Specificity Determinants in Neurotrophin-3 and Design of Nerve Growth Factor-Based trkC Agonists by Changing Central β-Strand Bundle Residues to Their Neurotrophin-3 Analogs[†]

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ABSTRACT: Neurotrophic factors mediate their signal by binding to specific cell surface receptors of the trk family. The binding sites of neurotrophin-3 (NT-3) and nerve growth factor (NGF) to their preferred receptors trkC and trkA, respectively, were previously determined by mutational analyses. These and other studies showed that trkA can discriminate between NGF and NT-3 primarily by recognition of their N-terminal residues. The mechanism of trkC discrimination, however, remained unclear, especially since the most important residue in NT-3 involved in binding to trkC, R103, is conserved in all neurotrophins. In this study residues that are part of the central β-strand bundle of NT-3 and are not conserved among the neurotrophins were grafted onto NGF and tested for recruitment of trkC affinity. Exchange of NGF residues at positions 18, 20, 23, 29, 84, and 86 by their NT-3 counterparts resulted in NGF variants that bound to trkC, while maintaining their affinity to trkA, and were able to induce autophosphorylation and differentiation of PC12 cells expressing trkC. These variants show that the amino acid at position 23 (glycine in NGF, threonine in NT-3) is critical for trkC recognition while other residues fine tune the specificity of NT-3 for trkC. The results demonstrate the importance of nonconserved residues of the central β-strand bundle region for the interaction of NT-3 with trkC and emphasize the different mechanism of specificity determination that is employed in the NT-3/trkC and NGF/trkA ligand/receptor pairs.

The neurotrophins form a highly homologous family of growth factors that are important for survival and maintenance of neurons during developmental and adult stages of the vertebrate nervous system [for review see Snider (1994)]. The family consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT4/5), and neurotrophin-6 (NT-6) (Barde, 1991; Götz et al., 1994). The neurotrophins mediate their signal into the cell by specific interaction with members of the *trk* gene family. Binding of the neurotrophins induces autophosphorylation of the trk receptors which triggers the subsequent steps in the signal transduction cascade (Kaplan & Stephens, 1994).

NGF interacts exclusively with trkA (Kaplan et al., 1991) while BDNF and NT4/5 bind to trkB (Ip et al., 1993). NT-3 signals preferably through trkC but can also bind to trkA and trkB with lower affinity (Lamballe et al., 1991; Urfer et al., 1994) (Figure 1). Thus, the most stringent member of the trk receptors in terms of specificity (trkC) interacts exclusively with the most promiscuous ligand (NT-3) of the neurotrophin family. The elucidation of the structural determinants for neurotrophin specificity is important for understanding the function and evolution of this family of growth factors. Since the neurotrophins have become candidates for therapeutics for a variety of neurodegenerative diseases, knowledge of the structural mechanism of neurotrophic specificity and function will help develop novel neurotrophin-based therapeutics.

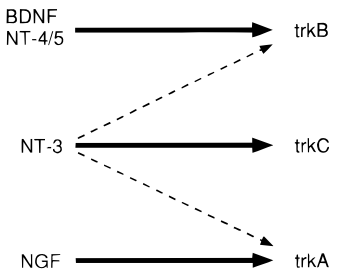

FIGURE 1: Specificities of neurotrophin/trk receptor interactions.

The three-dimensional structures of several neurotrophins have been resolved by X-ray crystallography (McDonald et al., 1991; Holland et al., 1994; Robinson et al., 1995). Extensive mutational analyses of human NT-3 (Urfer et al., 1994) and mouse and human NGF (Ibáñez et al., 1993; Shih et al., 1994) defined the binding sites for trkC and trkA, respectively. In NGF the N-terminal residues contribute significantly to affinity for trkA (Shih et al., 1994) and provide the most important determinants for specificity (Ibáñez et al., 1993; Urfer et al., 1994). For NT-3 it has been demonstrated that the epitope for trkC is formed by residues in the central β-strand bundle region but does not include residues from nonconserved loops or the first six residues of the N-terminus (Urfer et al., 1994). However, a nonconserved β-hairpin loop encompassing residues 40−

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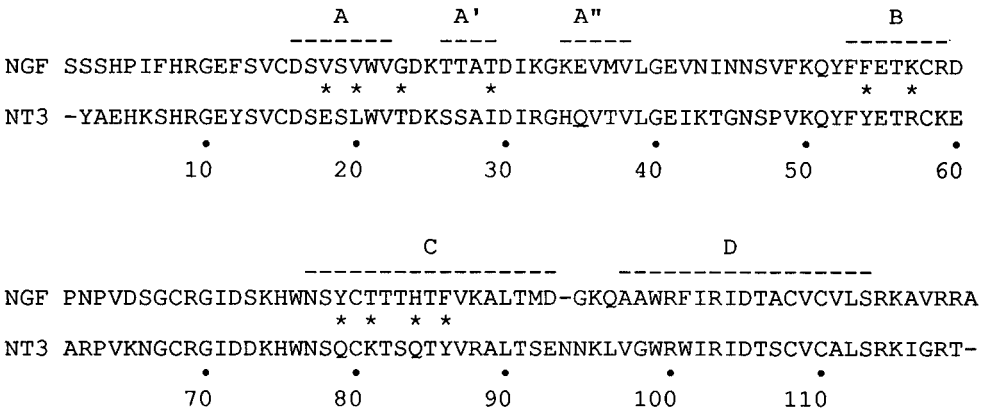

FIGURE 2: Sequence alignment of human NGF and human NT-3. Residue numbers refer to the NGF sequence throughout the paper. Asterisks highlight NGF residues which were mutated in the variants analyzed in this study. Bars indicate locations of β-strands in the X-ray structure of murine NGF (McDonald et al., 1991).

49 (NGF residue numbers will be used throughout the text) has been proposed to mediate trkA/trkC specificity (Ilag et al., 1994), though this loop does not contribute to NT-3 binding to trkC (Urfer et al., 1994). Since the trkC binding site on NT-3 is dominated by residues in the central β-strand bundle region, it was previously hypothesized that trkC recognizes this set of residues to distinguish NT-3 from the other neurotrophins (Urfer et al., 1994). In order to test this hypothesis, a set of five residues located in this structural region was transferred from human NT-3 to human NGF (Figure 2). The resulting NGF variant G23T/V18E/V20L/T81K/H84Q (NGF12) bound to trkC, maintained its affinity to trkA, and stimulated autophosphorylation and differentiation of PC12 cells expressing trkC. Further mutagenesis revealed that the most important determinants for specific trkC binding are located at positions 23 and 84 and that residues at positions 18, 20, 29, and 86 fine tune specificity for trkC. The results demonstrate the importance of non-conserved residues of the central β-strand bundle region for the interaction of NT-3 with trkC, emphasize the different mechanism of specificity determination that is employed in the NT-3/trkC and NGF/trkA ligand/receptor pairs, and support the proposal that the overall structure of neurotrophins, in contrast to short amino acid "active site" segments, may determine neurotrophin specificity (Suter et al., 1992).

EXPERIMENTAL PROCEDURES

Mutagenesis and Recombinant DNA Manipulations. NGF and NT-3 were previously cloned, sequenced, and subcloned into a vector which allows for production of double- and single-stranded DNA in *Escherichia coli*, as well as expression of the neurotrophins in a mammalian system under control of the cytomegalovirus promoter (Rosenthal et al., 1990). Mutagenesis on this vector was performed according to the method of Kunkel (1985). After transformation into the *E. coli* strain XL1-Blue (Stratagene, San Diego, CA), colonies were screened for the presence of the desired mutation by sequencing double-stranded DNA using the Sequenase version 2.0 kit (U.S. Biochemical Corp., Cleveland, OH). The entire DNA sequence coding for the mature NGF and NGF variants was verified for all positive clones. Double-stranded DNA was isolated from XL1-Blue with the QIAGEN DNA purification kit (Qiagen Inc., Chatsworth, CA).

Expression of Wild-Type and Variant Neurotrophins. Plasmid DNA containing either the NGF or variant coding sequences was introduced into the human fetal kidney cell line 293 by calcium phosphate precipitation (Gorman et al., 1990). The 75% confluent cells were cotransfected with 10 μg of plasmid DNA and 1 μg of AdVA plasmid per 15 mm cell culture dish and incubated for 15 h in serum-containing medium. Then the medium was removed and exchanged with serum-free medium (PSO4) supplemented with 10 mg/L recombinant bovine insulin, 1 mg/L transferrin, and trace elements. Supernatant was collected after 48 and 96 h, concentrated approximately 20-fold with Centriprep-10 filtration units (Amicon, Beverly, MA), and sterile filtered.

Quantification of Neurotrophin Variants. The specific NGF ELISA[1] was based on a protein A-purified polyclonal antiserum from guinea pig (a gift of Dr. D. Sinicropi, Genentech) and followed standard ELISA procedures. A polyclonal serum was used in order to reduce the potential for differential cross-reactivity of variants to the antibodies. The standard curve was determined using purified recombinant NGF.

The amounts of NGF variants after concentration varied between 0.3 and 30 μg/mL. The ELISA assay did not detect any NGF in supernatants from mock transfected cells. For each set of expressions of NGF variants, a wild-type NGF expression was performed and quantified by ELISA in parallel in order to obtain a comparative wild-type concentration for receptor binding studies. All variants were expressed, quantified, and assayed at least twice.

Iodination. Purified recombinant human NT-3, BDNF, and NGF (Genentech) was iodinated as described (Urfer et al., 1994). Usually, 20 μg of the neurotrophins were iodinated to specific activities ranging from 2000 to 3000 Ci/mmol. The labeled material was stored at 4 °C and used within 2 weeks of preparation.

Binding Assays. Receptor immunoadhesin proteins were constructed using human trkA and trkC extracellular domains fused to immunoglobulin constant domains (Shelton et al., 1995). Binding assays were performed as described (Shelton et al., 1995) using a 96-well plate format. The final concentration of labeled neurotrophin in each well was approximately 30 pM for trkA and trkC binding assays. Variants were assayed for binding affinity to the trkA and trkC receptor at least twice for each of the multiple expressions. This procedure allowed estimation of the error in affinity determination for each of the variants. All data

[1] Abbreviations: ELISA, enzyme-linked immunosorbent assay; IC50, concentration of neurotrophin variant resulting in 50% inhibition of binding of native neurotrophin.

were analyzed by applying a four-parameter fit procedure on the data set with the Kaleidagraph software package. Binding results in Table 1 are expressed as $IC50_{mut}/IC50_{wt}$.

Stimulation of trk Receptor Autophosphorylation on PC12 Cell Lines by Neurotrophic Factors. Approximately 1×10^7 PC12 cells (Tsoulfas et al., 1993) were treated at 37 °C for 5 min with 100 ng/mL neurotrophin. NP-40 plate lysis and immunoprecipitation with an anti-trkA-specific polyclonal antiserum (a gift from Dr. Louis Reichardt, University of California, San Francisco) or anti-trkC-specific polyclonal antiserum 656 (Urfer et al., 1994) was performed as previously described (Tsoulfas et al., 1993). The phosphotyrosine content was analyzed by Western blot using monoclonal antibody 4G10 as previously described (Soppet et al., 1991; Tsoulfas et al., 1993). All tyrosine autophosphorylation assays were performed at least twice for each neurotrophin assayed.

Differentiation Assays on PC12 Cells Expressing trkC. Approximately 10^3 PC12 cells expressing trkC (Tsoulfas et al., 1993, 1996) were plated onto 35 mm collagen-coated tissue culture dishes containing a total of 2 mL of medium. PC12/trkC cells were assayed at neurotrophin concentrations ranging from 250 pg/mL to 100 ng/mL. The proportion of neurite-bearing cells was determined by counting the number of cells containing processes at least twice the length of the cell body after 3 days. All neurite extension assays were performed at least twice.

RESULTS

Design of an NGF Variant That Binds to trkC. The complete mutational analysis of human NT-3 led to a detailed view of the binding epitope of NT-3 for its receptor trkC (Urfer et al., 1994). The binding site is dominated by a single residue, R103 (Figure 3A). Analysis of the structural vicinity of R103 revealed additional residues important for the NT-3/trkC interaction: K81 and Q84 on β-strand C, T23 on the loop connecting β-strands A and A′, and, with smaller effects, the two conserved residues E55 and R57 on β-strand B (Figures 2 and 3A). In mouse NGF, T81 and H84, the residues analogous to NT-3 K81 and Q84 (Figures 2 and 3B), have been shown to be involved in NGF binding to trkA (Ibáñez et al., 1993), and it is therefore possible that they contribute to the specificity. In human NGF, V18, G23, T81, and H84 have been shown to be involved in binding (Dr. J. Winslow, Genentech, unpublished results). The other nonconserved NT-3 residue, T23, is located in an area that is conserved within each member of the neurotrophins across species but is divergent between NT-3, BDNF, and NGF. Near T23 and located on the same face of the molecule are E18 and L20 (Figure 3A). Although they are not directly involved in binding to trkC, their structurally very different counterparts in NGF (V18 and V20) (Figure 3B) and BDNF (I18 and E20) may prevent binding to trkC. This suggested that the NGF residues V18, V20, G23, T81, and H84 and their respective counterparts in NT-3 are involved in determining specificity for trkC (Urfer et al., 1994). Therefore, variants of NGF that carried the NT-3 amino acids at these positions were constructed and analyzed for recruitment of trkC binding.

Mutations in the Central β-Strand Bundle of NGF Result in Variants That Bind to trkC. The variants NGF1 and NGF2 carried the mutations T81K/H84Q and G23T/V18E/V20L, respectively. The five point mutations were combined in the variant NGF12. These three variants, as well as NGF and NT-3, were expressed and assayed for their ability to bind to the trkC extracellular domain. In competitive displacement binding assays, the NT-3 wild type displayed an affinity of 21.0 ± 4.9 pM for trkC while NGF bound to this receptor with an affinity reduced by 3587-fold compared to NT-3 (Table 1). The variants NGF1 and NGF2 bound to trkC with 1036-fold and 291-fold lower affinity than NT-3, respectively (Table 1); this represents gains of affinity to trkC, when compared to NGF, of 3.5-fold and 12-fold, respectively. When the mutations in NGF1 and NGF2 were combined in the variant NGF12, the affinity to trkC was substantially increased in a synergistic manner. This variant bound to trkC with only 14.7-fold reduced affinity compared to NT-3, which represents a 244-fold increase of affinity when compared to NGF (Table 1).

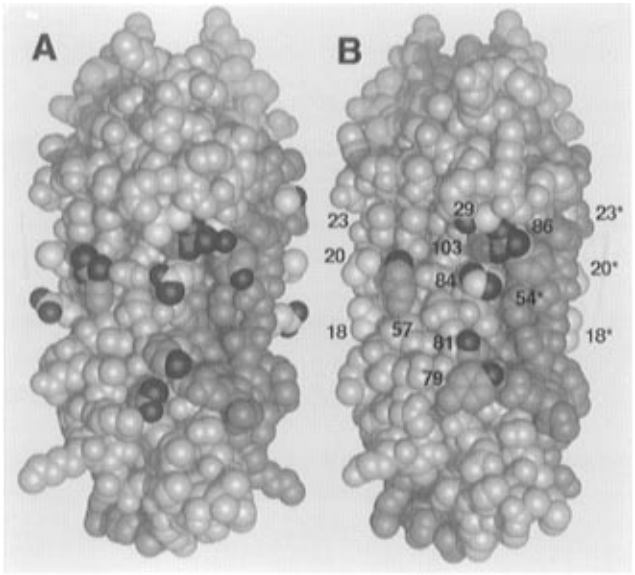

FIGURE 3: Model of human NT-3 (A) and crystal structure of murine NGF (B). The two monomers of each neurotrophin are shown in light gray and blue-gray; residue numbers in the NGF gray monomer are denoted with an asterisk. For highlighted residues, side chain oxygen atoms are in red and side chain nitrogen atoms are in blue. Residue 103 (Arg in both NGF and NT-3) is in purple. NGF residues which were replaced with their NT-3 counterparts and affected binding and specificity are in yellow; residues which did not affect binding and specificity are in green. The first residue seen in the NGF crystal structure (residue 10) is in brown. The variable β-hairpin loop (residues 40−49) previously proposed to affect specificity (Ilag et al., 1994) is shown in cyan.

All NGF variants, NGF, and NT-3 were assayed for trkA binding. NGF displayed an affinity of 33.9 ± 7.5 pM while NT-3 bound with 137-fold reduced affinity compared to NGF (Table 1). This reduction in affinity is in agreement with earlier results (Urfer et al., 1994). The variants NGF1, NGF2, and NGF12 bound to trkA with 0.7-fold, 2.1-fold, and 1.5-fold reduced affinity, respectively (Table1). This demonstrates that the changes in NGF2 resulted in a slight loss of affinity to trkA while changes in NGF1 led to a small increase in affinity. When NGF1 and NGF2 were combined in NGF12, the affinity of NGF12 to trkA was additive; in contrast the affinity of NGF12 to trkC was synergistic when compared to NGF1 and NGF2 (Table 1).

Importance of Individual Residues for trkC Specificity. In order to determine the importance for specificity of individual residues that were changed in NGF12, each of these residues was changed back to the NGF sequence. Variants NGFR1, NGFR2, NGFR3, NGFR4, and NGFR5 (Table 1) tested the

Table 1: Relative Affinities of NGF Variants to trkC and trkA[a]

	residue no.								receptor		
variant	18	20	23	29	79	81	84	86	trkA $IC50_{mut}/IC50_{NGF}$	trkC $IC50_{mut}/IC50_{NT-3}$	trkC $IC50_{mut}/IC50_{NGF12}$
NGF	V	V	G	T	Y	T	H	F	1.0 ± 0.1	3587 ± 771	
NT-3	*E*	*L*	*T*	*I*	*Q*	*K*	*Q*	*Y*	137 ± 43	1.0 ± 0.2	
NGF1	V	V	G	T	Y	*K*	*Q*	F	0.7 ± 0.2	1036 ± 184	
NGF2	*E*	*L*	*T*	T	Y	T	H	F	2.1 ± 0.6	291 ± 75	
NGF12	*E*	*L*	*T*	T	Y	*K*	*Q*	F	1.5 ± 0.7	14.7 ± 4.8	1.0
NGFR1	*E*	*L*	G	T	Y	*K*	*Q*	F	1.1 ± 0.2	3560 ± 875	242
NGFR2	V	*L*	*T*	T	Y	*K*	*Q*	F	0.9 ± 0.2	10.5 ± 2.5	0.7
NGFR3	*E*	V	*T*	T	Y	*K*	*Q*	F	1.6 ± 0.2	37.2 ± 10.1	2.5
NGFR4	*E*	*L*	*T*	T	Y	T	*Q*	F	1.5 ± 0.3	14.8 ± 5.1	1.0
NGFR5	*E*	*L*	*T*	T	Y	*K*	H	F	2.2 ± 0.4	113 ± 40	7.7
NGF123	*E*	*L*	*T*	*I*	Y	*K*	*Q*	F	1.2 ± 0.1	17.9 ± 7.3	1.2
NGF124	*E*	*L*	*T*	T	*Q*	*K*	*Q*	*Y*	1.1 ± 0.2	7.8 ± 2.0	0.5
NGF125	*E*	*L*	*T* + F54*Y*/K57*R*	T	Y	*K*	*Q*	F	1.2 ± 0.3	14.0 ± 5.4	0.9
NGF1234	*E*	*L*	*T*	*I*	*Q*	*K*	*Q*	*Y*	1.3 ± 0.2	4.9 ± 1.5	0.3
NGF126	*E*	*L*	*T*	T	Y	*K*	*Q*	*Y*	1.6 ± 0.1	3.3 ± 0.8	0.2
NGF127	*E*	*L*	*T*	T	*Q*	*K*	*Q*	F	1.0 ± 0.1	33.0 ± 6.0	2.2
NGF130	V	*L*	*T*	T	Y	T	*Q*	*Y*	1.0 ± 0.1	4.5 ± 0.7	0.3
NGF131	V	*L*	*T*	*I*	Y	T	*Q*	*Y*	1.1 ± 0.1	3.3 ± 0.9	0.2

[a] Affinities to trkA and trkC are shown relative to NGF and NT-3, respectively. The IC50 values were 33.9 ± 7.5 pM ($n = 12$) for NGF binding to trkA and 21.0 ± 4.9 pM ($n = 16$) for NT-3 binding to trkC. The results for variant affinities are expressed as the average of at least four independent binding experiments using proteins from two different expressions ± SD. NT-3 residues are shown in *italic* type.

contribution to specificity of G23, V18, V20, T81, and H84, respectively. Variant NGFR1 lost most of its ability to interact with trkC, NGFR3 and NGFR5 had their affinities significantly reduced, while NGFR2 and NGFR4 had affinities to trkC similar to NGF12 (Table 1). These data suggested that the most important specificity determinants for trkC binding are T23/G23, Q84/H84, and L20/V20 (NT3/NGF).

NGF12 Variants with Increased Affinity to trkC. Comparison of the model of human NT-3 (Urfer et al., 1994) and the X-ray structure of mouse NGF (McDonald et al., 1991) revealed several residues close to the main specificity determinants that differ between the two molecules. In order to further increase the affinity of NGF12 to trkC, some of these residues were changed to the analogous NT-3 amino acids. Adding the changes F54Y/K57R to NGF12 did not improve trkC binding (NGF125, Table 1). In contrast, addition of Y79Q/F86Y did enhance binding 2-fold compared to NGF12 (NGF124, Table 1). Individually changing these two residues in the NGF12 background showed that the F86Y exchange was a beneficial one while the Y79Q exchange was detrimental (NGF126 and NGF127, Table 1). Finally, the change T29I was evaluated. In the NGF12 background, T29I effected a slight decrease in binding (compare NGF12 versus NGF123, Table 1), but in the NGF124 and NGF126 backgrounds it slightly improved binding (compare variants NGF124 versus NGF1234 and NGF130 versus NGF131, Table 1). This may be due to interaction of the side chains at positions 29, 86, and 103 since residue 103 is situated between residues 29 and 86 (Figure 3). Alternatively, side chains at positions 29 and 86 may interact with the same amino acid in trkC and thereby influence one another via the trkC amino acid.

On the basis of these data, two additional variants were designed. Variant NGF131 contained the five changes V20L, G23T, T29I, H84Q, and F86Y and bound to trkC with an affinity that is only 3.3-fold reduced compared to NT-3. These changes did not affect trkA binding (Table 1). NGF126 and NGF131 bind equally well to trkC and trkA. Both variants have the mutations V20L, G23T, H84Q, and F86Y in common; NGF126 has the additional mutations V18E and T81K while NGF131 has the additional mutation T29I. This suggests that the changes V20L, G23T, H84Q, and F86Y in NGF are the minimum required for recruitment of trkC binding. Indeed, NGF130, which possesses these four mutations, bound to trkC similar to NGF126 and NGF131 (Table 1).

Induction of trkC Signaling by NGF Variants. PC12 cells that were engineered to constitutively express rat trkC respond to NT-3 by induction of strong autophosphorylation of trkC and formation of neurite extensions (Tsoulfas et al., 1993). Purified NGF (NGF/P) as well as the supernatant of NGF-expressing 293 cells (NGF/U) resulted in a strong signal for trkA autophosphorylation (Figure 4A) but did not induce autophosphorylation of trkC (Figure 5A). Purified NT-3 (NT-3/P) and the supernatant of NT-3-expressing 293 cells (NT-3/U) induced autophosphorylation of trkC (Figure 5A) but not trkA (Figure 4A). As expected from the affinity of NGF12 for trkC, this variant resulted in a strong signal for trkC autophosphorylation (Figure 5A) while maintaining its ability to elicit autophosphorylation of trkA (Figure 4A). The rather low affinity for trkC of the variants NGF1 and NGF2 is reflected in the weak signal in trkC autophosphorylation (Figure 5A). However, both variants still induced autophosphorylation of the trkA receptor with only slightly reduced activity when compared to NGF (Figure 4A).

Variants NGFR1 and NGFR5 were assayed for induction of autophosphorylation of PC12/trkC cells, and NGFR1 resulted in a very weak signal while the NGFR5 response was between that of NGF12 and NGFR1 (Figure 5B). These results correlate with the determined affinites of the variants for trkC. Variants of NGF12 (NGF123, NGF124, NGF125, and NGF1234) that further increased the affinity to trkC resulted in signals for trkC autophosphorylation that were similar to that elicited by NT-3 (Figure 5B). The three variants which bound best to trkC—NGF126, NGF130, and NGF131—also elicited strong signals for autophosphorylation of trkC (Figure 5C) as well as of trkA (Figure 4B). These results demonstrate that the NGF variants that had increased affinity to trkC were also able to interact with this receptor

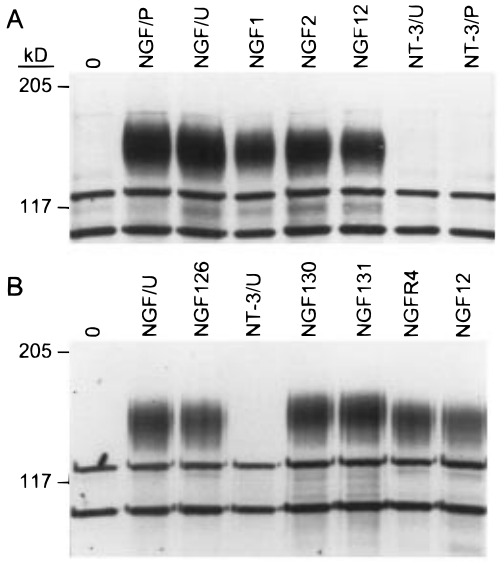

FIGURE 4: Tyrosine phosphorylation of trkA in PC12 cells expressing rat trkC. Cells were treated with 100 ng/mL quantities of the respective neurotrophin for 5 min. Lysates were equalized for cell protein, immunoprecipitated with an anti-trkA-specific polyclonal antiserum, and electrophoresed on 7.5% SDS−polyacryamide gels. Tyrosine phosphorylation was detected using an anti-phosphotyrosine mAb 4G10. Abbreviations: NGF/P, purified NGF; NGF/U, concentrated supernatant of NGF-expressing 293 cells; NT-3/P, purified NT-3; NT-3/U, concentrated supernatant of NT-3 expressing 293 cells; 0, mock-treated 293 cells. (A) and (B) show results from two separate experiments using the neurotrophins listed above each lane.

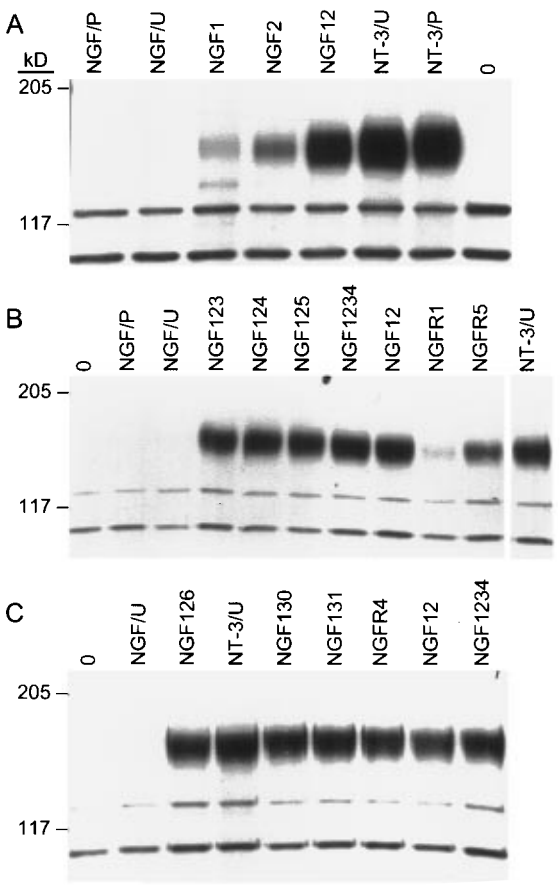

FIGURE 5: Tyrosine phosphorylation of trkC in PC12 cells expressing rat trkC. Cells were treated with 100 ng/mL quantities of the respective neurotrophin for 5 min. Lysates were equalized for cell protein, immunoprecipitated with the anti-trkC-specific antiserum 656, and electrophoresed on 7.5% SDS−polyacryamide gels. Tyrosine phosphorylation was detected using an anti-phosphotyrosine mAb 4G10. Abbreviations: NGF/P, purified NGF; NGF/U, concentrated supernatant of NGF-expressing 293 cells; NT-3/P, purified NT-3; NT-3/U, concentrated supernatant of NT-3 expressing 293 cells; 0, mock-treated 293 cells. (A), (B), and (C) show results from three separate experiments using the neurotrophins listed above each lane.

in the context of a model neuronal-like cell line. Furthermore, it is important to note that these cells also express trkA and that both receptors (trkA and trkC) compete for the multifunctional ligands. While it is possible that the NGF variants could elicit formation of a trkA/trkC heterodimer, this might not lead to transphosphorylation of the two receptors (Canossa et al., 1996).

Results from induction of neurite outgrowth in PC12/trkC cells are consistent with the binding and autophosphorylation assays (Table 2). While PC12/trkC cells possess both trkA and trkC, it has been shown previously that NT-3 leads to a significant induction of neurites during the first 3 days after application of neurotrophin whereas NGF does not; however, at 10 days NGF and NT-3 induce similar neurite outgrowth (Tsoulfas et al., 1996). Hence the PC12/trkC cells were evaluated for neurites after 3 days. Those variants which showed reduced binding to trkC also showed reduced response. NGF12 exhibited a dose−response which was shifted to higher values compared to native NT-3, and those variants with the poorest binding (NGF1, NGF2, NGFR1, and NGFR5) did not reach maximal response even at the highest dose tested (Table 2). NGF126 was as potent in neurite induction as native NT-3 (Table 2), and though variants NGF126, NGF130, and NGF131 were equivalent in trkC binding (Table 1), NGF126 was more efficacious in inducing neurites, reaching maximal response at 1 ng/mL compared to 10 ng/mL for NGF130 and NGF131 (Table 2). Notably, these NGF variants could induce neurites through trkC in an environment where trkA competes with trkC for their binding. At 10 days, the NGF12, NGF1, and NGF2 variants acted similarly as NGF in inducing neurites (data not shown) as would be expected from the binding of these variants to trkA (Table 1).

Table 2: Induction of Neurite Outgrowth in PC12 Cells Expressing Rat trkC

	concentration of neurotrophin in medium			
variant	250 pg/mL	1 ng/mL	10 ng/mL	100 ng/mL
NT-3	46 ± 6[a]	74 ± 4	73 ± 5	71 ± 11
NGF	0	0	0	0
NGF1	0	0	0	9 ± 1
NGF2	14 ± 3	35 ± 8	42 ± 9	48 ± 12
NGF12	16 ± 8	25 ± 6	54 ± 9	59 ± 7
NGFR1	4 ± 1	3 ± 2	7 ± 1	13 ± 5
NGFR5	5 ± 3	13 ± 5	23 ± 5	46 ± 16
NGF126	35 ± 6	70 ± 9	72 ± 3	69 ± 10
NGF130	26 ± 4	46 ± 3	69 ± 5	71 ± 4
NGF131	20 ± 6	49 ± 7	68 ± 2	67 ± 7

[a] Values are the percent of counted cells that carried neurites which were at least twice the length of the cell body.

DISCUSSION

Rational Design of an NGF Variant with NT-3-like Activity. The neurotrophins transduce their signal into the cell by interaction with the trk receptor tyrosine kinases (Kaplan & Stephens, 1994). The neurotrophins and the trks both form highly homologous protein families. Within each family the different members probably have similar struc-

tures, but individual members of the two families interact with each other in a very specific manner. This inherent specificity of neurotrophins is necessary for their biological function, and therefore information on the mechanisms of specificity determination contributes to an understanding of function and evolution of the neurotrophin family.

Molecular modeling and alanine scanning mutagenesis of human NT-3 (Urfer et al., 1994) and domain deletions/swaps of the human trks (Urfer et al., 1995) determined the binding epitopes of this ligand/receptor system. The former study revealed that the binding site of NT-3 for its receptor trkC is dominated by residue R103, with additional determinants in its vicinity. The binding site extends around the central β-strand barrel and, in contrast to the NGF binding site for trkA, does not include residues from loops and the first six residues of the N-terminus (Figure 3). Nonconserved residues that are part of the binding site include T23, K81, and Q84. Residue T23, together with L18 and E20, is located in an area which is conserved across all species within each of the members of the neurotrophin family, but is divergent between NT-3, NGF, and BDNF. Therefore, these five residues seemed to be reasonable candidates for specificity determinants in NT-3 for trkC binding. In order to test their importance for binding to trkC, residues in NGF (V18, V20, G23, T81, and H84) were changed to their corresponding NT-3 amino acids (E18, L20, T23, K81, and Q84), and the resulting protein, NGF12, was analyzed for recruitment of trkC binding and trkC-mediated biological activities. NGF12 was able to bind to trkC, induce autophosphorylation of trkC expressed on PC12 cells, and did not lose affinity to trkA (Table 1, Figures 4 and 5).

The Change from Glycine 23 to Threonine Dominates the Recruitment Effect in NGF12. Additional variants of NGF12 in which each of the mutated residues was individually changed back to the original NGF amino acid (i.e., E18V, L20V, T23G, K81T, Q84H) demonstrated that in NT-3 the most important determinant for trkC specificity is T23 followed by Q84 and L20. The change from glycine to threonine in NGF may introduce a side chain that is critical in binding to trkC; alternatively, the G23T change might effect a change of the backbone conformation and thereby influence the conformation of side chains in its vicinity. At position 84 the change from histidine to glutamine removes a potentially charged residue which might be repulsive to trkC or the glutamine side chain may be required for specific hydrogen bonding. Finally, the preference for leucine over valine at position 20 may be due to rather stringent spatial requirements of a hydrophobic interaction at the binding site.

In addition to T23, Q84, and L20, the amino acid at position 86 is important for neurotrophin specificity to trkC. Adding the NT-3 residue to NGF12 effected a 4.5-fold improvement in binding to trkC while maintaining binding to trkA (variant NGF126, Table 1). Residue 86 is proximal to the most important determinant for binding to trkC, R103 (Figure 3), further confirming the dominance of this structural region for trkC recognition. In NGF residue 86 is a phenylalanine and in NT-3 it is a tyrosine, suggesting that the side chain hydroxyl group may be involved in a specific hydrogen bond required for trkC recognition.

Finally, residues at positions 18 and 29 may fine tune the specificity of NT-3 for trkC. The effect at position 29 seems to be dependent on the character of the amino acid at position 86. When the change T29I was introduced into NGF12 (which has F86), trkC binding was slightly reduced, whereas when T29I was introduced into two other variants (which have Y86), the binding was slightly improved (compare NGF124 versus NGF1234 and NGF130 versus NGF131, Table 1). Similar to residue 86, residue 29 is proximal to the important R103 (Figure 3). At position 18, the mutation V18E contributed only slightly to trkC binding (cf. NGF12 and NGFR2, Table 1). However, comparison of induction of neurite outgrowth by NGF126 and NGF130 shows that inclusion of the V18E mutation improved induction of neurite outgrowth; NGF126, which includes V18E, elicited neurite outgrowth equivalent to native NT-3 (i.e., reaching maximal response at 1 ng/mL) whereas NGF130 required 10-fold more neurotrophin to elicit the maximal response (10 ng/mL) (Table 2).

In summary, the major specificity determinants on NT-3 for trkC include L20, T23, Q84, and Y86. Residues E18 and I29 may play a minor role in specificity. The six residues form two clusters: I29, Q84, and Y86 are proximal to R103, the most important residue involved in trkC binding, and E18 and T23 are located together but distant from the R103 cluster (Figure 3). This suggests that trkC uses at least two unique, spatially distant sites to discriminate between the various neurotrophins.

Mechanisms of Specificity Determination in the Neurotrophin Family. Earlier mutational analyses of rodent and human neurotrophins proposed that the first few residues of the N-terminus of NGF are important for specific binding to trkA (Ibáñez et al., 1993; Urfer et al., 1994; Shih et al., 1994). NT-3 variants that carried the N-terminal residues from NGF were constructed, and the resulting molecules MNTS-1 and PNT-1 indeed acquired affinity to trkA similar to NGF (Urfer et al., 1994; Ibáñez et al., 1993). This implies that the specificity of neurotrophin binding to trkA is dominated by the N-terminal residues and that the remainder of the NT-3 molecule is fully compatible with binding to trkA. Furthermore, the observation that MNTS-1 did not lose its affinity to trkC implies that the N-terminal residues of NT-3 are not involved in trkC recognition, a conclusion that was corroborated by site-directed mutagenesis of the NT-3 N-terminus (Urfer et al., 1994). The present work establishes the importance of residues located in the central β-strand bundle for function and specificity of NT-3. Nonconserved amino acids of NT-3 that were previously shown to be involved in trkC binding by site-directed mutagenesis (Urfer et al., 1994)—T23 and Q84—can be substituted in NGF and recruit trkC binding. In addition, nonconserved residues in NT-3—E18, L20, I29, and Y86—which apparently do not contribute to the binding of NT-3 to trkC (Urfer et al., 1994) can also contribute to the specificity of the NT-3/trkC interaction, though exhibiting a reduced effect compared to T23 and Q84. Replacing these six residues in NGF with their NT-3 counterparts maintains full affinity to trkA, suggesting that trkA does not utilize these specific amino acid positions to discriminate between NGF and NT-3. Hence, in NT-3 residues in the central β-strand bundle impart specificity whereas in NGF the N-terminal residues impart specificity.

Based on sequence conservation in NGF from different species, an earlier study proposed that Y79, T81, and H84 could be mediating the interaction of NGF with trkA (Ibáñez et al., 1993). This same study also found that replacing these three residues in NGF with those from BDNF (Q79, R81, Q84) resulted in a chimeric neurotrophin that could activate trkB. In contrast, replacing Y79Q and T81R did not activate

trkB (Ibáñez et al., 1993), suggesting that only residue 84 is important for trkB activation. In the present study, it was determined that residue 81 did not affect binding or specificity for trkA or trkC (cf. NGF12 and NGFR4, Table 1) and residue 84 was important for trkC specificity but not for trkA specificity (cf. NGF12 and NGFR5, Table 1). The present study also determined that NGF residue 79 was not involved in trkA specificity whereas for trkC the native human NGF residue (Y79) was slightly preferred compared to the human NT-3 Q79 (NGF127, Table 1). Taken together, the data from these two studies suggest that position 84 plays a role in specificity for trkB and trkC, but not trkA.

The results presented here show that grafting a specific set of central β-strand bundle residues from human NT-3 onto human NGF could recruit binding to the noncognate receptor (trkC) while maintaining the affinity for the cognate receptor (trkA). Therefore, the determinants for specificity of the neurotrophins to the different trk receptors are not only located in homologous positions in loop regions as proposed previously (Ilag et al., 1994; Ryden & Ibáñez, 1996) but also involve residues in the central β-strand bundle. Our data suggest a mechanism of specificity determination similar to that proposed for the homologous families of gonadotropins and gonadotropin receptors (Moyle et al., 1994) where the luteinizing hormone receptor recognizes regions on the luteinizing hormone that are distinct from the ones that the homologous follitropin receptor recognizes on follitropin.

Due to the high sequence homologies within the neurotrophins and the trks it is very likely that both families coevolved from common ancestors. An ancestral neurotrophin may have used the central β-strand bundle region around R103 for binding. Neurotrophins with unique trk specificities then could have diverged from the ancestral neurotrophin by acquiring repulsive and/or attractive forces on the neurotrophin surfaces encompassing the N-terminus, the variable loops, and the central β-strand barrel. In this respect it is important to note that the specificity profile of a present day neurotrophin could be changed by a single point mutation (Urfer et al., 1994).

Administration of neurotrophins in models of nerve lesions has been shown to be beneficial for regeneration and survival of neurons (Sendtner et al., 1992; Yan et al., 1992). Neuronal populations involved in neurodegenerative disorders may express more than one trk receptor, and therefore administration of molecules with multiple specificities, such as MNTS-1 (Urfer et al., 1994), PNT-1 (Ibáñez et al., 1993), and NGF126, could be advantageous compared to administration of a single monospecific neurotrophin or a cocktail of monospecific neurotrophins. For example, the various members of the neurotrophin family may have different pharmacokinetics, and therefore the behavior of neurotrophin cocktails could be difficult to predict or control.

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