Neurotrophins mediate their signals through two different receptors: the family of receptor tyrosine kinases, Trks, and the low affinity pan-neurotrophin receptor p75. Trk receptors show more restricted ligand specificity, whereas all neurotrophins are able to bind to p75. One important function of p75 is the enhancement of nerve growth factor signaling via TrkA by increasing TrkA tyrosine autophosphorylation. Here, we have examined the importance of p75 on TrkB- and TrkC-mediated neurotrophin signaling in an MG87 fibroblast cell line stably transfected with either p75 and TrkB or p75 and TrkC, as well as in PC12 cells stably transfected with TrkB. In contrast to TrkA signaling, p75 had a negative effect on TrkB tyrosine autophosphorylation in response to its cognate neurotrophins, brain-derived neurotrophic factor and neurotrophin 4/5. On the other hand, p75 had no effect on TrkB or TrkC activation in neurotrophin 3 treatment. p75 did not effect extracellular signal-regulated kinase 2 tyrosine phosphorylation in response to brain-derived neurotrophic factor, neurotrophin 3, or neurotrophin 4/5. These results suggest that the observed reduction in TrkB tyrosine autophosphorylation caused by p75 does not influence Ras/mitogen-activated protein kinase signaling pathway in neurotrophin treatments.

The survival and differentiation of neurons in the peripheral nervous system are dependent on neurotrophic factors, which are secreted by the target tissues. The first discovered family member of the closely related neurotrophins is nerve growth factor (NGF) (1). Other members are brain-derived neurotrophic factor (BDNF) (2, 3), neurotrophin 3 (NT-3) (4–8), and neurotrophin 4/5 (NT-4/5) (9–11). The primary event in neurotrophin signaling is the specific activation of receptor tyrosine kinases of the Trk family. Trk receptors have restricted ligand-binding specificities, NGF being a preferred ligand to TrkA, BDNF, and NT-4/5 to TrkB and NT-3 to TrkC (9, 11–14). NT-3 can activate also TrkA and TrkB, although at much higher neurotrophin concentrations than TrkC (reviewed in Refs. 15–17). Neurotrophin binding to a specific Trk receptor causes receptor dimerization and the activation of the intrinsic tyrosine kinase domain of Trk. The initial substrate of the Trk kinase is the receptor itself, which results from tyrosine phosphorylation of the other subunit of Trk dimer (18). A tyrosine-phosphorylated Trk recruits, phosphorylates, and activates a variety of adapter molecules that initiate downstream signaling cascades. Phosphotyrosine residues of activated Trk-receptors serve as specific recognition sites for effector molecules that contain a Src homology 2 domain. Among the proteins that interact with tyrosine-phosphorylated TrkA are the enzymes phospholipase Cγ (PLCγ) and phosphoinositols 3-kinase and the adapter protein Shc (19–22). Each of these molecules activates distinct signaling pathways that may have different functions. The Ras/mitogen-activated protein kinase (MAPK) pathway initiated by Shc or PLCγ is involved in differentiation, while the phosphoinositols 3-kinase pathway is important for survival. It appears that in response to NGF, MAPKs like extracellular signal-regulated kinase 2 (ERK2), are cooperatively regulated by Shc and PLCγ in TrkA expressing cell lines (21, 22). A major consequence of Ras activation in cells is threonine/tyrosine phosphorylation and activation of ERKs and Ras appears to be an indispensable element in NGF-promoted ERK activation. In NGF-treated PC12 cells, ERK becomes phosphorylated at a threonine and a tyrosine residue (23) leading to activation and translocation of ERK to the nucleus (24). In the nucleus it phosphorylates its target molecules including several transcription factors, as well as another family of kinases, the ribosomal S6 kinases (summarized in Refs. 25–27).

A second neurotrophin receptor, p75, binds all neurotrophins (28–30). It belongs to the tumor necrosis factor family of receptors, which also includes Fas, CD40, CD30, and CD27 (31). The p75 receptor has a number of distinct effects on Trk function. It alters the ligand binding specificity of Trks and enhances the proportion of TrkA receptors binding NGF with high affinity (13, 32, 33). Co-expression of TrkA with an excess of p75 dramatically increases the rate of association of NGF with Trk, in effect increasing the binding affinity (34). Another effect of p75 on TrkA is to enhance NGF-induced TrkA tyrosine kinase activity. Introduction of p75 into MAH sympathoprogenitor cells expressing TrkA increases NGF-stimulated TrkA autophosphorylation (35) and inhibition of NGF binding to p75 on PC12 cells reduces NGF-mediated TrkA autophosphorylation (36). p75 also has a number of Trk-independent effects on cell processes. These include retrograde transport of neurotrophins (37, 38), the promotion of Schwann cell migration (39), and the activation of the transcription factor NF-κB (40). In the presence of NGF, p75 can also either rescue cells from apoptosis or trigger the apoptotic cascade (31, 41, 42). The latter may be due...
to elevated sphingomyelin hydrolysis leading to increased intracellular ceramide level (43)

Although the collaboration of p75 and TrkA has been characterized by several groups, the influence of p75 on TrkB and TrkC mediated signaling is more poorly understood. A truncated form of p75 lacking most of the cytoplasmic domain can collaborate with TrkA and TrkB, but not with TrkC, in a mouse fibroblast cell line expressing endogenous NGF and BDNF but not NT-3 (44). p75 is also needed for neural differentiation in avian neural crest cultures expressing tyrosine kinase-deficient TrkC (45). Recently Bibel et al. (46) using co-immunoprecipitation reported the physical interaction of p75 with all Trk receptors having a hemagglutinin epitope at their N-terminal end. They also found that the transient expression of p75 in a fibroblast cell line stably expressing TrkB reduced the TrkB autophosphorylation induced by NT-4/5 and NT-3, but not by BDNF. In light of these observations we have studied the function of p75 on TrkB and TrkC mediated signaling in a mouse fibroblast cell line stably expressing both p75 and TrkB or TrkC receptors as well as in PC12 cells stably transfected with TrkB and revealed a dual function of p75 in Trk-mediated neurotrophin signaling.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture—The G418-resistant MG87 mouse fibroblast cell lines stably transfected with rat TrkB or TrkC cDNA were from Regeneron Pharmaceuticals, Inc. These cell lines were stably transfected with 9 µg of rat p75 cDNA and 1 µg of histidinol selection plasmid, and selected for 10 days with 500 µg/ml G418 (Life Technologies, Inc.) and 2.5 mM histidinol (Sigma). To confirm the expression of p75 in the TrkB and TrkC lines, clones were lysed with RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% deoxycholic acid, 0.1% SDS) supplemented with protease inhibitors (Complete™, Roche Molecular Biochemicals) and subjected to a Western blot analysis. Blots were probed with rabbit anti-p75 (Promega) and rabbit anti-pan-Trk (Santa Cruz Biotechnology) antibodies. Horseradish peroxidase-conjugated goat anti-rabbit antibody (Sigma) was used as a secondary antibody. The expression of Trk and p75 was detected with the enhanced chemiluminescence reagent (DuPont) and by exposure to x-ray film (X-Omat AR, Kodak). Selected clones were tested by enzyme-linked immunosorbent assay (47) and Northern blot analysis (data not shown) to exclude the endogenous expression of NGF, BDNF, NT-3, and NT-4/5, which could potentially interfere with the results. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with antibiotics and 0.1% of bovine serum albumin, fraction V (Amersham Pharmacia Biotech) in the presence or absence of protein A purified REX antibody (20 µg/ml) (48), a generous gift from Dr. Louis F. Reichardt) or NGF (1 µg/ml) for 30 min at 37 °C and treated with 0–100 ng/ml NGF (25.5 S, Harlan Bioproducts for Science, Inc.), BDNF, NT-3, or NT-4/5 (Regeneron Pharmaceuticals, Inc.) for 5 (TrkB) or 7 (ERK2) min under the same conditions. Thereafter, cells were washed once with ice-cold phosphate-buffered saline, lysed with RIPA buffer supplemented with 1 mM sodium orthovanadate (Sigma) and protease inhibitors at 4 °C for 20 min, centrifuged, and the supernatants transferred to a fresh tube. Protein concentration was measured using bicinchoninic acid protein assay (Pierce) according to the manufacturer’s instructions. For Trk phosphorylation assays, equal amounts of protein were immunoprecipitated with pan-Trk antibody and protein A-Sepharose (Amersham Pharmacia Biotech) for 2 h at 4 °C. Immunocomplexes were washed three times with ice-cold RIPA buffer and resuspended in 30 µl of Laemmli sample buffer (49). For ERK2 assays, equal amount of whole cell lysates were analyzed. After boiling for 5 min, proteins were separated by 7.5 (Trk) or 12% (ERK2) SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting. Blots were probed with mouse monoclonal anti-phosphotyrosine 4G10 antibody (Upstate Biotechnology) or mouse monoclonal anti-pERK antibody (Santa Cruz Biotechnology), the secondary antibody being horseradish peroxidase-conjugated donkey anti-mouse antibody (Fierce). The phosphorylation of Trk and ERK2 was detected with enhanced chemiluminescence reagents (NEB Life Science Products Inc.) and by exposure to x-ray film. The results were analyzed by densitometry.

RESULTS

Tyrosine Autophosphorylation of TrkB and TrkC in Response to Neurotrophins—In fibroblast cell lines transfected with TrkB or TrkC cognate neurotrophins are known to induce the autophosphorylation of the Trk receptors with BDNF, NT-3, and NT-4/5 being preferred ligands for TrkB and NT-3 for TrkC (50). To determine which ligands are able to induce the tyrosine autophosphorylation of TrkB and TrkC in the stably transfected MG87 cell lines expressing either TrkB or TrkC, the lines were exposed to the four neurotrophins in the presence or absence of stably transfected p75. As expected, TrkB was phosphorylated in response to 100 ng/ml BDNF, NT-3, and NT-4/5 both with and without p75 expression (Fig. 1, a and b). In the presence of p75 the tyrosine phosphorylation level of TrkB was similar in response to all three neurotrophins but in the absence of p75 the phosphorylation level was lower in response to NT-3 compared with BDNF and NT-4/5. There are two potential explanations for this observation. Either p75 increases TrkB phosphorylation in response to NT-3 or p75 decreases TrkB phosphorylation in response to BDNF and NT-4/5. The data in the next section show that the latter explanation is correct. TrkC was uniquely phosphorylated in response to NT-3 only in the absence of p75 (Fig. 1C). Interestingly, a slight tyrosine phosphorylation of TrkC was observed in response to BDNF and NT-4/5. The data in the next section show that the latter explanation is correct. TrkC was uniquely phosphorylated in response to NT-3 only in the absence of p75 (Fig. 1C). Interestingly, a slight tyrosine phosphorylation of TrkC was observed in response to BDNF and NT-4/5. The data in the next section show that the latter explanation is correct. TrkC was uniquely phosphorylated in response to NT-3 only in the absence of p75 (Fig. 1C).

The Effect of p75 on TrkB and TrkC Tyrosine Autophosphorylation in Response to Neurotrophins—p75 has been reported to enhance TrkA mediated signaling by increasing the tyrosine autophosphorylation of TrkA in PC12 and MAH cells (35, 36). Recently, p75 was observed to reduce the tyrosine autophosphorylation of TrkB in response to NT-3 and NT-4/5 in the A293 cell line stably transfected with TrkB and transiently with p75 (46). We examined the influence of p75 on TrkB and TrkC mediated signaling with the Trk tyrosine phosphorylation assay in stably transfected MG87 cells. The neurotrophin binding to p75 was blocked with either the REX antibody (48) or an excess of NGF. The positive influence of REX and non-specific rabbit IgG antibodies on TrkB and TrkC phosphorylation was excluded using Western blotting (data not shown). The blocking of BDNF binding to p75 with the REX antibody caused an increased tyrosine autophosphorylation of TrkB over a wide range of BDNF concentrations suggesting an inhibitory effect of p75 on BDNF-induced TrkB mediated signaling (Fig. 2a).

Since NGF is known to bind to p75 but not to TrkB or TrkC, and it was not able to induce TrkB or TrkC phosphorylation in the MG87 cell lines (Fig. 1) we used its p75 blocking capacity in the A293 cell line stably transfected with TrkB and transiently with p75 (46). We examined the influence of p75 on TrkB and TrkC mediated signaling with the Trk tyrosine phosphorylation assay in stably transfected MG87 cells. The neurotrophin binding to p75 was blocked with either the REX antibody (48) or an excess of NGF. The positive influence of REX and non-specific rabbit IgG antibodies on TrkB and TrkC phosphorylation was excluded using Western blotting (data not shown). The blocking of BDNF binding to p75 with the REX antibody caused an increased tyrosine autophosphorylation of TrkB over a wide range of BDNF concentrations suggesting an inhibitory effect of p75 on BDNF-induced TrkB mediated signaling (Fig. 2a). Since NGF is known to bind to p75 but not to TrkB or TrkC, and it was not able to induce TrkB or TrkC phosphorylation in the MG87 cell lines (Fig. 1) we used its p75 blocking capacity in BDNF treatment using a TrkB/p75 expressing cell line. Blocking p75 with NGF gave similar results as with the REX antibody blocking (Fig. 2b).

The blocking of NT-4/5 binding to p75 with NGF caused a reduced TrkB tyrosine phosphorylation, which was more prominent at lower neurotrophin concentrations but was not observed at high NT-4/5 concentration (100 ng/ml, Fig. 2c). Interestingly, p75 had no influence on NT-3 induced TrkB tyrosine phosphorylation (Fig. 2d).

In contrast to the TrkB expressing cell line, TrkC in the TrkC expressing line was significantly tyrosine autophosphorylated only in response to NT-3 (Fig. 1, c and d). Therefore, the influence of p75 on TrkC phosphorylation was examined using only NT-3 treatment. The inhibition of NT-3 binding to p75
with REX antibody did not have any influence on TrkC phosphorylation over a wide range of NT-3 concentrations (Fig. 3). Based on these observations we suggest that p75 does not play a role in TrkB or TrkC tyrosine autophosphorylation in response to NT-3.

All three Trk receptors are expressed at the highest levels in the nervous system (14, 51, 52), whereas the expression of p75 is not so highly restricted (53). Therefore, we wanted to confirm our results in a more neuronal-like environment and for that purpose PC12 cells stably transfected with TrkB cDNA were used. TrkA was not phosphorylated in response to BDNF, NT-3, or NT-4/5 in PC12 cells (Ref. 47 and data not shown). The phosphorylation status of TrkB was determined in the presence or absence of REX antibody in BDNF and NT-4/5 treatments. In both instances the blocking of neurotrophin binding to endogenously expressed p75 increased the autophosphorylation of TrkB (Fig. 4), which is consistent with the results obtained from stably transfected MG87 fibroblast cell lines. We also tested the influence of p75 on TrkA phosphorylation mediated by NGF treatment. We were able to use the same PC12/TrkB cell line because PC12 cells express endogenous TrkA in addition to p75. As previously reported (36), the blocking of NGF binding to p75 with REX caused a reduced autophosphorylation of TrkA at relatively low concentrations of NGF (Fig. 4).

**ERK2 Tyrosine Phosphorylation in Response to Neurotrophins**—To analyze downstream effects of p75 on TrkB and TrkC mediated signaling, we determined the tyrosine phosphorylation status of ERK2 in MG87 cells stably expressing both p75 and either TrkB or TrkC. Cells were treated with BDNF, NT-3, or NT-4/5 for 7 min and whole cell lysates were analyzed by Western blotting using phospho-ERK specific antibody. In the TrkB expressing cell line, BDNF and NT-4/5 were able to induce the phosphorylation of ERK2 to a similar extent (Fig. 5a). On the other hand, NT-3 although able to induce ERK2 phosphorylation, was less effective than BDNF and NT-4/5, even though the level of TrkB autophosphorylation is the same in the presence of p75 in response to all three neurotrophins (Fig. 1b). More surprisingly, the addition of NGF to abolish the binding of other neurotrophins to p75 had no effect on the level of ERK2 phosphorylation induced by these three neurotrophins (Fig. 5a). This suggests that the observed reduction in TrkB autophosphorylation caused by p75 does not affect the Ras/MAPK signaling pathway. In the TrkC expressing cell line only NT-3 was able to increase the phosphorylation of ERK2 (Fig. 5b). Again, the blocking of p75 had no influence on ERK2 phosphorylation in this cell line.
DISCUSSION

The neurotrophins are distinguished by receptor-signaling systems of two different transmembrane proteins, the Trks and p75. Although at least some of the biological actions of a neurotrophin are mediated solely by its interaction with a Trk receptor, p75 has been shown to collaborate with Trks to modify their activation. In this work, we found that in MG87 fibroblasts, p75 decreases the autophosphorylation of TrkB when induced by BDNF or NT-4/5 whereas p75 has no influence on NT-3-induced TrkB or TrkC activation. The collaboration of p75 with BDNF- or NT-4/5-induced TrkB and TrkC is, therefore, the opposite of what has been reported with NGF-induced TrkA. For example, coexpression of p75 with TrkA in a sympathetic adrenal cell line (MAH) led to an 8-fold increase in tyrosine autophosphorylation of TrkA (35). Consistent with this result is the blocking of NGF binding to p75 in rat primary sympathetic neurons (54) and in PC12 cells caused a reduced TrkA phosphorylation and also a decrease in the induction of the expression level of an immediate early gene, c-fos (36).

Additionally, BDNF binding to p75 in PC12 cells was shown to result in a reduced TrkA signaling (55).

In contrast, transient expression of p75 in A293 cells stably transfected with TrkB has no effect on TrkB phosphorylation induced by BDNF, whereas NT-3 and NT-4/5 caused reduced TrkB phosphorylation (46). Only the result with NT-4/5 agrees with the data we have obtained. Possible reasons behind these
different results may be differences in the transfection status of p75 (transient versus stable) and/or in the cell lines used in the experiments. It should be noted that we were able to obtain the same results in PC12 cells that we obtained in MG87 cells, i.e., suppression of BDNF and NT-4/5-induced activation of TrkB by p75 and, as a control, p75 enhanced activation of TrkA by NGF at lower concentrations.

In this MG87 fibroblast cell line NT-3 was the dominant ligand for TrkC and p75 had no effect on the activation of TrkC by NT-3. What was noticeable about the introduction of p75 into TrkC expressing cell line was a relaxation in the absolute specificity of TrkC for NT-3. In the presence of p75 both BDNF and NT-4/5 activated TrkC to a slight extent. The possibility of TrkB contamination in this cell line was excluded by Western blotting using a TrkB specific antibody (TRB (48), data not shown).

Mechanisms Involved in the Collaboration of p75 and Trks—There are at least two general mechanisms whereby the two neurotrophin receptors can collaborate at the level of the receptors. In the first, p75 acts to bind and pass on a neurotrophin such as NGF to TrkA. If the transfer reaction is as rapid as the initial binding to p75 then the relatively slow on rate of NGF binding to TrkA is converted to a more rapid on rate, increasing the affinity of the p75-TrkA complex for NGF (34, 36). The existence of a high molar ratio of p75 to TrkA argues in favor of this mechanism. In the second, changes in conformation in one or both receptors are thought to regulate the affinity of neurotrophin binding. A model based on the allostERIC properties of the p75 receptor has been described in detail by Bothwell (56). The finding that p75 interacts directly with a Trk (46) suggests that the second model may be correct. Binding of a neurotrophin to p75 can now be thought of as directly affecting the conformation and, therefore, the affinity of a Trk receptor and this type of regulation could work to increase or decrease Trk affinity.

Alternatively, or in addition, p75 and the Trks could indirectly interact to modify Trk phosphorylation through their
signaling cascade. For example, elevation of ceramide, one of the signaling molecules activated by p75 (43), has been shown to locally inhibit sympathetic axonal growth (57) while McPhee and Barker (55) have identified a ceramide activated serine-threonine kinase that could potentially down-regulate TrkB-mediated growth signals through TrkA phosphorylation.

Interestingly, truncated p75 lacking most, but not all, of the intracellular domain of the receptor still has the ability to enhance both TrkA and TrkB phosphorylation in the MG87 cell line that expresses NGF and BDNF (44). These results suggest that changes in receptor-binding site conformation that occur in the absence of the intracellular domain may be of primary importance. The truncated p75 had no effect on TrkC phosphorylation in the absence of exogenously added NT-3. Since these cells do not express endogenous NT-3, it is possible that the autocrine expression of a neurotrophin (NGF and BDNF in this instance) can affect p75/Trk interaction.

The inability of p75 to affect exogenous NT-3 binding to TrkB and TrkC could arise for at least two reasons. First, NT-3 binding to TrkB, TrkC, and/or p75 causes no change in the conformation of the complex. If this is correct then TrkB and TrkC behave differently in this regard than TrkA. This difference has been noted before. There are differences in the neurotrophin-binding domains of the Trks and these differences distinguish TrkA from TrkB and TrkC but not the latter two from each other (58). Furthermore, Canossa et al. (59) found that TrkB could transphosphorylate TrkC and vice versa, in COS cells but that TrkA did not take part in this phenomenon. Perhaps the failure of p75 to modify NT-3 binding to TrkB and TrkC fits into the same pattern. The other explanation for the lack of effect of p75 on NT-3-induced TrkB or TrkC phosphorylation is that there is no interaction between the signaling mechanisms of the receptors.

Important aspects of the p75/Trk interaction are their physiological implications. The ability of p75 to enhance TrkA activation demonstrated in a number of in vitro experiments as described above is reflected in the properties of sensory neurons from p75 knock-out mice. These neurons show a reduced sensitivity to low concentrations of NGF compared with wild type mice (60). It appears, therefore, that these neurons rely on the enhanced activation of TrkA by p75 to respond to the low levels of NGF present at certain stages of development. Although the binding of BDNF to p75 has been shown, for example, to inhibit sympathetic axonal growth in the pineal gland, this phenomenon is mediated directly through p75 signaling (61, 62) rather than by inhibition of TrkA signaling. As far as we are aware, potential systems in which the physiological consequences of the inhibition of TrkB and TrkC signaling through p75 have not been studied.

p75 Does Not Influence the Phosphorylation Status of ERK2—As one attempt to determine if the reduced activation of TrkB and TrkC are due to changes in the signaling components of the receptors we analyzed the phosphorylation status of ERK2, one of the commonest signaling intermediates, after neurotrophin treatment. We found that its phosphorylation status was not affected by blocking neurotrophin binding to p75. Lachance et al. (54) also noted, in results consistent with these observations, that p75 reduced TrkA phosphorylation but had no effect on NGF-mediated survival in sympathetic neurons. The phosphorylation of tyrosine 490 (Tyr490) in TrkB activates the Ras/MAPK pathways by forming a recognition site for the Sce adaptor protein (20, 22). In addition to this, the binding of PLCγ to the activated TrkA at Tyr785 is sufficient to induce ERK activity in response to NGF (22). Our observation that the ERK2 phosphorylation level is similar despite the blocking of the neurotrophin binding to p75 suggests that, at least, Shc or PLCγ is activated in response to BDNF and NT-4/5 even though the total tyrosine phosphorylation of TrkB is reduced by p75. Most likely, the reduced TrkB tyrosine phosphorylation does not influence the Ras/MAPK signaling pathway but uses another cascade in the signal transduction. Also the invariable phosphorylation status of TrkB, TrkC, and ERK2 after NT-3 treatment suggest that under these circumstances either p75 does not play a role in NT-3-mediated Trk activation or that its influence is mediated by a Trk tyrosine phosphorylation independent pathway.

It appears that the role of p75 in neurotrophin activation is far more complex than was initially assumed. The presence of p75 can act in a positive (binding of NGF to TrkA) or negative (binding of BDNF or NT-4/5 to TrkB) fashion or have no effect at all (binding of NT-3 to TrkB or TrkC) in Trk mediated activation. How p75 affects the downstream signaling from the Trks is part of the puzzle that is actively under investigation.

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