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Role of Variable β-Hairpin Loop in Determining Biological Specificities in Neurotrophin Family*

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The neurotrophins are members of a family of structurally and functionally related neurotrophic factors that control the development and maintenance of vertebrate neurons. The crystal structure of nerve growth factor (NGF), the prototypic member of this family, contains three pairs of anti-parallel β-strands connected by β-hairpin loops, which contain most of the variable residues among the four neurotrophin proteins. Recently, amino acid residues in these variable loop regions have been implicated in the interaction between NGF and its signal-transducing receptor TrkA. In NGF, residues 40–49 (variable region II) span a very flexible and solvent-accessible β-hairpin loop that is highly variable between different neurotrophins. To investigate the role of this domain in determining biological specificities in the neurotrophin family, we constructed a series of chimeric molecules by exchanging this variable region among three neurotrophins (*i.e.* NGF, neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4)) that bind and activate three different Trk receptors (*i.e.* TrkA, -C, and -B, respectively). The chimeric molecules were analyzed for their ability to activate different Trk receptor subtypes and to promote the survival of subpopulations of peripheral neurons expressing specific types of Trk mRNAs. Exchange of the 40–49 variable loop region between NGF and NT-3 resulted in molecules capable of activating both TrkA and TrkC receptors and of rescuing neurons containing TrkA and TrkC mRNAs, indicating that this loop plays an important role in determining biological specificities in these two neurotrophins. Furthermore, variable region II from NT-4 conferred the ability to differentiate nnr5 PC12-TrkB cells to a chimeric NT-3 molecule that was originally incapable of eliciting a response in these cells. In contrast, exchanges between NGF and NT-4 did not suffice to generate molecules with broader biological specificities, suggesting that other regions in these molecules are also required. Our results support the evolutionary relationships between the three polypeptides deduced from structural comparisons.

The neurotrophins are non-covalently linked homodimers of two highly basic 118-residue-long polypeptide chains. The neurotrophin family includes four structurally and functionally related members: NGF,[1] brain-derived neurotrophic factor (BDNF), NT-3, and NT-4, with a varied repertoire of biological activities influencing the generation, differentiation, survival, and regeneration of neurons (1–8). The neurotrophins share 50% of their amino acid sequence, and the regions of similarity and variation are clustered, indicating probable regions of structural and functional importance (9). The neurotrophins interact with two classes of receptors on responsive cells, a protein tyrosine kinase-type of receptor (members of the Trk family of receptors) (10–16) and a smaller receptor, distantly related to the tumor necrosis factor and CD40 receptors, containing a short cytoplasmic tail of unknown function (the p75 low affinity NGF receptor, $p75^{LNGFR}$) (17, 18). The four neurotrophins display specific sets of biological activities on peripheral and central neurons. This specificity correlates to some extent with their selective interaction with the different members of the Trk family of tyrosine kinase receptors. Thus, whereas NGF binds only to TrkA, BDNF and NT-4 exclusively interact with TrkB while NT-3 interacts with TrkC and, to a lesser extent, also with TrkA and TrkB. In contrast, all neurotrophins are able to bind with similar affinities to $p75^{LNGFA}$ (5, 19, 20). The three-dimensional crystal structure of NGF has recently been determined (21). The NGF protomer has a somewhat elongated structure with three pairs of anti-parallel β-strands connected by β-hairpin loop structures, which concentrate most of the variable residues among the four neurotrophins. These variable sequences have been grouped into seven different regions termed NH_2 (residues 1–9, NGF numbering), I (residues 23–35), II (residues 40–49), III (residues 59–66), IV (residues 79–88), V (residues 94–98), and COOH (residues 111–118), respectively (22, 23). Residues 32, 34, and 95 in NGF form a cluster of positive charges involved in the contact to $p75^{LNGFR}$ (24).

Previous results from structure-function studies have shown that specific combinations of variable regions I, III, IV, and V from NGF and BDNF resulted in chimeric molecules with a broader spectrum of neurotrophic activities than those of the two wild type proteins (22). Replacement of variable region II drastically reduced the level of protein production; thus, the importance of this region could not be assessed in those experiments. More recent studies have shown that in NGF and BDNF, discontinuous stretches of amino acid residues group together on one side of the neurotrophin dimer, forming a continuous surface responsible for binding to and activation of TrkA and TrkB receptors (23). Two symmetrical surfaces are formed along the 2-fold axis of the neurotrophin dimer, providing a model for ligand-mediated receptor dimerization. Residues 45–49 from variable region II localize in this binding

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§ Professor Håkan Persson passed away May 16, 1993 during the preparation of this study. His energy and inspiration will always be with us.

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[1] The abbreviations used are: NGF, nerve growth factor; NT-3, neurotrophin-3; NT-4, neurotrophin-4; BDNF, brain-derived neurotrophic factor; wt, wild type; chim, chimera.

surface, suggesting that they could constitute structural determinants of biological specificity in the neurotrophin family.

Here, we report the construction and characterization of stable chimeric neurotrophin molecules with exchanges in variable region II. A novel *in vitro* bioassay for the study of Trk subtype-specific neurotrophic activities is used, which evaluates the survival of subpopulations of peripheral neurons expressing specific types of Trk mRNAs. Exchange of variable region II between NGF and NT-3 and between NT-3 and NT-4, but not between NGF and NT-4, allowed broader biological specificities, suggesting that this loop region is an important determinant of biological specificity in the neurotrophin family.

EXPERIMENTAL PROCEDURES

Cells, Antisera, Plasmids, and Site-directed Mutagenesis—NIH3T3 fibroblasts expressing rat TrkA (NIH3T3 TrkA) or TrkB (NIH3T3 TrkB) were kindly provided by Luis Parada (NCI, Frederick, MD). MG3T3 fibroblasts expressing rat TrkC (MG3T3 TrkC) were kindly provided by George Yancopoulos (Regeneron Pharmaceuticals Inc., Tarrytown, NY). nnr5 PC12-TrkB cells are NGF non-responding mutant PC12 cells stably transfected with a TrkB cDNA, which only respond to BDNF and NT-4, and were kindly provided by Lloyd Greene (Columbia University, NY). Anti-TrkB polyclonal antiserum 443, which recognizes TrkA, TrkB, and TrkC, was kindly provided by Dr. David Kaplan (NCI). Anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology Inc. (New York). For the synthesis of riboprobes, fragments derived from chicken TrkA (kindly provided by Dr. Pantelis Tsouflas, NCI), TrkB (kindly provided by Dr. Tom Large, Cleveland, OH), and TrkC (kindly provided by Dr. Finn Hallböök, Biomedical Center, Uppsala, Sweden) cDNAs were subcloned into pBluescript KS$^+$ (Stratagene). Fragments containing the prepro-coding sequences from the rat NGF (25), rat NT-3 (4), *Xenopus* NT-4 (5), and rat NT-4 (8) genes were cloned into pBluescript KS$^+$. Single-stranded DNA from these plasmids was used as template for oligonucleotide-based site-directed mutagenesis as described by Kunkel (26), and the replacements were confirmed by nucleotide sequence analysis. For protein expression, DNA inserts containing the desired replacements were subcloned in the expression plasmid pXM as previously described (27).

Production and Quantitation of Recombinant Proteins—COS cells grown to about 70% confluency were transfected with 25 μg of plasmid DNA/100-mm dish using the DEAE-dextran-chloroquine protocol (28). To correct for differences in the amounts of recombinant protein produced by the different constructs, 35-mm dishes transfected in parallel were maintained in the presence of 100 μCi/ml [^{35}S]cysteine (Amersham Corp.). Aliquots of conditioned media were then analyzed by SDS-polyacrylamide gel electrophoresis, and the amounts of recombinant protein in the different samples were equilibrated after densitometer scanning of the corresponding autoradiograms as previously described (29). The absolute amount of wt NGF protein was determined by quantitative immunoblotting of conditioned media using an anti-NGF antibody and by measurement of biological activity in cultured sympathetic ganglia using standards of purified mouse NGF. The data obtained from these analyses were then used to determine the protein concentration in the samples containing wt NT-3, wt NT-4, and chimeric proteins. Wild type NGF, NT-3, and NT-4 were produced at high levels in COS cell-conditioned medium (150, 140, and 80 ng/ml, respectively). Chimeric molecules NGF II/NT-3, NGF II/NT-4, chim NT-3, and NT-4 II/chim NT-3 were also produced at high levels (250, 140, 60, and 50 ng/ml, respectively). Chimeric molecules NT-3 II/NGF and NT-4 II/NGF were produced at lower levels (10 and 14 ng/ml, respectively). All assays were performed using equivalent amounts of recombinant wild type and chimeric neurotrophins.

Binding Assays—Purified mouse NGF was labeled with ^{125}I by the lactoperoxidase method to an average specific activity of 1×10^8 cpm/μg. NIH3T3 TrkA were used at 2–10 × 10^6 cells/ml. Steady state binding was measured in competition assays as previously described (27) using serial dilutions of conditioned media containing equivalent amounts of wt or chimeric proteins.

Phosphorylation Assays—A confluent 10-cm plate containing about 10^7 NIH3T3 TrkA, NIH3T3 TrkB, or MG3T3 TrkC cells was treated for 5 min at 37 °C with wt or chimeric factors, subsequently lysed with 1 ml of ice-cold Nonidet P-40 lysis buffer, and analyzed for phosphorylation of Trk proteins by immunoprecipitation and Western blotting as previously described (23).

	40 49
rat NGF	GEVNINNSVF
rat NT-3	..IKTG..PV
xenopus NT-4	S.IQTLTGPL

FIG. 1. **Alignment of residues in variable region II (40–49 in NGF) from rat NGF and the homologous residues of rat NT-3 (3, 4) and *Xenopus* NT-4 (5).**

Biological Assays—Stimulation of neurite outgrowth from explanted chick embryonic day 9 (E9) sympathetic ganglia was assayed as previously described (30). A novel *in vitro* bioassay for the study of Trk subtype-specific neurotrophic activities was developed. Survival of specific subpopulations of developing peripheral neurons expressing different variants of Trk mRNAs was assayed in explanted or dissociated cultures of E8 chick dorsal root and sympathetic ganglia. Neurons were cultured for 48 h on poly-L-ornithine and laminin-coated 35-mm wells in the presence or absence of trophic factors. RNA was then extracted by homogenization in guanidine isothiocyanate and sodium acetate, pH 4.0, followed by phenol/chloroform extraction and ethanol precipitation. Prior to RNA extraction, a small amount of *in vitro* synthesized RNA complementary to each riboprobe was added to the guanidine isothiocyanate buffer to be used as recovery standard. Total RNA was analyzed for the presence of chicken TrkA, TrkB, or TrkC mRNAs using a very sensitive RNase protection assay (Ambion Inc.). Samples were then separated in 4% polyacrylamide denaturing gels and exposed to x-ray films with intensifying screens at −70 °C.

nnr5 PC12-TrkB cells plated in 24-well plates coated with rat collagen were incubated with conditioned media containing equivalent amounts (50 ng/ml final) of recombinant wild type and chimeric proteins. 48 h after plating, the percentage of cells bearing fibers longer than 2 cells in diameter was microscopically determined in five different fields of each well.

RESULTS

Using site-directed mutagenesis, chimeric molecules were generated by exchanging residues 40–49 (NGF numbering) between NGF and NT-3, ligands of the tyrosine kinase receptors TrkA and TrkC, respectively. In NGF, variable region II was replaced with that from NT-3, thus generating chimera NT-3 II/NGF, and in NT-3 variable region II was replaced with that from NGF, generating chimera NGF II/NT-3. These replacements resulted in the mutation of 6 residues in this domain of both NGF and NT-3 (Fig. 1). Wild type and mutated sequences were transiently expressed in COS cells (see "Experimental Procedures"). To correct for differences in the amounts of recombinant protein produced by the different constructs, 35-mm dishes transfected in parallel were maintained in the presence of 100 μCi/ml [^{35}S]cysteine. Aliquots of conditioned media were then analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2*A*), and the relative amounts of recombinant protein produced were established after densitometer scanning of the corresponding autoradiograms. The absolute amount of wt NGF protein was determined by quantitative immunoblotting of conditioned media, and combined with data from the metabolic labeling, it was used to determine the absolute protein concentration in the samples containing wt NT-3, wt NT-4, and chimeric proteins. In previous studies, replacement of region II in NGF with that from BDNF drastically reduced the level of protein produced, suggesting that foreign sequences in this hairpin loop in NGF may affect the synthesis and/or stability of the molecule. Chimera NT-3 II/NGF was produced at low but detectable levels (10 ng/ml) compared with wt NGF (150 ng/ml) (Fig. 2*A*). Chimera NGF II/NT-3 was produced at high levels (250 ng/ml), comparable with those obtained with wt NT-3 (140 ng/ml) (Fig. 2*A*). All assays were performed using equivalent amounts of recombinant wild type and chimeric neurotrophins.

Stimulation of NIH3T3-TrkA fibroblasts with wt NGF caused autophosphorylation of TrkA receptors (Fig. 3*A*). In contrast, stimulation with wt NT-3 resulted in only moderate lev-

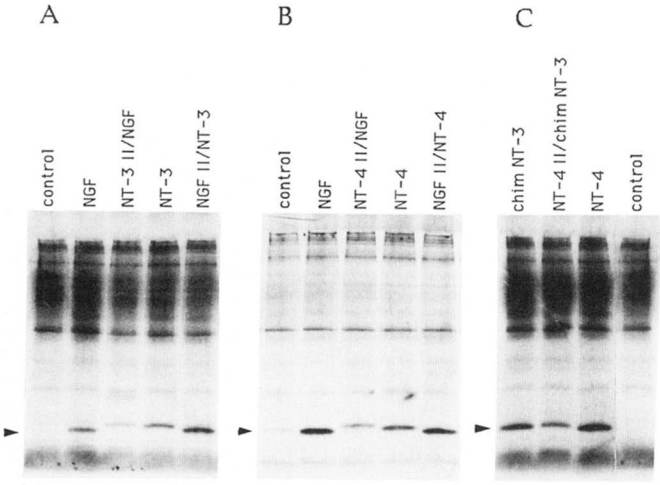

FIG. 2. **Production of wild type and chimeric neurotrophins in COS cell-conditioned media.** Metabolically labeled conditioned media of COS cells transfected with the indicated constructs was subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. The relative amounts of recombinant protein produced were established after densitometer scanning of appropriate exposures of the autoradiograms shown in *panels A*, *B*, and *C*. The absolute amount of wt NGF protein was determined by quantitative immunoblotting of conditioned media from dishes transfected in parallel, and combined with data from the metabolic labeling, it was used to determine the absolute protein concentration in the samples containing wt NT-3, wt NT-4, and chimeric proteins. All assays were performed using equivalent amounts of recombinant wild type and chimeric neurotrophins.

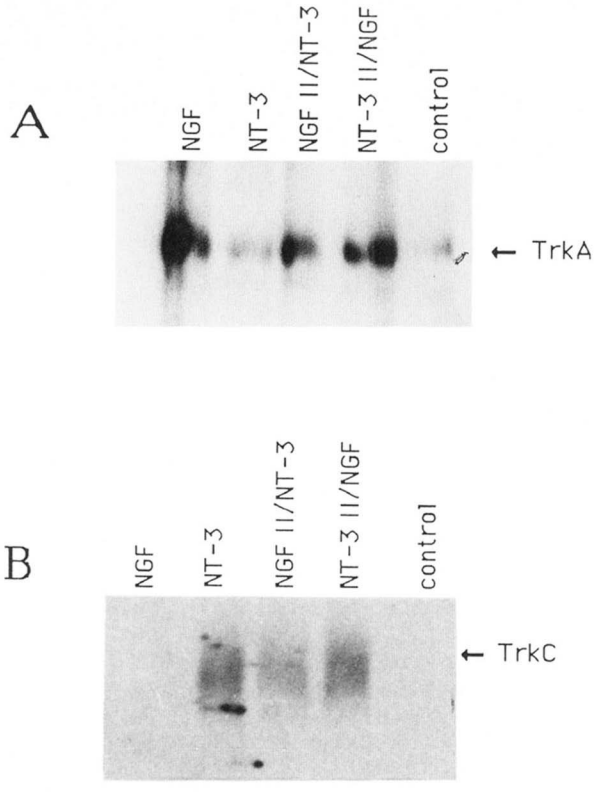

FIG. 3. **Tyrosine phosphorylation of TrkA (*A*) and TrkC (*B*) receptors stimulated by NGF, NT-3, and chimeric molecules NGF II/NT-3 and NT-3 II/NGF.** TrkA- and TrkC-expressing NIH3T3 fibroblasts were treated with 50 ng/ml of the indicated recombinant proteins and assayed for tyrosine phosphorylation. *Arrows* in *A* and *B* indicate the migration of phosphorylated TrkA ($p140^{trk}$) and TrkC ($p145^{trkC}$), respectively.

els of TrkA activation. However, replacement of region II in NT-3 with that from NGF allowed higher levels of TrkA activation, similar to those obtained with wt NGF (Fig. 3*A*). Treatment of MG3T3-TrkC fibroblasts with NT-3, but not with NGF, resulted in activation of TrkC receptors on these cells (Fig. 3*B*). Replacement of region II in NGF with that from NT-3 now allowed the chimeric molecule to stimulate TrkC autophosphorylation (Fig. 3*B*).

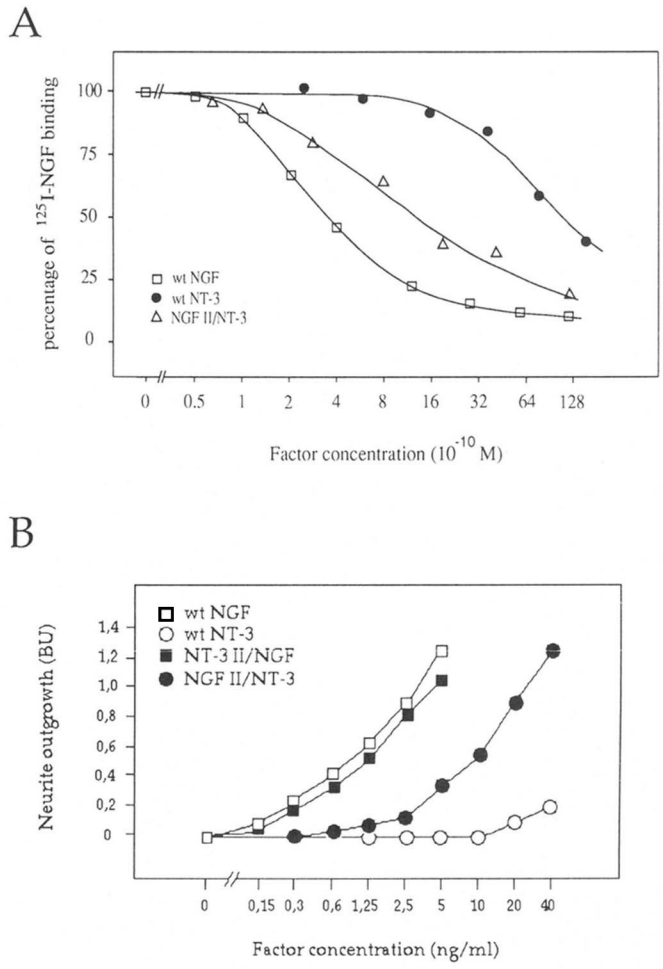

FIG. 4. **Binding to TrkA-expressing NIH3T3 fibroblasts (*A*) and biological activities in embryonic sympathetic neurons (*B*) of wt NGF, wt NT-3, and chimeric molecules NGF II/NT-3 and NT-3 II/NGF.** *A*, serial dilution of wt and chimeric factors were assayed for their ability to displace ^{125}I-NGF from receptors on NIH3T3 fibroblasts expressing TrkA. Medium from mock-transfected cells failed to displace ^{125}I-NGF from these cells. Each *point* represents the mean of triplicate determinations. Standard deviation was at or generally below ±10%. *B*, serial dilution of wt and chimeric factors were assayed for their ability to stimulate neurite outgrowth from explanted E9 chick sympathetic ganglia. Data from three determinations varied by ±20% of the average values reported here. *BU*, biological units.

In the case of the chimera NGF II/NT-3, its ability to activate TrkA correlated with an increased binding affinity to this receptor compared with the parental molecule, as shown in a competition binding assay (Fig. 4*A*). The affinity of NGF II/NT-3 to TrkA allowed dose-dependent stimulation of neurite outgrowth from explanted E9 chick sympathetic ganglia, which respond to NGF but not to NT-3 (Fig. 4*B*). To assess Trk subtype-specific biological activities, a sensitive RNase protection assay was developed to measure the amount of chicken TrkA, TrkB, or TrkC mRNAs in neuronal cultures after a 48-h incubation period in the presence of different trophic molecules. Dissociated neurons from E11 chick sympathetic ganglia survive in the presence of NGF, and the response to NT-3 at this developmental stage is much weaker (31). In these cultures, chimera NGF II/NT-3 rescued TrkA mRNA-containing neurons at similar levels than wt NGF (Fig. 5*A*). In contrast, only low

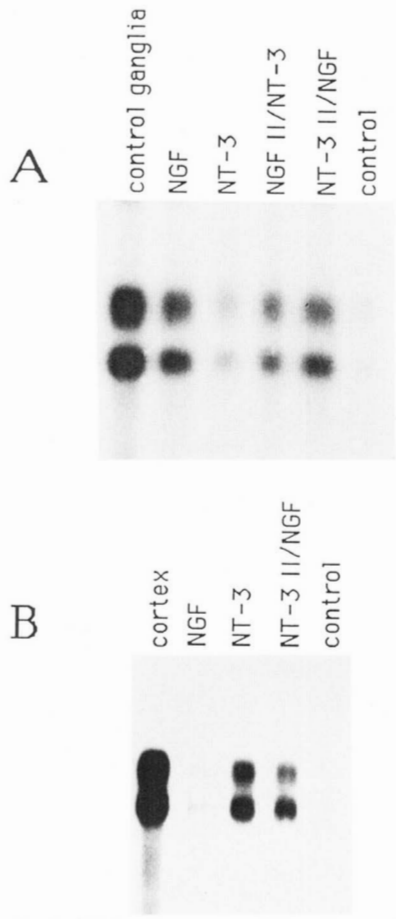

FIG. 5. **Survival of Trk subtype-specific subpopulations of neurons in embryonic peripheral ganglia with wt NGF, wt NT-3, and chimeric molecules NGF II/NT-3 and NT-3 II/NGF.** *A*, RNase protection analysis of TrkA mRNA in cultures of explanted E11 chick sympathetic ganglia 48 h after treatment with the indicated factors. RNA from cells treated with conditioned medium from mock-transfected cells (*control*) was used as negative control. Control ganglia were analyzed immediately after dissection. Both *bands* correspond to probe specifically protected by TrkA mRNA. *B*, RNase protection analysis of TrkC mRNA in cultures of explanted E8 chick dorsal root ganglia 48 h after treatment with the indicated factors. RNA from cells treated with conditioned medium from mock-transfected cells (*control*) was used as negative control. Chick cortex poly(A)$^+$ RNA (*cortex*) is used here as positive control. Both *bands* correspond to probe specifically protected by TrkC mRNA.

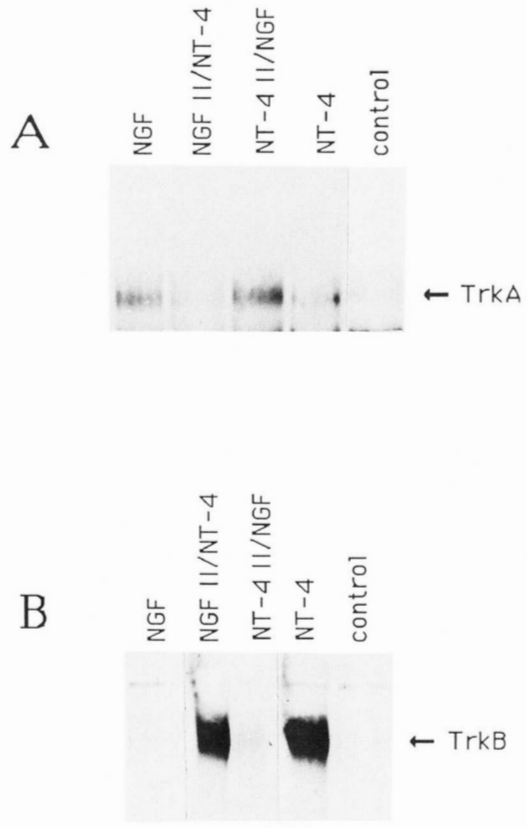

FIG. 6. **Tyrosine phosphorylation of TrkA (*A*) and TrkB (*B*) receptors stimulated by NGF, NT-4, and chimeric molecules NGF II/NT-4 and NT-4 II/NGF.** TrkA- and TrkB-expressing NIH3T3 fibroblasts were treated with 50 ng/ml of the indicated recombinant proteins and assayed for tyrosine phosphorylation. *Arrows* in *A* and *B* indicate the migration of phosphorylated TrkA (p140^{trk}) and TrkB (p145^{trkB}), respectively.

levels of TrkA mRNA were rescued by the parental molecule (wt NT-3) in these cultures. Chimera NT-3 II/NGF retained the ability to rescue TrkA mRNA-containing neurons, despite the replacement of variable region II with sequences from NT-3 (Fig. 5*A*). Embryonic chick dorsal root ganglia contain neurons responsive to all neurotrophins. Using a TrkC-specific riboprobe, survival of TrkC mRNA-containing neurons could be seen after 48 h of culture in the presence of NT-3. In contrast, NGF failed to rescue survival of these neurons (Fig. 5*B*). In agreement with its ability to stimulate TrkC autophosphorylation, chimera NT-3 II/NGF allowed survival of TrkC mRNA-containing neurons at levels comparable with those of wt NT-3 (Fig. 5*B*). These results show that exchange of variable region II between NGF and NT-3 generates chimeric molecules capable of cross-activating TrkA and TrkC receptors and promoting survival of neurons expressing either TrkA or TrkC mRNAs, demonstrating that residues in this loop are involved in determining the biological specificities of these two molecules.

We further analyzed the importance of this variable loop and constructed chimeric molecules exchanging residues 40–49 between NGF and NT-4, ligands of TrkA and TrkB receptors, respectively. In NGF, variable region II was replaced with that from NT-4, thus generating chimera NT-4 II/NGF, and in NT-4, variable region II was replaced with that from NGF, generating chimera NGF II/NT-4. These replacements resulted in the mutation of 9 residues in this domain of both NGF and NT-4 (Fig. 1). Wild type NT-4 and chimeras NT-4 II/NGF and NGF II/NT-4 were produced at 80, 14, and 140 ng/ml, respectively, in COS cell-conditioned media (Fig. 2*B*). All assays were performed using equivalent amounts of recombinant wild type and chimeric neurotrophins. In contrast to the exchanges between NGF and NT-3, no cross-activation of TrkA or TrkB receptors was observed in either of the two chimeras (Fig. 6, *A* and *B*). These results correlated with the performance of these molecules in biological assays. Chimera NGF II/NT-4, like its parental molecule wt NT-4, failed to induce neurite outgrowth from explanted sympathetic ganglia (Fig. 7*A*). On the other hand, chimera NT-4 II/NGF showed a similar potency in this assay when compared with the parental molecule wt NGF (Fig. 7*A*), indicating that replacement of variable region II from NGF with sequences from NT-4 did not interfere with the formation of a productive complex between NGF and the TrkA receptor. As expected, wt NT-4 rescued TrkB mRNA-containing neurons from developing chicken dorsal root ganglia (Fig. 7*B*). Chimera NGF II/NT-4 was equally active (Fig. 7*B*), suggesting that NT-4, like NGF, can accept changes in variable region II without loss of biological activity. However, chimera NT-4 II/NGF, like its parental molecule wt NGF, failed to promote survival of these neurons (Fig. 7*B*).

Given that NT-4 is structurally closer to NT-3 than to NGF (5, 8), we tested the possibility that variable region II from NT-4

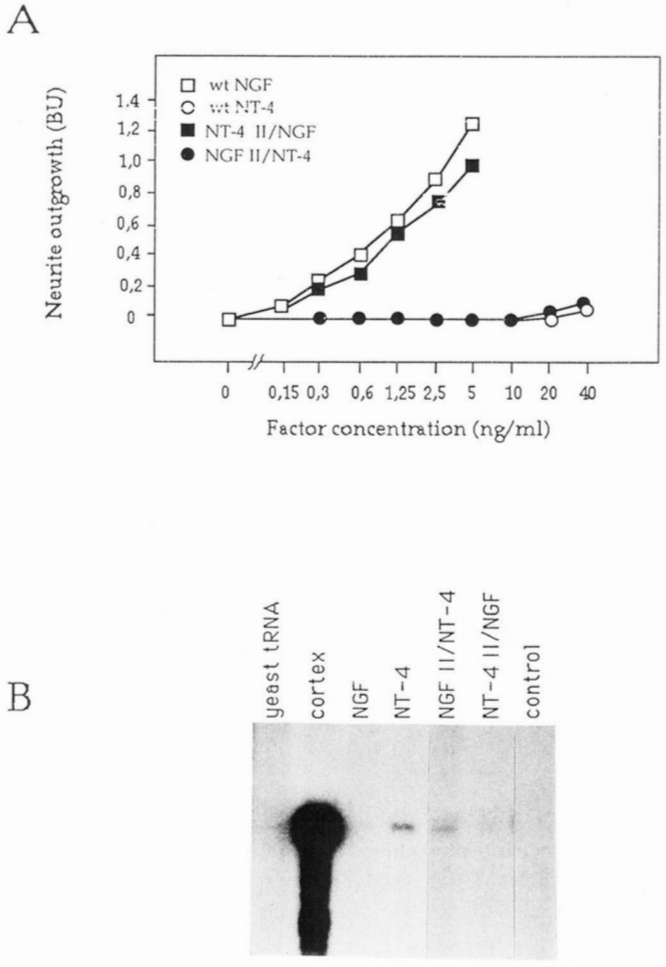

FIG. 7. **Biological activities of wt NGF, wt NT-4, and chimeric molecules NGF II/NT-4 and NT-4 II/NGF in embryonic peripheral ganglia.** *A*, serial dilution of wt and chimeric factors were assayed for their ability to stimulate neurite outgrowth from explanted E9 chick sympathetic ganglia. Data from three determinations varied by ±20% of the average values reported here. *B*, RNase protection analysis of TrkB mRNA in cultures of explanted E8 chick dorsal root ganglia 48 h after treatment with the indicated factors. RNA from cells treated with conditioned medium from mock-transfected cells (*control*) was used as negative control. Chick cortex poly(A)$^+$ RNA (*cortex*) and yeast tRNA are used here as positive and negative controls, respectively. *BU*, biological units.

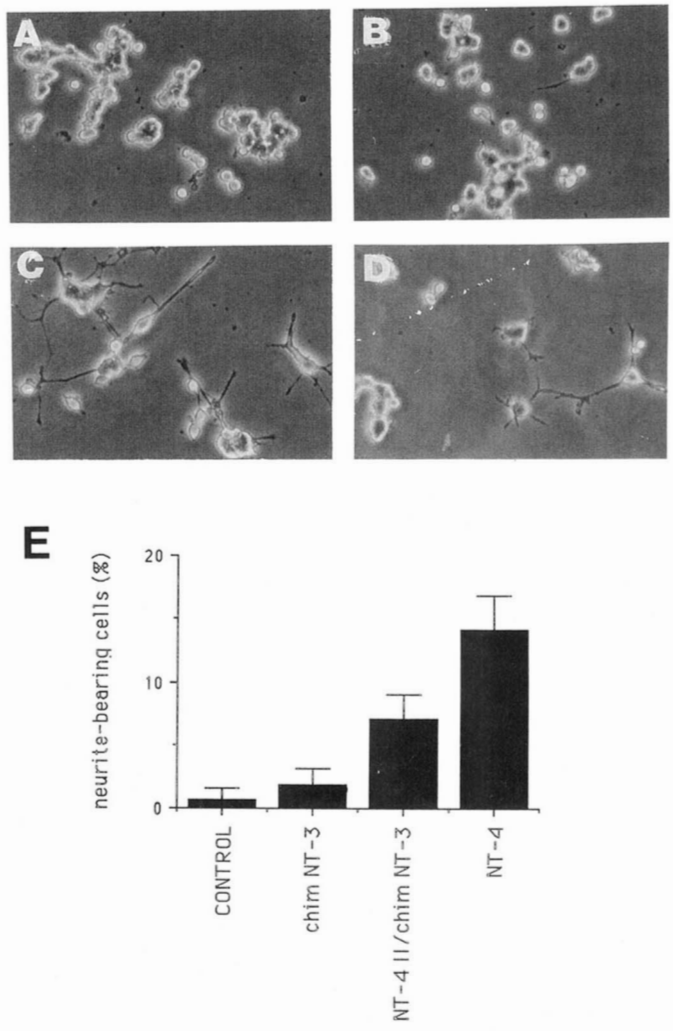

FIG. 8. **Variable region II from rat NT-4 confers TrkB-specific biological activity in nnr5 PC12-TrkB cells to a chimeric NT-3.** Phase contrast photomicrographs of nnr5 PC12-TrkB cells stimulated for 48 h with control medium (*A*) or 50 ng/ml of recombinant chim NT-3 (*B*), wild type rat NT-4 (*C*), or chimeric molecule NT-4 II/chim NT-3 (*D*). *E*, percentage of nnr5 PC12-TrkB cells bearing neurites longer than two cells in diameter in cultures treated with the indicated recombinant neurotrophins. *Values* represent the mean of five fields ±S.D.

may be able to confer to NT-3 the ability to activate TrkB in NT-4-responsive cells. Neither wild type NT-3 nor a chimeric NT-3 with residues 94–99 replaced with sequences from BDNF (chim NT-3) (23) was able to promote significant neurite outgrowth from nnr5 PC12-TrkB cells (NGF-non-responsive mutant PC12 cells stably transfected with a TrkB cDNA) (Fig. 8, *A* and *B*). In contrast, wild type rat NT-4 readily differentiated the cells into a neuron-like morphology (Fig. 8*C*). Replacement of variable region II in chim NT-3 with region II from rat NT-4 resulted in a molecule (NT-4 II/chim NT-3) capable of promoting outgrowth from nnr5 PC12-TrkB cells with a time course and extent comparable with those produced by wild type NT-4 (Fig. 8, *D* and *E*). Chimeric molecule NT-4 II/chim NT-3 was produced at high levels in COS cell-conditioned medium (Fig. 2*C*). The fact that the response to this molecule did not reach the level obtained with wt NT-4 suggests that other regions in the NT-4 molecule may also be required for full activation of TrkB receptors in nnr5 PC12-TrkB cells.

DISCUSSION

In this study, we have analyzed the importance of a highly variable β-hairpin loop in determining biological specificities in the neurotrophin family. Exchange of this variable region between NGF and NT-3, but not between NGF and NT-4, resulted in chimeric molecules capable of activating two different Trk receptors and displayed broader biological specificities on responsive neurons. The fact that residues in variable region II from NGF allowed a chimeric NT-3 to activate TrkA indicates that these residues are part of the binding site of NGF to the TrkA receptor. However, when placed in NT-4, this region did not result in broader specificities, suggesting that other residues in the NT-4 protein may interfere with the interaction between the chimera and the TrkA receptor. Thus NT-4, in contrast to NT-3, may have structural constrains in regions outside variable region II that prevent the formation of a productive complex with TrkA. It is interesting to note that NT-4, like BDNF, has an Arg residue at position 97, which, when introduced in NGF or NT-3, has been shown to interfere with the ability of these molecules to activate TrkA (23). In addition, NT-4 is the only known neurotrophin with a positive charge (Lys) at position 83, 1 additional residue in variable region I, and 3 additional residues in the NH_2 terminus, all regions previously shown to be implicated in binding to and activation of the TrkA receptor (23).

On the other hand, residues in variable region II may only be part of a more extended binding surface and therefore not sufficient to allow broader specificities in certain structural contexts. In agreement with this, recent studies have shown that in NGF and BDNF, the binding site to Trk receptors is formed by discontinuous stretches of amino acid residues that, in the NGF crystal structure, appear grouped on one side of the dimer, delineating an extended surface of binding (23). In agreement with our present results, residues 45–49 (included in variable region II) were shown to contribute to this surface, reinforcing the idea that they participate in direct interactions with Trk receptors. The fact that variable region II from NGF did not allow a chimeric NT-4 to bind and activate TrkA could therefore also be due to the fact that residues important for TrkA binding in regions other than 40–49 may be absent in the NT-4 protein. Interestingly, structural comparisons based on alignments of primary sequences of neurotrophins have revealed a closer relationship between NGF and NT-3 than between NGF and neurotrophins activating TrkB receptors, namely BDNF and NT-4 (5). In these comparisons, NT-3 appears to be equally related to both NGF and NT-4 (or BDNF) in agreement with its ability to partially activate TrkA and TrkB receptors in non-neuronal cells (14, 15, 32, 33). Thus, several residues important for binding of NGF to TrkA (*e.g.* Ile-31, His-8) are also conserved in NT-3. Therefore, replacement of short segments of residues from the binding surface of the NT-3 protein with NGF sequences may thus result in a chimeric molecule with extended biological specificities. In contrast, a greater number of residues may need to be replaced in the binding surface of NT-4 to achieve NGF specificities in this molecule. However, this hypothesis would predict that exchange of variable region II between the more closely related NT-3 and NT-4 (as opposed to NGF and NT-4) should result in chimeric molecules with broader biological specificities. Support for this hypothesis was obtained from the demonstration that variable region II from NT-4 was able to confer the ability to differentiate nnr5 PC12-TrkB cells to a chimeric NT-3 that was originally incapable of eliciting a response in these cells. Thus, together, our results indicate that this variable loop contains residues that contribute to the specificity of activation of all three subtypes of Trk receptors by the neurotrophins.

In variable region II, NGF and NT-3 differ in 6 amino acid residues (Fig. 1). At two of these positions (42 and 49) both molecules conserve the hydrophobic character of the residue. However, at position 48 Val in NGF is replaced by Pro in NT-3. This change is expected to alter the conformation of the loop by reducing the length of β-strand B, which in NGF extends from positions 47 to 58 (21). In NGF, the remaining 3 variable residues are located at the tip of the loop (positions 43–45) (21) and comprise a motif (NIN) that is characteristic of this neurotrophin and conserved in all mammalian, avian, and amphibian NGFs described to date (34–36). Similarly, the KTG motif at equivalent positions in NT-3 is unique to this neurotrophin. Together with our previous results obtained with chimeric molecules between NGF and BDNF (23), these data suggest that in these neurotrophins, residues at positions 43–45 and 48 are making contact with cognate Trk receptors and contribute to the biological specificity of these molecules. It should be pointed out that these results also indicate that other regions of the neurotrophin molecule are also necessary for full, monospecific biological activity. The fact that the exchanges between NGF and NT-3 gave reciprocal results indicates that determinants of binding to and activation of different Trk receptors are located in homologous positions in different neurotrophins. These data reinforce the idea that a parallel evolution of cognate neurotrophins and Trks has developed specific contacts through different residues in the same variable regions of the ligands.

In conclusion, our results demonstrate the importance of residues in region 40–49 in directing the specificity of the contact to and activation of Trk receptors by neurotrophins and suggest that in the neurotrophin family, specificity is determined by a set of residues that allows both active and permissive interactions with a complementary surface in the receptor binding site.

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