Transphosphorylation of the Neurotrophin Trk Receptors*

Marco Canossa, Giorgio Rovelli, and Eric M. Shooter†
From the Department of Neurobiology, Stanford University School of Medicine, Stanford, California 94305-5401

The potential for the activation of one Trk receptor by ligand binding to another Trk receptor was explored by determining if transphosphorylation on tyrosine residues can occur between receptors. For most of these experiments, functional chimeric receptors were used that contained the extracellular domain of the human type 2 tumor necrosis factor receptor and the transmembrane and cytoplasmic domains of rat TrkA, TrkB, or TrkC and that, when activated by the tumor necrosis factor, mediated the nerve growth factor-like biological activities in PC12 cells. Cotransfection experiments in COS-7 cells and fibroblasts showed that the presence of different extracellular regions, intermolecular transphosphorylation of homologous cytoplasmic domains occurred between TrkA or TrkB and their cognate chimeras. Heterologous transphosphorylation between TrkB and TrkC kinase domains was also observed when one partner was a chimeric receptor; however, TrkA did not transphosphorylate the TrkB or TrkC kinase domains of chimeric receptors or act as a transphosphorylation substrate for these two receptors. The failure of TrkA to take part in transphosphorylation reactions with TrkB and TrkC was confirmed using the natural receptors. Trk receptor transphosphorylation occurs in the two non-neuronal cell types, but TrkA is excluded from these reactions.

The nerve growth factor (NGF)1 is a prototype for a structurally related family of neurotrophic factors, the neurotrophins. Three other members of this gene family have been identified, brain-derived neurotrophic factor (BDNF), neurophin-3 (NT-3), and neurophin-4/5 (reviewed in Refs. 1–3). The neurotrophins share ~50% amino acid homology, but they exhibit characteristic patterns of activity. For instance, NGF, BDNF, and NT-3 can support the survival of embryonic sensory dorsal root ganglial neurons, but only NGF can effectively support sympathetic neurons (4, 5) from undergoing programmed cell death. Conversely, embryonic placode-derived sensory neurons are sustained by both BDNF and NT-3, but not by NGF (6–8). This selectivity is believed to depend on the presence of specific cell-surface receptors with tyrosine kinase activity, known as Trk receptors. A family of three related receptors, TrkA, TrkB, and TrkC, has been characterized. In neurons, NGF, BDNF, and NT-3 are the preferred ligands for TrkA, TrkB, and TrkC, respectively, while all the neurotrophins bind to a common receptor, designated p75NGFR (where NGFR is NGF receptor) (reviewed in Refs. 1–3). The ligand-induced dimerization of TrkA tyrosine kinase receptors leads to activation of the tyrosine kinase (9). Receptor phosphorylation occurs by a dimerization mechanism whereby the two partner molecules representing the dimer phosphorylate each other (10). It is known that the presence of homologous intra- or extracellular domains is sufficient to mediate transphosphorylation between some heterologous receptors (11–13), while intermolecular transphosphorylation can also occur between two different but related receptors (14, 15). Furthermore, the transphosphorylation involving an inactive tyrosine kinase receptor can lead to partial restoration of the functional internalization of this receptor (12) or to amplification of the response by an active receptor (12, 16).

Dose-response survival experiments with the various neurotrophins suggest that some neurons express more than one type of Trk. Furthermore, it has been demonstrated that subsets of dorsal root ganglial neurons coexpress TrkB with either TrkA or TrkC, suggesting that the neurotrophin activity can overlap in the same cell population (17). The colocalization of TrkA, TrkB, or TrkC receptor mRNA has also recently been reported in primary sensory neurons (18). It was of interest to determine if the homologous kinase domains of the various Trk receptors represent compatible substrates for heterologous transphosphorylation, permitting one neurotrophin to activate more than one Trk receptor. For this purpose chimeric, TNFR2-Trk receptors, which differ in mobility from the corresponding Trk receptors, have been used. Accordingly, active or inactive Trk receptors were coexpressed in COS-7 cells and fibroblasts with inactive or active chimeric TNFR2-Trk receptors, respectively, and the effects of one or more of the neurotrophins on receptor phosphorylation were determined. Intermolecular transphosphorylation occurred between homologous Trk receptors in these cells, while a restricted TrkB/TrkC heterologous transphosphorylation was observed.

MATERIALS AND METHODS

Construction of Chimeric Receptors—Two sets of chimeric receptors between human TNFR2 (a gift of Werner Lesslauer and Hansruedi Loetscher, Hoffmann-La Roche Ltd., Basel, Switzerland) and rat TrkB and TrkC (gifts of George Yancopoulos, Regeneron Pharmaceuticals Inc., Tarrytown, NY) were constructed. The cDNA coding regions of rat TrkA, TrkB, and TrkC and human TNFR2 receptors were subcloned into pRc/CMV and pCDM8 mammalian expression vectors (Invitrogen). Using these as substrate, a two-step polymerase chain reaction (PCR) strategy was employed to build chimeric receptors between the extracellular domain of human TNFR2 and the transmembrane and cytoplasmic domains of rat TrkB and TrkC (TB and TC, respectively).
Mutants in which the transmembrane domain of TrkB or TrkC was replaced by the transmembrane domain of rat TrkB or TrkC (TB- and TC- respectively) were constructed. Kinase-negative receptor mutants (TrkA-K, Trk-BK, and Trk-C-K) that carry an alanine instead of a lysine in the ATP-binding domain were constructed by PCR overlapping extension mutagenesis using full-length or chimeric receptors as substrate. The PCRs were performed as described previously (19) using specific oligonucleotides (PCR-mate, Applied Biosystems Inc.) complementary to the first 17-mer and the last 17-mer of each amplified fragment. We have synthesized oligonucleotides complementary to the following: the first 17-mer including the start of the C-terminal ATP-binding domain (ATG) of TrkA, TrkB, or TrkC; the last 17-mer of the human TNFR2 joined with the first 17-mer of the transmembrane domains of rat TrkB and TrkC; 5′-AAGGGACACTGGCAGCATCTCCTCTGGTCTATGC-3′ (primer 1) and 5′-GGACACTGGCAGCATCTCCTCTGGTCTATGC-3′ (primer 2); and 5′-AAGGGACACTGGCAGCATCTCCTCTGGTCTATGC-3′ (primer 3). Oligonucleotides complementary to the second 17-mer of the extracellular domain of human TNFR2 joined with the first 17-mer of the transmembrane domains of rat TrkB and TrkC, 5′-TCCAGGTTGCTGTCGCGGGCACTGAAGGGAGACA-3′ (primer 13); and 5′-AGGTGGGTTGGCACTGGCAGCATCTCCTCTGGTCTATGC-3′ (primer 14), respectively. Oligonucleotides complementary to the first 17-mer of the extracellular domain of human TNFR2, 5′CCGTCGCCGT-3′ (primer 1); to the last 17-mer of the extracellular domain of human TNFR2, 5′TCATCATGACCCAGGT/ACCAAAAGTGAGACAG-3′ (primer 4), and 5′-TCACTATGAGCCACGGTTAGTGTCGCTGTCGCGGCACTGAAGGGAGACA-3′ (primer 5), and 5′-TCACTGAGCGACCATGTCGCTGTCGCGGCACTGAAGGGAGACA-3′ (primer 6); to the last 17-mer of the carboxyl-terminal region of TrkB or TrkC (TB- and TC-, respectively) were joined together. The resulting cDNAs of the TB and TC constructs were subcloned into plasmid pCRTAII (Invitrogen) and sequenced. The mutations were introduced into TrkA, TB, or TC protolytic domains by PCR overlapping extension mutagenesis using primers 4–6 and 12–14 were generated in order to amplify both the DNA chains of the substrates. Every single fragment was generated in 2.5 ml of medium in 35-mm plastic dishes. 48 h after electroporation, the cells were treated with TNF or NGF, and dose-response curves were performed using the following concentrations: 0.1, 1, 2.5, 5, 10, 25, and 40 ng/ml. The number of cells bearing neurites was determined after 48 h; only cells presenting neurites of at least two cell diameter were scored.

Cell Survival Assay—Cytosine β-arabinofuranoside (Cytosine β-arabinofuranoside (ara-C)) was used for the incubation of the electroporated PC12 cells with chimeric molecules. Transfected PC12 cells were plated in 96-well plates at a density of 1 × 104 cells/well and incubated for 3 days with cytosine β-arabinofuranoside (DMEM and 50 ng/ml either TNF or NGF. Viable cells were stained by adding 10 μl of MTT (Promega) directly into the culture medium and incubating for 4 h at 37 °C; the dye was solubilized for absorbance measurements at 560 nm.

RESULTS

Expression and Autophosphorylation of Chimeric Receptors

Chimeric receptors consisting of the extracellular domain of human TNFR2 and the transmembrane and cytoplasmic domains of TrkB and TrkC were made as described previously (19) and were designated as TB and TC, respectively. Using TB and TC (Figs. 1A and 2A) as templates, three classes of mutants were made. In the first, the transmembrane domain of TrkB or TrkC was substituted with the corresponding region of TNFR2 (designated TB-TM and TC-TM, respectively). In the second, a lysine residue essential for the tyrosine kinase activity of TrkB or TrkC located in the conserved motif of the ATP-binding domain was mutated to an alanine (designated TB-A and TC-A, respectively). In the third, a lysine residue essential for the tyrosine kinase activity of TrkB or TrkC located in the conserved motif of the ATP-binding domain was mutated to an alanine (designated TB-A and TC-A, respectively). The chimeras were cloned into the expression vector pCDM8 (26), and the synthesis of the proteins was assayed by expression in COS-7 cells. The ratios of the plasmids used in the cotransfection were varied to achieve a similar level of expression of the two receptors in these (Figs. 1B and 2B) and other experiments. Transfected cells were lysed, and the receptors were immunoprecipitated using a monoclonal antibody directed against the extracellular domain of TNFR2 (anti-TNFR2) (27). All the chimeric receptors were expressed and migrated on SDS-PAGE as a doublet with an apparent molecular mass of ∼65–70 kDa (Figs. 1B and 2B). Although
Fig. 1. Schematic representation, protein expression, and tyrosine phosphorylation of the TB set of chimeric molecules. A, graphic representation of the TB set of chimeras. The extracellular domains of the human TNFR2 receptors are shown as black boxes; rat TrkB kinase domains are represented by white boxes. The extracellular domain of TNFR2 and the transmembrane and cytoplasmic domains of TrkB represent the prototypic molecule designated as TB (lanes 1 and 2). The transmembrane domain of TNFR2 is represented as an extension of the extracellular region (black box) into the cell membrane (TB-TM) (lanes 3 and 4). The lysine-mutated construct is indicated with a K (TB-K) (lanes 5 and 6). The carboxyl-terminal deletion of the last 15 amino acid residues is represented by the missing vertical bar under the kinase domain (TB-Δ) (lanes 7 and 8). B, protein expression of the TB set of chimeras. Chimeric molecules were transiently expressed in COS-7 cells; lysed cells were immunoprecipitated using anti-TNFR2 and resolved by SDS-polyacrylamide gel electrophoresis on 8% acrylamide gels. The blot was electrophoretically transferred to nitrocellulose, probed with anti-TNFR2, and developed by the ECL detection system. C, autophosphorylation of the TB set of chimeras. Transfected COS-7 cells were treated for 5 min with (lanes 1, 3, 5, and 7) or without (lanes 2, 4, 6, and 8) 50 ng/ml human TNF, and the phosphotyrosine contents were analyzed by immunoblotting with anti-pTyr.

Fig. 2. Schematic representation, protein expression, and tyrosine phosphorylation of the TC set of chimeras. Graphic representation, protein expression, and autophosphorylation activity were determined as described for the TB set of chimeras in the legend of Fig. 1.

there was variability in the proportion of the two receptor bands when expressed in COS-7 cells, this did not affect the interpretation of the data. The appearance of two bands may result from incomplete processing of the receptors (28); for the Trk receptors, this could also result from differences in glycosylation.

Receptors were evaluated for function by measuring the TNF-dependent phosphorylation of the tyrosine kinase domains. The TNF incubation time was optimized to result in maximal autophosphorylation of the chimeras (data not shown). The TB and TC chimeras showed a TNF-dependent increase in phosphotyrosine content of both bands of the doublet, with probably a greater relative increase for the upper band (Figs. 1C and 2C, lane 2) that was comparable to the phosphorylation observed by expressing wild-type TrkB and TrkC in COS-7 cells (28). The transmembrane domain of TNFR2 cannot functionally replace this domain in the Trk receptors.

For experiments to be described later, we also constructed an inactive TrkA carrying a single mutation in the kinase domain (designated TrkA-K) (see Fig. 5). As with the mutants described above, TrkA-K did not show any ligand-mediated autophosphorylation when expressed in COS-7 cells (data not shown). The construction of the remaining chimeric receptors, consisting of the extracellular domain of TNFR2 and the transmembrane and cytoplasmic domains of TrkA (EGF-TrkA) (28), the C-terminal deletions (TB-Δ and TC-Δ) prevented receptor autophosphorylation, confirming that this region is important for tyrosine kinase activity. Surprisingly, the TB-TM and TC-TM chimeras were also inactive, indicating that the transmembrane domain of TNFR2 cannot functionally replace this domain in the Trk receptors.

Characterization of Biological Activity of Chimeras

PC12 cells express TrkA and p75NGFR receptors and respond to NGF by acquiring a sympathetic neuron-like phenotype and surviving in the absence of serum. PC12 cells expressing ectopic TrkB or TrkC receptors show a similar response to BDNF and NT-3, respectively (23, 29, 30). Since PC12 cells treated with human TNF do not show any apparent biological response (19), we used PC12 cells as a model system to characterize the...
A and B). These data indicate that the ligand-mediated phosphorylation of the tyrosine kinase domains of TrkB and TrkC receptors, like that of TrkA (19), is necessary and sufficient to induce survival and a neuron-like phenotype in PC12 cells.

**Intermolecular Transphosphorylation between Trk Receptors and Chimeras**

Homologous Transphosphorylation between Trk and Chimeric Receptors in COS-7 Cells—Given the homology between the various Trk receptors, especially in their intracellular kinase domains, it was of interest to determine if Trk heterodimers form and become autophosphorylated. J Ing et al. (9) previously demonstrated that homologous inactive TrkA receptors are tyrosine-phosphorylated when coexpressed with TrkA and the latter activated with NGF. Therefore, our first experiments examined whether a similar transphosphorylation could occur between Trk receptors when only the cytoplasmic and transmembrane domains of these receptors were homologous. The pairs of receptors used initially were active TrkA with inactive chimeric TA-K receptors and chimeric TA with inactive TrkA-K receptors. Corresponding experiments were also carried out with TrkB and TrkC. The cotransfected COS-7 cells were treated with the appropriate neurotrophin or TNF, lysed, and immunoprecipitated using a pan-Trk antiserum that recognizes the C-terminal amino acids common to all Trk and chimeric receptors. Because of their different molecular masses, the full-length and chimeric receptors were readily separated by electrophoresis, and differences in phosphotyrosine content were detected by Western blotting with an anti-phosphotyrosine antibody (anti-pTyr). The state of tyrosine phosphorylation of active Trk receptors and inactive chimeric receptors (or vice versa) can therefore be compared directly.

The kinase-deficient TA-K receptor displayed a very low level of phosphorylation when expressed alone (Fig. 4A, lane 1), but a higher level when cotransfected with TrkA (lane 2). More significantly, not only was the level of TrkA phosphorylation increased on the addition of NGF, as anticipated, but so was the level of phosphorylation of TA-K (Fig. 4A, lane 3; see also Fig. 7A, lanes 1 and 3). In the converse experiments, the effect of coexpression of the chimeric TA receptor with full-length kinase-deficient TrkA-K was examined. Expression of TrkA-K by itself did not result in autophosphorylation of the receptor (Fig. 5, lane 1). Coexpression of TrkA-K with TA resulted in significant phosphorylation of both receptors in the absence of TNF (Fig. 5, lane 2). The addition of TNF increased the phosphorylation of the chimeric TA receptor, again as anticipated, and this was accompanied by an increase in the phosphoryla-
transphosphorylation of TrkA-K (Fig. 5, lane 3). Both bands of the two receptors were affected. These data suggest that transphosphorylation on tyrosine residues occurs between TrkA and the inactive chimeric receptor or between the active chimeric receptor and inactive TrkA. Note that an inactive receptor monomer can be transphosphorylated by an active receptor monomer irrespective of whether the extracellular domain is homologous. That the transphosphorylation results with the chimeric receptors are the same as with the normal receptors also suggests that the extracellular TNF domains may not exert any selective positive or negative influence on the heterologous interactions compared with the normal Trk extracellular domains.

When TrkB was coexpressed with inactive TB-K, the level of phosphorylation of the latter receptor increased along with that of TrkB on addition of BDNF (Fig. 4B, lane 5), suggesting that TrkB forms heterodimers with and transphosphorylates inactive chimeric TB-K. In contrast, no increase in the phosphorylation of TC-K coexpressed with TrkC was observed on addition of NT-3 (Fig. 4C, lanes 6 and 7) even though the phosphorylation of TrkC itself was significantly increased. Whether this is because the constitutive levels of phosphorylation of TC-K when cotransfected with TrkC are already high or because heterodimers of active TrkC and inactive TC-K do not form cannot be determined from these experiments. The converse experiments investigating whether TNF-activated TB or TC can transphosphorylate inactive TrkB or TrkC, respectively, have not been done.

Heterologous Transphosphorylation between Trk and Chimeric Receptors in COS-7 Cells—The second series of experiments explored whether transphosphorylation can occur between different Trk receptors. Coexpression of neither TrkB nor TrkC with TA-K resulted in enhanced phosphorylation of the latter on addition of BDNF or NT-3, respectively (Fig. 4A, lanes 5 and 7), in spite of the increased phosphorylation of TrkB by BDNF (lanes 5) and of TrkC by NT-3 (lane 7). In a similar vein, neither TB-K nor TC-K was phosphorylated when expressed with NGF-activated TrkA (Fig. 4B, lane 3; and C, lane 3). Furthermore, TNF-activated TB or TC failed to transphosphorylate kinase-deficient TrkA-K (Fig. 5, lanes 4 and 6, respectively). Therefore, transphosphorylation does not occur between TrkA and TrkB or between TrkA and TrkC.

A somewhat different picture emerges with TrkB and TrkC. NT-3 activated TrkB transphosphorylated TB-K (Fig. 4B, lane 7), although BDNF-activated TrkB does not appear to transphosphorylate TC-K (Fig. 4C, lane 5). Whether the high level of constitutive phosphorylation of TC-K cotransfected with TrkB and TrkC hides its transphosphorylation again cannot be determined. These data suggest that a heterodimer can form between the receptor monomers of TrkC and TB-K, with the inactive kinase domain of TB-K being a substrate for heterogenous transphosphorylation by TrkC.

Homologous and Heterologous Transphosphorylation between TrkB and Chimeric Receptors in Fibroblasts—The high constitutive phosphorylation of the Trk and chimeric receptors in COS-7 cells that interfered with the detection of transphosphorylation led to a search for another non-neuronal cell in which receptor expression did not result in constitutive receptor phosphorylation. The NIH-3T3 fibroblasts turned out to be such a cell line. Coexpression of TrkB with the active chimeric TA, TB, or TC receptor was achieved without constitutive phosphorylation of any of the receptors (Fig. 6, lanes 1, 3, and 5). Activation of TrkB with BDNF resulted in the expected significant phosphorylation of this receptor in each of the three different transfected cells (Fig. 6, lanes 2, 4, and 6). As noted in COS-7 cells, the activation of TrkB failed to transphosphorylate the chimeric TA receptor (Fig. 6, lane 2), but it did lead to transphosphorylation of the chimeric TB receptor (lane 4). In addition, the transphosphorylation of the chimeric TC receptor by activated TrkB was observed (Fig. 6, lane 6). These results obtained in fibroblasts extend the observations made in COS-7 cells and, taken together, show that the active kinase domains of TrkB and TrkC can transphosphorylate the inactive domain of the other receptor even when the extracellular domains are not the same.

Intermolecular Transphosphorylation between TrkA and TrkB or TrkC Receptors

The failure of TrkB or TrkC, activated by BDNF and NT-3, respectively, to transphosphorylate TA-K may be due either to some malfunctioning of this chimeric receptor that prevents interpretation of the result or to a real restriction on the ability of TA-K to take part in transphosphorylation reactions with the other two receptors. The finding that NGF-activated TrkA fails to transphosphorylate chimeric TB-K or TC-K receptors that do take part in transphosphorylation reactions suggests, but does not prove, that the second explanation is more likely. It has now been possible to confirm this second explanation with the natural Trk receptors. To study transphosphorylation between the Trk receptors themselves requires antibodies that specifically immunoprecipitate a single Trk receptor. One such antibody exists that immunoprecipitates active TrkA or inactive TrkA-K. The NIH-3T3 fibroblasts turned out to be such a cell line. Coexpression of either TrkA or TrkA-K because this is an inactive receptor; see also Fig. 5, lane 1). With this antibody, it is possible to examine whether activated TrkB or TrkC can transphosphorylate inactive TrkA-K. The latter receptor was chosen over active TrkA because NT-3 can activate TrkA, but not TrkA-K. The upper panel in Fig. 7A shows the level of expression of TrkA and/or TrkA-K in the cotransfection experiments with the various pairs of receptors. The level of protein expression of cotransfected TrkA/TrkA-K is higher than that for the other two pairs because the latter contain only TrkA-K. This experiment shows that TrkA is significantly expressed along with TrkA-K. The expression of TrkB and TrkC in the corresponding transfected cells was noted by first precipitating the cell lysate with pan-Trk antibody and then measuring the constitutive phosphorylation of TrkB or TrkC and the higher phosphorylation of activated TrkB or TrkC with the phosphotyrosine antibody. These phosphorylation levels were substantial (data not shown). The lower panel in Fig. 7A shows the constitutive and activated phosphorylation levels of TrkA when coexpressed with TrkA-K. More important, it shows that the immunoprecipitate obtained...
were recognized by this antibody.

and indicated that neither inactivated nor phosphorylated TrkB and TrkC (shown).

by transfecting COS-7 cells with TrkA-K (lanes 1 and 2), TrkB (lanes 3 and 4), or TrkC (lanes 5 and 6). Transfected cells were treated with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) NGF, BDNF, or NT-3, respectively, and the cell lysate was immunoprecipitated with the TrkA-specific antibody. TrkA and TrkA-K receptor protein expression levels (upper panel) and phosphotyrosine content (lower panel) were determined as described in the legend of Fig. 1. Aliquots of the cell lysate of each sample were used to immunoprecipitate TrkB and TrkC receptors using pan-Trk antibody, and receptor tyrosine phosphorylation was determined (data not shown). B, the selectivity of the TrkA-specific antibody was determined by transfecting COS-7 cells with TrkA-K (lanes 1 and 2), TrkB (lanes 3 and 4), and TrkC (lanes 5 and 6). Transfected cells were treated with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) NGF, BDNF, or NT-3, respectively, and the cell lysate was immunoprecipitated with the TrkA-specific antibody. TrkA-K receptor protein expression levels (upper panel) and phosphotyrosine content (lower panel) were determined and indicated that neither inactive nor phosphorylated TrkB and TrkC were recognized by this antibody.

with the specific anti-TrkA antibody from the activated TrkB/TrkA-K or TrkC/TrkA-K or TrkC/TrkA-K pair contained no phosphorylated TrkA-K. Therefore, even when the natural Trk receptors are used, no transphosphorylation of TrkA-K by activated TrkB or TrkC is observed.

**DISCUSSION**

Properties of Chimeric Receptors—As was observed with the chimeric TA receptors, activation of the tyrosine kinase domains of TB and TC with TNF is sufficient to mediate biological activities characteristic of neurotrophins. Mutated chimeric TA (19), TB, and TC receptors with inactive tyrosine kinase domains (TA-K, TB-K, and TC-K, respectively) failed to mediate any biological responses.

One of the differences that sets the Trk receptors apart from the other tyrosine kinase receptors is the short carboxyl-terminal tail of only 15 amino acid residues. Deletion mutants of chimeric EGF-TrkA receptors have previously indicated that this region is essential for conferring tyrosine kinase activity and high affinity binding to phospholipase C-γ (28). Similarly, we found that TNF failed to cause autophosphorylation of the chimeric TB-3C and TC-ΔC receptors, which lack this region, and found no TNF-dependent neurite extension and cell survival when they were transfected in PC12 cells. These results suggest that the carboxyl-terminal tail is also essential for the activity of TrkB and TrkC, and it seems not to be involved in negative control of the tyrosine kinase domain as has been reported for other tyrosine kinase receptors (35–37).

The transmembrane domains of the tyrosine kinase receptors can usually be exchanged in chimeric receptors without affecting their signal transduction capacity (12, 38, 39). Also, Trk oncogenes lacking the transmembrane domain of TrkA are oncogenic, suggesting that this region is not required for the activation of the tyrosine kinase domain (3). In contrast, exchanging the transmembrane domains of the Trk receptors with the corresponding regions of TNFR2 in TB-TM and TC-TM produced nonfunctional receptors. TNF treatment of these mutants induced neither autophosphorylation in COS-7 cells nor neurite outgrowth and survival in PC12 cells. These findings differ from the results obtained with the chimeric receptor constructed between the extracellular and transmembrane domains of type 1 TNFR (TNFR1; p55-TNFR) and the cytoplasmic domain of TrkA (19), where the transmembrane domain from TNFR1 worked equally as well as the same domain from TrkA. It should be noted, however, that these chimeric receptors (TNFR1-TrkA) were much less efficient at autophosphorylation and signal transduction than the TNFR2-TrkA receptor. Since the cytoplasmic regions of TNFR2 lack the characteristic consensus sequences found in a tyrosine kinase domain, it is possible that its transmembrane domain might be unable to properly transduce the signals leading to receptor dimerization and autophosphorylation.

Transphosphorylation of Trk Receptors with the Same Kinase Domains—The results show that transphosphorylation of inactive TrkA and inactive TA-K receptors by TNF-activated chimeric TA and NGF-activated TrkA receptors, respectively, occurs in COS-7 cells in spite of different extracellular domains in the receptor pairs. Similarly, BDNF-activated TrkB transphosphorylates inactive TB-K receptors in COS-7 cells and active TB receptors in fibroblasts. It seems likely that this phenomenon should extend to TrkC and the corresponding chimeric TC receptors, although the effect may well have been masked by the high constitutive phosphorylation of these receptors in COS-7 cells. These data fit with the previously observed transphosphorylation of inactive TrkA receptors by NGF-activated TrkA (19) and with the other examples in fibroblasts, in which a ligand-induced phosphorylation of an active tyrosine kinase receptor leads to transphosphorylation of an inactive but homologous tyrosine kinase domain in a second receptor.

Transphosphorylation of Trk Receptors—Transphosphorylation of an active chimeric TC receptor by BDNF-activated TrkB in fibroblasts and of an inactive TB receptor by NT-3-activated TrkC in COS-7 cells suggests that the kinase domains of TrkB and TrkC are sufficiently homologous to permit the formation of heterodimers under certain conditions in spite of different ligand-binding domains. Similar results have been reported using a chimeric EGF-insulin receptor, where transphosphorylation of both the insulin receptor (identical intracellular but different extracellular domains) and the EGF receptor (identical extracellular and homologous intracellular domains) was observed (11, 12). The erbB-2 receptor is also a substrate for the EGF-activated EGF receptor in mammary carcinoma cells (13), and the Bek and Flg fibroblast growth factor receptors can transphosphorylate each other in fibroblasts (15). What is perhaps unique and unusual about the chimeric TNFR2-Trk receptors used here is that the ligand TNF causes trimerization of the TNF receptors (40) rather than dimerization as with the neurotrophins and the Trk receptors or with EGF and insulin and the EGF and insulin receptors, respectively. Nevertheless, transphosphorylation is still readily observed. Surprisingly, TrkA does not take part in transphosphorylation reactions with TrkB or TrkC in fibroblasts or COS-7 cells, even though significant homology exists between the kinase domains of these receptors. This result was observed with the natural Trk receptors as well as the chimeric receptors. It is interesting that TrkA stands out from TrkB and TrkC in other ways. While the temporal and spatial expression of TrkB and TrkC in the central nervous system is widespread and similar, that of TrkA is...
highly restricted and different (30). TrkB and TrkC are expressed in multiple forms, including truncated receptors (3), while TrkA, apart from one splice variant (41), is not. Furthermore, although the immunoglobulin-like domains close to the transmembrane domain of the TrkB and TrkC receptors are the unique binding sites for BDNF and NT-3, respectively, the situation with the NGF-binding site on TrkA is more complex (31).

The two conclusions of this work are that Trk receptor transphosphorylation is observed in COS-7 cells and fibroblasts and that TrkA is excluded from these reactions. The nature of the mechanisms that prevent TrkA from forming heterodimers has not been addressed in this work and need to be clarified before the data can be extrapolated to neurons. Another approach to this problem will be to use the neurotrophin heterodimers that have been shown to form efficiently in cells that express more than one neurotrophin and that are stable enough to allow their purification (42–44). These heterodimers display biological activity (42, 43), and whether this involves the formation of Trk heterodimers will be a question of great interest.

Acknowledgments—We are very grateful to David Kaplan for generously providing the pan-Trk antiseraum; Stuart C. Feinstein for providing the TrkA-specific antibody; Werner Lesslauer and Hansruedi Loetscher for providing TNF, TNFR2, and anti-TNFR2 antibody; George Yancopoulos for providing full-length rat TrkB and TrkC; and Verity and John Heymach for helpful discussions.

REFERENCES

1. Meakin, S. O., and Shooter, E. M. (1992) Trends Neurosci. 15, 323–331
2. Chao, M. V., and Hempstead, B. L. (1995) Trends Neurosci. 18, 321–326
3. Barbacid, M. (1993) in Molecular Genetics of Nervous System Tumors (Levine, A. J., and Schmidt, H. H., eds) pp. 123–135, John Wiley & Sons, Inc., New York.
4. Chun, L. L., and Patterson, P. H. (1977) J. Cell Biol. 75, 705–711
5. Greene, L. A. (1977) Dev. Biol. 68, 96–105
6. Hohn, A., Lenbrock, J., Bailey, K., and Barde, Y.-A. (1990) Nature 344, 339–341
7. Thoenen, H., and Barde, Y. A. (1980) Physiol. Rev. 60, 1284–1325
8. Lindsay, R. M., Thoenen, H., and Barde, Y.-A. (1993) Dev. Biol. 152, 319–328
9. J. J. S. Tapley, P., and Baradac, M. (1992) Neuron 9, 1067–1079
10. Ullrich, A., and Schlessinger, J. (1990) Cell 61, 203–212
11. Ballotti, R., Lammers, R., Scmeca, J.-C., Dull, T., Schlessinger, J., Ullrich, A., and Van Obberghen, E. (1989) EMBO J. 8, 3303–3309
12. Lammers, R., Van Obberghen, E., Ballotti, R., Schlessinger, J., and Ullrich, A. (1990) J. Biol. Chem. 265, 16886–16890
13. Herbst, R., Lammers, R., Schlessinger, J., and Ullrich, A. (1991) J. Biol. Chem. 266, 19903–19916
14. King, C. R., Borelo, I., Belot, F., Comoglio, P., and Schlessinger, J. (1988) EMBO J. 7, 1647–1651
15. Bellot, F., Crumley, G., Kaplow, J. M., Schlessinger, J., Jaye, M., and Dione, C. A. (1991) EMBO J. 10, 2849–2854
16. Hack, N., Sue-a-Quan, A., Mills, G. B., and Skorecki, K. L. (1993) J. Biol. Chem. 268, 26441–26446
17. McMahon, S. B., Armanini, M. P., Ling, L. H., and Phillips, H. S. (1994) Neuron 12, 1161–1171
18. Verge, V. M. K., Wetmore, C., and Hockfeld, T. (1993) Soc. Neurosci. Abstr. 19, 1177
19. Rovelli, G., Heller, R. A., Caanossa, M., and Shooter, E. M. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 8717–8721
20. Gluzman, Y. (1981) Cell 23, 175–182
21. Greene, L. A., and Tishler, A. S. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2424–2428
22. Luthman, H., and Magnusson, G. (1983) Nucleic Acid Res. 11, 1295–1305
23. Squinto, S. P., Stitt, T. N., Atrich, T. H., Davis, S., Blanco, S. M., Radziejewski, C., Glass, D. J., Masakiowski, P., Furth, M. E., Valenzuela, D. M., DiStefano, P. S., and Yancopoulos, G. D. (1991) Cell 65, 885–893
24. Deleted in proof
25. Deleted in proof
26. Seed, B. (1987) Nature 329, 840–842
27. Hohnmann, H. P., Remy, R., Brockhaus, M., and van Loon, A. P. G. M. (1989) J. Biol. Chem. 264, 14927–14934
28. Obermeier, A., Halfter, H., Wismiuller, K. H., Jung, G., Schlessinger, J., and Ullrich, A. (1993) EMBO J. 12, 933–941
29. Valenzuela, D. M., Maisonnier, P. C., Glass, G., Rusas, E., Nuene, L., Kong, Y., Gies, D. R., Stitt, T., Ip, N. Y., and Yancopoulos, G. D. (1993) Nature 360, 963–974
30. Tcholias, P., Soppe, D., Escandor, E., Tesserall, N., Mendoza-Ramirez, J., Rosenthal, A., Nikolts, K., and Parada, L. F. (1993) Nature 10, 975–990
31. Urfer, R., Tsoufas, P., O'Connell, L., Shilton, D. L., Parada, L. F., and Presta, L. G. (1993) EMBO J. 12, 2795–2805
32. Deleted in proof
33. Deleted in proof
34. Deleted in proof
35. Russell, D. S., Gherzi, R., Ohnson, E. L., Chou, C.-K., and Rosen, O. M. (1987) J. Biol. Chem. 262, 11833–11840
36. Honegger, A. M., Dull, T. J., Bellot, F., Van Obberghen, E., Szapary, D., Schmidt, A., Ullrich, A., and Schlessinger, J. (1988) EMBO J. 7, 3045–3052
37. Khazaile, K., Dull, T. J., Graf, T., Schlessinger, J., Ullrich, A., Beug, H., and Vennstrom, B. (1988) EMBO J. 7, 3061–3071
38. Riedel, H., Dull, T. J., Honegger, A. M., Schlessinger, J., and Ullrich, A. (1988) EMBO J. 8, 2943–2954
39. Lee, J., Dull, T. J., Lax, I., Schlessinger, J., and Ullrich, A. (1989) EMBO J. 8, 167–173
40. Banner, D. W., D'Arcy, A., Janes, W., Gentz, R., Schoenfeld, H. J., Broger, C., Loetscher, H., and Lesslauer, W. (1993) Cell 73, 401–416
41. Barker, P. A., Lomen-Hoerth, C., Gensch, E. M., Meakin, S. O., Glass, D. J., and Shooter, E. M. (1993) J. Biol. Chem. 268, 1–8
42. Jungbluth, S., Bailey, K., and Barde, Y. A. (1993) Eur. J. Biochem. 221, 677–685
43. Radziejewski, C., and Robinson, R. C. (1993) Biochemistry 32, 13350–13356
44. Heymach, J. V., and Shooter, E. M. (1995) J. Biol. Chem. 270, 12297–12304