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Tyrosine 785 is a major determinant of Trk-substrate interaction

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Interaction of the nerve growth factor (NGF) receptor/Trk with cellular substrates was investigated by transient co-overexpression in human 293 fibroblasts using ET-R, a chimeric receptor consisting of the epidermal growth factor receptor (EGF-R) extracellular ligand binding domain and the Trk transmembrane and intracellular signal-generating sequences. The chimera was fully functional, and associated with and phosphorylated phospholipase Cγ (PLCγ), ras GTPase-activating protein (GAP) and the non-catalytic subunit of phosphatidylinositol-3′-kinase, p85, in a ligand-dependent manner. Deletion of 15 C-terminal amino acids, including tyrosine 785 (Y-785) abrogated receptor and substrate phosphorylation activities. Mutation of Y-785 to phenylalanine somewhat impaired receptor phosphorylation activity, which was reflected in reduced GAP and p85 phosphorylation. In contrast, ET-YF phosphorylation of PLCγ was significantly reduced, while the high affinity association potential with this substrate was abrogated by this point mutation *in vitro* and in intact cells. Furthermore, a tyrosine-phosphorylated synthetic C-terminal peptide competitively inhibited Trk cytoplasmic domain association with PLCγ. Thus, the short C-terminal tail appears to be a crucial structural element of the Trk cytoplasmic domain, and phosphorylated Y-785 is a major and selective interaction site for PLCγ.

Key words: nerve growth factor/signal transduction/tyrosine phosphorylation

Introduction

The missing link between nerve growth factor (NGF) and cellular signalling mechanisms that lead to the formation of neurites has been identified by the recent finding that the proto-oncogene product gp140^{trk} (Trk) (Martin-Zanca *et al.*, 1989), a transmembrane receptor with tyrosine kinase activity, is a functional receptor for this neurotrophic factor (Kaplan *et al.*, 1991a,b; Klein *et al.*, 1991). Receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGF-R), platelet-derived growth factor receptor (PDGF-R) and macrophage colony stimulating factor receptor (CSF-1R) are known to function as molecular switches that transduce extracellular binding to their cognate ligand into intracellular events, ultimately resulting in DNA synthesis and cell proliferation or differentiation (reviewed in Ullrich and Schlessinger, 1990; Cantley *et al.*, 1991). Ligand binding induces receptor dimerization (Schlessinger, 1988; Ullrich and Schlessinger, 1990) and autophosphorylation, followed by association and tyrosine phosphorylation of a specific subset of cellular protein substrates. Among these are the RTK substrates phospholipase Cγ (PLCγ), GTPase-activating protein (GAP) and the p85 subunit of phosphatidylinositol 3′-kinase (PI3′-K), which are thought to be involved in distinct intracellular signal transduction pathways. Different RTKs appear to utilize distinct sets of signalling polypeptides to exert their final effects. For example, PLCγ is a substrate for the tyrosine kinases of PDGF-R (Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989; Margolis *et al.*, 1990), fibroblastic growth factor receptors (FGF-Rs) (Burgess *et al.*, 1990; Mohammadi *et al.*, 1991) and EGF-R (Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989; Nishibe *et al.*, 1989), but not for CSF-1R (Downing *et al.*, 1989) and insulin receptor (Nishibe *et al.*, 1990). In addition, GAP has been found to be a substrate for EGF-R and PDGF-R (Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990), but not for basic FGF-R (Molloy *et al.*, 1989).

PLCγ, GAP and p85 are thought to bind to phosphotyrosine (PY) residues on their respective phosphorylated, therefore activated, RTKs via their src homology region 2 (SH2) domains (Moran *et al.*, 1990; Cantley *et al.*, 1991; Koch *et al.*, 1991). Variations in SH2 domains, together with different amino acid sequences surrounding PY residues, are likely to be responsible for specific substrate binding to particular PY residues on distinct receptors (Koch *et al.*, 1991). For example, Y-992 and Y-1068 on human EGF-R and Y-766 on chicken FGF-R (Flg) have been identified as high affinity binding sites for PLCγ SH2 domains (Mohammadi *et al.*, 1991, 1992; Peters *et al.*, 1992; Rotin *et al.*, 1992). Similarly, tyrosine residues in the C-tail of mouse and human βPDGF-R appear to be involved in receptor-PLCγ interactions (Rönnstrand *et al.*, 1992; Seedorf *et al.*, 1992).

Although accumulating evidence points to Trk as the only component of the high affinity, signal-generating NGF-R (Klein *et al.*, 1991; Weskamp and Reichardt, 1991; Ibáñez *et al.*, 1992; Meakin *et al.*, 1992), it is not yet entirely clear whether the low affinity NGF-R, p75^{LNGFR}, is necessary in addition to Trk to form a high affinity binding site for NGF and to elicit a full biological response (Hempstead *et al.*, 1990, 1991; Ragsdale and Woodgett, 1991). To address this question further and to investigate the significance of structural domains of Trk signal generation, we utilized a receptor chimera approach that had been instrumental in previous studies of RTK function (Riedel *et al.*, 1986; Lammers *et al.*, 1989; Seedorf *et al.*, 1991). The ET-R

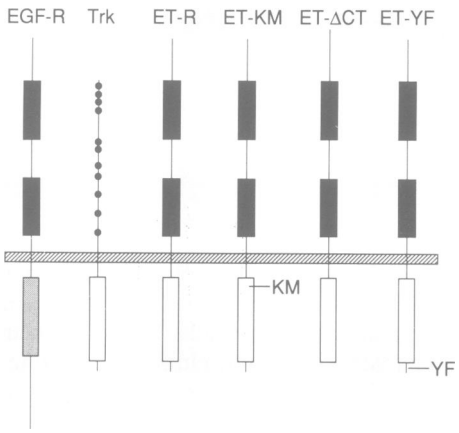

Fig. 1. Schematic diagram of receptors. EGF-R extracellular domain cysteine-rich regions are shown as black boxes; black circles in the extracellular domain of Trk indicate single cysteine residues. Tyrosine kinase domains are represented by shaded (EGF-R) and open (Trk) boxes.

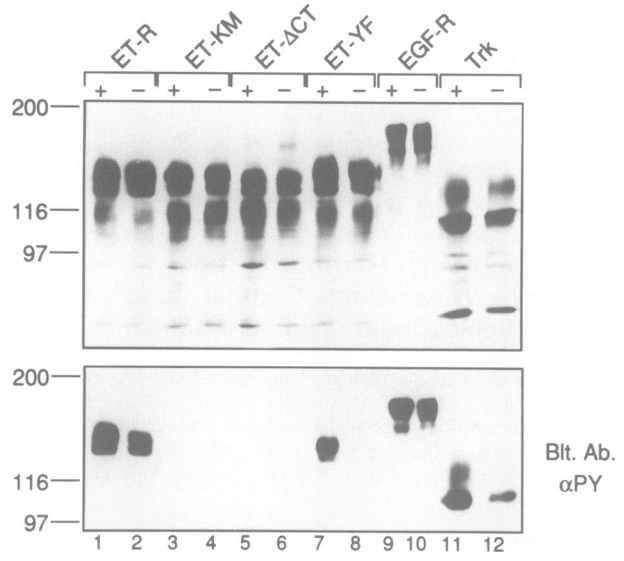

Fig. 2. Expression and tyrosine phosphorylation of ET-R and mutants. 293 cells transiently expressing ET-R, ET mutants, or parent receptors (EGF-R and Trk) and metabolically radiolabelled with [^{35}S]methionine were stimulated with EGF (EGF-R, ET-R and mutants; +) or NGF (Trk; +), or left unstimulated (−), lysed and then immunoprecipitated with mAb 108.1 (αEGF-R) (lanes 1−10) or ATC (αTrk) (lanes 11 and 12). Samples were subjected to 7.5% SDS−PAGE and electrophoretically transferred to nitrocellulose. Expression levels were monitored by autoradiography (upper panel). Phosphotyrosine contents of the same receptor proteins were analyzed by immunoblotting with mAb 5E2 (αPY) (lower panel).

chimera, consisting of EGF-R extracellular sequences and Trk transmembrane and cytoplasmic domains, was fully active in ligand-dependent auto- and substrate phosphorylation functions, which further confirmed that $p75^{LNGFR}$ is dispensable for NGF signalling through the Trk system. The signalling potential of ET-R was established by demonstrating that PLCγ, GAP and p85 are substrates of the Trk tyrosine kinase. Surprisingly, deletion of the 15 amino acid C-terminal tail rendered the Trk kinase inactive with respect to autophosphorylation as well as substrate phosphorylation. Most importantly, our data demonstrate that the C-terminal-most tyrosine residue of Trk, Y-785, is an essential part of the PLCγ-binding site.

Results

Expression and autophosphorylation activities of ET-R and mutants

To examine the signalling capacity of Trk, we constructed a chimeric receptor, ET-R, which consists of the EGF-R extracellular domain fused to the transmembrane and cytoplasmic sequences of the NGF receptor, Trk (Figure 1). In addition, three mutations were introduced into the ET-R background, yielding a kinase-negative point mutant, ET-KM, a C-terminal truncation mutant, ET-ΔCT, and a point mutant, ET-YF, containing a phenylalanine in place of the C-terminal domain tyrosine residue at position 785 (Figure 1).

Expression plasmids coding for ET-R, ET-KM, ET-ΔCT and ET-YF were transiently transfected into a human fibroblast cell line, 293. Metabolic radiolabelling with [^{35}S]methionine and immunoprecipitation demonstrated that all chimeric receptors were synthesized, each appearing as two bands in SDS−PAGE (Figure 2). The upper bands, presumably representing the fully processed receptors, migrated at molecular weights of 142 kDa (ET-R and point mutants) and 141 kDa (ET-ΔCT). In each case, the lower bands migrated at 130 kDa and probably represent high mannose oligosaccharide-bearing receptors, which fail to exit the endoplasmic reticulum because they are preferentially localized inside the cells.

EGF addition to intact transfected cells resulted in a significant increase in autophosphorylation activity of ET-R and ET-YF (Figure 2, lower panel, lanes 1, 2, 7 and 8). The phosphorylation activity of ET-YF was somewhat lower than that of ET-R, indicating that the C-terminal tyrosine might represent an autophosphorylation site. As expected, ET-KM did not display any kinase activity, yet surprisingly, phosphorylation of ET-ΔCT could also not be detected.

ET-R/EGF-R transphosphorylation

Receptor phosphorylation is thought to occur by intermolecular transphosphorylation between dimerized or oligomerized receptors in response to ligand stimulation (Schlessinger, 1988; Honegger *et al.*, 1989). Transphosphorylation studies employing EGF-R−insulin receptor chimerae (Ballotti *et al.*, 1989; Lammers *et al.*, 1990) or an EGF-R−c-kit chimera (Herbst *et al.*, 1991) have suggested that both homologous extracellular or cytoplasmic domains may be sufficient to mediate receptor dimerization and transphosphorylation.

Receptor coexpression experiments in 293 cells demonstrated that transphosphorylation can occur even between RTKs as distantly related as Trk and EGF-R. As shown in Figure 3 (lanes 7−10), the kinase-deficient EGF-R-KA mutant (Honegger *et al.*, 1987) was phosphorylated on tyrosines when coexpressed with the Trk tyrosine kinase of ET-R, suggesting transphosphorylation, whereas, interestingly, transphosphorylation of kinase-negative ET-KM by EGF-R was not detectable. Thus, Trk cytoplasmic sequences do not appear to be a substrate for the EGF-R tyrosine kinase in intact cells, while the reverse is possible.

Tyrosine phosphorylation of cellular proteins by ET-R

Distinct substrate specificities of different RTKs result in receptor- and cell type-specific pleiotropic responses (for

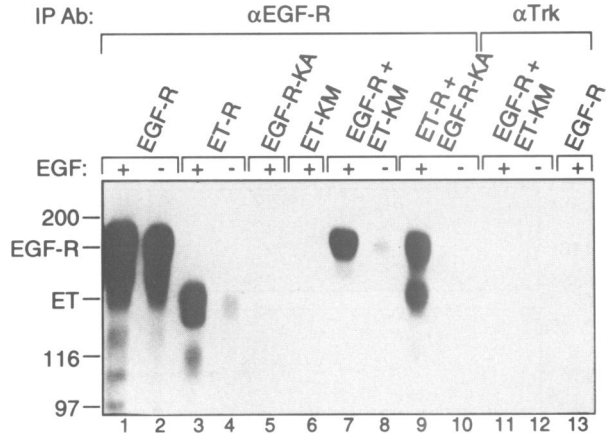

Fig. 3. Receptor transphosphorylation. 293 cells transiently expressing the respective receptors or coexpressing combinations of wild-type and kinase-negative receptors were treated with EGF (as well as with Na-vanadate) (+) or left untreated (−), lysed and then immunoprecipitated with either mAb 108.1 (αEGF-R) or polyclonal antiserum ATC (αTrk). Samples were subjected to 6% SDS−PAGE and electrophoretically transferred to nitrocellulose. Phosphotyrosine-containing proteins were detected with mAb 5E2 (αPY) and visualized by the ECL system.

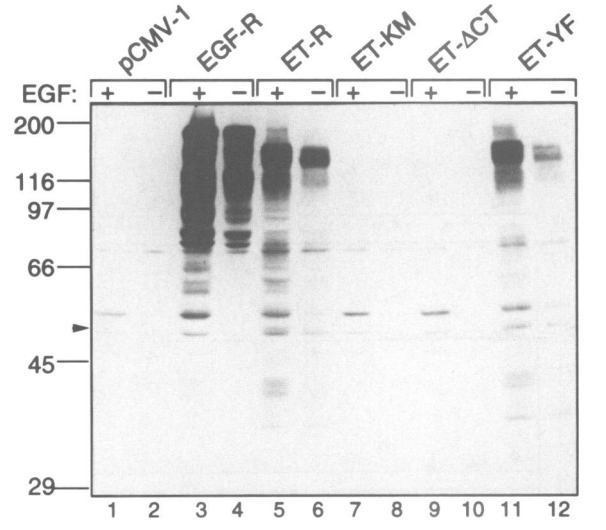

Fig. 4. Tyrosine phosphorylation of cellular proteins. 293 cells transiently transfected with expression vector pCMV-1 as a negative control or expression constructs for EGF-R, ET-R or ET mutants, were stimulated with EGF (+) or not (−) and lysed. Lysates were precleared and mixed with sample buffer, and proteins were then separated by 9.5% SDS−PAGE. After transfer to nitrocellulose, tyrosine-phosphorylated proteins were detected by immunoblotting with mAb 5E2 (αPY) and visualized with the ECL system. ET-R specific p52 is indicated by an arrowhead.

review, see Cantley *et al.*, 1991; Glenney, 1992; Schlessinger and Ullrich, 1992). To investigate the molecular basis of Trk-specific signal generation, we first determined the total spectrum of substrates phosphorylated by the EGF-stimulated Trk tyrosine kinase of ET-R.

Lysates of transfected, EGF-stimulated 293 cells were subjected directly to SDS−PAGE and analyzed for tyrosine-phosphorylated proteins by immunoblotting with anti-phosphotyrosine mAb 5E2 (αPY) (Figure 4). Comparison of the substrate phosphorylation patterns produced by the Trk (lanes 5 and 6) and EGF-R (lanes 3 and 4) tyrosine kinases revealed similarities and some clear differences. Four proteins in the range of 76−95 kDa were much more strongly phosphorylated by EGF-R than by ET-R. Conversely, four proteins in the range 35−52 kDa, designated p35, p40, p41 and p52, appeared to be predominantly phosphorylated by ET-R. These results demonstrated distinct substrate phosphorylation characteristics for Trk and EGF-R kinases under comparable experimental conditions.

The phosphorylation pattern of ET-YF was virtually identical to that of ET-R, except for a decrease in phosphorylation activity, which especially affected the higher molecular weight region but did not appear to influence the bands below 50 kDa. This was consistent with the reduced autophosphorylation level of ET-YF relative to ET-R (Figure 2). Surprisingly, the truncation mutant ET-ΔCT lacked both auto- and substrate phosphorylation function, while this was expected for the ET-KM mutant receptor. Thus, these experiments indicate that the C-terminal 15 amino acids of Trk are necessary for the receptor function in terms of autophosphorylation as well as substrate phosphorylation.

Receptor interactions with PLCγ, GAP and p85 in intact cells

To investigate interactions between Trk and PLCγ, we cotransfected 293 cells with expression plasmids for ET-R or ET mutants and PLCγ. Prior to EGF stimulation, [^{35}S]methionine-labelled cells were treated with sodium vanadate for 1 h to prevent dephosphorylation by phosphotyrosine phosphatases (Swarup *et al.*, 1982). As controls, cells were transfected with the pCMV-1 vector or expression constructs for ET-R or PLCγ alone, and treated as described for cotransfected cells. Receptors were immunoprecipitated with anti-EGF-R extracellular domain mAb 108.1 using a quarter of each lysate. A quarter of the same lysate was used in each case for immunoprecipitation of PLCγ with polyclonal antiserum CT-PLCγ, and half of each lysate was immunoprecipitated with αPY mAb 5E2.

The αPY immunoblot of the αEGF-R immunoprecipitates (Figure 5, upper panel) demonstrated clearly that PLCγ was coprecipitated with and tyrosine-phosphorylated by ET-R (lanes 7 and 8). This was confirmed by an αPLCγ immunoblot of αPY immunoprecipitates (middle panel, lane 7). Receptor phosphorylation was required, since both ET-KM and ET-ΔCT had no effect (lanes 9−12), and the basal tyrosine phosphorylation of ET-R led to virtual abolition of PLCγ association and phosphorylation (lane 8). Interestingly, coprecipitation of PLCγ with Trk cytoplasmic sequences was abrogated by the single amino acid substitution in ET-YF (Figure 5, lanes 13 and 14). Visualization of ^{35}S-labelled proteins on the immunoblot by autoradiography revealed that the phosphorylation differences were not due to variability in the expression levels of either PLCγ (Figure 5, lower panel) or the receptors (data not shown). A slight shift of the upper band representing PLCγ (lower panel, lane 13) indicated that a small fraction of the molecules may have been phosphorylated by ET-YF, which was not detectable by the αPY antibody. This was confirmed by αPLCγ precipitation and detection with αPY antibody (not shown), and suggested that additional low affinity binding sites may be present in the Trk kinase domain which mediate interaction with the substrate sufficiently to result in tyrosine phosphorylation of the substrate.

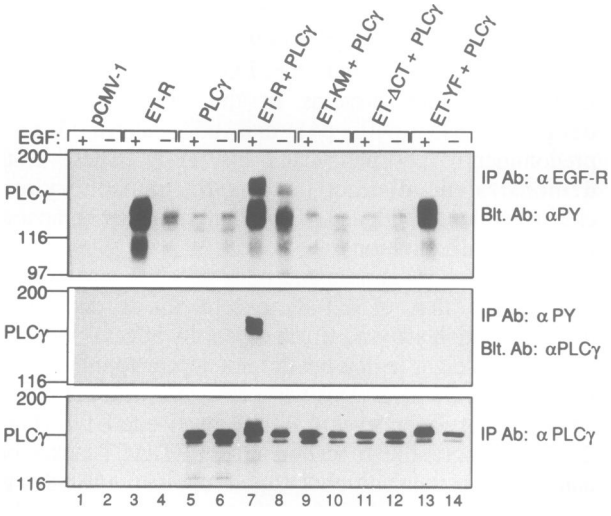

Fig. 5. PLCγ tyrosine phosphorylation and coprecipitation. 293 cells were transfected with pCMV-1 or cDNA constructs for ET-R or PLCγ as negative controls, or cotransfected with PLCγ and ET-R, ET-KM, ET-ΔCT or ET-YF. To monitor expression levels, cells were metabolically radiolabelled with [^{35}S]methionine. Prior to lysis, cells were pretreated with Na-vanadate as well as stimulated with EGF where indicated (+). A quarter of each precleared lysate was immunoprecipitated with mAb 108.1 (αEGF-R), a quarter with polyclonal antiserum CT-PLCγ (αPLCγ) and half with mAb 5E2 (αPY). Precipitates were subjected to 6% SDS−PAGE and then electrophoretically transferred to nitrocellulose. 108.1-precipitated proteins were immunoblotted with mAb 5E2 (upper panel), and 5E2-precipitated proteins with CT-PLCγ (middle panel). An autoradiograph is shown of CT-PLCγ precipitates (lower panel).

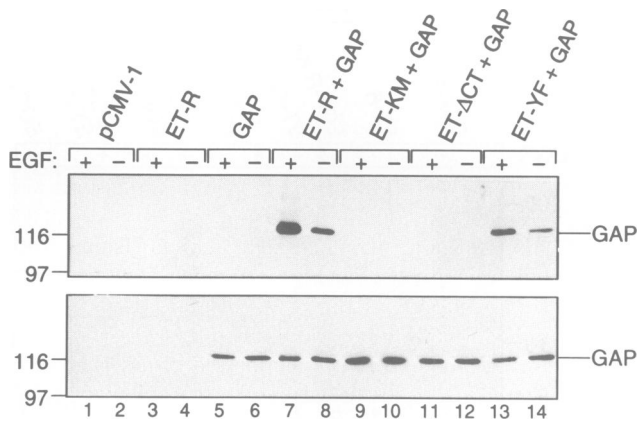

Fig. 6. GAP tyrosine phosphorylation and coprecipitation. 293 cells were transfected with pCMV-1 or cDNA constructs for ET-R or GAP as negative controls, or cotransfected with GAP and ET-R, ET-KM, ET-ΔCT or ET-YF. Prior to lysis, [^{35}S]methionine-labelled cells were pretreated with Na-vanadate as well as stimulated with EGF where indicated (+). Precleared lysates were immunoprecipitated with polyclonal antiserum CT-GAP (αGAP). Precipitates were subjected to 7.5% SDS−PAGE and electrophoretically transferred to nitrocellulose. Phosphotyrosine-containing proteins of the CT-GAP precipitates were detected by immunoblotting with mAb 5E2 (αPY) (upper panel). An autoradiograph monitoring expression levels is shown for CT-GAP precipitates (lower panel).

To determine whether GAP is also a substrate for the Trk tyrosine kinase, we performed an analogous 293 cell coexpression experiment. Prior to lysis, [^{35}S]methionine-labelled cells were treated with sodium vanadate, and where indicated, stimulated with EGF. Lysates were halved and either immunoprecipitated with anti-EGF-R extracellular domain mAb 108.1 or the polyclonal antiserum CT-GAP (αGAP), respectively. Figure 6 shows the αPY immunoblot and the autoradiograph of αGAP precipitates. GAP was tyrosine-phosphorylated by ET-R and to a lesser yet significant extent by ET-YF (Figure 6, upper panel, lanes 7, 8, 13 and 14). Weak coprecipitation with GAP was observed for ET-R (lane 7) but not for ET-YF under our experimental conditions. Equal expression of GAP and receptors in the respective lanes (Figure 6, lower panel and data not shown) rules out the possibility that effects were due to unequal expression levels.

We next examined the suitability of p85 as a Trk substrate. Figure 7 shows the result of a coexpression experiment performed analogously to that described for PLCγ and GAP. The αPY blot of proteins immunoprecipitated with polyclonal antiserum CT-p85 (αp85) revealed tyrosine phosphorylation of p85 by ET-R and ET-YF. In contrast to PLCγ and similar to GAP, phosphorylation of p85 by ET-YF was not significantly reduced, in comparison to ET-R. Moreover, comparable amounts of receptors ET-R and ET-YF were coprecipitated with p85 in addition to other proteins. It should be noted, however, that endogenous 293 cell p85 apparently associated with and coprecipitated ET-R (lane 3). Interestingly, p85 tyrosine phosphorylation was not detected in this lane, which suggests that phosphorylation

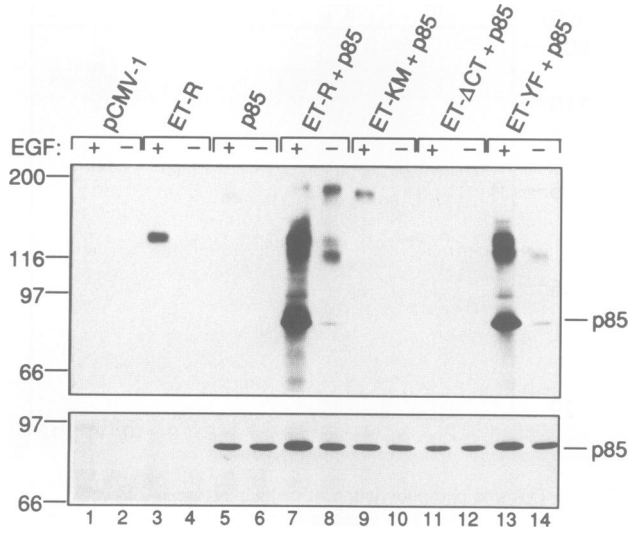

Fig. 7. p85 tyrosine phosphorylation and coprecipitation. 293 cells were transfected with pCMV-1 or cDNA constructs for ET-R or p85 as negative controls, or cotransfected with p85 and ET-R, ET-KM, ET-ΔCT or ET-YF. Prior to lysis, [^{35}S]methionine labelled cells were pretreated with Na-vanadate as well as stimulated with EGF where indicated (+). Precleared lysates were immunoprecipitated with polyclonal antiserum CT-p85 (αp85). Precipitates were subjected to 7.5% SDS−PAGE and electrophoretically transferred to nitrocellulose. CT-p85 precipitates were immunoblotted with mAb 5E2 (αPY) (upper panel). An autoradiograph of the CT-p85 precipitates monitoring p85 expression levels is also shown (lower panel).

of this PI3′-K subunit may not be detectable under these stoichiometric circumstances. Additional phosphotyrosine-containing proteins that were coprecipitated with p85 are of unknown identity. Thus, in contrast to PLCγ, the Trk C-terminal-most tyrosine residue appears not to play a major role in the interaction with p85.

In vitro association of PC12 cell proteins with chimeric receptors

To obtain further information about the substrate specificity of Trk in a more relevant environment, the association of endogenous proteins from neuron-like PC12 pheochromocytoma cells with ET receptors was examined. For this purpose, unlabelled lysates of receptor-expressing 293 cells were mixed with equal amounts of [^{35}S]methionine-labelled PC12 cell lysates, and the mixtures were immunoprecipitated with anti-EGF-R mAb 108.1. For comparison, EGF-R was analyzed in parallel. After separation of proteins by SDS–PAGE, the gel was processed for autoradiography. The signals obtained correspond to proteins coprecipitated with the respective receptors as a consequence of specific association.

EGF-R and ET-R displayed distinct association patterns, demonstrating clear differences in substrate affinity of EGF-R and Trk cytoplasmic domains (Figure 8A). The strong bands in lanes 2 and 3 could represent endogenous rat EGF-R from PC12 cells, which associate more efficiently with the unlabelled human EGF-R than with ET-R, and are not due to significant cross reaction of mAb 108.1 with rat EGF-R because of the absence of this band in lanes 4–9. The most striking signal was a strong band migrating at 145 kDa, which was exclusively associated with stimulated ET-R (Figure 8A, lane 4). Very faint 145 kDa bands appeared in the lanes corresponding to unstimulated ET-R and stimulated EGF-R only after long exposure times.

Analysis of the immunoprecipitates by immunoblotting and probing with the polyclonal antiserum CT-PLCγ identified the 145 kDa band as PLCγ (Figure 8B, lane 4) and demonstrated that the affinity of PLCγ for Trk cytoplasmic sequences was remarkably higher (>100×) than for EGF-R, which is well known to phosphorylate this substrate with high stoichiometry. The extraordinarily high affinity of Trk for PLCγ is also obvious from the fact that about the same amount of PLCγ was coprecipitated with ET-R from the reaction mixture as was precipitated by an excess of polyclonal αPLCγ antiserum (CT-PLCγ) (data not shown). Interestingly, replacement of the most C-terminally located tyrosine residue (Y-785) of Trk with phenylalanine (ET-YF) completely abrogated this high affinity PLCγ association. This, together with the results from the receptor/PLCγ coexpression experiment (see Figure 5), strongly suggested that Y-785 of Trk is indispensable for high affinity binding to PLCγ. Receptor phosphorylation was monitored by reprobing the immunoblot with anti-PY mAb 5E2 (Figure 8B, lower panel). Coomassie staining of proteins in the gels ruled out the possibility that the effects described above were due to different receptor expression levels (data not shown).

Inhibition of PLCγ binding to ET-R by a synthetic tyrosine-phosphorylated peptide

To investigate further whether Y-785 of Trk is the binding site for PLCγ, we performed an *in vitro* association experiment, as described above, with ET-R and a [^{35}S]methionine-labelled PC12 cell lysate, and added a pentadecapeptide identical in sequence to the Trk C-terminus in which the tyrosine residue corresponding to Trk-Y-785 was phosphorylated. The control experiment included a non-phosphorylated peptide of the same sequence.

At a concentration of 10 nM, the phosphopeptide significantly reduced the amount of PLCγ that coprecipitated with ET-R (Figure 9, lane 4). Substrate–receptor association

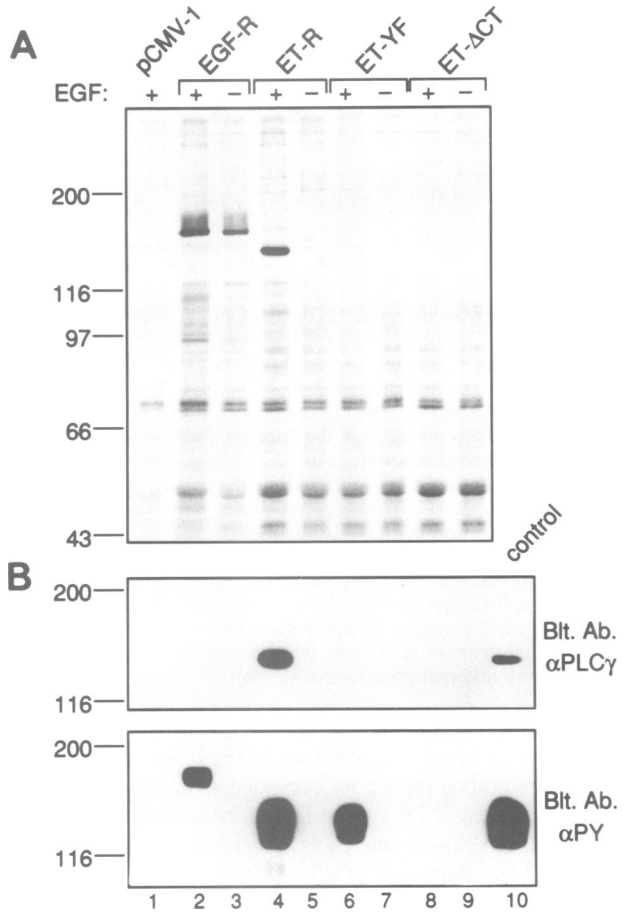

Fig. 8. *In vitro* association with PC12 cell proteins. Receptor-expressing 293 cells were treated with EGF (as well as with Na-vanadate) where indicated (+) and lysed, and the precleared lysates were mixed with equal aliquots of precleared lysate from [^{35}S]methionine-labelled PC12 cells. Receptors were immunoprecipitated with mAb 108.1, the samples halved and separately electrophoresed on 7.5% SDS. One gel was processed for autoradiography (**A**) to visualize coimmunoprecipitated proteins from labelled PC12 cell lysate, whereas proteins of the other gel were electrophoretically transferred to nitrocellulose and probed with polyclonal antiserum CT-PLCγ (αPLCγ) and with mAb 5E2 (αPY) (**B**). Each sample contains receptor from a 10 cm dish of subconfluent 293 cells mixed with labelled lysate of a 15 cm dish of subconfluent PC12 cells. The control lane differs from ET-R (+) lane only in that 293 cell lysate containing autophosphorylated ET-R was left without radiolabelled PC12 cell lysate. The difference in the signal intensity of PLCγ bands between control and ET-R (+) lanes demonstrates that associated PLCγ is mainly derived from PC12 cells.

was further decreased at increasing phosphopeptide concentrations, and was completely abolished to undetectable amounts at 500 nM (Figure 9, lane 10). In contrast, under the same conditions the nonphosphorylated peptide had no capacity to inhibit PLCγ binding (Figure 9). Comparable effects, however, were obtained at concentrations four orders of magnitude higher than that employed for the phosphopeptide (data not shown). These results demonstrate that the C-terminal sequence of Trk, containing phosphorylated Y-785, is the binding site for PLCγ.

Discussion

Spatial and temporal organization of multicellular organisms requires a tightly controlled balance between cell growth and

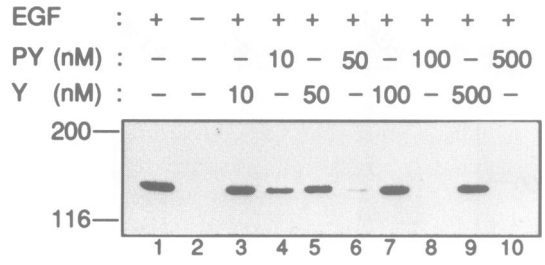

Fig. 9. Phosphotyrosine peptide inhibition of PLCγ binding to ET-R. ET-R-expressing 293 cells were treated with EGF (as well as as with Na-vanadate) where indicated (+) and lysed, and the precleared lysates were mixed with precleared lysate from PC12 cells. Then different amounts of phosphopeptide (PY) QALAQAPPVY(PO_3H_2)LDVLG or nonphosphorylated peptide (Y) of the same sequence were added. ET-R was immunoprecipitated with mAb 108.1, the precipitates were separated by 7.5% SDS−PAGE and electrophoretically transferred to nitrocellulose. Amounts of coprecipitated PLCγ were detected with αPLCγ polyclonal antiserum (CT-PLCγ). [Lysates from seven 15 cm dishes of subconfluent PC12 cells as well as lysates from nine 10 cm dishes of subconfluent, ET-R expressing and stimulated 293 cells were pooled and equal aliquots of both pools were mixed. The amount of ET-R from one 10 cm dish of unstimulated 293 cells (lane 2) was equal to ET-R amounts in all other lanes as judged by Ponceau S-staining of transferred proteins (not shown)].

differentiation. Cell surface receptors with tyrosine kinase activity, such as the receptors for EGF, PDGF, insulin and CSF-1, are crucial regulatory components in these processes. Recently, a new RTK subfamily has been described which consists of the closely related Trk, TrkB and TrkC receptors (reviewed in Barbacid *et al.*, 1991). These structurally similar tyrosine kinases are functional receptors for the neurotrophic factors, NGF, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), respectively (reviewed in Meakin and Shooter, 1992). All of these receptor−ligand pairs are implicated in neuronal survival and differentiation.

While there is agreement that high affinity receptor binding is a prerequisite for the induction of neurotrophic biological effects (Green *et al.*, 1986; Weskamp and Reichardt, 1991; Meakin and Shooter, 1992), there is presently no consensus as to the role of the low affinity NGF-R, $p75^{LNGFR}$, in NGF binding. One model suggests that a heterodimeric Trk/$p75^{LNGFR}$−receptor complex comprises the high affinity NGF binding site (Hempstead *et al.*, 1990, 1991; Ragsdale and Woodgett, 1991). Another hypothesis is that Trk alone, possibly in the form of a homodimer, is the only component required for high affinity NGF binding (Klein *et al.*, 1991; Weskamp and Reichardt, 1991; Ibáñez *et al.*, 1992; Meakin *et al.*, 1992).

In this report we have investigated the signal generation capacity of Trk by examining its ability to interact with cellular substrates. For the activation of the Trk tyrosine kinase, we replaced the extracellular ligand binding domain of Trk with the extracellular domain of EGF-R. The resulting chimeric receptor, ET-R, was faithfully synthesized and transported to the cell surface. Moreover, interaction with EGF clearly stimulated the tyrosine kinase activity contained within the Trk cytoplasmic domain (Figure 4). Since a number of earlier studies had demonstrated that the signalling potential and substrate specificity of RTKs are determined exclusively by their cytoplasmic sequences (Ballotti *et al.*, 1989; Pandiella *et al.*, 1989; Riedel *et al.*, 1989; Lev *et al.*, 1990; Herbst *et al.*. 1991), we were able to obtain important clues regarding the signalling capacity of the wild type (wt) Trk system. The validity of our findings was substantiated by experiments with wt Trk, which yielded analogous results and will be presented elsewhere. These results support the notion that the Trk kinase domain is sufficient for neurotrophic factor signalling.

To investigate Trk signalling characteristics, we analyzed total cell protein tyrosine phosphorylation catalyzed by ET-R and, for comparsion, that catalyzed by EGF-R in intact 293 cells (Figure 4). Four proteins of currently unknown identity, p35, p40, p41 and p52, were preferentially phosphorylated by Trk, in comparison with the EGF-R. Further comparisons with other RTKs will be necessary to evaluate the role of these substrates in Trk signal definition. In addition, 293 cells clearly represent a heterologous system, and the availability and concentrations of these protein substrates may differ in Trk-expressing neurons.

PLCγ, GAP and the noncatalytic subunit of PI3′-K, p85, were the first cellular proteins to be identified as RTK substrates. As yet, however, none of the more downstream signalling pathways have been elucidated conclusively, and the cell responses connected to them are unknown. In spite of the ubiquitous expression of these signal mediators, it is possible that the corresponding signalling pathways fulfil different roles in different cell types. This is the case, for example, in the Ras system, which triggers mitogenesis in many cell types and differentiation of PC12 cells (Bar-Sagi and Feramisco, 1985; Hagag *et al.*, 1986).

We therefore examined the potential role of PLCγ, GAP and p85 as Trk substrates in a transient expression system. All three proteins associated with ET-R, as judged by their coimmunoprecipitation, and were tyrosine-phosphorylated in a ligand-dependent fashion. Therefore our data indicate that the NGF-R/Trk is capable of coupling to PLCγ-, GAP- and PI3′-K-mediated pathways for the transduction of cellular signals. In the case of PLCγ, our results are consistent with previous findings of NGF-stimulated PLCγ phosphorylation in PC12 cells (Kim *et al.*, 1991; Vetter *et al.*, 1991). In addition, a trpE−PLCγ fusion protein containing SH3 and both SH2 domains of PLCγ has been shown to associate with $p70^{trk}$ (Ohmichi *et al.*, 1991a), an oncogenic version of Trk originally found in a human colon carcinoma (Martin-Zanca *et al.*, 1986), and with wild type Trk (Ohmichi *et al.*, 1991b). Similarly, a trpE−GAP fusion protein containing SH3 and both SH2 domains of GAP was found to associate weakly with $p70^{trk}$, but no association with Trk could be detected (Ohmichi *et al.*, 1991a,b). Recently, Li *et al.* (1992) demonstrated that NGF causes an increase in GAP activity towards Ras in PC12 cells. The increase in GAP activity was even stronger in Trk-overexpressing PC12 cells, suggesting a connection between Trk and GAP. Because tyrosine phosphorylation of GAP and association with activated Trk could not be demonstrated (Li *et al.*, 1992), these authors proposed serine/threonine phosphorylation of GAP as an alternative mechanism for GAP activation. We have shown here that Trk tyrosine kinase activation leads to tyrosine phosphorylation of GAP, which provides a link between NGF stimulation of the Trk signalling pathway and initiation of a signalling cascade which involves GAP and Ras.

Carter and Downes (1992) recently demonstrated NGF activation of PI3′-K in PC12 cells. However, no evidence was obtained for direct association of this enzymatic activity with the Trk receptor. Our findings that p85 associates tightly

with ET-R and is phosphorylated in an EGF-dependent fashion strongly suggest a direct interaction of PI3′-K with the Trk cytoplasmic domain, and thus regulation of its enzymatic activity by NGF. These observations are in accordance with the recently reported findings of Solthoff *et al.* (1992).

A great deal of progress has been made in terms of the identification of RTK interaction sites with substrate molecules involved in signalling pathway initiation or regulation. Surprisingly, the factors that have been identified to date that bind to RTK cytoplasmic sequences with high affinity all contain src-homologous (SH2) regions. These SH2 sequences include structural determinants that recognize phosphorylated tyrosines, which are flanked by sequences that apparently define both the specificity and affinity of the interaction. Interestingly, the better characterized sites for RTK–substrate interactions are all located within regions that were suggested early on to be involved in RTK-specific activities due to their hydrophilic nature and divergent sequence characteristics (Yarden and Ullrich, 1988). This includes the kinase-insertion region of subclass III RTKs, which in the case of PDGF-R and $p145^{c\text{-}kit}$ contain major binding sites for GAP and p85 (Fantl *et al.*, 1992; Kashishian *et al.*, 1992), and the C-tail regions of EGF-R, PDGF-R and FGF-R, which contain phosphotyrosine residues that are recognized by PLCγ SH2 domain structures (Mohammadi *et al.*, 1991, 1992; Peters *et al.*, 1992; Rotin *et al.*, 1992; Seedorf *et al.*, 1992). The apparent generality of receptor–SH2 domain interactions and the current lack of evidence for RTK-specific substrates, further underscores the question of how RTK-specific and signalling parameters are defined.

Our experiments and those of others indicate that Trk-mediated signals involve factors such as PLCγ, GAP and p85, which are also utilized by other receptors with different biological roles. Comparative analysis of proteins from PC12 cells that associate with Trk and EGF-R cytoplasmic sequences revealed clear differences, which may reflect the distinct roles these receptors play in these cells. Ligand-induced association with ET-R was observed most strikingly for PLCγ and with lower intensity for polypeptides of 85, 91, 104 and 114 kDa, while the EGF-R association pattern included bands at 94, 97, 101, 107 and 111 kDa (Figure 8A). In striking contrast, strong association of EGF-R with PLCγ, under identical conditions, was not detected even though PLCγ clearly serves as a substrate for this RTK (Margolis *et al.*, 1989). This indicates an affinity difference of $>100\times$ between these two RTKs for PLCγ.

To assess the role of the Trk C-terminal tail region, which is unusually short (15 amino acids) and contains a single tyrosine residue at position 785, we examined mutants bearing either a deletion of the entire sequence, ET-ΔCT, or a replacement of the tyrosine with phenylalanine, ET-YF. Surprisingly, deletion of 15 amino acids in ET-ΔCT resulted in complete loss of the kinase function. This was similar to recently reported observations with a PDGF-R deletion mutant of 115 C-terminal residues (Seedorf *et al.*, 1992), but different from EGF-R mutants, which preserve their kinase activity even after a loss of over 200 C-terminal amino acids. These findings suggest conformation differences in the cytoplasmic domains of different RTK subclasses and a major role of Trk C-terminal sequences in the stabilization of a functional three-dimensional structure.

Especially informative was the ET-YF mutant. Although ET-YF retained its capacity for auto- and substrate phosphorylation, 293 cell overexpression experiments revealed that its ability to coprecipitate with PLCγ had been lost. The PC12 cell protein association (Figure 8A) and phosphopeptide competition (Figure 9) experiments confirmed this finding and further emphasized the central role of the PY-785 determinant in Trk-PLCγ interaction. Interestingly, the loss of high affinity binding in ET-YF did not result in complete abrogation of PLCγ phosphorylation, suggesting the existence of other low affinity binding sites in the Trk cytoplasmic domain.

At this point, we cannot explain the basis for the remarkable affinity difference for PLCγ between Trk and EGF-R, nor the contribution of flanking region sequence determinants. The Trk Y-785 flanking sequences and recently identified PLCγ binding sites in the PDGF-Rβ (Rönnstrand *et al.*, 1992) do not confirm V/LXXXXEYL/I as a consensus sequence, as suggested previously on the basis of the EGF-R and FGF-R binding sites for PLCγ (Mohammadi *et al.*, 1992; Rotin *et al.*, 1992). However, our findings support the hypothesis that enzymatic parameters and structural determinants defining receptor affinities for a common set of substrates, in conjunction with possible cell type- and receptor-specific signalling pathways, form the basis of RTK-characteristic signals. Further work will be necessary to investigate the biological significance of NGF-stimulated PLCγ phosphorylation and its role in the processes of nerve cell differentiation and survival that are mediated by Trk.

Materials and methods

Construction of chimeric ET-R and ET mutants

Plasmid CVNHERc (Riedel *et al.*, 1988), containing the full-length cDNA of human EGF-R, and pLTRgagactrk (generously given by N.Hynes), containing the cDNA of a human actin–trk fusion oncogene, were used as cDNA sources for restriction fragments and as polymerase chain reaction (PCR) templates for the fusion of the EGF-R extracellular domain to the transmembrane and intracellular sequences of Trk, thereby generating ET-R.

The coding sequence for the EGF-R extracellular domain was primarily constituted from a 1680 bp *Xba*I–*Apa*I restriction fragment. The remaining 418 bp were generated by PCR technology, ligated to the larger fragment via the *Apa*I site, and simultaneously cloned into an *Xba*I–*Eco*RV-linearized BluescriptII KS(+) vector (Stratagene).

The coding sequence for the Trk intracellular domain was mainly constituted from a 1100 bp *Nar*I–*Eco*RI restriction fragment. The remaining 364 bp, also containing the transmembrane coding region, were generated by PCR technology, ligated to the larger fragment via the *Nar*I site, and simultaneously cloned into a *Sma*I/*Eco*RI-linearized pT7T3 18U vector (Pharmacia).

The complete cDNA for the EGF-R extracellular domain and the Trk transmembrane and intracellular domains were recovered from the Bluescript vector by digestion with *Xba*I and *Pvu*I, and from the pT7T3 vector by digestion with *Sca*I and *Eco*RI, respectively, ligated and cloned into a *Xba*I/*Eco*RI-linearized BluescriptII KS(+) vector, creating chimeric ET-R cDNA. At the E–T fusion point, the codon for the last EGF-R extracellular amino acid, serine, is directly joined by the codon for the first Trk transmembrane amino acid, threonine.

For transient expression studies, the chimeric ET-R cDNA was subcloned as an *Xba*I–*Hin*dIII fragment into the polylinker of the cytomegalovirus promoter based pCMV-1 expression vector (Eaton *et al.*, 1986). The PCR-derived sequences, including the E–T fusion point, were subsequently sequenced.

In order to construct various mutants, the *Nar*I–*Eco*RI Trk cDNA restriction fragment was finally cloned into a M13mp18 vector (Boehringer Mannheim).

In vitro mutagenesis was performed according to Taylor *et al.* (1985) using the 'oligonucleotide-directed *in vitro* mutagenesis system version 2'

(Amersham) with the following 18mer oligonucleotides: 5′-pTGGCTG-TCATGGCACTGA-3′ for ET-KM, a kinase-negative receptor mutant, which carries a methionine instead of the conserved lysine in the ATP-binding site (K-538 of Trk); 5′-pCCCGGCTGTAAGCCCTGG-3′ for ET-ΔCT, a receptor mutant that lacks the 15 C-terminal amino acids defined as the Trk C-terminus (Martin-Zanca *et al.*, 1989); 5′-pCTCCTGTCTT-CCTGGATG-3′ for ET-YF, a receptor mutant in which the only C-terminally located tyrosine residue (Y-785 of Trk) has been replaced by phenylalanine.

After verifying the mutations by sequencing, suitable fragments, each carrying one of the mutations, were cloned into the ET-R background.

Other expression plasmids

In addition to the chimeric ET receptors, human EGF-R and human Trk were also expressed in 293 cells in some experiments. The genetic constructs used in these cases were pCMV-1-HERc and pCMV-1-trk, containing the full-length EGF-R cDNA and Trk, respectively. The full-length Trk cDNA was assembled from the *Nar*I−*Eco*RI restriction fragment from pLTRgagactrk and an 859 bp *Bam*HI−*Nar*I plus an 810 bp *Bam*HI−*Eco*RI restriction fragment, both from pLM6 (Martin-Zanca *et al.*, 1989), kindly provided by Mariano Barbacid. The full-length Trk cDNA was integrated in an *Eco*RI-linearized pCMV-1 expression vector. For receptor/substrate coexpression experiments, cDNA sequences coding for PLCγ, GAP and p85 were subcloned into pCMV-1.

Cell culture, transient expression and in vitro association

Human embryonic kidney fibroblasts (293; ATCC CRL 1573) were grown in DMEM, 4500 mg glucose/liter (Gibco) containing 9% fetal calf serum (FCS; Gibco), and 2 mM L-glutamine. 30−35 h prior to transfection, 1.5, 3.5 and 10 × 10^5 cells were seeded into a well of a six-well dish, into 6 cm dishes and 10 cm dishes, respectively. Transfections were then carried out using the calcium phosphate coprecipitation technique according to the protocol of Chen and Okayama (1987) with a total of 4, 8 and 16 μg CsCl gradient-purified plasmid DNA per well or dish, respectively. 12−18 h after addition of precipitates, cells were washed once with DMEM, and DMEM containing 0.5% FCS was then added. To quantify expression levels directly, MEM with Earle's salt, without L-methionine (Gibco), containing 4500 mg glucose/l, 2 mM L-glutamine and 0.5−1.0% FCS, was used instead of DMEM, and cells were metabolically radiolabelled overnight with 40 μCi [^{35}S]methionine/ml (1000 Ci/mmol; Amersham).

Cells were stimulated with 150 ng/ml EGF (or NGF) 10 min prior to lysis (where mentioned, sodium orthovanadate (Na-vanadate) was added to a final concentration of 1 mM 1 h before stimulation). After stimulation, cells were lysed on ice with 0.3−1.5 ml (depending on the size of the well or dish) 'lysis buffer' (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM sodium pryophosphate, 10% glycerol, 1% Triton X-100, 10 mM NaF, 1 mM Na-vanadate, 1 mM phenylmethylsulfonyl fluoride, 250 μM *p*-nitrophenylphosphate, 100 μM ATP, 10 μg/ml aprotinin and 10 μg/ml leupeptin). [The inclusion of ATP in the Lysis buffer was compensated for by an excess of sodium pyrophosphate, and had no effect on the results obtained.] 10 min later, lysates were transferred to microcentrifuge tubes and precleared by centrifugation at 12 500 *g* for 20 min at 4°C.

For immunoprecipitations, 20 μl of protein A−Sepharose (Pharmacia; prewashed in 20 mM HEPES pH 7.5, 0.1 mg/μl) and the appropriate antiserum were added to the cleared lysate and incubated for 3 h on a rotor at 4°C. Precipitates were washed three times with 1.5 ml 'Washing buffer' (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM sodium pyrophosphate, 10% glycerol and 0.1% Triton X-100). SDS sample buffer was added, and the samples were boiled for 5 min before loading on SDS−polyacrylamide gels.

For analysis of total cellular proteins, 45 μl of sample buffer was added directly to 90 μl of precleared lysates (from 5 × 10^4 cells) and the mixture was boiled for 15 min.

After separation by SDS−PAGE, proteins were electrophoretically transferred to nitrocellulose filters. For immunoblot analysis, filters were preincubated 1 h with 5% milk powder solution in TBST (20 mM Tris pH 7.5, 150 mM NaCl, 0.02% Tween 20), washed with TBST, incubated overnight with antibody, washed three times and incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibody. Immunoblots were developed using the ECL system (Amersham; Thorpe *et al.*, 1985). In order to reprobe proteins with another antibody, filters were incubated for 2 h in Strip buffer at 50°C (62.5 mM Tris, pH 6.8, 100 mM β-mercaptoethanol, 2% SDS).

For *in vitro* association experiments, receptor-expressing 293 cells were treated with EGF and Na-vanadate (where indicated) and lysed, and the precleared lysates mixed with equal aliquots of precleared lysate from [^{35}S]methionine-labelled PC12 cells. The mixed lysates were immunoprecipitated with anti-receptor antibody as described above, except that incubation on the rotor was extended to 5 h. Proteins were fractionated by 7.5% SDS−PAGE, and either transferred to nitrocellulose for immunoblotting or processed for autoradiography: fixed for 1 h in 40% methanol/10% acetic acid, stained for 20 min in 0.025% Coomassie G 250, destained in 10% acetic acid, dried and exposed to film (Kodak X-Omat).

PC12 cells were grown in DMEM with 4500 mg glucose/liter containing 2 mM L-glutamine, 9% FCS and 4.5% horse serum (Boehringer Mannheim).

Antibodies

The antibodies recognizing the human EGF-R extracellular domain (108.1), phosphotyrosine (5E2), and the C-termini of PLCγ (CT-PLCγ), GAP (CT-GAP) and p85 (CT-p85) have been described previously (Herbst *et al.*, 1992). Additionally, ATC, a polyclonal rabbit antibody against a peptide corresponding to the last 15 amino acids of the Trk C-terminus (see below), was employed.

Peptides

Tyrosine-phosphorylated and nonphosphorylated pentadecapeptides corresponding in sequence to the C-terminus of Trk (QALAQAPPV-YLDVLG) were synthesized as described by Kitas *et al.* (1989) and analyzed by ion spray mass spectrometry.

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