cells were incubated before lysis for 10 min with 30 ng/ml PDGF-BB or, in the case of Trk-overexpressing cells, 100 ng/ml NGF. Equal amounts of total cellular protein were analysed by immunoprecipitation with monoclonal antibody B2 (Rönnstrand *et al.*, 1988) against the extracellular domain of PDGF-R or with the polyclonal antibody Ab-1 (Oncogene Science) raised against a C-terminal Trk peptide. After SDS−PAGE, receptor expression and autophosphorylation were determined by immunoblotting with Ab-1 (Figure 2, upper panel) and reprobing with the monoclonal antiphosphotyrosine antibody 5E2 (Fendly *et al.*, 1990) (lower panel). Taking into account the variance in expression levels, the PT-R chimaera and all PT-YF mutants displayed comparable autophosphorylation activities, with the exception of the triple mutant PT-Y3F, which, presumably due to the loss of the three tyrosine phosphorylation sites, reacted only weakly with the 5E2 antibody. As expected, PT-KM was autophosphorylation defective and, therefore, PC12 cells expressing this mutant were used as a negative control in our differentiation assays.

The effects of Trk cytoplasmic domain mutations on recombinant virus-infected PC12 cells were evaluated by visual examination of the frequency and length of ligand-induced neurite outgrowth in direct comparison with PC12/PT-R and PC12/Trk controls. As shown in Figure 3, upon stimulation with PDGF, PC12/PT-R but not PC12/PT-KM cells readily differentiated. Under identical conditions, PC12 cells expressing one each of the various PT-YF mutants displayed clear differences in their neuronal differentiation responses after PDGF stimulation (Figure 3 and Table I). The same was true for cells expressing PT-Y785F or PT-Y751F, which differentiated upon PDGF stimulation to the same extent as the positive control cells, indicating that elimination of the PLCγ- or the p85/PI3′-K binding site did not affect Trk-mediated induction of neurite outgrowth. In contrast, the differentiation signal of the Trk cytoplasmic domain was severely impaired by mutation of the SHC binding site tyrosine 490, as shown by the phenotype of PC12/PT-Y490F cells, which developed only few neurites of significantly reduced length when treated with PDGF. Mutation of the binding sites for both SHC and PI3′-K in PT-Y751/490F had no further effect on reducing the PDGF-induced differentiation response, but rather enhanced it somewhat, indicating the possibility of PI3′-K signalling pathway involvement in negative regulation. The differentiation response to PDGF stimulation was even more defective in PC12 cells expressing the receptor chimaera PT-Y785/490F, which lacked both SHC and PLCγ binding sites, or PT-Y3F, missing all three docking sites for the substrates investigated. A slight but significant decrease in neurite

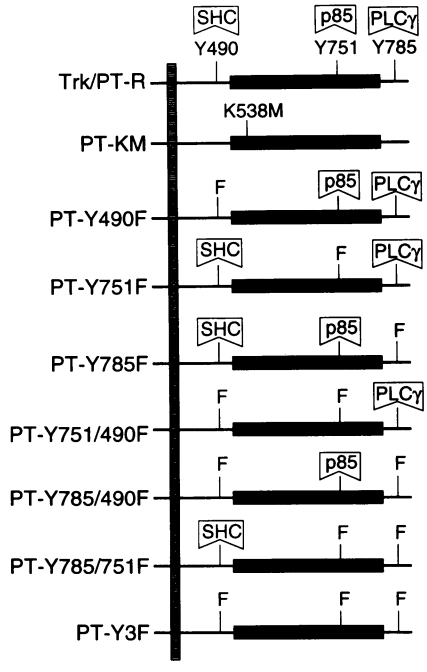

Fig. 1. Schematic representation of substrate-binding capacities of receptor constructs. The cytoplasmic portions of the PT-R chimaera mutants are shown to the right of the schematically indicated plasma membrane. Black boxes denote the tyrosine kinase domain. PT-Y3F is an abbreviation for PT-Y490/751/785F.

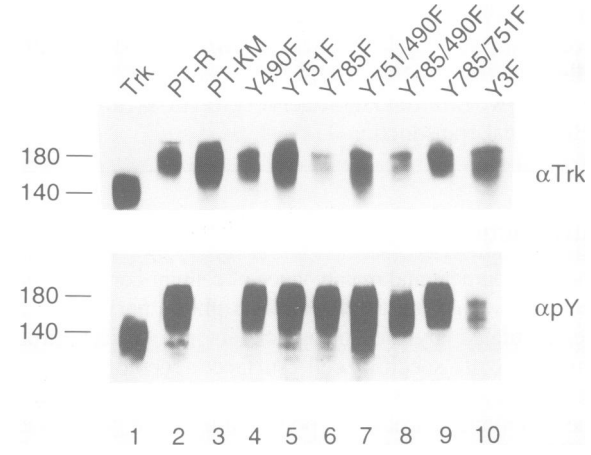

Fig. 2. Expression of PT receptors and Trk in PC12 cells. PC12 cells were infected with retroviruses containing cDNAs for Trk, PT-R, PT-KM and PT receptors bearing either one, two or all three YF mutations at amino acid positions 490, 751 and 785. After G418 selection and propagation, ~10^7 cells of each line were stimulated with 30 ng/ml PDGF-BB (lanes 2−10) or 100 ng/ml NGF (lane 1) for 10 min and collected in lysis buffer. Receptors were immunoprecipitated with either monoclonal antibody B2 (Rönnstrand *et al.*, 1988) against the human βPDGF-R extracellular domain (lanes 2−10) or with an affinity-purified polyclonal antibody against the C-terminus of Trk (Ab-1; Oncogene Science) (lane 1). Precipitated proteins were separated by 7.5% SDS−PAGE, immunoblotted with Ab-1 (upper panel) and reprobed with monoclonal α-phosphotyrosine antibody 5E2 (Fendly *et al.*, 1990) (lower panel).

outgrowth was evident with cells expressing the PT-Y785/751F receptor, which retained the capacity to bind SHC, but lacked sites of association for both PLCγ and PI3′-K. Thus, compared with the single-site mutants PT-Y785F and PT-Y751F, SHC-initiated signalling pathways seemed to be less potent in transducing a Trk-specific signal if PLCγ and PI3′-K pathways were disconnected.

The observed effects were not due to different receptor expression levels because PC12/PT-Y785F cells, which displayed the lowest expression level, fully differentiated upon addition of PDGF. Moreover, several independently derived cell lines expressing different levels of the same receptors reproducibly presented essentially the same biological response. In addition, while transphosphorylation of endogenous Trk by overexpressed chimaeric receptors did not occur (not shown), normal differentiation of all cell lines was inducible by NGF through activation of endogenous receptor, which demonstrated that all cell lines had retained their full differentiation capacity. Furthermore, there was no significant induction of neurites in the absence of ligand,

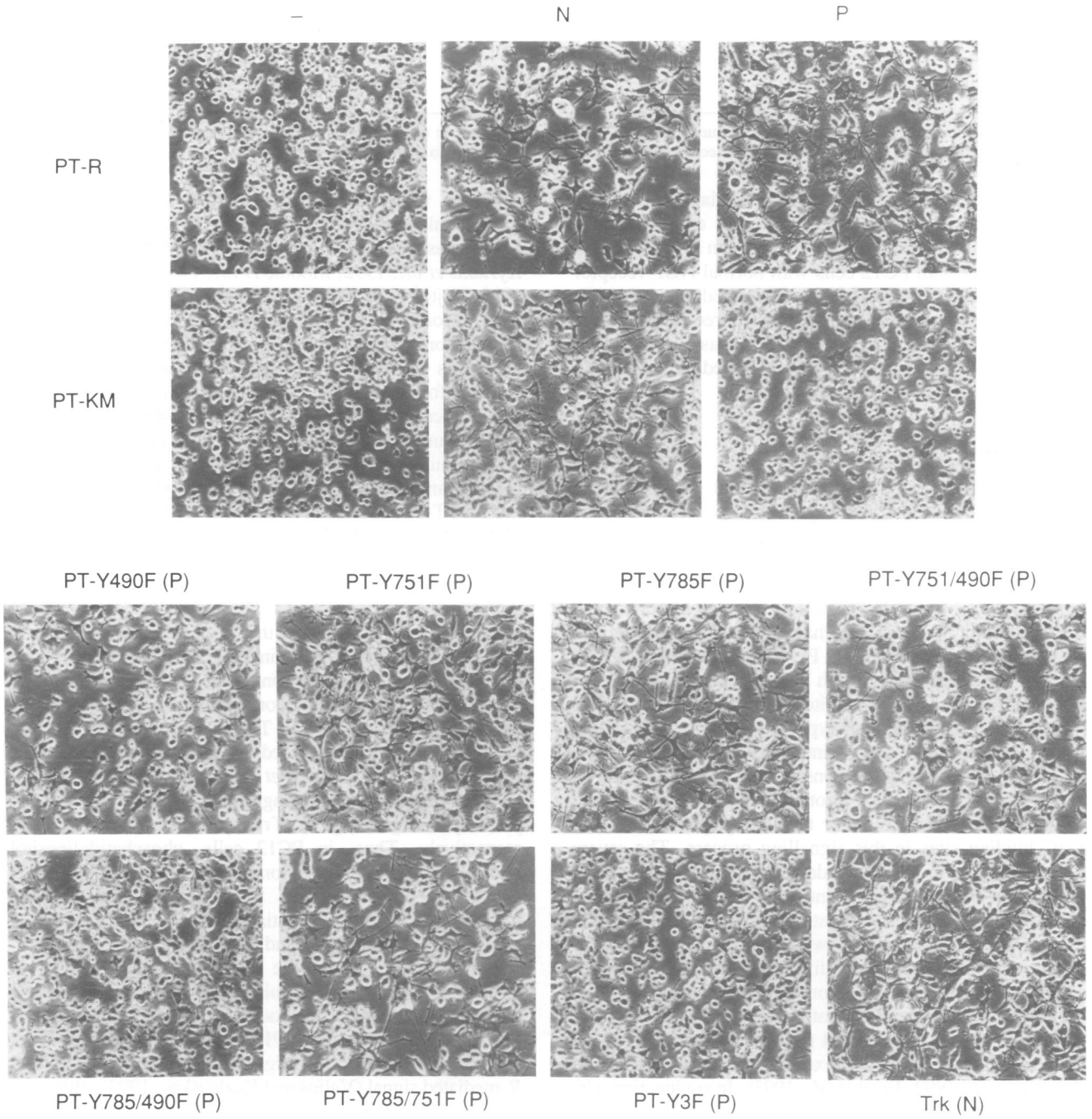

Fig. 3. Differentiation of PC12 transfectants. PC12 cells stably overexpressing PT receptors (or overexpressing Trk) were seeded into six well dishes and 1 day later either no ligand (−), NGF (N) or PDGF-BB (P) were added to final concentrations of 100 and 30 ng/ml, respectively. Photographs of the cells were taken 0, 1 and 3 days after addition of ligand. Medium and ligand were replaced every 2 days. Depicted photographs show cells 3 days after addition of ligand, are representative of 2–4 independently generated polyclonal cell lines per receptor construct and represent those cell lines for which receptor expression levels are presented in Figure 1. Experiments were performed twice and each time receptor expression levels were monitored.

Table I. Differentiation of PC12 cell transfectants

Receptor	Binding capacity			Differentiation response to	
	PLCγ	PI3′-K	SHC	PDGF	NGF
PT-R	+	+	+	++++	++++
PT-KM	−	−	−	−	++++
PT-Y490F	+	+	−	+	++++
PT-Y751F	+	−	+	++++	++++
PT-Y785F	−	+	+	++++	++++
PT-Y751/490F	+	−	−	++	++++
PT-Y785/490F	−	+	−	+/−	++++
PT-Y785/751F	−	−	+	+++	++++
PT-Y3F	−	−	−	+/−	++++
Trk	+	+	+	−	++++

The degree of differentiation, as judged by the frequency of cells (10^5) bearing neurites in combination with the number and length of the neurites, is given on an arbitrary scale ranging from − (no detectable differentiation) to ++++ (maximal observed differentiation).

indicating that the observed effects are mediated exclusively by the activated chimaeric receptors. As expected, cells overexpressing Trk did not differentiate upon PDGF addition but, compared with PC12 cells with normal NGF-receptor expression levels, displayed an accelerated rate of NGF-dependent differentiation, consistent with recently reported findings (Hempstead *et al.*, 1992). This was also found for PC12/PT-R cells, which fully differentiated within 24−48 h of PDGF treatment (data not shown).

Discussion

Growth, differentiation, movement and the metabolic homeostasis of cells are regulated by ligand-induced pleiotropic signals that are generated by autophosphorylated receptor tyrosine kinases through the interaction with cellular SH2 domain-containing signal transducers (Schlessinger and Ullrich, 1992). While this basic concept is well established, the question of how an RTK-characteristic signal is defined remains poorly understood. In PC12 cells, the NGF-R/Trk, but not the epidermal growth factor receptor, is able to promote the extension of neurites from cell bodies and thus induce a differentiated phenotype, which *in vivo* is essential for the establishment and maintenance of nerve functions.

Our experimental observations with PC12 cells expressing a variety of Trk SH2 protein binding site mutants demonstrate distinct roles for the PLCγ, p85/PI3′-K and SHC binding sites in this signalling process. The severe neurite outgrowth signalling defect displayed by the SHC association-incompetent PT-mutant receptor chimaera in PC12/PT-Y490F cells suggests an essential role for this protein and the respective downstream signalling pathway in the regulation of neuronal differentiation. SHC has been implicated in linking receptor and cytoplasmic tyrosine kinases to the Ras signalling pathway by virtue of the ability of its tyrosine-phosphorylated form to bind to GRB-2, the connector protein for the Ras exchange factor SOS (Rozakis-Adcock *et al.*, 1992; Egan *et al.*, 1993). In conjunction with previous reports of neurite outgrowth stimulation by SHC overexpression (Rozakis-Adcock *et al.*, 1992), constitutive Ras activation (Bar-Sagi and Feramisco, 1985) and Raf overexpression (Wood *et al.*, 1993), our results provide strong evidence that SHC binding to Trk/Y490 initiates an intracellular cascade of protein−protein interactions, likely involving GRB-2/Sem5, SOS and Ras, which directly connects with the downstream Ser/Thr kinases Raf, microtubule-associated protein (MAP) kinase kinase, and MAP kinase (MAPK) (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Moodie *et al.*, 1993; van Aelst *et al.*, 1993; Vojtek *et al.*, 1993; Warne *et al.*, 1993; Zhang *et al.*, 1993). Moreover, our Trk signalling domain mutants provide support for a complementary or co-operative role of PLCγ, which, as we reported earlier, exhibits a remarkably high affinity for this RTK (Obermeier *et al.*, 1993a). The significance of the PLCγ binding site Tyr785 for Trk-specific signal transduction is revealed, on the one hand, by the double-mutant receptor PT-Y785/490F, which is unable to mediate any appreciable neurite outgrowth, and the PT-R mutant PT-Y751/490F, which is able to bind PLCγ, but not SHC or PI3′-K, yet still mediates a moderate differentiation response. Activation of this signal transducer by NGF/Trk may, among possibly other effects related to cytoskeletal rearrangements (Goldschmidt-Clermont *et al.*, 1991; Shariff and Luna, 1992), recruit the protein kinase C (PKC) signalling system through an increase in membrane-proximal diacylglyercol concentrations and eventually lead to modulation of MAPK function, as suggested by a previous report (Gotoh *et al.*, 1990). This downstream effect might be mediated by PKC-induced activation of the Raf kinase (Sozeri *et al.*, 1992; Kolch *et al.*, 1993), which then could be considered a converging point of SHC and PLCγ pathways originating at Trk tyrosines 490 and 785, respectively. Thus, in PC12 cells, phosphatidylinositol hydrolysis and PKC activation may co-operate with the Ras signalling pathway, and may be involved in the implementation of differentiation-related changes in the cytoskeletal organization and physiology of the cell.

A significant role for the PLCγ signalling pathway in PDGF-induced growth regulation is suggested by recent experiments in which reintroduction of its tyrosine binding site into a PI3′-K, GAP, PTP1D and PLCγ binding-deficient receptor mutant restored a significant portion of the PDGF-R-mediated signal (Valius and Kazlauskas, 1993), although, as previously shown, mutation of the PLCγ binding site had no effect on PDGF-induced DNA synthesis (Rönnstrand *et al.*, 1992). Analogously, the role of PLCγ in Trk-specific signalling was not obvious from experiments with the PLCγ binding site mutant Y785F, but only from those with an 'add-back' mutation of the PLCγ binding site to a receptor lacking association sites for PI3′-K, SHC and PLCγ.

In view of previous reports that suggested Src as an essential element in neuronal differentiation of PC12 cells (Kremer *et al.*, 1991) and the recently unravelled chain of events of the mitogenic signal (Rozakis-Adcock *et al.*, 1992; Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Moodie *et al.*, 1993; Egan *et al.*, 1993; van Aelst *et al.*, 1993; Vojtek *et al.*, 1993; Warne *et al.*, 1993; Zhang *et al.*, 1993), our findings raise the possibility of a link between the Src signalling system and the pathway triggered by SHC/Trk, and possibly PLCγ/Trk, interactions. Such a co-operative signal amplification scenario may, in fact, be critical for the generation of a more sustained activation of PLCγ and downstream signal transducers like Ras and MAP kinase (Qiu and Green, 1992; Traverse *et al.*, 1992) and could be the feature that distinguishes the Trk signal from that of the EGF-R in PC12 cells. In summary, our findings provide a basis for the understanding of the complex molecular mechanisms that define receptor signals necessary for differentiation of neuronal cells.

Materials and methods

Construction of PT receptors

An *Eco*RI/*Mse*I restriction fragment containing the cDNA sequence for human βPDGF-R extracellular domain was fused to a cDNA sequence coding for the transmembrane and intracellular domains of rat Trk, which was generated by polymerase chain reaction (PCR) from a full-length rat Trk cDNA cloned into the *Xba*I site of the pCMV-1 (Obermeier *et al.*, 1993b) polylinker. For fusion, an *Mse*I site was introduced by the 5′ PCR primer sequence without changing the amino acid sequence. The chimaeric cDNA was subcloned as a 3.3 kb *Eco*RI fragment into pCMV-1 and into the retroviral vector pLEN (Adam *et al.*, 1991; pLEN is a derivative of pLAEN, modified by removing ADA by T.v. Rüden). The full-length Trk cDNA was subcloned via *Eco*RI into pLEN. Mutagenesis of PT-R cDNA was performed in the f1-origin-containing pCMV-1 vector using a modified version of the method described by Kunkel (Kunkel, 1985; Kunkel *et al.*, 1987) employing M13K07 helper phages (Pharmacia). Oligonucleotides 5′-GGTATCACTGAAGAACTGTGGGTTCTC-3′ (Y490F), 5′-GCGCAT-GATGGCGAAGACATCAGGAGG-3′ (Y751F), 5′-CAGAACGTC CAGGAAACTCGTGGCGC-3′ (Y785F) and 5′-CTCCTTCAGTGC-CTTGACAGCCACCAG-3′ (KM) were used to generate PT-YF mutants and PT-KM. After mutagenesis, PT-cDNA fragments were subcloned via *Eco*RI into pLEN.

Generation of PC12 transfectants and cell culture

Ecotrophic retroviruses were generated with the help of PA317 and GP+E-86 producer cell lines essentially as described previously (Redemann *et al.*, 1992). In the case of pLEN-PT-Y785F and pLEN-PT-Y785/490F, ecotrophic viruses were generated by transient transfection of 293 cells (ATCC CRL 1573) with pLEN constructs together with pSV-Ψ⁻-E-MLV helper virus DNA (Muller *et al.*, 1991) in a 5:3 ratio using the calcium phosphate co-precipitation technique (Chen and Okayama, 1987); 48 h after transfection, virus-containing supernatants were collected. Pure retrovirus-containing cell-free supernatants from G418-selected GP+E-86 or 293 cells were then added to PC12 cells and incubated twice for four hours in the presence of polybrene (4 μg/ml; Aldrich). Infected PC12 cells were subsequently shifted from DMEM, 4500 g/l glucose (Gibco), 5% FCS (Gibco), 10% HS (Boehringer Mannheim) to DMEM, 2.5% FCS, 10% HS from platelet-poor plasma (Sigma), and selected for at least 4 weeks with G418. PC12 cells were generally grown at 37°C, 8% CO_2 on collagen (type I, from rat tail; Sigma) coated plastic dishes (Nunc).

Immunoblotting

For analysis of receptor expression, stimulated cells were lysed in 1% Triton X-100-containing lysis buffer as previously described (Obermeier *et al.*, 1993b), and protein concentrations of precleared lysates determined by a micro BCA protein assay (Pierce) and adjusted accordingly. Immunoprecipitation of receptors, SDS–PAGE and immunoblotting procedures were carried out essentially as previously described (Obermeier *et al.*, 1993b). Briefly, lysates were centrifuged to remove insoluble materials and the supernatants incubated with anti-βPDGF-R extracellular domain antibody B2 (Rönnstrand *et al.*, 1988) or anti-Trk antibody (Ab-1, Oncogene Science) and protein A–Sepharose. After extensive washing, the immunoprecipitates were separated by 7.5% SDS–PAGE and the proteins in the gel were electrophoretically transferred onto nitrocellulose membrane. The blot was incubated with Ab-1, followed by incubation with peroxidase-conjugated goat anti-rabbit immunoglobulins. The ECL system (Amersham) was used to visualize proteins recognized by the antibodies. After stripping antibodies off the blot, immunodetection procedures were repeated with antiphosphotyrosine antibody 5E2 (Fendly *et al.*, 1990) and goat anti-mouse immunoglobulin.

PC12 differentiation assays

PC12 transfectants were seeded into collagen-coated six-well dishes and after 1 day the medium was replaced by medium containing either 30 ng/ml PDGF-BB, 100 ng/ml NGF or no ligand. Thereafter, medium and ligands were replaced every 2 days. Photomicrographs of cells were taken at 0, 1 and 3 days after addition of ligand with a phase-contrast microscope (Nikon) supplemented with a camera.

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