A Second Determinant of Binding to the p75 Neurotrophin Receptor Revealed by Alanine-scanning Mutagenesis of a Conserved Loop in Nerve Growth Factor*

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In the neurotrophin family, variable regions contain solvent-accessible residues important for receptor binding specificity, whereas many of the conserved residues are buried in hydrophobic cores or in the dimer interface. A stretch of six amino acids (from Asp-72 to Asn-77) in nerve growth factor (NGF) represents an exception to this general rule. These residues are highly conserved and yet form an exposed hydrophilic loop region away from other known determinants of receptor binding. We have investigated the functional importance of this region in NGF using alanine-scanning mutagenesis. Individual mutation of Asp-72, Lys-74, or His-75 to alanine (mutants D72A, K74A, and H75A, respectively) reduced the binding affinity for the p75 neurotrophin receptor by 4–10-fold. Only the D72A mutant showed an additional impairment in binding to the TrkA receptor, which was accompanied by reduced biological activity in PC12 cells, indicating a structural and/or conformational effect of this mutation. Replacement of Ser-73 or Asn-77 with alanine (mutants S73A and N77A, respectively) had no measurable effects on receptor binding. The triple mutant K74A/H75A/N77A exhibited properties that were consistent with the combined effects of the individual mutations, namely impaired binding to p75 without deficits in its interaction with TrkA. In contrast, in the triple mutant D72A/S73A/K74A, the simultaneous replacement of Asp-72 and Lys-74 with alanine had a compensatory effect such that binding to both p75 and TrkA was comparable to that of wild-type NGF, despite the deficits seen in the individual replacements. This molecule, however, was produced at low levels, and its biological activity in sympathetic ganglion explants was reduced, which, together with results from TrkA phosphorylation assays, indicated a reduced stability during prolonged culture conditions. Taken together, these data reveal a second region of interaction with the p75 receptor in NGF with the positively charged residues Lys-74 and His-75 as candidate points of contact. In addition, Asp-72 appears to be a structurally important side chain for stabilizing the conformation of the loop through interactions with neighboring residues.

Neurotrophic factors are polypeptides important for the survival, maturation, and maintenance of vertebrate neurons (for an overview, see Ref. 1). Several families of molecules with neurotrophic activity have been identified, among which the neurotrophins are the best studied. Up to four neurotrophins have been isolated in any single vertebrate species: nerve growth factor (NGF),1 brain-derived neurotrophic factor, neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). They are disulfide-bridged homodimers composed of two 110-residue-long protomers. In the crystal structure of NGF, each protomer is composed of two pairs of anti-parallel β-strands joined by looped structures (2). The neurotrophins share with each other ∼50% of their amino acid sequence; segments of variability and identity are clustered, forming structurally distinct domains. Variable regions are found in the N and C termini, three β-hairpin loops, one turn, and one β-strand (3). In contrast, many of the conserved residues are buried in the dimer interface, indicating structural roles. Neurotrophins interact with two distinct classes of cell-surface receptors: members of the receptor tyrosine kinase Trk family and the low affinity neurotrophin receptor p75, which lacks intrinsic catalytic activity. Neurotrophin interaction with members of the Trk family is specific in that NGF binds to TrkA, brain-derived neurotrophic factor and NT-4 bind to TrkB, and NT-3 binds to TrkC. In contrast, all neurotrophins bind to p75 with similar affinity (Kd = 10⁻⁹ M), albeit with different kinetic constants (4, 5). Neurotrophin binding to Trks leads to receptor dimerization, autophosphorylation in tyrosine residues, and downstream signal transduction (6). A wealth of in vitro as well as in vivo data have convincingly demonstrated that Trks can mediate many of the effects normally elicited by neurotrophins in the absence of p75. The biological role of p75, in contrast, is less clear. p75 is distantly related to tumor necrosis factor receptor-1, the Fas antigen, and CD40. This receptor has been shown to play a role in ligand internalization and ligand discrimination as well as in enhancing NGF-mediated activation of TrkA (7). Furthermore, several reports indicate that p75 could play a role in the regulation of apoptosis (8–10). Moreover, recent evidence indicates that p75, at least in some non-neuronal cells in vitro, can function as a signal-transducing receptor in the absence of TrkA (reviewed in Ref. 11) via a pathway involving sphingomyelin hydrolysis (12, 13); activation of the transcription factor NF-κB (14); or the c-Jun N-terminal kinase (9).

The potential therapeutic applications of neurotrophins have stimulated efforts to define residues involved in receptor binding, with the ultimate goal of designing smaller peptide analogues with altered specificity and/or potency. Extensive mutational studies have been carried out to define these residues and to assess their contribution in receptor binding and biological activity (reviewed in Ref. 1). The binding surface of NGF to TrkA consists of several regions in the primary sequence in-

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1 The abbreviations used are: NGF, nerve growth factor; NT, neurotrophin.
cluding residues in the N terminus and in several variable loop regions. In the three-dimensional model of NGF, these residues delineate a continuous surface extending approximately parallel to the 2-fold axis of the NGF dimer (15). In contrast, the main functional epitope involved in NGF binding to p75 is more localized, primarily involving the positively charged residues Lys-32, Lys-34, and Lys-95 located in two spatially close β-hairpin loop regions in the “north end” of the NGF molecule (16).

Site-directed mutagenesis of these residues to alanine diminishes binding to p75 in an additive manner, although mutant molecules retain wild-type TrkA binding and activation. Interestingly, positively charged residues in homologous positions in brain-derived neurotrophic factor, NT-3, and NT-4 are also involved in binding to p75, indicating a general role for basic residues at these positions in the contact with this receptor (17). It should be noted, however, that although similar, these functional epitopes are not identical, and their differences could account for the differences in p75 binding kinetics observed among the neurotrophins (17).

At the opposite end of the NGF dimer, a loop region consisting of six exposed residues (Asp-72, Ser-73, Lys-74, His-75, Trp-76, and Asn-77) is highly conserved among all of the NGF sequences isolated to date from different vertebrate species. The corresponding regions in brain-derived neurotrophic factor, NT-3, and NT-4 also show a similar evolutionary conservation. The solvent accessibility of these residues ranges from 20–30% (Asp-72, His-75, and Trp-76) to 50–80% (Ser-73, Lys-74, and Asn-77), indicating moderate to high side chain exposure (2). The hydrophilicity, accessibility, and conservation of these residues suggest a common role for this loop region in all neurotrophins, possibly in modulating binding to cell-surface receptors. A previous study showed that mutation of Trp-76 to phenylalanine had no major effect on receptor binding and biological activity (18). A more recent study found reduced affinity for TrkA and decreased biological activity after replacement of His-75 with alanine (19). Intriguingly, an increased affinity for the p75 receptor in H75A mutant NGF was also reported.

We have investigated the functional importance of residues in this region in NGF using alanine-scanning mutagenesis. The resulting mutant NGF molecules were studied with respect to receptor binding affinity and biological activity in NGF-responsive cell lines and primary neurons. We found that replacement of either Lys-74 or His-75 with alanine affected the affinity of NGF for p75 but not for TrkA, indicating a second site of interaction with the p75 receptor. The conservation of these residues in other neurotrophins indicates that variations in charge and conformation in this region may also be critical for p75 binding in other members of the neurotrophin family.

MATERIALS AND METHODS

Production, Purification, and Quantification of Recombinant Proteins—A 771-base pair sequence coding for rat prepro-NGF was subcloned into pBluescript KS+ (Stratagene). Single-stranded DNA from this plasmid was used as template for oligonucleotide-based site-directed mutagenesis as described by Kunkel (25). Each mutation was confirmed by DNA sequencing. For protein expression in COS cells, DNA inserts containing the desired replacements were excised and subcloned into pX (Stratagene). COS cells grown to 70% confluency were transiently transfected with 25 μg of plasmid by the DEAE-dextran method. To correct for differences in production levels between the different constructs, parallel transfections were performed in 35-mm dishes in the presence of 1.3 units of Cellfection (Amersham Corp.). Conditioned medium was separated by SDS-polyacrylamide gel electrophoresis, and the amounts of recombinant protein in the different samples were equalized after densitometric scanning of the corresponding autoradiograms. The absolute amounts of wild-type NGF protein were determined by quantitative immunoblotting of conditioned medium and by measurement of biological activity in cultured sympathetic ganglia using standards of purified mouse NGF and were used to determine absolute protein levels in conditioned media containing mutant proteins. Triple mutants were purified using a combination of cation-exchange, size-exclusion, and reverse-phase high performance liquid chromatography essentially as described previously (17). Purified proteins were quantified on silver-stained gels using standards of commercial NGF. The purity of the most pure fraction was >90% as determined by silver staining of SDS-polyacrylamide gels.

RESULTS

Alanine-scanning Mutagenesis of a Conserved Loop Region in NGF—The functional importance of amino acid residues Asp-72, Ser-73, Lys-74, His-75, and Asn-77 in a conserved loop region in the “south end” of the NGF molecule (Fig. 1A) was analyzed by alanine-scanning mutagenesis. These residues are highly conserved in NGFs isolated from different vertebrate species as well as in other members of the neurotrophin family (Fig. 1B). Mutant molecules were generated by oligonucleotide-based site-directed mutagenesis. Expression plasmids coding for wild-type and mutant NGFs and a control plasmid were transiently transfected into COS cells. Single mutants obtained by alanine replacement migrated as distinct 13-kDa monomers on SDS-polyacrylamide gels and were expressed at levels comparable to those of wild-type NGF, with the exception of D72A and N77A, which were expressed at 50 and 60% of the level of wild-type NGF, respectively (Table I). (Mutants are abbrevi-
ated by the wild-type residue in one-letter code followed by the codon number from the amino acid sequence of rat NGF and the mutant residue.) The reduced production of these two mutant proteins may be due to differences in synthesis, secretion, and/or stability of the molecules (see, for example, Ref. 16).

Replacement of Conserved Charged Residues with Alanine Reduces Affinity for p75—The receptor binding affinity of each single mutant was determined in equilibrium displacement binding assays using $^{125}$I-NGF. Affinity was estimated by measuring the concentration of recombinant molecule required to displace 50% of the radioiodinated NGF (IC$_{50}$). Binding to p75 was assessed in a human melanoma cell line (A875) that expresses high levels of p75, but not detectable levels of TrkA (Fig. 2A and Table I). S73A and N77A were as effective as native protein in displacing $^{125}$I-NGF, with an IC$_{50}$ in the nanomolar range. In contrast, D72A, K74A, and H75A showed 4–10-fold increased IC$_{50}$ values, with D72A showing the most pronounced loss of affinity (Table I). These results indicate that modifications of charged residues in the south end of NGF affect binding to p75.

Impaired TrkA Binding and Reduced Biological Activity after Mutation of Asp-72 to Alanine—Affinity for TrkA was assessed in displacement binding assays using mouse 3T3 fibroblasts stably expressing TrkA. S73A, K74A, and H75A showed 4–10-fold increased IC$_{50}$ values, with D72A showing the most pronounced loss of affinity (Table I). These results indicate that modifications of charged residues in the south end of NGF affect binding to p75.

PC12 is a pheochromocytoma cell line that expresses p75 and TrkA and that differentiates into a sympathetic neuron-like cell upon NGF treatment, an effect mediated by TrkA (23). In PC12 cells, S73A, K74A, and H75A displayed biological activities indistinguishable from that of native NGF, in agreement with their wild-type binding affinity for TrkA (Fig. 3). D72A showed a reduced ability to induce neurite outgrowth in PC12 cells, which correlated with the reduced affinity of this mutant for TrkA. N77A was also somewhat impaired in its biological...
potency in PC12 cells, although it had normal affinity for TrkA in 3T3 cells. A dissociation between receptor binding and activation has also previously been observed in other mutant NGFs (15) and could result from a reduced stability of the mutated ligand in prolonged culture conditions or from a decreased ability to induce receptor dimerization.

Additive and Synergistic Effects of Multiple Alanine Replacements in the Conserved Loop Region of NGF—Mutations of single residues in this region suggested that charged residues could be directly or indirectly involved in the binding of NGF to its receptors. We next investigated the effects of multiple substitutions in the conserved loop of NGF by generating the triple mutants D72A/S73A/K74A and K74A/H75A/N77A. The latter was expressed at slightly lower levels than native protein (70% of wild-type NGF) in COS cells, whereas D72A/S73A/K74A was produced at 35% of wild-type NGF, in agreement with the reduced production of the single mutant D72A (see above and Table I). Large-scale preparations of these two mutant NGFs were made in COS cells, and recombinant protein was purified from conditioned media by a combination of gel-filtration, ion-exchange chromatography, and reverse-phase chromatography (17).

The receptor binding affinity of the triple mutants was assessed in displacement binding assays in A875 cells with serial dilutions of purified triple mutants. K74A/H75A/N77A had a substantially impaired affinity for this receptor. D72A/S73A/K74A displayed wild-type (wt) affinity. B, equivalent experiments were performed in TrkA-expressing fibroblasts. The same symbols are used as in A.

FIG. 2. Binding of mutant NGFs to p75 and TrkA receptors. Binding of single mutant and wild-type (wt) NGFs to p75 (A) and TrkA (B) was assessed in steady-state competitive binding assays. A, serial dilutions of transfected COS cell-conditioned medium containing equal amounts of the indicated recombinant molecules were incubated with A875 cells in the presence of $^{125}$I-NGF. Note the impaired binding of mutants D72A, K74A, and H75A. B, in TrkA-expressing fibroblasts, only D72A shows an impaired affinity for TrkA. The same symbols are used as in A. In both A and B, each point represents the mean of triplicate determinations from at least four independent experiments. Standard deviations were at or below 20% of the values reported. Medium from mock-transfected cells failed to displace $^{125}$I-NGF (not shown).

FIG. 3. Biological activity of wild-type and mutant NGFs in PC12 cells. The ability of single mutants and wild-type (wt) NGF protein to elicit neurite outgrowth in PC12 cells was determined. Cells were incubated in the presence of serial dilutions of transfected COS cell-conditioned medium containing equal amounts of the indicated factors. The percentage cells bearing neurites longer than two cell diameters was quantified after 48 h. Note that, in agreement with its reduced affinity for TrkA, the bioactivity of D72A was also reduced.

FIG. 4. Additive and synergistic effects of multiple alanine replacements in the conserved loop region of NGF. Steady-state competitive binding assays in cells expressing NGF receptors were performed as described in the legend to Fig. 2. A, affinity for p75 was assessed in A875 cells with serial dilutions of purified triple mutants. K74A/H75A/N77A had a substantially impaired affinity for this receptor. D72A/S73A/K74A displayed wild-type (wt) affinity. B, equivalent experiments were performed in TrkA-expressing fibroblasts. The same symbols are used as in A.
The biological activity of the purified triple mutants was first assayed in TrkA-expressing MG87-3T3 fibroblasts. When grown in serum-free medium, these cells survive and proliferate in response to NGF in a dose-dependent manner. In this assay, the activity of the K74A/H75A/N77A triple mutant was indistinguishable from that of wild-type NGF (Fig. 5A), in agreement with its native affinity for the TrkA receptor. Despite displaying wild-type affinity for TrkA, the D72A/S73A/K74A triple mutant had a reduced biological activity in the fibroblast assay. As pointed out above, a dissociation between receptor binding and bioactivity could result from a reduced stability of the mutated ligand in prolonged culture conditions (4 days in this case) or from a decreased ability of the mutant protein to induce receptor dimerization.

To distinguish between these two possibilities, a short-term assay was performed in which ligand-induced activation of the TrkA tyrosine kinase was evaluated by measuring the levels of receptor tyrosine autophosphorylation. TrkA-expressing MG-3T3 fibroblasts were exposed for 5 min to different concentrations of partially purified preparations of the D72A/S73A/K74A triple mutant or the H75A single mutant, which had not shown differences between receptor binding and bioactivity, and cell lysates were assessed for TrkA tyrosine autophosphorylation by immunoprecipitation and Western blotting. Both molecules was compared with standards obtained with purified NGF. Results are representative of at least two experiments. wild-type NGF, D72A/S73A/K74A, K74A/H75A/N77A.

![Graph A](image1.png)

**FIG. 5.** Triple mutants exhibit differential biological activity in TrkA-expressing fibroblasts. A, survival/growth assays in TrkA-expressing MG87-3T3 fibroblasts were performed in serum-free medium containing serial dilutions of purified factors. After a 4-day incubation, total cell content was assessed using a kit of reagents detecting cellular acid phosphatase. Cell lysates were measured in a spectrophotometer at 405. K74A/H75A/N77A retained biological activity at levels indistinguishable from those of wild-type (wt) protein. In contrast, D72A/S73A/K74A displayed a significant reduction in bioactivity despite its normal binding affinity for TrkA. Each point represents the mean ± S.D. of quadruplicate determinations. B, the tyrosine phosphorylation assay was carried out in TrkA-expressing MG87-3T3 fibroblasts. TrkA-expressing MG87-3T3 fibroblasts were exposed for 5 min to the indicated concentrations of the partially purified D72A/S73A/K74A triple mutant or the H75A single mutant, prior (no) or subsequent (4-day) to a 4-day preincubation of the factors at 37 °C. Following ligand stimulation, cell lysates were assessed for TrkA tyrosine autophosphorylation by immunoprecipitation and Western blotting. Phosphorylated TrkA is indicated (arrow). Note the reduction in activity subsequent to preincubation in the triple mutant.

![Graph B](image2.png)

**FIG. 6.** Biological activity of triple mutants in explanted embryonic sympathetic ganglia. The ability of the purified triple mutants and native NGF to induce neurite extension from explanted embryonic day 9 sympathetic ganglia was compared. Biological activity is expressed in semiquantitative scale in biological units by comparison with standards obtained with purified NGF. Results are representative of at least two experiments. wild-type NGF, D72A/S73A/K74A, K74A/H75A/N77A.

The ability of the purified triple mutant NGF molecules was compared in the sympathetic ganglion explant bioassay. Embryonic sympathetic neurons survive and differentiate, extending arborizing neurites in the presence of NGF. The neurites form a distinctive dose-dependent halo around the explant, and this biological effect can be expressed on a semiquantitative scale. The response elicited by the K74A/H75A/N77A mutant was comparable to that of wild-type NGF, in agreement with its near wild-type affinity for the TrkA receptor (Fig. 6). The D72A/S73A/K74A mutant had a much reduced biological activity in the sympathetic ganglion bioassay (Fig. 6), in agreement with results from the fibroblast assay.

**DISCUSSION**

In this study, we have investigated the functional importance of solvent-accessible residues in a conserved loop region of NGF by site-directed mutagenesis. We found that several charged residues in this region, notably Lys-74 and His-75, may be involved in the direct contact with the p75 receptor. Since these residues are located at the opposite end of the molecule relative to a first binding epitope found in previous studies (16), these data indicate an extended surface of interaction between NGF and p75. These data agree with the results of a mutagenesis study on NT-3 (24) in which several residues important for p75 binding, including a lysine residue at the equivalent position to Lys-74 in NGF, were also found in this region. It is interesting to note that most of the residues important for binding to p75 detected so far in point mutation
and His-75, do not participate directly in structurally important interactions.

The reduced biological activity of mutations of Asp-72 was accompanied by decreased expression levels in COS cells, indicating that replacements at this position may have effects on the structure and/or stability of the molecule. In agreement with this, we have also seen a markedly reduced production level in COS cells of a D72K mutant, and a 37 °C preincubation of the D72A/S73A/K74A triple mutant affected its subsequent ability to stimulate tyrosine phosphorylation of TrkA receptors. Replacement of the negative charge at this position is expected to disrupt the hydrogen bond to His-75 that stabilizes the loop and may conceivably affect the folding of the molecule. The fact that production, TrkA binding, and biological activity were normal after replacement of His-75 suggests that, in the absence of the histidine, other residues, perhaps Lys-74, may contribute to the stability of the loop. Interestingly, we found very similar effects after mutation of either Lys-74 or His-75, suggesting equivalent roles for these two adjacent residues.

Destabilization of the loop after replacement of Asp-72 may result in an aberrant position of the bulky residue Lys-74, thereby interfering with p75 and TrkA receptor binding via steric hindrance. In support of this interpretation, we found that the effect of the D72A mutation on receptor binding was alleviated if both Asp-72 and Lys-74 were simultaneously replaced (as in the D72A/S73A/K74A triple mutant). This triple mutation should have no effect on the net charge of the loop and could likely result in an increased solvent accessibility of the positively charged side chain of His-75 (Fig. 7), making it available for polar interactions with the p75 receptor. The fact that affinity for p75 was greatly reduced after simultaneous mutation of both Lys-74 and His-75 reinforces the importance of positively charged residues for ligand binding to this relatively acidic receptor.

Reduced biological activity in the absence of detectable effects on TrkA binding could be the result of a reduced stability during the prolonged culture conditions required for biological assays compared with binding assays. In the latter, ligand is exposed to receptor for only a few hours and at low temperatures. Alternatively, a reduced capacity of inducing TrkA dimerization in otherwise normal binding ligands would also result in decreased biological activity. Nonproductive binding to tyrosine kinase receptors has previously been seen in various mutant growth factors including several chimeric neurotrophins (15). In the case of the triple mutant D72A/S73A/K74A, the results of TrkA phosphorylation experiments showed a reduced activity of this molecule after, but not before, prolonged incubation at 37 °C, indicating a reduced protein stability.

In summary, the conformation of the conserved loop in NGF is likely to play a role in the interaction of NGF with its receptors as well as in the overall stability of the molecule. Exchange of positively charged residues with alanine in this exposed loop predominantly affected binding to p75. Together with previous studies, these results suggest an extended interaction between NGF and p75.

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