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Disruption of the Low Affinity Receptor-Binding Site in NGF Allows Neuronal Survival and Differentiation by Binding to the *trk* Gene Product

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Summary

Nerve growth factor (NGF), like many other growth factors and hormones, binds to two different receptor molecules on responsive cells. The product of the proto-oncogene *trk*, p140^{trk}, is a tyrosine kinase receptor that has been identified as a signal-transducing receptor for NGF, while the role of the low affinity NGF receptor, p75^{NGFR}, in signal transduction is less clear. The crystal structure of NGF has recently been determined, although structures involved in receptor binding and biological activity are unknown. Here we show that Lys-32, Lys-34, and Lys-95 form a positively charged interface involved in binding to p75^{NGFR}. Simultaneous modification of Lys-32 with either of the two other lysines resulted in loss of binding to p75^{NGFR}. Despite the lack of binding to p75^{NGFR}, these mutants retained binding to p140^{trk} and biological activity, demonstrating a functional dissociation between the two NGF receptors.

Introduction

The control of cell growth and differentiation requires specific factors that exert their effects via interaction with receptors on the surface of responsive cells. Despite the increasing number of growth and differentiation factors that have been discovered and characterized, the precise structures involved in binding and biological activity and the sequential and causal molecular events underlying the activation of multiple receptors are largely unknown.

Nerve growth factor (NGF) is a 118 amino acid polypeptide that controls the survival, development, and differentiation of the sympathetic nervous system, as well as parts of the sensory and central nervous systems (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980; Whittemore and Seiger, 1987; Thoenen et al., 1987). The biologically active form of NGF is a dimer of identical subunits, each of which is produced from a precursor molecule (Angeletti and Bradshaw, 1971; Angeletti et al., 1973). A cDNA clone for NGF was first isolated in the mouse (Scott et al., 1983). Subsequently, the NGF gene has been characterized in a number of other species including several mammals, birds, reptiles, and fishes (Schwarz et al., 1989; Hallböök et al., 1991). NGF belongs to a family of structurally and functionally related molecules, collectively known as neurotrophins, which includes three other members, brain-derived neurotrophic factor (BDNF) (Barde et al., 1982; Leibrock et al., 1989), neurotrophin-3 (NT-3) (Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990; Ernfors et al., 1990), and neurotrophin-4 (NT-4) (Hallböök et al., 1991; Ip et al., 1992).

NGF interacts with a low affinity receptor expressed on a variety of cell types of both neuronal and nonneuronal origin (Ernfors et al., 1988; Yan and Johnson, 1988; Heuer et al., 1990; Hallböök et al., 1990). This receptor is represented by a transmembrane glycoprotein of approximately 75,000 daltons (p75^{NGFR}) that binds NGF with a K_d of 10^{-9} M (Johnson et al., 1986; Radeke et al., 1987). However, high affinity binding (K_d = 10^{-11} M), restricted to a subpopulation of p75^{NGFR}-positive cells, is necessary to mediate the biological action of NGF (Banerjee et al., 1973; Herrup and Shooter, 1973; Sutter et al., 1979; Richardson et al., 1986). While the molecular relationship between the two receptor states is not entirely clear, several reports have indicated that the cytoplasmic domain of p75^{NGFR}, which lacks structural features known to mediate signal transduction in other receptors, is required for high affinity binding and signal transduction (Hempstead et al., 1989; Yan et al., 1991; Berg et al., 1991). It has recently been demonstrated that the proto-oncogene *trk* encodes a functional receptor for NGF (Kaplan et al., 1991a; Klein et al., 1991). The product of the *trk* proto-oncogene is a 140,000 dalton protein (p140^{trk}) that is a member of the tyrosine kinase family of transmembrane receptors (Martin-Zanca et al., 1991). Though it has been postulated that this protein participates in the primary signal transduction mechanism of NGF, there is considerable disagreement regarding the equilibrium binding constant of p140^{trk} for NGF. Whereas Klein et al. (1991) reported that p140^{trk} binds NGF with both low and high affinities, Kaplan et al. (1991a) and Hempstead et al. (1991) reported that p140^{trk} binds NGF with an affinity similar to that of p75^{NGFR} and that coexpression of both receptors is required for high affinity binding to occur.

A better understanding of the molecular mechanisms by which NGF exerts its biological effects could be provided by the study of structure–function relationships and the creation of NGF mutants with altered properties. Initial studies along this line have analyzed the functional importance of highly conserved amino acid residues in chicken NGF (Ibáñez et al., 1990). More recently, an analysis of chimeric molecules between NGF and BDNF has delineated regions involved in determining the biological speci-

A

	25 36
rat NGF	KTPAIDIKGKEV
mouse NGF	.....
human NGF	.....
bovine NGF	.....
guinea pig NGF	.....
chicken NGF	.....
xenopus NGF	..K.....
snake NGF	.....NT.

B

rat NGF	KTPAIDIKGKEV
rat BDNF	.K..V.MS.GT.
rat NT-3	..S.I..R.HQ.
xenopus NT-4	.R..V.DR..I.

Figure 1. Conservation of Amino Acid Residues 25 to 36 in NGFs from Different Species and in the Homologous Region of Different Neurotrophins

Amino acid residues are listed using the single letter code.
(A) Alignment of residues 25 to 36 from rat (Whittemore et al., 1988), mouse (Scott et al., 1983), human (Ullrich et al., 1983), bovine (Meier et al., 1986), guinea pig (Schwarz et al., 1989), chicken (Ebendal et al., 1986; Meier et al., 1986), Xenopus (Carriero et al., 1991), and snake (Selby et al., 1987) NGF.
(B) Alignment of residues 25 to 36 from rat NGF and the homologous residues of rat BDNF (Maisonpierre et al., 1990), rat NT-3 (Maisonpierre et al., 1990; Ernfors et al., 1990), and Xenopus NT-4 (Hallböök et al., 1991).

ficities of these two factors (Ibáñez et al., 1991a). Comparison of NGF genes from different species has revealed clusters of amino acid residues that are highly conserved across different groups of vertebrates. Among these conserved parts, the region spanning residues 25 to 36 is the most hydrophilic and therefore likely to be on the surface of the NGF molecule (Meier et al., 1986; Ebendal et al., 1989). Synthetic peptides designed from this sequence have been shown to inhibit the in vitro biological activity of NGF (Longo et al., 1990). In the present study, we have used site-directed mutagenesis combined with binding and biological assays to assess the functional importance of these amino acid residues. While this work was being completed, a high resolution three-dimensional crystal structure of NGF was completed (McDonald et al., 1991). The solved NGF structure shows that the amino acid residues analyzed in this study include a β-hairpin loop exposed on the outside arm of the NGF dimer (McDonald et al., 1991). Our results show that residues with a positively charged side chain within this region are responsible for the main contact between NGF and $p75^{NGFR}$. NGF molecules mutated in these positions do not bind to the $p75^{NGFR}$ but retain binding to the *trk* proto-oncogene product and biological activity, supporting the argument that $p140^{trk}$ alone is sufficient, at least in culture, to mediate biological activity of NGF in neuronal cells.

Results

Modification of Residues in the 25–36 Region Alters the Stability of the NGF Molecule

Alanine-scanning mutagenesis (Cunningham and Wells, 1989) was applied to map structurally and functionally important residues in the region between amino acid residues 25 and 36 of rat NGF. This region is highly conserved among different species of vertebrates (Figure 1A) and shows 50%–60% conservation in other members of the NGF family (Figure 1B). Mutant proteins were transiently expressed in COS cells. The yield of mutant protein production was assessed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of metabolically labeled polypeptides in conditioned medium of transfected cells in order to standardize for the amount of mutant protein used for receptor binding and biological assays. As shown in Table 1, the levels of mutant NGF proteins varied over a 10-fold range. Five of the mutant NGF proteins (K25A, A28Δ, D30A, G33Δ, and V36A) did not accumulate in the medium at detectable levels. Interestingly, these residues correspond to the five positions from this domain that are strictly conserved among the different members of the NGF family (Figure 1B). No protein was detected either after Lys-25 or Gly-33 was changed into the more similar amino acid residue Gln or Ala, respectively (Table 1). In contrast, the D30A and V36A mutants could be rescued by replacement into Asn and Leu, respectively, though at lower levels than those seen with the wild-type protein (Table 1). Lys-25 was also changed into Arg, the most conservative replacement possible at this position. This mutation allowed the detection of NGF protein at about 50% of the levels of the wild-type protein (Table 1).

The variations observed in the amounts of mutant protein may reflect differences in protein synthesis, stability,

Table 1. Relative Yield, Receptor Binding to PC12 Cells, and Specific Biological Activity of Wild-Type and Mutant NGF Proteins

Mutant Protein[a]	Yield[b]	Receptor Binding[c]	Biological Activity[c]
	% of Wild Type		
Wild type	100	100	100
K25A	–	–	–
K25Q	–	–	–
K25R	50	130	100
T26A	40	100	100
T27A	46	120	63
A28Δ	–	–	–
T29A	18	71	74
T26A+T27A+T29A	–	–	–
D30A	–	–	–
D30N	11	25	23
I31A	28	30	25
I31M	50	35	100
I31V	34	130	100
K32A	76	16	100
G33Δ	–	–	–
G33A	–	–	–
K34A	53	50	100
E35A	5[d]	85[e]	85[e]
K32A+K34A+E35A	65	<1	65
V36A	–	–	–
V36L	51	33	90

[a] Mutants are abbreviated by the wild-type residue (single amino acid designation), followed by its codon number and the mutant residue. Δ indicates that the corresponding residue was deleted.
[b] Steady-state levels calculated after SDS–PAGE of metabolically labeled conditioned medium. The dash indicates that the level of mutant protein was below detection (< 2% of wild-type NGF).
[c] Data from two dose-response experiments varied by ± 10% of the average values reported here.
[d] Data based on the fully processed form (see text for details).
[e] Data based on both processed and unprocessed forms (see text for details).

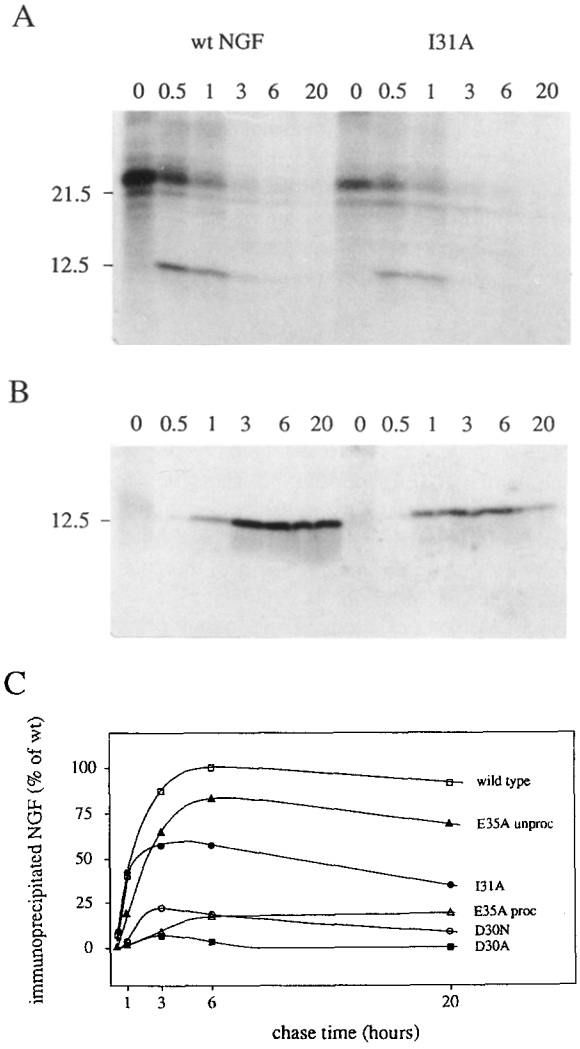

Figure 2. Stability of Wild-Type and Mutant NGF in COS Cells

(A) SDS–PAGE of immunoprecipitates from cellular extracts of pulse-labeled COS cells expressing wild-type NGF or I31A mutant NGF after different times of chase. Chase time in hours is indicated. Molecular size markers are in kilodaltons.

(B) SDS–PAGE of immunoprecipitates from conditioned medium of pulse-labeled COS cells expressing wild-type NGF or I31A mutant NGF after different times of chase. Chase time in hours is indicated. Molecular size marker is in kilodaltons.

(C) Levels of wild-type and mutant NGF proteins immunoprecipitated from conditioned medium of pulse-labeled COS cells after different times of chase. Data were obtained from scanning densitometry of X-ray films from protein gels like the one shown in (B).

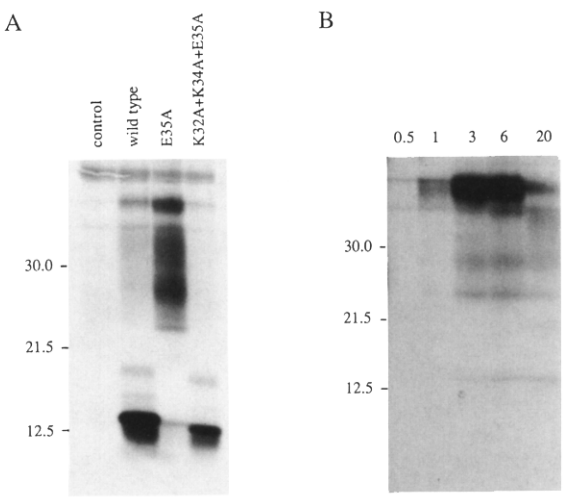

Figure 3. Effect of the E35A Mutation on the Processing of the NGF Propeptide

(A) SDS–PAGE of immunoprecipitates from conditioned medium of metabolically labeled mock-transfected COS cells (control) and cells expressing wild-type NGF, E35A, or K32A+K34A+E35A mutant NGF proteins. Molecular size markers are in kilodaltons.

(B) SDS–PAGE of immunoprecipitates from conditioned medium of pulse-labeled COS cells expressing E35A mutant NGF after different times of chase. Chase time in hours is indicated. Molecular size markers are in kilodaltons.

or secretion of individual polypeptides in COS cells. To discriminate between these possibilities, pulse-chase experiments were carried out, followed by immunoprecipitation and SDS–PAGE. After a 15 min pulse, a predominating 23K wild-type NGF precursor protein could be immunoprecipitated from cellular extracts (Figure 2A). Fully processed, mature 13K NGF was detected after 30 min of chase, and almost all of the intracellular NGF disappeared after 3 hr. The disappearance of intracellular NGF correlated with the appearance of NGF in the medium, which peaked 6 hr after the chase and remained at this level for at least 14 more hours (Figure 2B). The I31A mutant, which was produced at a level three to four times lower than wild-type NGF (Table 1), accumulated in the transfected cells to a similar extent as the wild-type protein (Figure 2A). However, lower levels of the I31A mutant were detected in the medium, and a drop of 50% was seen in the last 17 hr after the chase (Figure 2B), indicating a reduced stability of the I31A protein. Similarly, the amount of the D30N mutant protein, produced at 10-fold lower levels than wild-type NGF (Table 1), decreased significantly after 3 hr of chase (Figure 2C). In addition, very low levels of the D30A mutant protein could be seen after 3 hr of chase, although they dropped to undetectable levels in the following 12 hr (Figure 2C). The reduced half-lives of the I31A, D30N, and D30A mutant proteins, estimated to be 18, 12, and 3 hr, respectively, indicated that the reduced yields of these mutants were due to lower stability of these proteins in the conditioned medium. The greatly reduced peak levels seen after 3 hr of chase in the D30N and D30A mutants suggested that in this case protein synthesis could also be affected (Figure 2C).

Replacement of Glu-35 for Ala Affects the Processing of the NGF Protein

Fully processed, mature E35A mutant protein was detected in the conditioned medium at a level corresponding to 5% of wild-type NGF (Table 1). However, after immunoprecipitation, several higher molecular mass polypeptides (in the range of 23 to 34 kd) were seen that were only very weakly detected in the wild-type NGF sample (Figure 3A). Pretreatment of the conditioned medium at 70°C in the presence of 1% SDS and 1.5 M NaCl prior to immunopre-

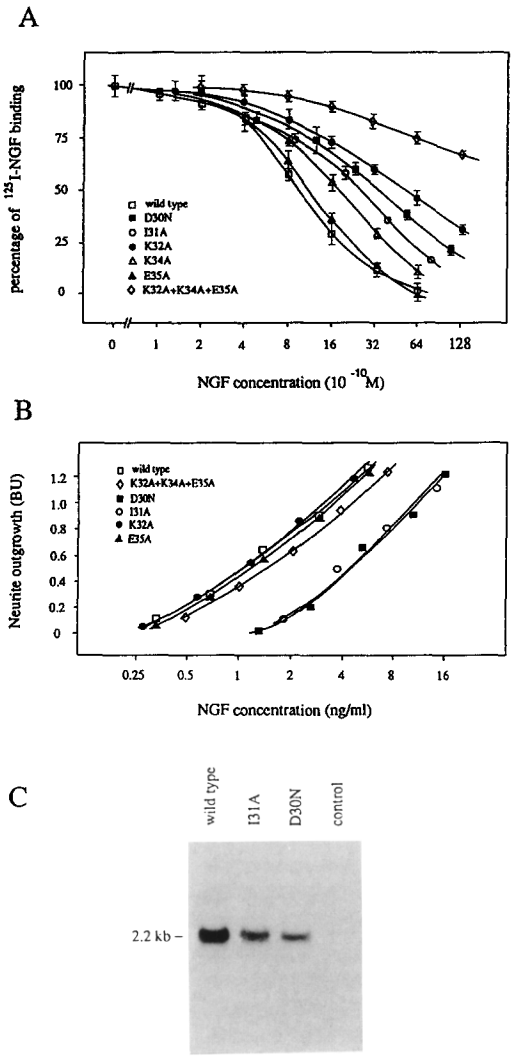

Figure 4. Competitive Receptor Binding and Biological Activities of Wild-Type and Mutant NGF in PC12 Cells

(A) Serial dilutions of transfected COS cell–conditioned medium containing equal amounts of wild-type (open squares), D30N (closed squares), I31A (open circles), K32A (closed circles), K34A (open triangles), E35A (closed triangles), and K32A+K3A+E35A (open diamonds) mutant NGF were assayed for their ability to displace ^{125}I-labeled NGF from receptors on PC12 cells. Medium from mock-transfected cells failed to displace ^{125}I-labeled NGF from PC12 cells. Each point represents the mean ± standard deviation of triplicate determinations.

(B) Dose response of neurite outgrowth stimulation from E9 chick sympathetic ganglia. Serial dilutions of transfected COS cell–conditioned medium containing equal amounts of wild-type (open squares), D30N (closed squares), I31A (open circles), K32A (closed circles), E35A (closed triangles), and K32A+K3A+E35A (open diamonds) mutant NGF were assayed for their abiliy to stimulate neurite outgrowth from E9 chick sympathetic ganglia. Data from three determinations varied by ± 10% of the average values reported here.

(C) c-*fos* mRNA induction in PC12 cells. Northern blot of total RNA from PC12 cells treated for 30 min with 10 ng/ml wild-type, I31A, and D30N mutant NGF or with conditioned medium from mock-transfected cells (control). Ten micrograms of total RNA from each sample was electrophoresed, blotted onto a nitrocellulose membrane, and hybridized to a radiolabeled rat c-*fos* gene fragment.

cipitation did not affect the polypeptide pattern immunoprecipitated from the E35A mutant (not shown), indicating that the higher molecular weight polypeptides did not represent unrelated proteins that coprecipitated with the E35A mutant. Instead, the size of these polypeptides suggests that they represent incompletely processed intermediates in the biosynthesis of the E35A protein. Pulse-chase experiments using this mutant revealed that both the incompletely processed and mature forms of this protein were very stable in the conditioned medium (Figures 2C and 3B).

Amino Acid Residues in the β-Hairpin Loop 30–34 Involved in Receptor Binding to PC12 Cells

Conditioned medium containing equal amounts of mutant NGF proteins was used to displace ^{125}I-NGF from its receptors on the NGF-responsive pheochromocytoma cell line PC12. Competitive binding assays were performed using concentrations of ^{125}I-NGF (~1.5 nM) at which 80% of the radiolabeled ligand associated with the cells is bound to low affinity NGF receptors (NGFRs) (Sutter et al., 1979). Concentrations of wild-type and mutant proteins required to displace 50% of the ^{125}I-labeled NGF from the PC12 cells (IC_{50}) were calculated (Table 1). The conservative replacement of Lys-25 for Arg or the replacement of either of the three Thr residues (26, 27, and 29) for Ala did not affect the affinity of the protein for receptors on PC12 cells (Table 1). However, a 3- to 4-fold reduction in binding affinity was observed when Asp-30 or Ile-31 was modified (Table 1 and Figure 4A). The importance of Ile-31 was further tested by replacement with Met (the residue that occurs at this position in BDNF) (Leibrock et al., 1989) and with Val. Interestingly, only the most conservative change (I31V) allowed a binding affinity similar to wild-type NGF (Table 1). A marked reduction of receptor binding was seen after replacement of Lys-32 with Ala, in which case the affinity was reduced approximately 6-fold compared with wild-type NGF (Table 1 and Figure 4A). Replacement of Lys-34 with Ala and Val-36 with Leu reduced binding to 50% and 45% of the wild type, respectively (Table 1 and Figure 4A). Surprisingly, the incompletely processed E35A mutant showed close to wild-type binding affinity (Table 1 and Figure 4A), indicating that intermediates in NGF biosynthesis can bind to NGFRs as efficiently as the mature protein.

Modification of Asp-30 and Ile-31 Reduces the Biological Activity of NGF

The specific biological activity of the mutant NGF proteins was first studied by assaying their ability to stimulate neurite outgrowth from E9 chick sympathetic ganglia (Levi-Montalcini and Angeletti, 1968; Ebendal, 1984, 1989). In agreement with their ability to displace ^{125}I-labeled NGF from PC12 cells, the biological activities of the mutants K25R, T26A, T27A, and T29A were all similar to the activity of wild-type NGF (Table 1). To test the possibility that the Thr residues could compensate their modification when changed individually, a triple mutant was generated where the three Thr residues were simultaneously replaced by

Ala. However, this mutant failed to accumulate in the medium of transfected cells at detectable levels (Table 1).

A 4-fold reduction of biological activity was seen with the D30N and I31A mutants (Table 1 and Figure 4B) that correlated with their respective receptor binding affinities (Table 1). To eliminate the possibility that the decreased activity was due to the reduced stability of these mutant molecules (see Figure 2C), induction of c-*fos* mRNA was tested in PC12 cells. It is well documented that maximal induction of c-*fos* mRNA in these cells takes place within 30–45 min after exposure to NGF (Milbrandt, 1986; Gizang-Ginsberg and Ziff, 1990), a time period that is 20 to 30 times shorter than the estimated half-lives of these molecules. A peak in c-*fos* mRNA was detected after 30 min exposure of PC12 cells to wild-type NGF (Figure 4C). Both the D30N and the I31A mutants induced maximal c-*fos* mRNA levels after 30 min that were, however, 3- to 4-fold lower than the maximal level obtained with wild-type NGF (Figure 4C).

Interestingly, four mutants with reduced binding affinities to PC12 cells (I31M, K32A, K34A, and V36L) showed wild-type levels of biological activity (Table 1 and Figure 4B). Thus, for the K32A mutant, the 6-fold reduction in binding did not affect its biological activity in the sympathetic ganglia (compare Figures 4A and 4B). In agreement with the receptor-binding data, the E35A mutant displayed wild-type levels of biological activity, despite the fact that it contained only ~5% of a correctly processed, mature protein (Table 1 and Figure 4B).

The Simultaneous Replacement of Lys-32, Lys-34, and Glu-35 by Ala Drastically Reduces Binding but Not Biological Activity of NGF

The three charged residues Lys-32, Lys-34, and Glu-35, where an individual mutation had no effect on the biological activity, were simultaneously replaced by Ala, thereby eliminating the charged side chains at these positions. Interestingly, this mutant protein was completely recovered as a fully processed protein in spite of its containing the E35A mutation (see Figure 3A). The triple mutation reduced binding of this protein to PC12 cells to less than 1% of that seen with the wild-type molecule (Table 1 and Figure 4A). The same result was obtained when the cells were preincubated with the mutant protein for 2 hr prior to the addition of ^{125}I-labeled NGF (not shown). However, the biological activity of the triple mutant in sympathetic ganglia was close to wild-type NGF activity (Table 1 and Figure 4B).

Next, neurite outgrowth was assayed in PC12 cells to test whether the loss of binding correlates with the biological activity in these cells (Figure 5A). The individual change of Lys-32, Lys-34, and Glu-35 to Ala did not significantly change the ability of the proteins to stimulate neurite outgrowth in spite of their different affinities to NGFRs on these cells. Moreover, the triple mutant (K32A+K34A+E35A) also elicited wild-type activity, despite its greatly reduced low affinity binding to PC12 cells (Figure 5A).

The possibility that the apparent discrepancy observed between binding and biological activity was due to a slower

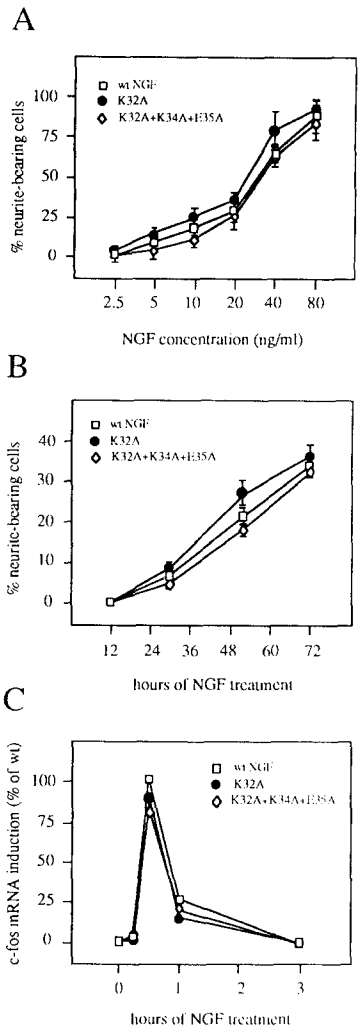

Figure 5. Biological Activities in PC12 Cells of Wild-Type NGF and NGF Mutants Deficient in Low Affinity Receptor Binding

(A) Dose response of neurite outgrowth stimulation in PC12 cells. Serial dilutions of transfected COS cell–conditioned medium containing equal amounts of wild-type (open squares), K32A (closed circles), and K32A+K3A+E35A (open diamonds) mutant NGF were assayed for their ability to stimulate neurite outgrowth from PC12 cells. Neurite outgrowth was scored after 5 days in culture as the percentage of cells bearing processes longer than 2 cell diameters per microscope field. Each point represents the mean ± standard deviation of triplicate determinations.

(B) Kinetics of neurite outgrowth stimulation in PC12 cells. PC12 cells were treated with 50 ng/ml wild-type (open squares), K32A (closed circles), or K32A+K3A+E35A (open diamonds) mutant NGF. At the indicated time intervals neurite outgrowth was scored as the percentage of cells bearing processes longer than 2 cell diameters per microscope field. Each point represents the mean ± standard deviation of triplicate determinations.

(C) Kinetics of c-*fos* mRNA induction in PC12 cells. PC12 cells were treated with 10 ng/ml wild-type (open squares,) K32A (closed circles), or K32A+K3A+E35A (open diamonds) mutant NGF. At the indicated time intervals total RNA was prepared and 10 μg from each sample was electrophoresed, blotted onto a nitrocellulose membrane, and hybridized to a radiolabeled rat c-*fos* gene fragment. Data from two experiments varied by ± 15% of the average values reported here.

receptor-mediated degradation was also examined. As seen with other peptide hormones that undergo receptor-mediated endocytosis (i.e., insulin), a reduced binding affinity may not always translate into a reduced biological activity when examined over a longer period of time. As a consequence of the reduced binding, mutant molecules may have a lower rate of receptor-mediated degradation that results in a slower but prolonged biological activity that can reach wild-type levels when integrated over a period of time. To investigate this possibility, the kinetics of both an early (c-*fos* mRNA induction) and a delayed (stimulation of neurite outgrowth) response in PC12 cells were studied. Despite their reduced binding affinities, both the K32A and the triple mutant induced c-*fos* mRNA and neurite outgrowth with the same time course and intensity as the wild-type molecule (Figures 5B and 5C).

Mutation of Lys-32 and Lys-34 Affects the Binding of NGF to p75^{NGFR} but Not to p140^{trk}

Receptor binding assays to PC12 cells were performed using high concentrations of ^{125}I-labeled NGF at which most of the observed binding is of the low affinity type (Sutter et al., 1979). However, since PC12 cells express both p75^{NGFR} and p140^{trk} (Herrup and Thoenen, 1979; Hosang and Shooter, 1985; Kaplan et al., 1991b), these results cannot clearly discriminate between the binding of the mutant NGFs to either one of these two molecules. Therefore, we next compared the binding affinities of the mutants K32A, K34A, E35A, and the triple mutant K32A+K34A+E35A with A875 cells, a human melanoma cell line that expresses high amounts of only p75^{NGFR} (Buxser et al., 1983), and with *rtrk* 3T3 cells, a fibroblast cell line that expresses only rat p140^{trk} (Kaplan et al., 1991a).

The replacement of the positively charged side chain of Lys-32 by the methyl group of Ala reduced the binding of the molecule to p75^{NGFR} to 5% of the binding seen with wild-type NGF (Table 2 and Figure 6). The change of Lys-34 into Ala reduced binding to A875 cells to 55% of the wild-type levels. However, the simultaneous replacement of Lys-32, Lys-34, and Glu-35 completely abolished the binding of the mutant molecule to p75^{NGFR} (Table 2 and Figure 6). The individual change of Glu-35 into Ala had no effect on the binding affinity to A875 cells (Table 2 and Figure 6), indicating that the loss of binding seen with the triple mutant was due to the modification of the positively charged residues Lys-32 and Lys-34.

Table 2. Relative Receptor Binding to A875 Cells and *rtrk* NIH 3T3 Cells of Wild-Type and Mutant NGF Proteins

Mutant Protein	Binding to A875 Cells (% of Wild Type)	Binding to *rtrk* NIH 3T3 Cells (% of Wild Type)
Wild type	100	100
K32A	5	100
K34A	55	90
E35A	100	100
K32A+K34A+E35A	No IC_{50}	55
K95A	55	80
K32A+K95A	<1	40
K32A+K34A+E35A+K95A	No IC_{50}	40

Data from three independent experiments varied by ± 10% of the average values reported here.

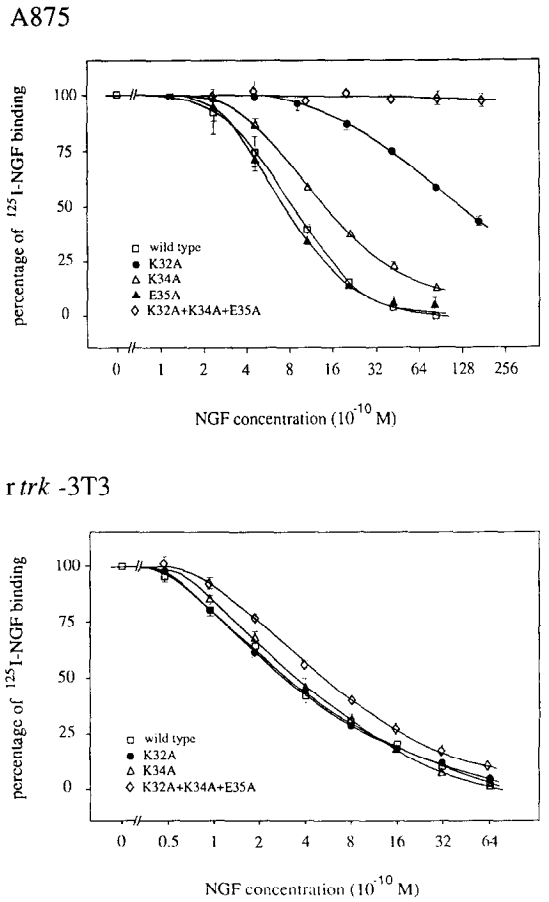

Figure 6. Competitive Receptor Binding of Wild-Type NGF and NGF Mutants Deficient in Low Affinity Receptor Binding to A875 Cells Expressing p75^{NGFR} and *rtrk* NIH 3T3 Cells Expressing p140^{trk}

Serial dilutions of transfected COS cell–conditioned medium containing equal amounts of wild-type (open squares), K32A (closed circles), K34A (open triangles), E35A (closed triangles), and K32A+K3A+E35A (open diamonds) mutant NGF were assayed for their ability to displace ^{125}I-labeled NGF from receptors on A875 or *rtrk* NIH 3T3 cells. Equivalent amounts of medium from mock-transfected cells failed to displace ^{125}I-labeled NGF from these cells. Each point represents the mean ± standard deviation of triplicate determinations.

Despite its 20-fold reduction in binding to p75^{NGFR}, the K32A mutant was indistinguishable from wild-type NGF in binding to p140^{trk} expressed on *rtrk* 3T3 cells (Table 2 and Figure 6). Similarly, the K34A and E35A mutants showed wild-type affinity to p140^{trk} (Table 2 and Figure 6). Interestingly, the triple mutant, which failed to displace ^{125}I-NGF from p75^{NGFR}, retained significant binding to p140^{trk}, at about 55% of the level seen with wild-type NGF (Table 2 and Figure 6).

Lys-95 Also Forms Part of the Receptor-Binding Site to p75^{NGFR}

The results with the Lys-32, Lys-34, and the triple mutant suggest that these two positively charged residues form

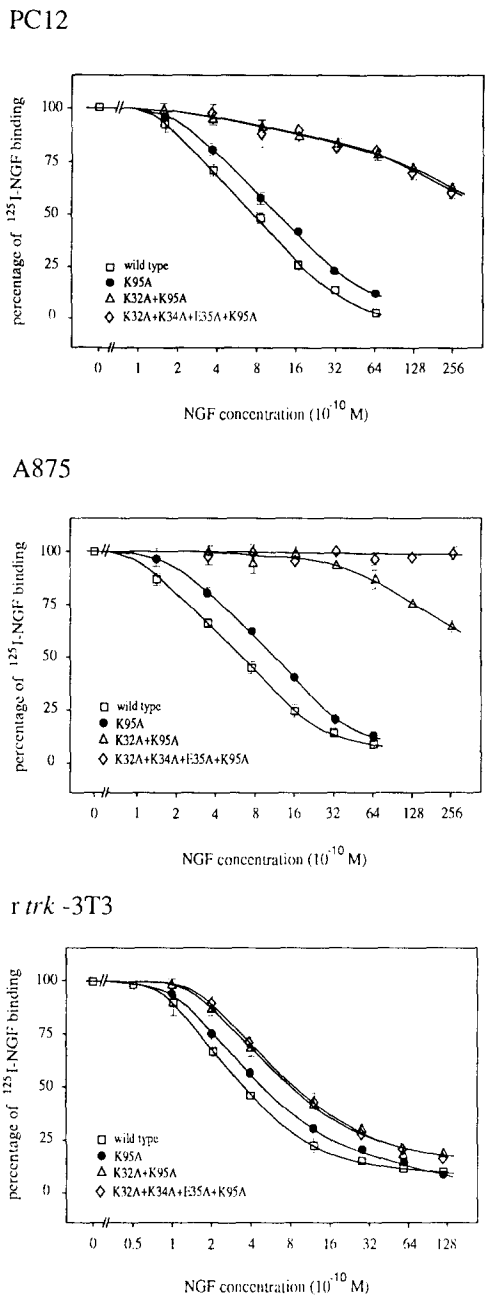

Figure 7. Effect of the K95A Mutation on Receptor Binding of NGF to Receptors on Different Cell Types

Serial dilutions of transfected COS cell–conditioned medium containing equal amounts of wild-type (open squares), K95A (closed circles), K32A+K95A (open triangles), and K32A+K3A+E35A+K95A (open diamonds) mutant NGF were assayed for their ability to displace ^{125}I-labeled NGF from receptors on PC12, A875, or *rtrk* NIH 3T3 cells. Equivalent amounts of medium from mock-transfected cells failed to displace ^{125}I-labeled NGF from these cells. Each point represents the mean ± standard deviation of triplicate determinations.

contact points between NGF and the p75^{NGFR} molecule. Examination of the NGF crystal structure with computer graphics revealed that another positively charged residue, Lys-95, is spatially close to Lys-32 and Lys-34. As in the case of the other two residues, Lys-95 is also fully exposed and does not participate in secondary interactions. To test the possibility that Lys-95 could also take part in the contact to the p75^{NGFR} molecule, this residue was replaced by Ala. A double mutant K32A+K95A and a quadruple mutant K32A+K34A+E35A+K95A were also generated. The K95A mutant showed 65% binding to PC12 cells compared with wild-type NGF (Figure 7). However, combination of K95A with K32A or with K32A+K34A+E35A drastically reduced binding to PC12 cells to 0.7% of wild-type levels (Figure 7). The reduction of low affinity binding to PC12 cells correlated with loss of binding to p75^{NGFR} expressed on A875 cells (Table 2 and Figure 7). In the case of the quadruple mutant, no IC_{50} could be calculated. However, despite their inability to bind to p75^{NGFR}, these mutants retained the ability to displace ^{125}I-labeled NGF from p140^{trk} expressed on fibroblasts (Table 2 and Figure 7) and promoted neurite outgrowth from sympathetic neurons (Figure 8A) at significant levels.

The triple mutant K32A+K34A+E35A and the double mutant K32A+K95A offer a possibility to examine the role of p75^{NGFR} in neuronal survival. Dissociated sympathetic neurons from the rat superior cervical ganglion were tested for survival after 3 days in culture. Less than 5% of the cells survived in the presence of medium from mock-transfected cells or in normal medium when compared with wild-type NGF or purified mouse NGF (Figure 8B). However, in cultures treated with the mutant NGFs, the extent of neuronal survival was identical to that seen with the wild-type protein (Figure 8B).

Discussion

Amino Acid Residues Involved in Stability, Receptor Binding, and Biological Activity in the Loop Region 25–36 of NGF

This report describes an analysis of highly conserved residues in a hydrophilic region of NGF by site-directed mutagenesis. While this study was being completed, the crystal structure of the NGF dimer was determined (McDonald et al., 1991). A 2.3 Å resolution revealed a novel structure consisting of three antiparallel pairs of β strands and four loop regions that contain almost all the variable residues observed between different NGF-related molecules. One of these loops corresponds to the residues analyzed in the present study and includes a β-hairpin turn (residues 30 to 34). Our results show that residues in region 25–36 are important for stability, receptor binding, and biological activity of the NGF molecule (Figure 9A).

Lys-25 was shown to play an important structural role since only the closely related Arg, but not Ala or Gln, could replace Lys at this position to form a stable protein. In agreement with this, the crystal structure revealed that Lys-25 makes a side chain hydrogen bond to Glu-55 that is important for the correct folding of the NGF protein (McDonald et al., 1991). Deletion of Ala-28 prevented the accumulation of NGF protein in the conditioned medium, indicating a structural role for this position.

Several important hydrogen-bonding side chains are buried in the NGF subunit, including Asp-30 (McDonald et al., 1991). Our results showed that the half-life of the NGF molecule is reduced about 20 times when Asp-30 is re-

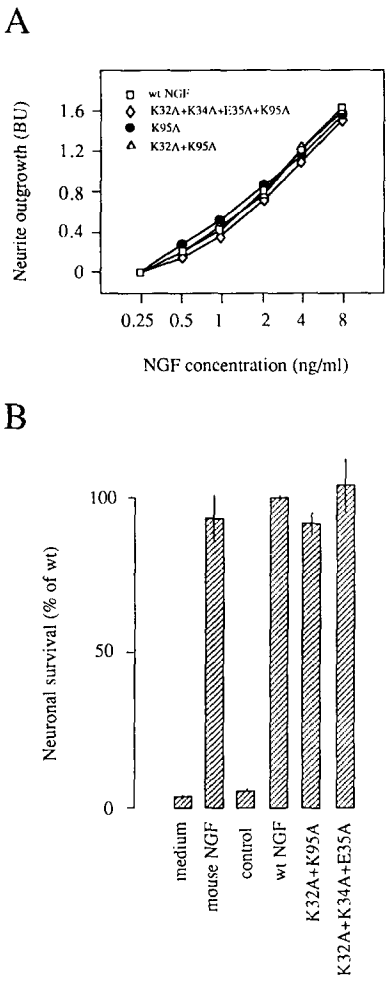

Figure 8. Biological Activities in Sympathetic Neurons of Wild-Type NGF and NGF Mutants Deficient in Binding to $p75^{NGFR}$

(A) Dose response of neurite outgrowth stimulation from E9 chick sympathetic ganglia. Serial dilutions of transfected COS cell–conditioned medium containing equal amounts of wild type (open squares), K95A (closed circles), K32A+K95A (open triangles), and K32A+K34A+E35A+K95A (open diamonds) mutant NGF were assayed for their ability to stimulate neurite outgrowth from E9 chick sympathetic ganglia. Data from three determinations varied by ± 10% of the average values reported here.

(B) Neuronal survival of dissociated sympathetic neurons. Dissociated neurons of the superior cervical ganglion from postnatal day 1 rats were incubated in medium alone, medium containing 10 ng/ml purified mouse NGF, medium from mock-transfected COS cells (control), or in medium containing 10 ng/ml wild-type, K32A+K95A, or K32A+K34A+E35A mutant NGF. Cell survival was determined after 3 days in culture by scoring the number of surviving neurons in three random areas of the well (corresponding to about 200 cells in the cultures treated with NGF). Results are presented as percentage relative to wild-type NGF ± standard deviation.

placed by Ala, a residue that would prevent the proposed hydrogen bond from the side chain of Asp-30 to the main chain at Lys-34. The reduced recovery and half-life of the D30N mutant show that Asn can work at this position albeit at a lower efficiency. On the other hand, elimination or alanine replacement of Gly-33 resulted in loss of recovery of NGF protein, probably owing to a reduced stability of the molecule. Glycine at this position allows the formation of a turn by having main-chain torsion angles outside the allowed range for amino acids with a side chain (Sibanda et al., 1989). Taken together, the results with the Asp-30 and Gly-33 mutants suggest that these residues play a structural role in the stabilization of the β-hairpin loop 30–34 and that their modification may have functional effects through changes in the conformation of the loop (Figure 9A). The high conservation of these positions in other members of the NGF family suggests that these residues could play a similar role in the other three neurotrophins.

As a result of the turn at 30 to 34, the hydrophobic Ile-31 becomes exposed on the surface of the NGF molecule. Replacement of this residue by Ala reduced both receptor binding in PC12 cells and biological activity. Interestingly, only biological activity but not receptor binding was rescued after replacement into Met, whereas wild-type binding and biological activity were seen after change into Val. In addition, preliminary results showed a 5-fold reduction in binding to $p140^{trk}$ in the I31A mutant but wild-type levels in I31M. Taken together these results suggest a role for the nonpolar side chain of Ile-31 in both biological activity that correlates with binding to $p140^{trk}$ and low affinity binding (Figure 9A).

Replacement of Glu-35 for Ala resulted in the production of incompletely processed polypeptides in the range of 23 to 34 kd that were shown to have similar receptor binding affinity and biological activity as the fully processed, wild-type molecule. The fact that an in vitro synthesized full-length NGF precursor of 35K was previously shown to have very low levels of biological activity suggests that removal of some N-terminal sequences may be important for the activation of the NGF precursor (Edwards et al., 1988). Our results also demonstrate that, in addition to conserved domains in the NGF propeptide (Suter et al., 1991), residues in the mature molecule also play a role in the biosynthesis of fully processed, mature NGF.

Replacement of the nonpolar side chain at Val-36 with Leu was also shown to affect receptor binding to PC12 cells. In contrast to Ile-31, Val-36 is deeply buried in the NGF monomer and it appears to be involved in the formation of the hydrophobic core of the NGF subunit (McDonald et al., 1991). The fact that Leu, but not Ala, could replace Val at this position indicates the importance of the hydrophobic contribution of Val-36 to the core of the molecule and suggests that the reduced binding of the V36L mutant probably reflects structural rearrangements required to accommodate the larger Leu side chain at this position.

Receptor-Binding Site of NGF to the $p75^{NGFR}$

The crystal structure of NGF revealed a cluster of exposed positively charged side chains close to and around the β-hairpin loop 30–34 (Figures 9B and 9C) (McDonald et al., 1991). It is possible that the high overall negative charge observed for the $p75^{NGFR}$ (an estimated pI of 4.4 [Radeke et al., 1987]) may require a complementary ionic interaction from the highly basic NGF dimer (pI 9.3) in this region. The results presented here provide strong support to the notion that these positively charged amino acid residues serve as the main points of contact between NGF and $p75^{NGFR}$. Several lines of evidence support this hypothesis: First,

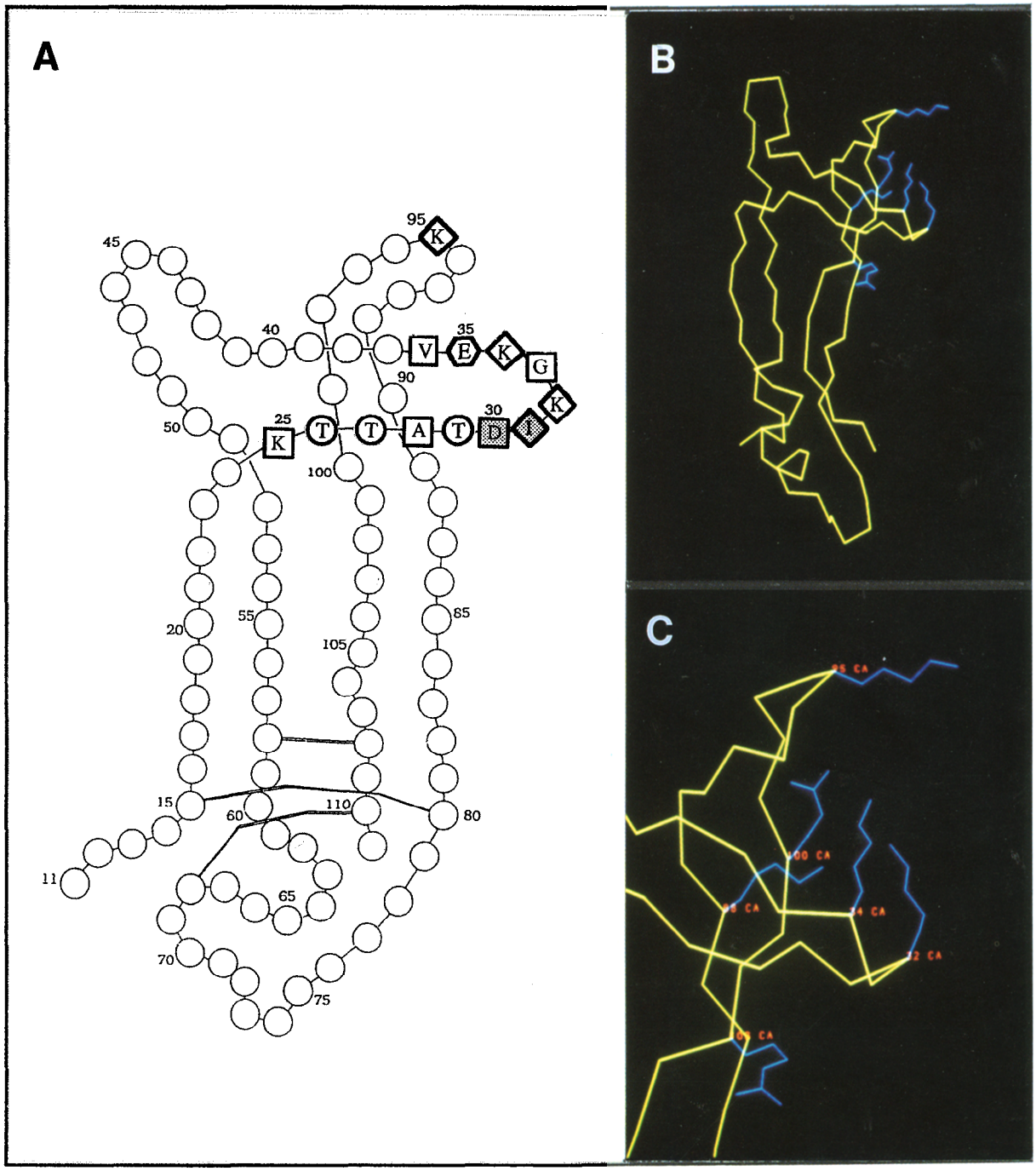

Figure 9. Functional Dissection of the Receptor-Binding Site of NGF to $p75^{NGFR}$

(A) Schematic representation of the three-dimensional structure of the NGF monomer (McDonald et al., 1991). Side chains involved in structure (open squares), processing (open hexagon), and binding to $p75^{NGFR}$ (open diamonds) are indicated. A stippled symbol indicates that modification of the corresponding residue affected biological activity. Double lines at the bottom indicate disulphide bridges. Amino acid residues are numbered from the amino terminus of the mature rat NGF protein.

(B) Computer graphics representation of the NGF monomer. The carbon α chain is yellow; amino- and carboxy-terminal ends are on the left and right sides of the picture, respectively. The side chains of Lys-32, Lys-34, and Lys-95, involved in the contact to $p75^{NGFR}$, and Arg-100, Arg-103, and Lys-88, contributing to the formation of a positively charged interface, are in blue.

(C) Close up of the receptor-binding site region shown in (B). The α carbons of Lys-32, Lys-34, Lys-88, Lys-95, Arg-100, and Arg-103 are labeled.

as revealed by the crystal structure, Lys-32, Lys-34, and Lys-95 are highly exposed (50%–70% side chain solvent accessibility), and their side chains do not have a structural role in the molecule (McDonald et al., 1991). Second, as shown here, replacement of Lys-32 for Ala reduced by 6-fold the affinity of the mutant to receptors on PC12 cells under low affinity binding conditions. Third, the simultaneous replacement of Lys-32, Lys-34, and Glu-35 further

reduced low affinity binding to PC12 cells to less than 1% of that seen with wild-type NGF. This was not due to the E35A mutation, since replacement of Glu-35 for Ala did not change the affinity of binding. Fourth, replacement of Lys-95 had a synergistic effect when combined with K32A, reducing the binding to PC12 cells to almost undetectable levels. Fifth, in all cases, the loss of low affinity binding to PC12 cells correlated with loss of binding to $p75^{NGFR}$ expressed on A875 cells. In the cases of the triple mutant K32A+K34A+E35A and the double mutant K32A+K95A, binding to $p75^{NGFR}$ was completely abolished or reduced 150-fold, respectively. And sixth, despite the loss of binding to $p75^{NGFR}$, all mutant NGFs retained binding to $p140^{trk}$ and biological activity, further demonstrating that the loss of low affinity binding was not due to drastic alterations in conformation of the mutant proteins.

The synergistic effects observed with the multiple lysine mutants indicate that these positively charged residues cooperate in the formation of an interface for binding to $p75^{NGFR}$ (Figures 9B and 9C). Lys-32 appears to be making the strongest contact, followed by Lys-34 and Lys-95, which are probably responsible for the residual binding observed in the K32A mutant. Additional positively charged residues, like the previously studied Arg-100 and Arg-103 (Ibáñez et al., 1990) and perhaps Lys-88, may also contribute to the binding interface (Figures 9B and 9C). The loss of binding to $p75^{NGFR}$ in the K32A+K34A+E35A and the K32A+K95A mutants suggests that a minimal number of positive charges are required on the surface of the NGF molecule to provide a stable contact with $p75^{NGFR}$. This model does not rule out the possibility that other types of contacts such as the hydrophobic residue Ile-31 may also contribute to stabilize the association between NGF and $p75^{NGFR}$.

The other three known neurotrophins can also bind to the low affinity NGFR (Rodriguez-Tébar et al., 1990; Ernfors et al., 1990; Squinto et al., 1991; Hallböök et al., 1991). Lys-95 is conserved in all four proteins described so far, and, in NT-3 and NT-4, Lys-32 is replaced by Arg, another positively charged amino acid residue. Lys-34 is also conserved in NT-4. However, in BDNF, Lys-32 and Lys-34 are replaced by Ser and Gly, respectively. Interestingly, the spatially close loop of residues 93 to 96 in BDNF has three consecutive positively charged residues that may compensate for the absence of Lys-32 and Lys-34. In support of this hypothesis, a chimeric NGF molecule that has residues 23 to 35 (variable region I) replaced by the corresponding residues in BDNF (Ibáñez et al., 1991a) showed a 10-fold reduction of low affinity binding to PC12 cells (C. F. I., unpublished data). The low affinity binding was restored in another chimeric molecule that contains both variable region I and residues 94 to 98 (variable region V) from BDNF (C. F. I., unpublished data), indicating that the three positively charged residues at positions 95, 96, and 97 in BDNF can indeed compensate for the lack of Lys-32 and Lys-34. Although both NGF and BDNF appear to compete equally for binding to $p75^{NGFR}$, this receptor also recognizes differences between the two ligands that are reflected, in the case of BDNF, by positive cooperativity and slower dissociation kinetics (Rodriguez-Tébar et al., 1990). It therefore appears that BDNF and NGF are recognized by $p75^{NGFR}$ as similar, albeit not identical, structures. Our results offer a structural explanation for the observed differences between NGF and BDNF and suggest that other neurotrophins may interact with $p75^{NGFR}$ through the same region.

Loss of Binding to $p75^{NGFR}$ Has No Effect on the Biological Activities of NGF

Recently, the product of the *trk* proto-oncogene has been shown to constitute a functional receptor for NGF (Kaplan et al., 1991a; Klein et al., 1991). NGF binding to $p140^{trk}$ results in rapid phosphorylation of this molecule and stimulation of its tyrosine kinase activity (Kaplan et al., 1991a, 1991b; Klein et al., 1991). In contrast, the role of $p75^{NGFR}$ in signal transduction has remained elusive. Recently, it was reported that the cytoplasmic domain of this receptor is involved in mediating neuronal differentiation (Yan et al., 1991) and NGF-induced tyrosine phosphorylation (Berg et al., 1991) in PC12 cells. However, other recent studies have shown that polyclonal antibodies against $p75^{NGFR}$ abolish NGF binding to this molecule and some of the high affinity binding but do not inhibit biological responses to NGF (Weskamp and Reichardt, 1991). Recent reports using cell lines expressing $p140^{trk}$ have demonstrated that in the presence of NGF this receptor molecule can mediate survival and mitotic proliferation of fibroblasts in the absence of $p75^{NGFR}$ (Cordon-Cardo et al., 1991). These studies could not rule out the possibility that binding to $p75^{NGFR}$ could be important in mediating NGF responses in neurons and neuron-like cell lines. It has also recently been shown that the *trk* proto-oncogene can rescue NGF responsiveness in mutant NGF-nonresponsive PC12 cell lines (Loeb et al., 1991). However, these cells still expressed substantial levels of $p75^{NGFR}$ and therefore do not allow for assessing whether the presence of this molecule was required for the observed functional effects. Our results using mutated NGFs show that binding to $p75^{NGFR}$ is not required for induction of early gene expression, such as that of c-*fos*, or for neuronal differentiation of PC12 cells. Moreover, neither neurite outgrowth nor neuronal survival of cultured sympathetic neurons, which express both $p75^{NGFR}$ mRNA and protein (Ernfors et al., 1988; Yan and Johnson, 1988) and *trk* mRNA (G. B., unpublished data), was affected by the loss of binding to $p75^{NGFR}$. Our results cannot discard the possibility that in these cells NGF binds through a different binding site to a new pocket created by a heterodimer of $p75^{NGFR}$ and $p140^{trk}$ (Hempstead et al., 1991). Alternatively, free $p75^{NGFR}$ could still contact complexed NGF-$p140^{trk}$ and in some way cooperate in signal transduction. However, $p75^{NGFR}$–$p140^{trk}$ complexes have so far not been detected in cross-linking experiments performed with either PC12 cells or sensory neurons under conditions that allowed detection of $p75^{NGFR}$ or $p140^{trk}$ homodimers (Meakin and Shooter, 1991). The fact that our mutant molecules retained binding to $p140^{trk}$ strongly supports the argument that the observed biological activities were mediated by this receptor molecule alone.

Recently, a region in human interleukin 2 that binds to

the low affinity α chain (p55) of the interleukin 2 receptor was reported (Sauvé et al., 1991). Interestingly, despite their lack of binding to p55, all interleukin 2 analogs retained substantial binding to the intermediate-affinity p70 subunit (β chain) of the receptor complex and exhibited significant biological activity (Sauvé et al., 1991). Our results with NGF mutants have a remarkable parallel to those seen for interleukin 2 and suggest that this type of interaction may constitute a general strategy shared by other growth factors and cytokines that make contact with multiple receptors.

In conclusion, the results presented in this study identify amino acid residues in NGF that are responsible for the main contact between this molecule and $p75^{NGFR}$. Mutated molecules that do not bind to $p75^{NGFR}$ but still retain binding to $p140^{trk}$ and biological activity show for the first time that the *trk* proto-oncogene product alone is sufficient to mediate a response to NGF in cultured neuronal cells and open up new and unique possibilities to unravel the role of both $p75^{NGFR}$ and $p140^{trk}$ in mediating the biological activities of NGF.

Experimental Procedures

DNA Cloning and Site-Directed Mutagenesis

A 770 bp EcoRI fragment containing the pre-proNGF coding sequence from the rat NGF gene (Whittemore et al., 1988) was cloned into pBluescript KS+ (Stratagene). Single-stranded DNA from this plasmid was used as template for oligonucleotide-based site-directed mutagenesis, as decribed by Kunkel (1985) and detailed in Ibáñez et al. (1990). The replacements were confirmed by nucleotide sequence analysis by the chain termination method (Sanger et al., 1977). For protein expression, DNA inserts containing the desired replacements were then subcloned in pXM (Yang et al., 1986).

Production and Quantitation of Recombinant Proteins

COS cells grown to about 70% confluency were transfected with 25 μg of plasmid DNA per 100 mm dish using the DEAE–dextran–chloroquine protocol (Luthman and Magnusson, 1983). To correct for differences in the amounts of recombinant protein produced by the different constructs, 35 mm dishes transfected in parallel were grown in the presence of 100 μCi/ml ^{35}S-cysteine (Amersham). Aliquots of conditioned medium were then analyzed by SDS–PAGE, and the amounts of recombinant protein in the different samples were equilibrated after densitometer scanning of the corresponding autoradiograms as previously described (Ibáñez et al., 1991b). The absolute amount of wild-type NGF protein was assessed by quantitative immunoblotting of conditioned medium and by measurement of biological activity in cultured sympathetic ganglia using standards of purified mouse NGF (Ibáñez et al., 1990, 1991b). The data obtained from these analyses were then used to determine the protein concentration in the samples containing mutant proteins.

Pulse Chase and Immunoprecipitation

Forty-eight hours after transfection cells were incubated in cysteine-free medium for 4 hr. The cells were then pulse labeled with 1 mCi/ml ^{35}S-cysteine for 15 min. The chase was performed by replacing the labeling medium with complete medium fortified with 2 mg/ml cold cysteine. Parallel wells were harvested at different times, and cell extracts and conditioned medium were immunoprecipitated with a polyclonal rabbit antiserum (rabbit no. 30) against mouse NGF (Ebendal et al., 1989) and analyzed by SDS–PAGE under reducing conditions as previously described (Ibáñez et al., 1990, 1991b).

Binding Assays

Mouse NGF was labeled with ^{125}I by the chloramine-T method to an average specific activity of 3×10^7 cpm/μg. Rat PC12 cells (Greene and Tischler, 1976), human A875 cells (Buxser et al., 1983), and mouse *rtrk* 3T3 cells (Kaplan et al., 1991a) were used at 2×10^6 to 10×10^6 cells/ml. Steady-state binding was measured in competition assays performed at 37°C using 1.5×10^{-9} M ^{125}I-labeled NGF and serial dilutions of conditioned medium containing equivalent amounts of wild-type or mutated NGF protein. All components were added at the same time, and the cells were collected by centrifugation after equilibrium was reached (1–2 hr incubation). Control experiments using medium from mock-transfected COS cells showed that other proteins present in the conditioned medium had no effect on the binding of ^{125}I-NGF to the cells. Nonspecific binding was measured in a parallel incubation to which at least a 1000-fold molar excess of unlabeled NGF was added. All results were corrected for this nonspecific binding, which was always less than 10% of total binding. The concentration of each mutant and wild-type NGF that gave 50% binding (IC_{50}) was determined, and relative binding was calculated using the relationship: (mutant IC_{50}/wild-type IC_{50}) × 100.

Biological Assays

Serial dilutions of conditioned medium containing equivalent amounts of recombinant protein (in the range of 0.2 to 20 ng/ml) were assayed for biological activity on explanted chick embryonic day 9 sympathetic ganglia as previously described (Ebendal, 1984, 1989). Fiber outgrowth was scored on a semiquantitative scale in biological units (BU) by comparison with standards obtained with purified mouse NGF, for which 1 BU is equivalent to approximately 5 ng/ml. The concentration of each NGF protein that gave 0.5 BU in this scale was determined and used to calculate the relative activity compared with that obtained with wild-type NGF.

PC12 cells plated in 35 mm wells coated with poly-D-lysine were incubated with serial dilutions of conditioned medium containing equivalent amounts of recombinant protein. At different time intervals, the percentage of cells bearing fibers longer than 2 cell diameters was determined microscopically.

Induction of c-*fos* mRNA was measured by quantitative Northern blot analysis of total mRNA from PC12 cells treated with dilutions of conditioned medium containing equivalent amounts of recombinant wild-type and mutant NGF. Total RNA was extracted as previously described (Ibáñez et al., 1990). Ten micrograms of total RNA was electrophoresed in a 1% agarose gel containing 0.7% formaldehyde and transferred to nitrocellulose membranes. The filters were then hybridized with an [α-^{32}P]dCTP radiolabeled rat c-*fos* gene fragment (Curran et al., 1987) and washed at high stringency. The amount of c-*fos* mRNA was determined by densitometer scanning of autoradiograms.

Dissociated neurons of the superior cervical ganglion from postnatal day 1 rats were cultured in 35 mm wells coated with poly-D-lysine at a density of 30,000 cells/well. Serial dilutions of conditioned medium containing equivalent amounts of recombinant protein were added at the time of plating, and neuronal survival was determined after 72 hr by phase-contrast microscopy.

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