Mutational studies of conserved residues in the dimer interface of nerve growth factor

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Abstract

An understanding of the structure–function relationship of nerve growth factor (NGF) requires precise knowledge of all the residues and regions that participate in NGF receptor binding, receptor activation, and biological activity. Seven recombinant human NGF mutants having alanine substituted for residues located either in the NGF dimer interface or β -strand region were studied to determine the role of each amino acid residue in NGF biological activity. F86A, T91A, R100A, and R103A remained nearly fully active with 61, 120, 91, and 73% of wild-type activity, respectively, in the PC12 cell bioassay. Hydrophobic core and dimer interface residues Y52, F53, and F54 were studied in more detail. Y52A and F54A were expressed in very low levels, suggesting that these two residues may be important for protein stability. Y52A retained full biological activity (91%). F53A had a 20- and 70-fold reduction in biological activity and TrkA phosphorylation, respectively, with only a 5- to 10-fold effect on TrkA binding and no effect on low-affinity receptor binding. F54A had significantly decreased TrkA phosphorylation and biological activity (40-fold). The results suggest that F53 and F54 may play a structural role in TrkA receptor activation subsequent to binding.

Keywords: dimer; hydrophobic interaction; mutant, nerve growth factor; receptor binding; recombinant; structure; subunit contacts

Neurotrophins are a family of structurally and functionally related proteins which support survival, maintenance and differentiation of neurons. Nerve growth factor (NGF) is a member of a neurotrophin family and was the first neurotrophin to be identified (Levi-Montalcini & Hamburger, 1953; Levi-Montalcini, 1987). NGF supports the survival and differentiation of developing and mature sympathetic and neural crest-derived sensory neurons in the peripheral nervous system and survival of basal forebrain cholinergic neurons in the central nervous system (Thoenen & Barde, 1980; Levi-Montalcini, 1987; Thoenen et al., 1987). Other neurotrophins (BDNF, NT-3, NT-4/5) support similar and different neuronal populations, depending upon the distribution of the Trk family of tyrosine kinase receptors (Ebendal, 1992; Barbacid, 1994; Lindsay et al., 1994).

The biological form of NGF is a homodimer composed of two noncovalently bound 118-amino acid polypeptide chains (Angeletti & Bradshaw, 1971). The dimer is quite stable with a dissociation constant less than 10^{-13} M (Bothwell & Shooter, 1977). The crystal structure of murine β NGF dimer at 2.3 Å resolution (McDonald et al., 1991; Holland et al., 1994) reveals that the molecule has a thin, flat, elongated shape. Each monomer contains two pairs of antiparallel β -strands down the elongated axis and a shorter pair of antiparallel β -strands at an angle to the other two. Three β hairpin loops (29–35, 43–48, and 92–98) are located at the “north” end of the molecule, whereas a reverse turn (59–66) and three intrachain disulfide bonds are found in the “south” end. The dimer is held together in the dimer interface by interaction of hydrophobic residues from the long β -strands. This interaction may be essential for biological activity because no defined monomer retaining biological activity has been prepared. The large twist in each pair of β -strands allows the formation of a hydrophobic core. Members of neurotrophin family (BDNF, NT3, NT4/5) share about 55% amino acid sequence identity (Ebendal, 1992). The highly conserved residues, which are mainly located in the β -strand region, play a structural role in maintaining the proper conformation of the molecule. The

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variable residues among the different neurotrophins are concentrated within the loops and within the amino and carboxyl termini. These variable regions were suggested to play a role in determining the specificity of binding to different high-affinity NGF receptors (Ibanez et al., 1991; McDonald et al., 1991) and have been confirmed by mutagenesis (see Ibanez, 1994 for review).

NGF initiates biological function by binding to and activation of cell surface receptors. Two types of neurotrophin receptors have been identified: the p75 low-affinity NGF/neurotrophin receptor (Johnson et al., 1986; Radeke et al., 1987) and the Trk tyrosine kinase receptor family (Martin-Zanca et al., 1989; Kaplan et al., 1991a, 1991b). TrkA was demonstrated to play a major role in mediating NGF signal transduction and functional responses (Hempstead et al., 1991; Allsopp et al., 1993; Rovelli et al., 1993). The p75 receptor binds to all the known neurotrophins with similar low affinity, but with different rates of association and dissociation (Rodriguez-Tebar et al., 1990, 1992; Hallbook et al., 1991). The role that p75 plays in the formation of high-affinity binding and intracellular signaling is complex.

To understand the structure-function relationships of NGF requires precise knowledge of sequences that participate in receptor binding, activation, and biological activity. Numerous mutational and immunochemical studies have been used to characterize the regions and amino acid residues of NGF that are important for receptor binding, activation, and biological activity. The amino acid residues involved in TrkA binding include the amino terminus (Kahle et al., 1992; Ibanez et al., 1993; Shih et al., 1994; Woo et al., 1995), the carboxyl terminus (Luo & Neet 1992; Drinkwater et al., 1993), multiple residues in northern loop regions (Ibanez et al., 1993; Ilag et al., 1994), determinants in the southside loops (Nanduri et al., 1994), and histidine residues H75 and H84 (S.B. Woo & K.E. Neet, unpubl. data). The p75 binding site is mainly mediated by a group of positively charged residues (lysine residues K32, K34, and K95) in the north loop region (Ibanez et al., 1992).

The roles of amino acid residues in the NGF dimer interface and β -strand region in NGF structure-function relationship have not yet been well characterized. In this paper, we studied seven human NGF point mutants, which are located either in the NGF dimer interface or in the β -strand region (Kinemage 1). The baculovirus system was used for expression in *Spodoptera frugiperda* 21 (Sf21) insect cells and the resultant mutants partially purified. We have demonstrated that F53 and, perhaps, F54 play a structural role in determining activation of TrkA phosphorylation and, hence, biological activity.

Results

Expression of recombinant NGF protein in Sf21 cells and partial purification

Seven human recombinant NGF (rNGF) single point mutants were generated by site-directed mutagenesis. The amino acid residues Y52, F53, F54, F86, T91, R100, and R103 of human NGF were individually replaced with alanine. NGF mutants were expressed in Sf21 insect cells using the baculovirus expression system. All of the mutants were properly proteolytically processed into the mature 13-kDa NGF molecule (data not shown). Y52A and F54A were produced at about 10% that of wild type,

whereas F53A was produced at about 50% that of wild type. The expression levels of the other four mutants were similar to wild type (Table 1). F53A and R103A were partially purified by CM52 cation exchange chromatography to about 70% purity (data not shown) for subsequent study.

Biological activity of mutant NGF

The biological activities of the human NGF mutants were tested by their ability to stimulate neurite outgrowth in the PC12 pheochromocytoma cell line. F53A and F54A showed reduced biological activity with a shift in the EC_{50} (50% effective concentration) of 20-fold and 40-fold, respectively. Other mutants showed similar levels of biological activity to the wild type (Fig. 1; Table 1).

Binding of F53A to both classes of receptors

The reduction in biological activity of the F53A and F54A mutants might be due to either decreased binding or activation of the TrkA receptor. Low expression levels precluded testing of F54A for receptor binding. To determine the total binding affinity of the F53A mutant to NGF receptors, PC12 cells were incubated with 100 pM [125 I] NGF with varying concentrations of competitive ligand for 40 min at 37 °C. This PC12 receptor-binding profile reflects binding largely of the low-affinity type, because the number of p75 receptors in PC12 cells is about 10–20-fold greater than TrkA (Vale & Shooter, 1985; Woodruff & Neet, 1986). F53A showed close to wild-type binding affinity (Fig. 2).

To measure the ability of mutants to compete with high-affinity binding in PC12 cells, a cold chase-stable binding assay, which is usually correlated with high-affinity binding, was performed. PC12 cells were incubated with 100 pM of [125 I] NGF in the presence of 1 nM and 0.1 nM of competing mutant rNGF for 40 min at 37 °C, followed by cold chase at 0.5 °C for 30 min with 4,000-fold excess of unlabeled mouse NGF. All of the fast dissociation component is dissociated within a few seconds and the slow dissociation component remains stably bound during the chase period (Landreth & Shooter, 1980; Vale & Shooter, 1985). In the presence of 1 nM and 0.1 nM wild-type

Fig. 1. Dose-response curve of rNGF in PC12 bioassay. Serial dilutions of rNGF were assayed for their ability to stimulate PC12 cell neurite outgrowth in defined medium 48 h after rNGF treatment.

Table 1. Levels of protein expression and EC_{50} values for human rNGF in the PC12 bioassay

rNGF	Relative expression level ^a (%)	Biological activity (EC_{50}) ^b	Relative activity (%)
Wild type	100	55 pM	100
Y52A	12	60 pM	91
F53A	49	1 nM	5.5
F54A	12	2 nM	2.7
F86A	96	90 pM	61
T91A	88	45 pM	120
R100A	100	60 pM	91
R103	112	75 pM	73

^a Relative expression levels were calculated from the amount of rNGF protein detected by immunoblot analysis. The level of expression of wild type was about 400 μ g/L Sf21 culture.

^b EC_{50} values represent averages of two dose-response experiments.

NGF, about 22% and 58% cold chase-stable binding remained, respectively, whereas about 55% and 83% cold chase-stable binding remained in the presence of 1 nM and 0.1 nM F53A (Fig. 3). The result indicated that the F53A mutant has a reduced high-affinity binding to NGF receptors on PC12 cells.

In order to further discriminate the binding affinity of the mutants to either one of the two receptors, binding assays were done with the A875 cell line, a human melanoma cell line that expresses only p75 and the Sf21 cell line that expresses only TrkA (Stephens et al., 1994). F53A had reduced TrkA binding by about 4–5-fold (Fig. 4). However, F53A showed similar binding affinity to wild-type NGF with the p75 receptor on the A875 cell line (Fig. 5). The binding affinity of F53A to p75 and TrkA was consistent with total binding to PC12 cells and cold chase-stable binding with PC12 cells, respectively. The overall binding results suggested that mutation at F53 affected binding affinity

Fig. 2. Competitive binding assay to receptors on PC12 cells. Wild-type rNGF and rNGF mutants were assayed for their ability to displace [125 I] NGF from receptors on PC12 cells. PC12 cells (1×10^6 cells/mL) were incubated with 100 pM [125 I] NGF and various concentrations of wild-type rNGF or each mutant rNGF at 37 °C for 40 min. Data are corrected for nonspecific binding and presented as the mean and standard deviations (bars) of the ratio of bound to free ligand from six determinations.

Fig. 3. Cold chase-stable binding to NGF receptors on PC12 cells. PC12 cells were incubated with 100 pM [125 I] NGF and two concentrations (0.1 nM and 1 nM) of wild-type (wt) rNGF or rNGF mutants at 37 °C for 40 min and followed by a chase with 4 μ M unlabeled mouse β -NGF for 30 min at 0.5 °C. Data are corrected for nonspecific binding and presented as the mean and standard deviations (bars) of the cold chase-stable binding as a fraction of the total binding from six determinations. The asterisk indicates statistical significance from wt at $p < 0.01$.

to TrkA and high-affinity binding in PC12 cells but not p75 binding.

TrkA phosphorylation

The ability of the mutants to induce TrkA phosphorylation, an initial step of NGF-mediated signal transduction, was examined. PC12 cells were treated with 500 pM NGF for 15 min and TrkA phosphorylation was measured by specific immunoprecipitation of TrkA, SDS gel electrophoresis, and immunoblotting for phosphotyrosine. F53A and F54A showed a dramatically reduced ability to induce Trk phosphorylation (Fig. 6). This result suggested that reduction of biological activity of F53A and F54A mutants was correlated with the reduction in TrkA phosphorylation. For F53A, the time course and dose-response curve for

Fig. 4. Competitive binding assay to TrkA receptors ectopically expressed in Sf21 cells. Wild-type rNGF and rNGF mutants were assayed for their ability to displace [125 I] NGF from TrkA receptors expressed on Sf21 cells. Sf21 cells (5×10^5 cells/mL) were incubated with 100 pM [125 I] NGF and various concentrations of wild-type rNGF and each mutant rNGF at 0.5 °C for 2 h. Data are corrected for nonspecific binding and presented as the mean and standard deviations (bars) of the ratio of bound to free ligand from six determinations.

Fig. 5. Competitive binding assay to p75 receptor on A875 cells. Wild-type rNGF and rNGF mutants were assayed for their ability to displace [125 I] NGF from p75 receptors on A875 cells. A875 cells (5×10^5 cells/mL) were incubated with 100 pM [125 I] NGF and various concentrations of wild-type rNGF and mutant rNGF at 37 °C for 40 min. Data are corrected for nonspecific binding and are presented as the mean and standard deviations (bars) of the ratio of bound to free ligand from six determinations.

TrkA phosphorylation was also tested. Both wild type and F53A induced maximal TrkA phosphorylation at 5 min after NGF treatment. However, the phosphorylation levels induced by F53A were significantly lower than the maximal level obtained with wild-type NGF (Fig. 7). F53A was about 70-fold less potent in eliciting TrkA phosphorylation compared to wild type (Fig. 8). F53A showed a less pronounced loss in TrkA binding (4–5-fold lower) (Fig. 4), but had a dramatic effect on TrkA phosphorylation (70-fold lower), suggesting that this mutation might affect TrkA activation separately from binding.

MG139 fibroblast survival assay

In order to examine a biological response to TrkA in the absence of p75, F53A was assayed for the NGF-dependent survival of

Fig. 6. TrkA phosphorylation induced by wild-type and mutant rNGF. PC12 cells were treated with 500 pM rNGF for 15 min and assayed for tyrosine phosphorylation. The phosphorylated TrkA band was identified by anti-phosphotyrosine immunoblotting following anti-TrkA immunoprecipitation (see the Materials and methods). Molecular weight markers are shown on the left. Arrow indicates the position of phosphorylated TrkA. Lane 1, wild-type rNGF; lane 2, F53A; lane 3, F54A; lane 4, Y52A; lane 5, no NGF.

Fig. 7. Time course of TrkA phosphorylation induced by wild-type rNGF, F53A. PC12 cells were treated with 1 nM of the indicated rNGF. At the indicated time intervals, Trk phosphorylation was measured as described in Figure 6 and the Materials and methods. Arrow indicates the position of phosphorylated TrkA. Time course of Trk phosphorylation induced by wild-type rNGF (A) or F53A rNGF (B). Quantitation of the phosphorylated Trk band by densitometric scanning expressed in arbitrary units of optical density (C).

MG139 fibroblasts that ectopically express TrkA but have no p75. NGF acts as a survival factor in dose-dependent fashion in this cell line (Barker et al., 1993). Cells were grown for 4 days in defined media containing various concentrations of rNGF and survival was then quantitated using a proliferation/cytotoxicity assay (see the Materials and methods). A 40-fold shift to higher concentrations was observed in the dose response curve for F53A (Fig. 9). This finding correlates well with the Trk phosphorylation results, suggesting that the major deficit with F53A is in TrkA activation.

Fig. 8. Dose-response of TrkA phosphorylation induced by wild-type and F53A rNGF. **A:** PC12 cells were treated with indicated concentrations of rNGF for 15 min and Trk phosphorylation was measured as described in Figure 6 and the Materials and methods. **B:** Phosphorylated TrkA band was quantitated by densitometric scanning and the results are expressed in arbitrary units of optical density.

Binding of R103A to high- and low-affinity receptors

Although R100A and R103A exhibited no loss of biological activity (Fig. 1; Table 1), previous mutagenesis studies had suggested that R100 and R103 might participate in p75 binding (Ibanez et al., 1990). Therefore, detailed binding studies of

Fig. 9. Dose-response curve of rNGF-mediated survival of fibroblast MG139 cells expressing the TrkA receptor. Cells were grown for 4 days in defined medium containing various concentrations of rNGF from 1 pM to 1 nM and then assayed for survival using CellTiter 96 nonradioactive cell proliferative/cytotoxicity assay kit as described in the Materials and methods. The colorimetric reading (arbitrary units) is expressed relative to the largest value as 100%.

R103A were made. R103A showed affinity for total binding to PC12 cells, predominantly measuring low-affinity binding, that was very similar to that of wild-type rNGF (Fig. 2). R103A also displayed a cold chase-stable binding affinity comparable to that of wild-type rNGF (Fig. 3). These results were confirmed with binding measurements to cells containing only one or the other type of receptor. The binding affinity of R103A to TrkA, expressed in Sf21 cells, was similar to that of wild-type NGF (Fig. 4). R103A showed similar binding affinity to wild-type NGF with the p75 receptor on the A875 cell line (Fig. 5). Thus, the binding of R103A for p75 and TrkA on cells containing only these receptors was consistent with their total binding on PC12 cells and cold chase-stable binding on PC12 cells, respectively. We conclude, therefore, that R103A shows similar binding affinity for both receptors as does wild-type NGF.

Discussion

In this study, the role of seven residues of human NGF, which are well conserved in all species of NGF and often conserved in the other neurotrophins, were examined by site-directed mutagenesis. Among these residues, Y52, F54, and F86 are aromatic residues located in NGF dimer interface; F53 is located in the hydrophobic core of NGF monomer; and T91, R100, and R103 are located in the β -strand region (McDonald et al., 1991; Holland et al., 1994). Of the seven conserved residues mutated in this study, five (Y52A, F86A, T91A, R100A, R103A) had only small effects on biological activity in the PC12 bioassay. These residues may possibly be involved in other cellular functions that are not directly reflected in bioactivity, but that require conservation of the particular residue. The interface residues (orange) and the position of key mutated residues in the NGF dimer are indicated in Figure 10 and Kinemage 1.

Role of the hydrophobic core and dimer contact residues Y52, F53, F54, and F86

Mutants Y52A and F54A were produced at a very low levels (Table 1), in agreement with a previous finding that the corresponding Y51G mutant of chicken NGF was expressed in COS cells at an undetectable level (Ibanez et al., 1990). Some studies have shown that mutations in buried positions are often destabilizing and make the protein more sensitive to proteolysis (Pakula et al., 1986; Reidhaar-Olson & Sauer, 1988; Cunningham & Wells, 1989). Y52 and F54 contribute to the hydrophobic dimer interface (McDonald et al., 1991). Substitution of an aromatic residue with a smaller alanine residue may diminish the hydrophobic interaction, weaken the dimer affinity, and therefore affect the protein stability. Mutation of another dimer interface residue, W21, was also found to severely affect the protein expression level (Drinkwater et al., 1991; Ibanez et al., 1990). Because mutation of Y52 and F54 significantly affect the level of protein expression in Sf21 cells, these two residues may play an important role in determining the protein stability.

Y52A, although poorly produced, retained wild-type levels of biological activity as well as TrkA phosphorylation (Figs. 1, 6). This result is in agreement with previous chemical modification studies showing that nitration of Y52 does not affect mouse NGF biological activity (Frazier et al., 1973; Bradshaw et al., 1994). However, substitution of F54 with alanine dramatically affected both NGF biological activity and TrkA phosphoryla-

Fig. 10. Model of the NGF dimer. The “north” end is up in this figure and the “south” end (down) contains the disulfide knot region. Both the N- and C- termini are oriented downward. The hydrophobic dimer interface residues are shown in orange in the center of the model. Interface residues W21, F49, Y52, F54, F86, W99, and F101 are indicated at the level they occur in the molecule. F53 is colored white (not in dimer interface) and is just outside of F86 in this view. R103 (white) is located on the edge (surface) just below F54. The structure was drawn using QUANTA software on a Silicon Graphics workstation from the coordinates of NGF (McDonald et al., 1991) kindly provided by Dr. Alexander Wlodawer, NCI.

tion (Figs. 1, 6), indicating the importance of this residue to protein function. Insufficient F54A was produced for the receptor binding studies. F86 is also in the dimer interface; however, substitution of this residue by alanine has less than a twofold effect on activity (Fig. 1; Table 1), suggesting that F86, like Y52, is less critical in the hydrophobic interactions than F53 or F54.

The 20-fold reduction in the biological activity of F53A on PC12 cells (Fig. 1) could be due either to reduced affinity for one or both NGF receptors or to decreased TrkA receptor activation. Both components are affected by this mutation. The F53A mutant had reduced binding to TrkA by only 5-fold (Fig. 4) and reduced high-affinity binding to PC12 cells by about 10-fold (Fig. 3). Nevertheless, TrkA phosphorylation of F53A was reduced by 70-fold (Figs. 7, 8). Thus, the effect of F53A on TrkA phosphorylation was much greater than its effect on TrkA binding. Because TrkA phosphorylation was measured in PC12 cells expressing both p75 and TrkA receptors, the presence of p75 on the cells could potentially have affected phosphorylation more than binding. Therefore, the ability of F53A

to mediate NGF-dependent survival of MG139 fibroblasts (that contain only the TrkA receptor) was also examined. A 40-fold shift to higher concentrations in the dose response curve for F53A was also observed with MG139 cells (Fig. 9), comparable to the Trk phosphorylation dose response in PC12 cells (Fig. 8). Thus, the discrepancy between the effects on Trk binding and Trk phosphorylation appears to be due to a difference in activation of the receptor by the mutant and not due to the presence of p75. Because TrkA phosphorylation is the initial step in NGF signal transduction, the 20–30-fold reduction in the biological activity of F53A and F54A (Table 1) can be accounted for by their reduced ability to induce TrkA phosphorylation. A distinction between TrkA binding and phosphorylation has also been observed in some of the NGF/BDNF chimeric mutants, which retain TrkA binding but cannot induce TrkA phosphorylation as well (Ibanez et al., 1993). Variable region II (residue 41, 45–49) of NGF has been shown to play an important role in TrkA receptor phosphorylation (Ibanez et al., 1993). Therefore, some residues or regions may not be important for TrkA binding, but may play an important role in determining the conformation required for TrkA activation.

The side chain of F53 is buried in a hydrophobic core of the NGF monomer (Fig. 10 and Kinemage 1) near, but not in, the dimer interface (McDonald et al., 1991; Holland et al., 1994). F53 is therefore not likely to be directly involved in binding with NGF receptors. Also, the major structural regions for TrkA binding are located elsewhere (Ibanez et al., 1993; Shih et al., 1994). The gross conformation of F53A must have been maintained, because this mutant retained the ability to bind the p75 receptor on PC12 cells and on A875 cells with normal affinity (Figs. 2, 5). Binding to p75 can be sensitive to point mutations at other positions (Drinkwater et al., 1991; Ibanez et al., 1992, 1993). The fact that F53A retained binding affinity to low-affinity NGF receptors also suggests that this residue is not essential for low-affinity binding to the p75 receptor.

Replacement of phenylalanine with a smaller alanine residue may weaken the hydrophobic contribution of F53 to the core stabilization. Decreased TrkA binding and high-affinity binding to PC12 cells for F53A may be related to a consequent conformational change or to an indirect alteration of subunit interactions. Ibanez et al. (1993) have proposed, based on mutagenesis data, that the TrkA binding site is formed by residues contributed by both NGF monomers. This interpretation argues for the necessity of a dimeric molecule for TrkA binding. Therefore, a mutation that affects the NGF subunit interaction may possibly affect the receptor binding by loosening the dimer interface. Activation of receptors by ligand-induced receptor dimerization appears to be a general mechanism for protein tyrosine kinase receptors (Heldin, 1995). TrkA homodimer formation upon NGF binding and intermolecular transphosphorylation were observed in fibroblasts expressing TrkA (Jing et al., 1992). Trk receptor activation is proposed to be induced by neurotrophin-mediated dimerization of receptors, with a stoichiometry of one neurotrophin dimer per receptor dimer, followed by autophosphorylation of their tyrosine residues (Jing et al., 1992; Clary et al., 1994; Philo et al., 1994). Because both F53A and F54A have significantly altered TrkA phosphorylation, the effect introduced by these two mutations may be related to the destabilizing effect on NGF subunit interactions and a consequent effect on TrkA dimerization. In the simplest case of causing dimerization of a receptor through binding to iso-

gous sites, e.g., PDGF (Heldin, 1995), the integrity of the receptor dimer is directly dependent on maintenance of the ligand dimer. Assuming this model is true for NGF, then a weakening of the ligand dimer (NGF) might affect the proximity of the cytoplasmic tails in the looser receptor dimer (TrkA) without greatly affecting the ligand-receptor affinity. Thus, a mutation of a dimer interface residue, while keeping the receptor-ligand contacts intact, could affect the amplification of a signal, i.e., receptor autophosphorylation, rather than affecting binding, per se. F53 and F54 may play an important role in NGF dimer stability that may be required for efficient TrkA phosphorylation. Further studies of the F53A and F54A NGF mutants with regard to their thermodynamic stabilities (Timm & Neet, 1992; Timm et al., 1994), secondary and tertiary structure (Timm & Neet, 1992), and induction of TrkA receptor dimerization (Jing et al., 1992) are required to verify this hypothesis.

Role of R100, R103, and T91

The arginines at positions 100 and 103 are located in the long β -strand regions (McDonald et al., 1991; Holland et al., 1994). Ibanez et al. (1990) showed that replacement of the arginine equivalent to R100 by lysine had no effect either on binding to the PC12 cell low-affinity receptor or on biological activity, indicating that this conservative substitution was tolerated. However, when both R100 and R103 equivalent residues were replaced by glycine, both binding to PC12 cells and biological activity were reduced more than 90% (Ibanez et al., 1990). In our study, the individual point mutations of arginine to alanine in R100A or R103A retained full biological activity (Fig. 1). In addition, R103A displayed wild-type levels of binding affinity to both the NGF low affinity receptor and TrkA receptor (Figs. 2, 3, 4, 5). The difference between our results and the previous study indicates that either the double mutation or the more drastic substitution by glycine, in which ϕ and ψ dihedral angles may vary more greatly, is required for disruption of receptor binding and biological activity. Because R100 forms a side-chain hydrogen bond with T91 and R103 forms a hydrogen bond with D30 (Bradshaw et al., 1994), a disruption of these hydrogen bonds that seems to be tolerated. Substitution of alanine for threonine at position 91 similarly did not affect biological activity (Fig. 1), confirming that this H-bond is not essential. Interestingly, the R103A mutation in NT-3 completely disrupted binding and activation of TrkC (Urfer et al., 1994), suggesting a different mode of interaction for this neurotrophin-Trk pair. Finally, the R103A mutant of NGF retained the wild-type level of binding to NGF low-affinity receptors, as did R103A in NT-3 (Urfer et al., 1994), indicating that the side chain of R103 is not involved in formation of the positively charged interface (K32, K34, K95) for binding to NGF low-affinity receptors. Measurements from the molecular model indicate that R103 is about 13–21 Å from this positively charged surface area.

Materials and methods

Proteins and reagents

Mouse β NGF was purified from 7S NGF that had been isolated from submaxillary glands of adult male Swiss Webster mice (Woodruff & Neet, 1986). Purified human rNGF from baculovirus was provided by Cephalon, Inc.

Baculovirus coding for the human TrkA gene was kindly provided by Dr. D. Kaplan. Polyclonal anti-Trk antibody was from Oncogene Science. Monoclonal antiphosphotyrosine antibody 4G10 was from UBI. Polyclonal anti-NGF antibody was from Collaborative Research, Inc. Grace's medium and Dulbecco's modified Eagle's medium (DMEM) were from GibcoBRL. ExCell-400 and ExCell-401 were from JRH Biosciences. Protein-A Sepharose was from Pharmacia. 125 I and the enhanced chemiluminescence (ECL) kit were from Amersham.

The Sf21 cell line was obtained from Dr. B. Corsaro, Cornell University (Meyer et al., 1994). The MG139 fibroblast cell line was a kind gift from Dr. P. Barker. PC12 cells were from a line initiated by Greene and Tischler (1976).

The cDNA for the coding region of human NGF was constructed from two human genomic clones as follows. The *Hind* III-*Eco*R I fragment of p β N8D8 (Ullrich et al., 1983; American Type Culture Collection #57490) was inserted into pGBT-518 (Gold Biotechnology, Inc.). Oligonucleotide-directed site-specific mutagenesis to delete DNA sequences 5' to the start of the region encoding prepro-hNGF was carried out using the Alter-Gene™ kit (Gold Biotechnology, Inc.) following the manufacturer's instructions. Following sequence verification, this fragment was assembled with the 363-bp *Eco*R I-[*Mbo* II] (brackets indicate that the *Mbo* II site was converted to a blunt end using T4 polymerase) fragment from p β N8B9 (Ullrich et al., 1983; American Type Culture Collection #57488) to generate the cDNA for the entire prepro-human NGF. This construct was inserted either into pVL941 (Luckow & Summers, 1989; provided by Dr. M. Summers, Texas A&M) for the production of recombinant wild-type virus or into the phagemid pGEM-7Zf(+) (Promega, Inc.) for mutagenesis. Mutant human rNGF cDNAs were generated by oligonucleotide-directed mutagenesis (Kunkel et al., 1987), sequenced to confirm the nucleotide substitution, and subsequently reinserted into pVL941 for the production of recombinant virus as described (Meyer et al., 1994).

The following oligonucleotides were synthesized for the site-directed mutagenesis: Y52A, 5'-GTGTATTCAAACAGGCCCTTTTGAGACC; F53A, 5'-GTATTCAAACAGTACGCTT TTGAGACCAAG; F54A, 5'-TTCAAACAGTACTTTGCTGAGACCAAGTG; F86A, 5'-CACGACTCACACCGCTGTCAAGGCGCTG; T91A, 5'-CAAGGCGCTGGCCATGGATG; R100A, 5'-CAGGCTGCCTGGGCGTTTATCCGGATAG; R103A, 5'-CTGGCGGTTTATCG CGATAGATACGGCC.

Expression, partial purification, and quantitation of recombinant NGF

Sf21 insect cell culture and expression of rNGF were performed according to the protocol from Invitrogen Corporation. Sf21 cells were maintained in supplemented Grace's medium containing 10% heat-inactivated fetal bovine serum. For expression of rNGF, Sf21 cells in suspension (2×10^6 cells/mL) were infected with recombinant baculovirus at a multiplicity of infection of 2–4 plaque forming units per cell and expressed for 72 h at 27 °C in ExCell-401 containing 5% fetal bovine serum. Some mutants were partially purified by a CM52 cation exchange column according to Luo and Neet (1992). Crude culture supernatant of infected cells or partially purified rNGF was analyzed by 15% polyacrylamide SDS gel electrophoresis. The amount of rNGF were quantitated by western blot and densitometric scanning of

NGF immunoreactive bands. The concentration of rNGF was determined by comparing the area of signal corresponding to the monomer form of each rNGF protein with a known amount of purified wild-type human rNGF.

PC12 cell bioassay

Biological activity was measured by the ability of rNGF to stimulate PC12 cell neurite outgrowth in a defined medium bioassay. PC12 cells were cultured at 37 °C in DMEM containing 10% horse serum, 5% fetal bovine serum, 4.5 mg/mL glucose, 4.0 mM L-glutamine, 100 units/mL penicillin, 100 units/mL streptomycin, and 100 units/mL fungizone in humidified 10% CO₂. The bioassay was performed in defined medium (DMEM containing 5 µg/mL insulin-transferrin-selenium, 4 mM glutamine, 0.5 mg/mL bovine serum albumin (BSA), and antibiotics) as described by Reinhold and Neet (1989). Neurite-bearing cells were counted 48 h after NGF addition. A cell was determined to be a neurite-bearing cell if it had at least one process that was longer than a cell body. These experiments were repeated at least two times.

MG139 fibroblast survival assay

The survival of MG139 fibroblasts was assayed using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Barker et al., 1993) with slight modification. MG139 cells were originally derived from the parent MG87 fibroblast subline by stable transfection with full-length TrkA in the CDMX vector (Barker et al., 1993). Cells were seeded into 96-well plates in defined media at 5×10^3 cells/well. Varying concentrations of rNGF were added and cells cultured for four days. The number of surviving cells was determined by color formation of a formazan product using the CellTiter 96 nonradioactive cell proliferative/cytotoxicity assay kit (Promega, Inc.). Each experiment was replicated twice.

Binding assay

Mouse β NGF was iodinated using the lactoperoxidase method (Woodruff & Neet, 1986) to a specific activity of 50–60 cpm/pg mNGF. Binding assays with PC12 or A875 cells were performed in suspension as previously described (Woodruff & Neet, 1986; Luo & Neet 1992). For total binding, 1×10^6 PC12 cells/mL (PC12) or 5×10^5 (A875) were incubated with 100 pM [¹²⁵I]-mouse β NGF and varying concentrations of rNGF as competitor at 37 °C for 40 min. For cold chase-stable binding, PC12 cells were incubated with 100 pM [¹²⁵I] NGF and two concentrations (0.1 nM and 1 nM) of rNGF at 37 °C for 40 min and followed by a chase with 4 µM unlabeled mouse β NGF for 30 min at 0.5 °C. TrkA binding was performed as previously described (Woo et al., 1995) with Sf21 cells ectopically expressing TrkA (Stephens et al., 1994); 5×10^5 cells/mL were incubated with 100 pM [¹²⁵I]-NGF in the presence of varying concentrations of rNGF for 2 h at 0.5 °C. In all the above binding assays, cell-bound [¹²⁵I] NGF was separated from free [¹²⁵I] NGF by sucrose cushion centrifugation and bound and free [¹²⁵I] NGF determined separately. Nonspecific binding, which was determined by a parallel incubation with a 1,000-fold excess of unlabeled mouse NGF, accounted for less than 3% of total binding for the binding assays in PC12 cells and A875 cells and less than 15%

of total binding for the binding assays in Sf21 cells expressing TrkA. All the results were corrected for nonspecific binding. Each experiment was repeated two or three times with triplicate sedimentations each.

Trk phosphorylation

PC12 cells grown to about 80% confluency in a 10-cm dish were treated with the appropriate amount NGF for 5 min. Cells were washed with cold phosphate-buffered saline, lysed in 1 mL of RIPA buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 0.2% SDS, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 µg/mL leupeptin, 1 mM sodium orthovanadate), sonicated for 5 s, and rocked for 20 min at 4 °C. Insoluble material was removed by centrifugation at 4 °C for 10 min at 12,000 rpm. Protein concentrations in cell lysates were estimated using the Bradford protein assay kit (Bio-Rad) with BSA as the standard. Lysates containing equal amounts of protein were incubated with anti-Trk antibody (1 µg/mL) overnight at 4 °C and the immunocomplex was precipitated by incubation with protein-A Sepharose for 2 h at 4 °C. Immunoprecipitates were collected and washed twice with cold RIPA buffer and once with H₂O. Immunocomplexes were dissociated in Laemmli sample buffer by boiling for 5 min, electrophoresed on 7% SDS polyacrylamide gel and transferred to nitrocellulose. The filter was blocked with 2% BSA/TBST buffer (10 mM Tris, pH 7.5, 0.15 M NaCl, 0.1% Tween 20) for 1 h at room temperature, and incubated with a 1/1,000 dilution of anti-phosphotyrosine antibody overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated anti-mouse secondary antibody for 1 h at room temperature. Reactive bands were detected by ECL following the prescribed protocol.

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