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Functional analysis of mutant neurotrophins deficient in low-affinity binding reveals a role for p75^{LNGFR} in NT-4 signalling

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The neurotrophins mediate their effects through binding to two classes of receptors, a tyrosine kinase receptor, member of the Trk family, and the low-affinity neurotrophin receptor, p75^{LNGFR}, of as yet undefined signalling capacity. The need for a two-component receptor system in neurotrophin signalling is still not understood. Using site-directed mutagenesis, we have identified positively charged surfaces in BDNF, NT-3 and NT-4 that mediate binding to p75^{LNGFR}. Arg31 and His33 in NT-3, and Arg34 and Arg36 in NT-4, located in an exposed hairpin loop, were found to be essential for binding to p75^{LNGFR}. In BDNF, however, positively charged residues critical for p75^{LNGFR} binding (Lys95, Lys96 and Arg97) were found in a spatially close but distinct loop region. Models of each neurotrophin were built using the coordinates of NGF. Analysis of their respective electrostatic surface potentials revealed similar clusters of positively charged residues in each neurotrophin but with differences in their precise spatial locations. Disruption of this positively charged interface abolished binding to p75^{LNGFR} but not activation of cognate Trk receptors or biological activity in Trk-expressing fibroblasts. Unexpectedly, loss of low-affinity binding in NT-4, but not in BDNF or NT-3, affected receptor activation and biological activity in neuronal cells co-expressing p75^{LNGFR} and TrkB, suggesting a role for p75^{LNGFR} in regulating biological responsiveness to NT-4. These findings reveal a possible mechanism of ligand discrimination by p75^{LNGFR} and suggest this receptor may selectively modulate the biological actions of specific neurotrophin family members.

Key words: NGF/BDNF/NT-3/NT-4/Trk/site-directed mutagenesis

Introduction

The neurotrophins are a family of structurally and functionally related polypeptides that control the generation, differentiation, survival and maintenance of vertebrate neurons (Barde, 1989; Thoenen, 1991; Korsching, 1993; Persson and Ibáñez, 1993). The neurotrophin family presently consists of four proteins, i.e. nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), which share ~50% amino acid sequence identity (Angeletti, 1970; Leibrock *et al.*, 1989; Ernfors *et al.*, 1990; Hohn *et al.*, 1990; Jones and Reichardt, 1990; Kaisho *et al.*, 1990; Maisonpierre *et al.*, 1990; Berkemeier *et al.*, 1991; Hallböök *et al.*, 1991; Ip *et al.*, 1992). Each of these factors promotes the survival of a distinct set of embryonic neurons. Individual neurons may also be responsive to more than one neurotrophin at a given time or at subsequent times during development. The pattern of neurotrophin responsiveness in the developing nervous system is determined by the coordinated expression of specific neurotrophin receptors. Two classes of neurotrophin receptors have been described. The low-affinity neurotrophin receptor (p75^{LNGFR}) is a transmembrane glycoprotein initially isolated by its ability to bind NGF (Chao *et al.*, 1986; Radeke *et al.*, 1987), but subsequently shown to bind all members of the neurotrophin family with equal affinity (Rodriguez-Tébar *et al.*, 1990, 1992; Hallböök *et al.*, 1991). p75^{LNGFR} is structurally related to the tumour necrosis factor receptor and to CD40, and it contains a short cytoplasmic domain of unknown biochemical function. Recently, this receptor has been suggested to mediate activation of the sphingomyelin cycle by NGF (Dobrowsky *et al.*, 1994). Unlike p75^{LNGFR}, members of the Trk family of tyrosine kinase receptors exhibit ligand-dependent activation of endogenous tyrosine kinase activity and thus can clearly function as signalling receptors (Kaplan *et al.*, 1991a; Meakin and Shooter, 1992). The specificity of neurotrophin action is believed to be achieved in part by the selective interaction between members of the Trk family of receptors and the different neurotrophins. Thus p140^{trk} (TrkA) serves as a receptor for NGF (Kaplan *et al.*, 1991a,b; Klein *et al.*, 1991a), p145^{trkB} (TrkB) serves as a receptor for BDNF and NT-4 (Glass *et al.*, 1991; Klein *et al.*, 1991b, 1992; Soppet *et al.*, 1991; Squinto *et al.*, 1991; Ip *et al.*, 1993), while p145^{trkC} (TrkC) is the main receptor for NT-3 (Lamballe *et al.*, 1991), which can also interact, albeit to a lesser extent, with TrkA and TrkB (Cordon-Cardo *et al.*, 1991; Soppet *et al.*, 1991; Squinto *et al.*, 1991; Ip *et al.*, 1993).

Why two neurotrophin receptors? While the Trks are clearly signalling receptors and can mediate functional responses to neurotrophins in the absence of p75^{LNGFR}, the precise role of p75^{LNGFR} is still not clearly understood. This receptor was initially postulated to form part of a functional high-affinity NGF receptor complex (Hempstead *et al.*, 1989, 1991), and to mediate signal transduction and biological responses to NGF (Matsushima and Bogemann, 1990; Berg *et al.*, 1991; Yan *et al.*, 1991),

but later evidence disputed a direct functional role of this molecule (Weskamp and Reichardt, 1991; Birren *et al.*, 1992; Ibáñez *et al.*, 1992; Jing *et al.*, 1992). More recently, however, re-examination of the role of $p75^{LNGFR}$ in different systems has revealed functional aspects of this receptor which may reconcile data from previous studies. Mice deficient in $p75^{LNGFR}$ generated by gene targeting developed sensory deficits and had reduced numbers of sensory neurons in dorsal root ganglia (Lee *et al.*, 1992). In addition, embryonic dorsal root sensory and neonatal sympathetic neurons isolated from these animals displayed a decreased sensitivity to NGF (Davies *et al.*, 1993; Lee *et al.*, 1994b). These results suggest a role for $p75^{LNGFR}$ in modulating the biological potency of neurotrophins, a hypothesis that has recently gained support after the observation that overexpression of $p75^{LNGFR}$ can enhance NGF-induced tyrosine autophosphorylation of TrkA and accelerate neuronal differentiation in a sympathoadrenal progenitor cell line (Hantzopoulos *et al.*, 1994; Verdi *et al.*, 1994). This effect may in part be explained by the reported ability of $p75^{LNGFR}$ to increase the association rate of NGF binding when co-expressed with TrkA (Mahadeo *et al.*, 1994). Interestingly, a high $p75^{LNGFR}$/TrkA ratio appeared to be required for these effects, a parameter which had not been considered in previous reconstitution experiments. In fact, manipulations of the ratio between these two receptors in PC12 cells has been shown to result in altered binding affinity and cross-activation of TrkA by NT-3, suggesting that $p75^{LNGFR}$ may in addition assist in ligand discrimination (Benedetti *et al.*, 1993). Intriguingly, although normal for the most part, the sympathetic system of $p75^{LNGFR}$ knock-out mice failed to innervate some of its targets such as pineal and sweat glands (Lee *et al.*, 1994a). Because the deficits were only seen in the most distant targets of sympathetic innervation, it was speculated that $p75^{LNGFR}$ may be required for retrograde neurotrophic signalling to sympathetic neurons. In addition, these mice appear to also have specific deficits in the retrograde transport of NT-4 to neurons of dorsal root ganglia (DRG) (Curtis *et al.*, 1993).

The study of structure–function relationships in the neurotrophins offers an alternative approach to address some of the unresolved questions regarding neurotrophin function (Ibáñez, 1994). Because all neurotrophins can bind to $p75^{LNGFR}$, it was initially proposed that regions conserved among the different molecules may be involved in $p75^{LNGFR}$ binding. However, site-directed mutagenesis studies later revealed the essential role of variable amino acid residues in two spatially close β-hairpin loop regions of NGF (Ibáñez *et al.*, 1992). Lys32, Lys34 and Lys95 were found to form a positively charged interface involved in binding to $p75^{LNGFR}$. Lys32 appeared to be making the strongest contact, and simultaneous modification of this residue with either of the two other lysines resulted in loss of binding to this receptor. Despite the lack of binding to $p75^{LNGFR}$, these mutants retained binding to TrkA and biological activity, demonstrating a functional dissociation between the two NGF receptors (Ibáñez *et al.*, 1992). Unlike NGF, BDNF does not present positively charged residues in this region; Lys32 and Lys34 of NGF are replaced by Ser and Gly, respectively. NT-3 retains only one of the two positive charges in this loop but in the form of Arg, a residue bulkier than Lys, while in NT-4, both lysine residues are replaced by Arg. Thus, it is not clear whether homologous structural determinants represent binding sites to $p75^{LNGFR}$ in the different neurotrophins. Here we have investigated the structural requirements for binding to $p75^{LNGFR}$ in BDNF, NT-3 and NT-4 using chimeric and mutant neurotrophins generated by site-directed mutagenesis. Our analysis revealed similarities and differences in the way neurotrophins interact with $p75^{LNGFR}$ that bear functional implications for a role of this receptor in ligand discrimination and neurotrophin signalling.

	variable region I		variable region V
		32 34	95
		↓ ↓	↓
rat NGF	G--DKTTATDI**K**G**K**E		TDD**K**Q-AA
rat BDNF	TAA..K..V.MS.GT		M.S.**KR**IG
rat NT-3	T-...SS.I..**R**.HQ		SENN**K**LVG
rat NT-4	T--DRR..V.L**R**.**R**.		A.AQG**R**VG

Fig. 1. Alignments of amino acid sequences from variable regions I and V of NGF, BDNF, NT-3 and NT-4 (rat sequences). Numbering corresponds to the rat NGF sequences. Selected positively charged residues are highlighted in bold face.

Results

Determinants of binding to $p75^{LNGFR}$ in BDNF, NT-3 and NT-4

A hairpin loop region in NGF (variable region I) contains positively charged residues previously shown to be essential for the interaction of this neurotrophin with $p75^{LNGFR}$. BDNF lacks positively charged residues in this region (Figure 1), suggesting that positive charges are not involved in the binding of this neurotrophin to $p75^{LNGFR}$ or, alternatively, that binding determinants may be located elsewhere. The importance of this loop region in the interaction of BDNF with $p75^{LNGFR}$ was investigated using NGF/BDNF chimeric molecules generated by site-directed mutagenesis. A chimeric NGF molecule in which variable region I was exchanged with homologous sequences from BDNF (BDNF I/NGF) lacks positively charged residues (Lys32 and Lys34) essential for NGF binding to $p75^{LNGFR}$ (Ibáñez *et al.*, 1992). This molecule was produced in transiently transfected COS cells and concentrated conditioned medium was tested for its ability to displace [^{125}I]NGF from $p75^{LNGFR}$ expressed on A875 human melanoma cells. The chimeric molecule displayed low binding, with an IC_{50} that was 25-fold higher than that of wild-type (wt) NGF (Figure 2A and Table I). The N-terminal part of variable region I of BDNF contains two additional amino acid residues not present in any of the other three neurotrophins (Figure 1). Insertion of these two residues in an NGF skeleton may conceivably alter the orientation of the side chains in the loop. In order to assess the importance of this insertion for binding to $p75^{LNGFR}$, two additional chimeric molecules were constructed. In the first one, the first four residues of region I of NGF (positions 23–26) were replaced by the first six residues of region I of BDNF (chimera BDNF Ia/NGF). In the second one, the last 10 residues of region I of NGF (positions 26–35) were replaced by the last 10 residues of region I of BDNF (chimera BDNF Ib/NGF). The first

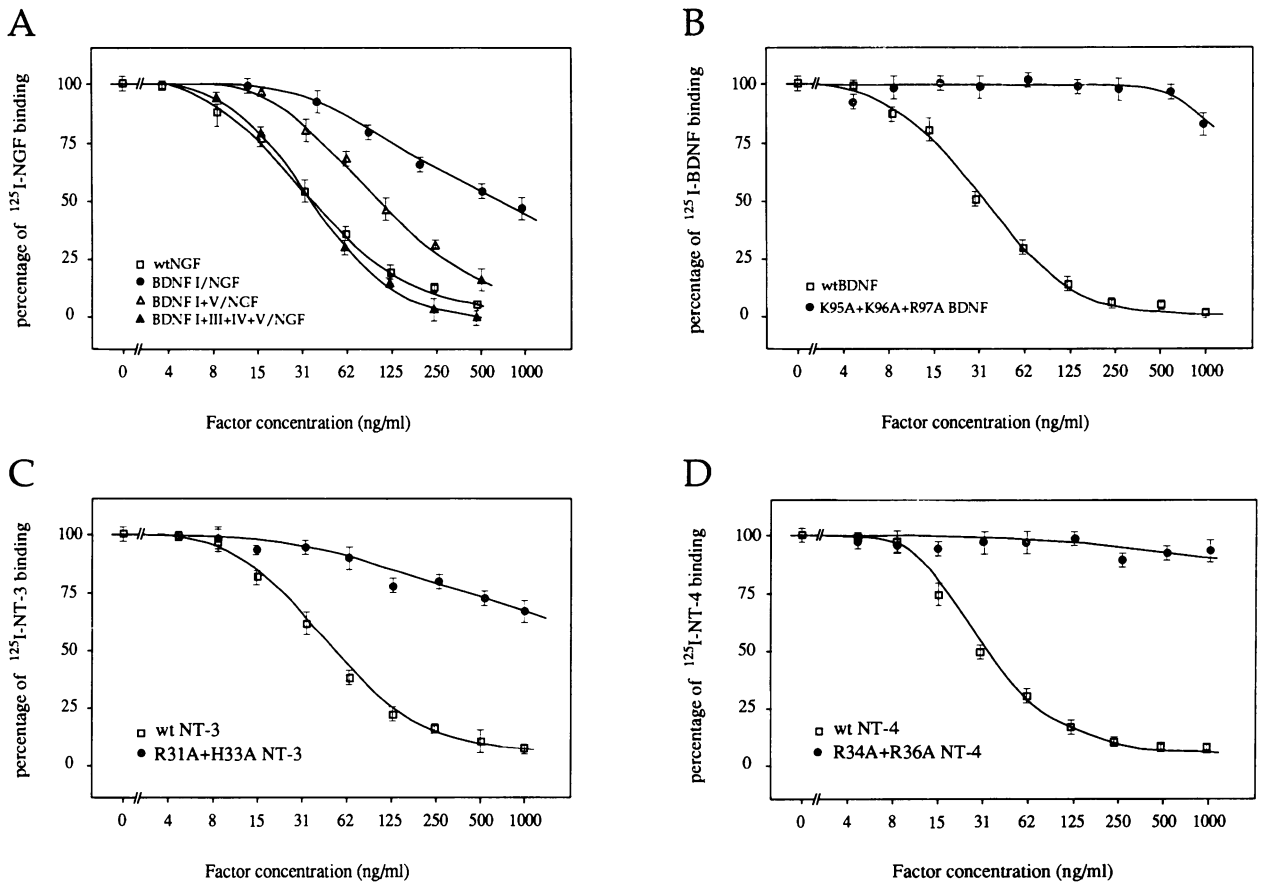

Fig. 2. Binding of mutant and wild type neurotrophins to p75^{LNGFR} expressed on A875 cells. (**A**) Serial dilutions of transfected COS cell conditioned medium containing equal amounts of wt NGF (□) and chimeric molecules BDNF I/NGF (●), BDNF I+V/NGF (△) and BDNF I+III+IV+V/NGF (◆) were assayed for their ability to displace [^{125}I]NGF from A875 cells. Medium from mock transfected cells failed to displace [^{125}I]NGF (not shown). Each point represents the mean ± SD of triplicate determinations. (**B**) Serial dilutions of purified wt BDNF (□) and mutant BDNF K95A+K96A+R97A (●) were assayed for their ability to displace [^{125}I]BDNF from A875 cells. Each point represents the mean ± SD of triplicate determinations. (**C**) Serial dilutions of purified wt NT-3 (□) and mutant NT-3 R31A+H33A (●) were assayed for their ability to displace [^{125}I]NT-3 from A875 cells. Each point represents the mean ± SD of triplicate determinations. (**D**) Serial dilutions of purified wt NT-4 (□) and mutant NT-4 R34A+R36A (●) were assayed for their ability to displace [^{125}I]NT-4 from A875 cells. Each point represents the mean ± SD of triplicate determinations.

chimera, which contains the insertion but retains the positive charges at positions 32 and 34, showed an IC_{50} very similar to that of wt NGF (Table I). This suggested that the two-residue insertion is not likely to affect the relative orientation of the side chains in loop region I and that it has no influence on the affinity of binding to p75^{LNGFR}. In contrast, replacement of the last 10 residues of this region (chimera BDNF Ib/NGF), which removes the positive charges, drastically reduced binding to p75^{LNGFR} to a level comparable with that obtained after replacement of the whole region I (Table I). This indicated that the positive charges are the important determinants of binding of NGF to p75^{LNGFR} in this region and that variable region I of NGF and BDNF are not functionally equivalent with respect to binding to this receptor. Interestingly, the absence of positive charges in region I of BDNF contrasts with the presence of three consecutive positively charged residues (Lys95, Lys96 and Arg97) in a different but spatially close loop region (variable region V) (Figure 1). This tripeptide is not found in any other member of the neurotrophin family described so far. In order to assess the role of these residues in binding to p75^{LNGFR}, a double chimeric molecule was constructed in which variable regions I and V in NGF were simultaneously replaced by corresponding segments from BDNF (chimera BDNF I+V/NGF). The replacement of region V partially restored the ability of the chimeric molecule to bind to p75^{LNGFR} (Figure 2A), suggesting that the introduction of additional positive charges in this loop compensated for the absence of positive charges in region I of the chimera. Further replacement of additional variable regions (chimera BDNF I+III+IV+V/NGF) completely restored p75^{LNGFR} binding to levels indistinguishable from wt NGF or wt BDNF (Figure 2A and Table I) and suggests that positively charged residues in loop region V may work more efficiently in a more BDNF-like backbone context. The role of positively charged residues Lys95, Lys96 and Arg97 in the binding of BDNF to p75^{LNGFR} was directly tested in a triple BDNF mutant in which the three positions were simultaneously replaced by Ala. This BDNF mutant (K95A+K96A+R97A) was produced in baculovirus-infected insect cells and subsequently purified to homogeneity by a combination of ion exchange, size exclusion and reverse-phase chromatography. Displacement binding assays in A875 cells demonstrated that the triple mutation completely abolished the capacity of the

Table I. Relative receptor binding to A875 cells of wild type (wt) and mutant neurotrophins

Protein	A875 binding (% of wild-type)
wt NGF	100
BDNF I/NGF	4
BDNF Ia/NGF	100
BDNF Ib/NGF	3
BDNF I+V/NGF	25
BDNF I+III+IV+V/NGF	100
wt BDNF	100
K95A+K96A+R97A BDNF	no IC_{50}
wt NT-3	100
K95A NT-3	85
R31A+H33A NT-3	<1
R31A+H33A+K95A NT-3	<1
wt NT-4	100
R34A+R36A NT-4	no IC_{50}

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

molecule to bind to $p75^{LNGFR}$ (Figure 2B and Table I), indicating that Lys95, Lys96 and Arg97 play a crucial role in the binding of BDNF to this receptor. The deficit in $p75^{LNGFR}$ binding was not due to effects of the mutation on the stability or conformation of the molecule (see below), and suggests that binding of BDNF to $p75^{LNGFR}$ also appears to be mediated by positively charged residues, although in a different spatial location than in NGF.

In contrast to BDNF, NT-3 contains one positively charged residue in variable region I, Arg31, while NT-4 has two Arg residues in this region, Arg34 and Arg36 (Figure 1). The role of these residues in binding to $p75^{LNGFR}$ was studied using mutant NT-3 and NT-4 molecules in which Ala simultaneously replaced each residue at these positions. A double NT-3 mutant (R31A+H33A) and a double NT-4 mutant (R34A+R36A) were generated by site-directed mutagenesis, produced in baculovirus-infected insect cells and subsequently purified to homogeneity as indicated above. Purified mutant NT-3 and NT-4 proteins were used to displace [^{125}I]NT-3 or [^{125}I]NT-4, respectively, from $p75^{LNGFR}$ binding sites on A875 cells. Both mutant proteins showed great deficits in binding to $p75^{LNGFR}$ (Figures 2C and D). The NT-3 mutant retained <1% of the affinity of wt NT-3, whereas the NT-4 mutant displayed no measurable IC_{50} in this assay (Table I). To test the importance of Lys95, a positively charged residue in variable region V of NT-3, a single NT-3 mutant (K95A) and a triple NT-3 mutant (R31A+H33A+K95A) were produced in COS cells. The single mutant displayed an affinity comparable with wt NT-3 (Table I), and the triple mutant showed no further deficits compared with the double NT-3 mutant R31A+H33A (Table I). Taken together, these results indicate that, as in NGF, the binding sites to $p75^{LNGFR}$ in NT-3 and NT-4 also contain positively charged residues in variable loop region I.

Mutant BDNF and NT-3 deficient in binding to $p75^{LNGFR}$ retain the ability to activate cognate Trk receptors and to elicit biological activities in Trk-expressing fibroblasts and neuronal cells

Since disruption of the $p75^{LNGFR}$ binding site in NGF has previously been shown not to affect NGF binding to TrkA or biological activity (Ibáñez *et al.*, 1992), we investigated the ability of the BDNF, NT-3 and NT-4 mutants to activate cognate Trk receptors and to elicit biological activities. Activation of TrkB and TrkC was tested in a tyrosine-phosphorylation assay using NIH3T3 fibroblast cell lines constitutively expressing these receptors. Despite their considerably reduced $p75^{LNGFR}$ binding, all mutants retained the capacity to promote autophosphorylation of cognate Trk receptors, indicating that the binding deficits of the mutant proteins were not due to major structural or conformational alterations of the molecules. The ability to induce Trk autophosphorylation was, however, affected differently in the different mutant proteins. The triple BDNF mutant was less potent (~5-fold) than wt BDNF in promoting TrkB autophosphorylation in fibroblasts (Figure 3A), suggesting that the mutation also partially affected the ability of the molecule to interact with TrkB. We have previously demonstrated that introduction of these positively charged residues in an NGF backbone allows interaction with TrkB (Ibáñez *et al.*, 1993), supporting the notion that these residues form part of the TrkB binding site in BDNF. The ability of mutant BDNF to initiate biological responses in TrkB-expressing MG87-NIH3T3 fibroblasts was also investigated. These fibroblasts are dependent on growth factors for survival and proliferation, and can be engineered to respond to exogenous neurotrophins by stable transfection with cognate Trk receptors (Glass *et al.*, 1991; Ip *et al.*, 1993). In agreement with its decreased potency in TrkB autophosphorylation, the ability of the triple BDNF mutant to promote survival and proliferation of TrkB-MG87 fibroblasts was also reduced (Figure 3B). The reduction in activity was of approximately the same magnitude as that observed in the autophosphorylation assay (compare Figure 3A and 3B). Because these cells lack $p75^{LNGFR}$, the partial deficits of the mutant molecule cannot be due to its inability to bind this receptor and most likely represent partial disruption of the TrkB binding site in BDNF.

The neurotrophin mutants deficient in low-affinity binding offer a possibility to examine the role of $p75^{LNGFR}$ in mediating biological activities of neurotrophins on cells co-expressing $p75^{LNGFR}$ and Trk receptors. The biological activity of the triple BDNF mutant on embryonic sensory neurons was tested in dissociated cultures of E8 chick DRG. This mutant was somewhat less potent (~3-fold) in promoting survival of these neurons compared with wt BDNF (Figure 3C). The biological activity of the BDNF mutant was also tested in nnr5PC12-TrkB cells, an NGF-non-responsive mutant PC12 cell line stably transfected with a TrkB cDNA that retains expression of $p75^{LNGFR}$ and responds to BDNF and NT-4 (L.Greene, unpublished results). The potency of the mutant BDNF in promoting neurite outgrowth from these cells was slightly reduced but not significantly different from wt BDNF (Figure 3D). The differences in specific activity between mutant and wt BDNF in sensory neurons do not correlate with the complete inability of the mutant molecule to bind $p75^{LNGFR}$ (Figure 2B). On the other hand, they are in agreement with the activity of the mutant in TrkB-MG87 fibroblasts, which do not express $p75^{LNGFR}$. Thus, the reduced potency of the mutant BDNF in neurons was likely due to partial

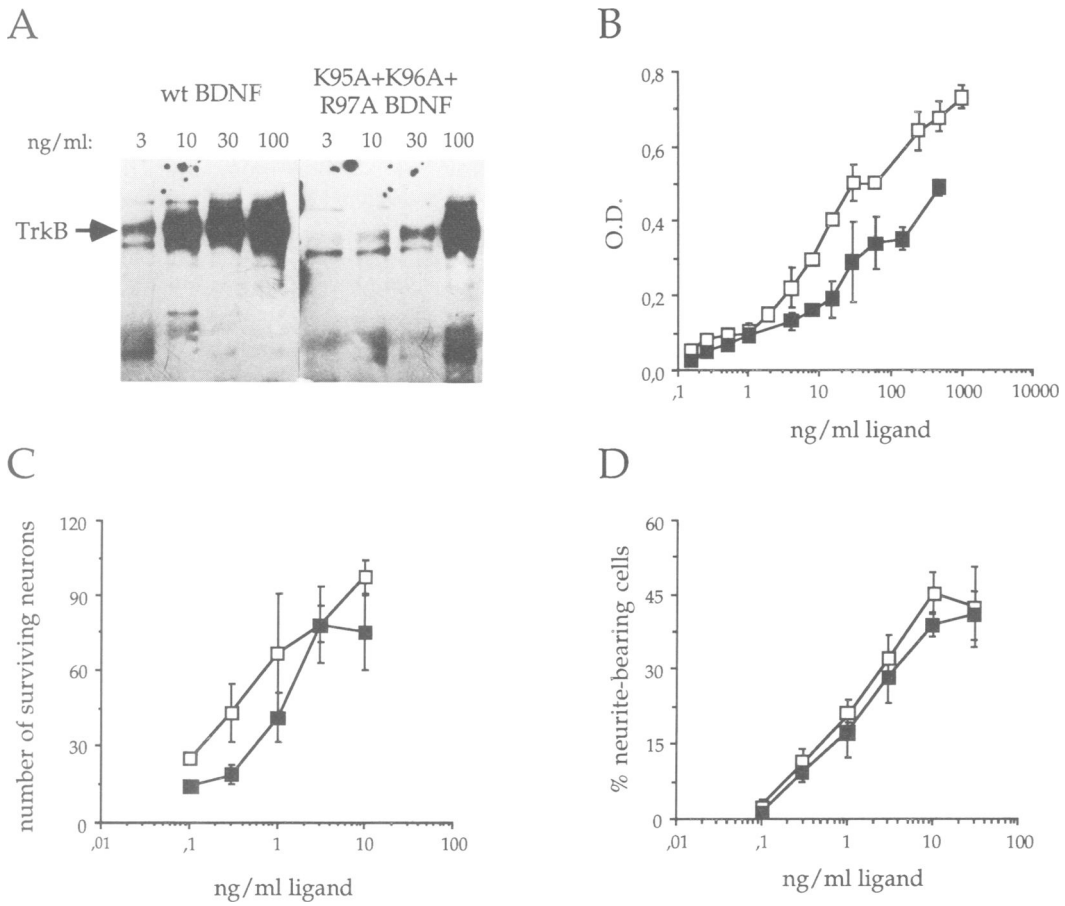

Fig. 3. TrkB activation and biological activities of mutant K95A+K96A+R97A and wt BDNF. (**A**) Serial dilutions of purified wt and mutant BDNF were assayed for their ability to stimulate tyrosine autophosphorylation of TrkB expressed in fibroblast cells. An autoradiogram of a phosphotyrosine blot is shown. The arrow indicates the migration of phosphorylated TrkB (p145^{trkB}). Similar results were obtained in a duplicate experiment. (**B**) Serial dilutions of purified wt (□) and mutant (■) BDNF were assayed for their ability to stimulate survival and growth of MG87 fibroblast expressing TrkB as assessed by metabolic labelling with MTT. Data are expressed as the average optical density (O. D.) of triplicate wells ± SD. (**C**) Serial dilutions of purified wt (□) and mutant (■) BDNF were assayed for their ability to promote survival of E8 chick DRG neurons. Data are expressed as the average number of surviving neurons in a defined area of triplicate wells ± SD. (**D**) Serial dilutions of purified wt (□) and mutant (■) BDNF were assayed for their ability to promote neurite outgrowth in nnr5PC12-TrkB cells. Data are expressed as the average percentage of neurite-bearing cells in triplicate wells ± SD.

disruption of its binding site to TrkB and not to the loss of binding to p75^{LNGFR}.

In contrast to BDNF, modification of residues in variable region I of NT-3 had no effect on the ability of the mutant molecule to induce TrkC autophosphorylation. A dose–response assay revealed no differences between the R31A+H33A NT-3 mutant and wt NT-3 (Figure 4A). In addition, the activity of the mutant in TrkC-MG87 fibroblasts was indistinguishable from wt NT-3 (Figure 4B), indicating that this mutation only affected binding to p75^{LNGFR}. The NT-3 mutant was tested in dissociated cultures of E8 chick DRG neurons and compared with wt NT-3. This mutant was able to promote survival of embryonic sensory neurons with a potency comparable with that of wt NT-3 (Figure 4C), in agreement with the results in TrkC-MG87 fibroblasts. Combined, these data suggest that, in BDNF and NT-3, as demonstrated previously for NGF, binding to p75^{LNGFR} is dispensable for neuronal differentiation and survival *in vitro*.

Disruption of binding to p75^{LNGFR} in NT-4 does not affect TrkB activation or biological activity in TrkB-expressing fibroblasts but reduces responsiveness in neuronal cells co-expressing TrkB and p75^{LNGFR}

Like its BDNF and NT-3 counterparts, the mutant NT-4 deficient in p75^{LNGFR} binding retained the ability to induce tyrosine autophosphorylation of TrkB expressed in fibroblasts (Figure 5A). In dose–response experiments, phosphorylation elicited by the NT-4 mutant was indistinguishable from wt NT-4. TrkB tyrosine phosphorylation was also similar between wt and mutant NT-4 in the human neuroblastoma line SH-SY5Y (data not shown), which expresses endogenous TrkB but not p75^{LNGFR} receptors (D.Kaplan, personal communication). In agreement with these data, dose–response curves of TrkB-MG87 fibroblast survival elicited by mutant or wt NT-4 were identical (Figure 5B), indicating that the mutation only affected binding to p75^{LNGFR}. Unexpectedly, however,

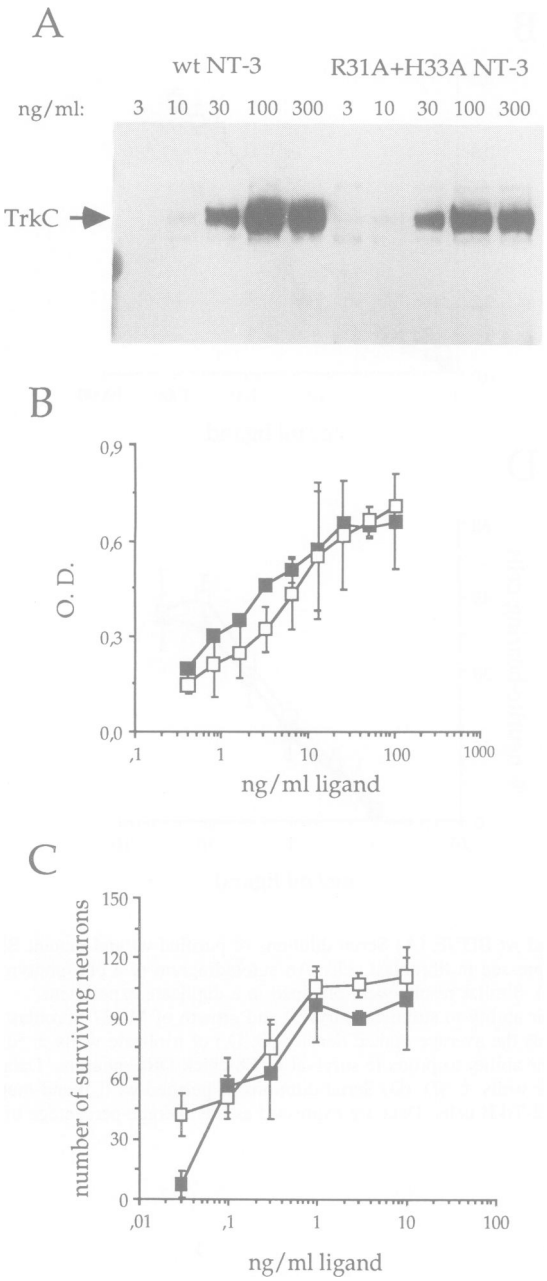

Fig. 4. TrkC activation and biological activities of mutant R31A+H33A and wt NT-3. (**A**) Serial dilutions of purified wt and mutant NT-3 were assayed for their ability to stimulate tyrosine autophosphorylation of TrkC expressed in fibroblast cells. An autoradiogram of a phosphotyrosine blot is shown. The arrow indicates the migration of phosphorylated TrkC ($p145^{trkC}$). Similar results were obtained in a duplicate experiment. (**B**) Serial dilutions of purified wt (□) and mutant (■) NT-3 were assayed for their ability to stimulate survival and growth of MG87 fibroblast expressing TrkC as assessed by metabolic labelling with MTT. Data are expressed as the average optical density (OD) of triplicate wells ± SD. (**C**) Serial dilutions of purified wt (□) and mutant (■) NT-3 were assayed for their ability to promote survival of E8 chick DRG neurons. Data are expressed as the average number of surviving neurons in a defined area of triplicate wells ± SD.

the ability of the mutant to promote neurite outgrowth from nnr5PC12-TrkB cells, expressing both TrkB and $p75^{LNGFR}$, was drastically reduced (up to 15-fold) (Figure 5C). A 30-fold reduction was also observed in the ability of the mutant to promote survival of E16 rat DRG neurons (Figure 5D). The deficits in biological activity of the mutant NT-4 in neuronal cells correlated with a 3- to 5-fold reduction in its ability to induce TrkB autophosphorylation in nnr5PC12-TrkB cells compared with wt NT-4 (Figure 5E). Since this mutant was indistinguishable from wt NT-4 in promoting TrkB autophosphorylation and biological activity in fibroblasts which did not express $p75^{LNGFR}$, these results suggest that the deficits of the mutant in sensory neurons and nnr5PC12 cells were due to the complete inability of the molecule to bind $p75^{LNGFR}$.

Discussion

Although all four neurotrophins bind $p75^{LNGFR}$ with equal affinity, residues that participate in this interaction do not appear to be conserved among the four proteins. Furthermore, increasing evidence indicates this receptor may recognize each of the four ligands as related, albeit not identical, structures (Rodriguez-Tebar *et al.*, 1992; Benedetti *et al.*, 1993). This poses interesting questions regarding the analysis of molecular recognition, namely which are the similarities and differences in the way neurotrophins interact with $p75^{LNGFR}$ and what are their functional implications?

Similarities: positively charged residues mediate binding of neurotrophins to $p75^{LNGFR}$

Combined with our previous results (Ibáñez *et al.*, 1992), the analysis presented here demonstrates that, in all members of the neurotrophin family, positively charged amino acid residues clustered in an exposed arm of the neurotrophin dimer are essential for binding to $p75^{LNGFR}$. This finding provides a structural explanation for the common interaction of the neurotrophins with this receptor. Based on the crystal structure of NGF (McDonald *et al.*, 1991), we constructed three-dimensional models of the other three neurotrophins and analysed the electrostatic surfaces for each (Figure 6). All four neurotrophins show a similar charge distribution on the surface. The binding site comprises loop regions I and V of the first protomer and extends through the dimer interface towards the second protomer (Figure 6). Highly conserved positively charged residues at positions 88 and 100 of the first protomer (NGF numbering) and at 25 and 50 of the second protomer extend the positively charged surface across the top of the dimer, forming a slightly concave pocket of positive charge. Two symetrically related binding sites are formed at each side of the neurotrophin dimer which may allow $p75^{LNGFR}$ dimerization upon ligand binding (Meakin and Shooter, 1991; Jing *et al.*, 1992). Three-dimensional models of the mutants deficient in $p75^{LNGFR}$ binding show that in each case side chains important for binding to $p75^{LNGFR}$ are located at the edge of the binding pocket, readily accessible for interaction with the receptor (Figure 6). Electrostatic interactions have previously been shown to be important for receptor binding in other growth factors (Wlodawer *et al.*, 1993; Wells, 1994). These are generally long range interactions that may serve to guide ligands to receptors to bind or collide in a productive fashion, and are therefore likely to affect the association rate of binding. The spatial location of the residues identified here suggests that they may be involved in the initial stages of ligand–receptor contact. The interaction

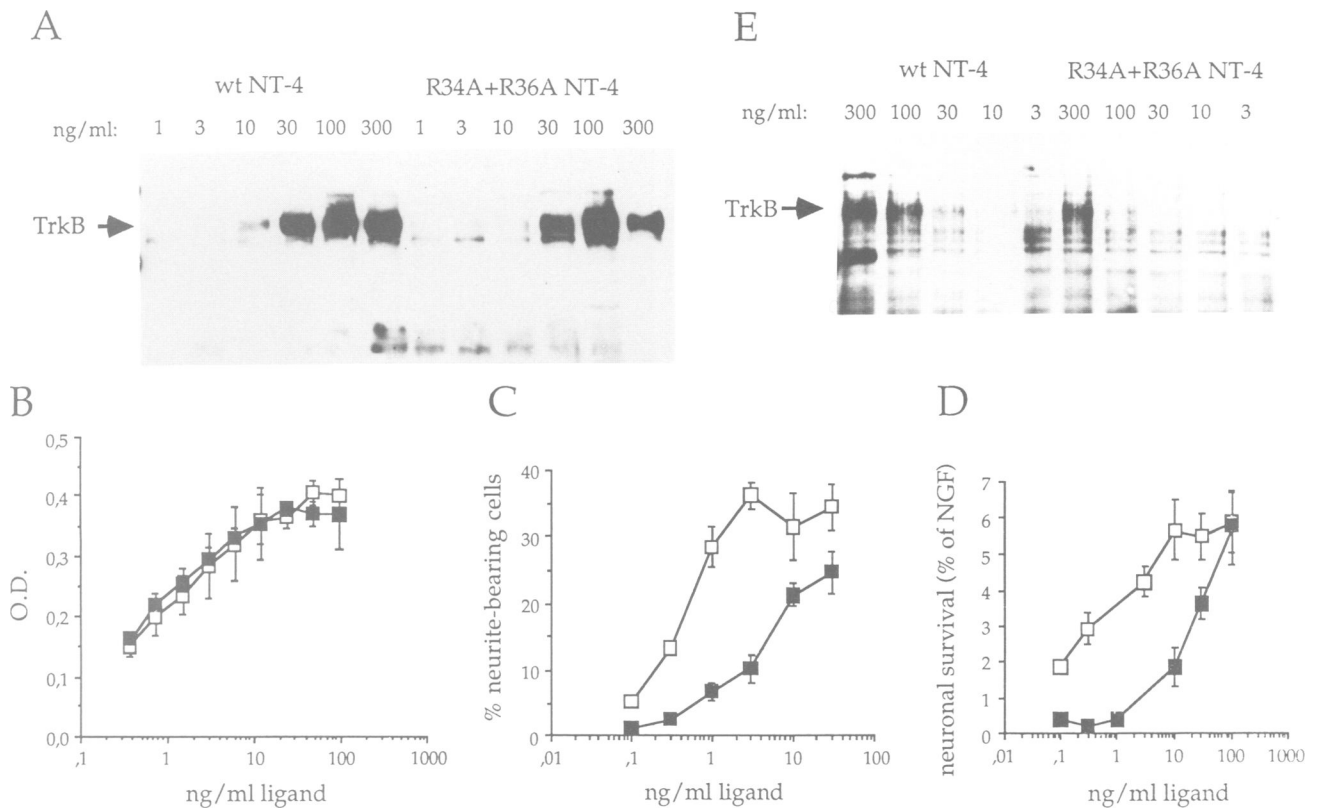

Fig. 5. TrkB activation and biological activities of mutant R34A+R36Aand wt NT-4. (**A**) Serial dilutions of purified wt and mutant NT-4 were assayed for their ability to stimulate tyrosine autophosphorylation of TrkB expressed in fibroblast cells. An autoradiogram of a phosphotyrosine blot is shown. The arrow indicates the migration of phosphorylated TrkB ($p145^{trkB}$). Similar results were obtained in a duplicate experiment. (**B**) Serial dilutions of purified wt (□) and mutant (■) NT-4 were assayed for their ability to stimulate survival and growth of MG87 fibroblast expressing TrkB as assessed by metabolic labelling with MTT. Data are expressed as the average optical density (O. D.) of triplicate wells ± SD. (**C**) Serial dilutions of purified wt (□) and mutant (■) NT-4 were assayed for their ability to promote neurite outgrowth in nnr5PC12-TrkB cells. Data are expressed as the average percentage of neurite-bearing cells in triplicate wells ± SD. (**D**) Serial dilutions of purified wt (□) and mutant (■) NT-4 were assayed for their ability to promote survival of E16 rat DRG neurons. Data are expressed as neuronal survival relative to that obtained with saturating concentrations (10 ng/ml) of NGF ± SD. (**E**) Serial dilutions of purified wt NT-4 and mutant NT-4 were assayed for their ability to stimulate tyrosine autophosphorylation of TrkB expressed in nnr5PC12-TrkB cells. The arrow indicates the migration of phosphorylated TrkB ($p145^{trkB}$).

could subsequently be stabilized by contacts through the rest of the positively charged pocket and/or other regions of the molecule. It should also be noted that, while the residues identified here define functional $p75^{LNGFR}$ epitopes on the neurotrophins, structural epitopes are likely to be far more extensive and may involve multiple Van der Waal and hydrogen bonding contacts. However, such determinants, taken individually, may not contribute sufficient binding energy to be easily identified from mutagenesis experiments. The mutations do not eliminate all the positive charges from the binding surface (Figure 6), suggesting that certain key positions require positive charges to allow binding to $p75^{LNGFR}$. An NGF chimeric molecule in which variable region V was replaced by homologous sequences from BDNF (Ibáñez *et al.*, 1991) contains positively charged residues from the binding interfaces of both NGF and BDNF. In this molecule, the additional positive charges, however, did not appear to increase its affinity for $p75^{LNGFR}$ (C.F.Ibáñez, unpublished results), suggesting that this receptor can only accept a limited number of electrostatic contacts in this region. Further insights await detailed evaluation of the effects of increased or decreased number of positive charges on the association and dissociation rates of binding to $p75^{LNGFR}$. The residues involved in $p75^{LNGFR}$ binding identified here are located in variable regions that are also important for determining the specificity of binding to Trk receptors (Ibáñez *et al.*, 1991, 1993). A shared binding surface for Trk and $p75^{LNGFR}$ would likely alter the ability of the neurotrophin to accept or tolerate amino acid changes. Because all members of the neurotrophin family have preserved a similar surface property for binding to $p75^{LNGFR}$, a need to maintain binding to this receptor may have imposed evolutionary constraints to the rate of divergence of neurotrophin variable regions and, therefore, to the generation of different biological specificities within the family.

Differences: distinct spatial arrangements of binding determinants and possible mechanisms of ligand discrimination

Although binding of all four neurotrophins to $p75^{LNGFR}$ is mediated through positively charged residues, the nature and spatial arrangement of the side chains involved varies among the different proteins. In NGF, NT-3 and NT-4, positively charged residues in region I extend the surface towards the edge of the molecule approximately perpendicular to the 2-fold symmetry axis (Figure 6). In BDNF, positively charged side chains in region V project outwards in the top portion of the dimer (Figure 6). In BDNF,

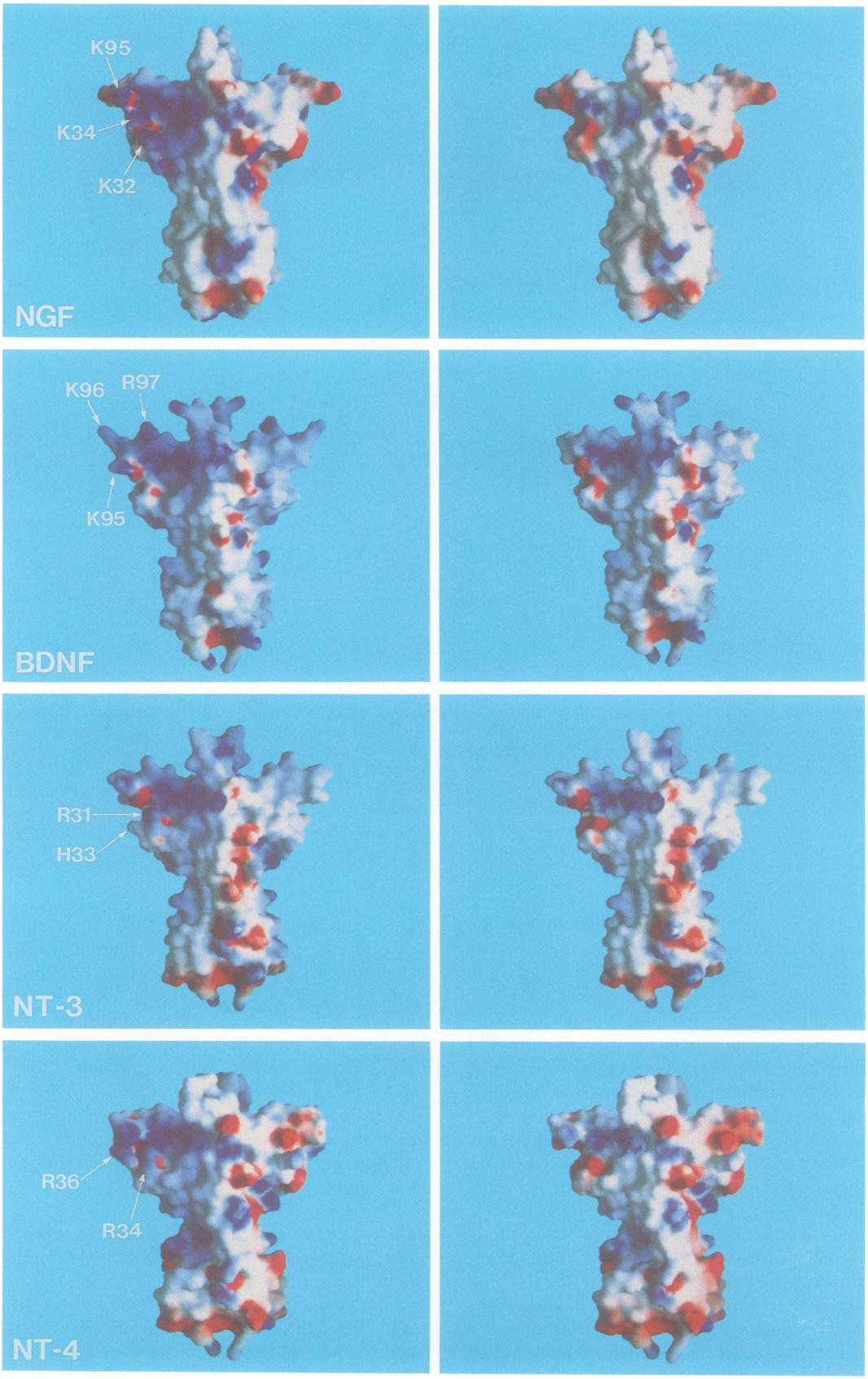

Fig. 6. Electrostatic potential surface of wild-type neurotrophin dimers (left column) and their respective mutants (right column) deficient in binding to $p75^{LNGFR}$. From top to bottom on the right column are mutant molecules K32A+K34A+E35A+K95A NGF, K95A+K96A+R97A BDNF, R31A+H33A NT-3 and R34A+R36A NT-4. Blue represents positive potential, red negative and white neutral. Positively charged residues participating in binding to $p75^{LNGFR}$ are labelled. Note the presence of a cluster of positive charges in the upper left corner of the wild-type molecules. Note also the disruption of the positively charged surface in the mutant forms defective in $p75^{LNGFR}$ binding. For details on the modelling rationale see Materials and methods.

these positively charged residues have previously been implicated in TrkB binding and activation (Ibáñez *et al.*, 1993), suggesting that p75^{LNGFR} and TrkB have partially overlapping binding sites in BDNF. Another positively charged residue in BDNF (Lys46) appears in close proximity to the binding site and therefore may also be involved in contact to p75^{LNGFR}. That positive charges can be located in different positions within the binding interface of different neurotrophins suggests that loop regions I and V may be quite flexible to allow appropriate alignment of electrostatic interactions during ligand–receptor contact. In support of this notion, recent analysis of different crystal forms of NGF established that, with the exception of the termini, mobile regions comprise the three variable loops clustered at the top of the molecule (Holland *et al.*, 1994). Interestingly, the two short strands connecting the turn at positions 30–34 (variable loop region I) appear to shift significantly between different crystal forms (Holland *et al.*, 1994), suggesting that determinants of binding to p75^{LNGFR} in the neurotrophins may be quite mobile.

Rodriguez-Tébar *et al.* (1990) reported that the dissociation rate from p75^{LNGFR} of BDNF was much slower than that of NGF, with an off rate of at least an order of magnitude smaller. More recently, Timm *et al.* (1994) reported changes in circular dichroism (CD) spectra that occur after complexing neurotrophins with a soluble p75^{LNGFR} extracellular domain, indicating that conformational changes accompany binding. Although similar overall, the shape and intensities of the differences in CD spectra varied depending on which neurotrophin was mixed with p75^{LNGFR}, suggesting that different conformational changes occur after complex formation with each neurotrophin (Timm *et al.*, 1994). Our results suggest that these kinetic and conformational differences between neurotrophins could be the result of distinct spatial arrangements of positive charges in their respective p75^{LNGFR} binding interfaces. These differences may subsequently be transmitted to cognate Trk receptors and/or the signalling machinery in order to influence ligand discrimination.

Role of p75^{LNGFR} in regulating biological responsiveness to NT-4

Functional analysis of neurotrophin mutants deficient in p75^{LNGFR} binding revealed that, for BDNF and NT-3, binding to this receptor was not necessary to promote survival or differentiation of neuronal cells co-expressing p75^{LNGFR} and cognate Trks. These results concur with our previous findings using analogous NGF mutant molecules (Ibáñez *et al.*, 1992). Mutant BDNF was somewhat less potent on cells expressing TrkB whether or not they expressed p75^{LNGFR}, suggesting that this mutation may also have partially affected the BDNF binding site to TrkB. Dose–response analysis of neuronal survival using a mutant NGF has recently indicated that disruption of binding to p75^{LNGFR} may, however, affect the potency of the molecule at low ligand concentrations (at or below the high-affinity K_d, 260 pg/ml) (C.F.Ibáñez, unpublished results). In addition, this mutant NGF was recently shown to have lower activity than wt NGF in promoting TrkA autophosphorylation and biological activity in a sympathoadrenal precursor cell line expressing both TrkA and p75^{LNGFR} (Verdi *et al.*, 1994). Thus, these observations suggest that binding to p75^{LNGFR} may nevertheless be important for neurotrophin responsiveness at low ligand concentrations.

In contrast to the other three neurotrophins, mutation of the p75^{LNGFR} binding site in NT-4 greatly reduced responsiveness in neuronal cells co-expressing p75^{LNGFR} and TrkB over a wide range of ligand concentrations. The ability to induce TrkB receptor autophosphorylation in nnr5PC12-TrkB cells was also reduced by the mutation, suggesting that the deficits in biological activity originate at the cell surface and may be due to reduced receptor activation. This result was surprising given the normal behaviour of the mutant NT-4 in fibroblast cells expressing only TrkB. Because both the nnr5PC12 and fibroblast TrkB-expressing lines were obtained by transfection of the same TrkB cDNA, the differences observed could not have been due to the presence of different TrkB isoforms in these cells. It is possible that other differences between these cells (lipid membrane composition, presence of other proteins, etc.) could influence neurotrophin responsiveness. However, no significant differences between the two cells were seen using an analogous BDNF mutant produced under similar conditions. Moreover, the deficits of the mutant NT-4 in nnr5PC12 and neuronal cells could not have been due to TrkB being in a more neuronal environment because no significant differences were seen between wt and mutant NT-4 in the neuroblastoma line SH-SY5Y, which expresses TrkB but not p75^{LNGFR}. In addition, the activity of the mutant NT-4 in basal forebrain neurons in a paradigm which does not depend upon p75^{LNGFR} was recently shown to be similar to wt NT-4 (W.Friedman, personal communication). Together, these data indicate that the behaviour of the NT-4 mutant is intrinsic to the effect of the mutation on its binding to p75^{LNGFR} and not to structural alterations or to inability to interact with TrkB on neuronal environments. Interestingly, recent studies have shown that retrograde transport of NT-4 to DRG is selectively dependent upon p75^{LNGFR} (Curtis *et al.*, 1993). Of all the neurotrophins, NT-4 transport was most sensitive to inhibition by co-injected p75^{LNGFR} blocking antibodies or soluble p75^{LNGFR} extracellular domain, and was most severely reduced in mice with a targeted disruption of the p75^{LNGFR} gene (Curtis *et al.*, 1993). Furthermore, NT-4, in contrast to BDNF, was only transported to motor neurons after axotomy, coincident with increased expression of p75^{LNGFR} in these cells (Curtis *et al.*, 1993). Together with the data presented here, these results indicate a distinct interaction between NT-4 and p75^{LNGFR} that appears to be required for effective TrkB activation and biological activity in cells co-expressing both receptors. Binding and functional assays have indicated that p75^{LNGFR} can cooperate with Trk receptors to increase the affinity of neurotrophin binding and/or the signalling efficiency (Hempstead *et al.*, 1991; Barker and Shooter, 1994; Hantzopoulos *et al.*, 1994; Mahadeo *et al.*, 1994; Verdi *et al.*, 1994). Several models have been presented to account for this effect. p75^{LNGFR} may increase binding of NGF to TrkA by increasing the local concentration of NGF around TrkA receptors (Barker and Shooter, 1994). This effect appears to be saturable and it has only been detected at concentrations below 25–100 ng/ml. Interestingly, the deficits of our mutant NT-4 in neuronal cells were particularly evident at concentrations below

30–100 ng/ml. Thus, NT-4, in contrast to the other neurotrophins, may be more dependent on a concentrative role of $p75^{LNGFR}$. This receptor may alternatively act by presenting the ligand in a favourable conformation for binding. Although both BDNF and NT-4 efficiently bind and activate TrkB (Ip *et al.*, 1993), NT-4 has been shown to be incapable of activating a point-mutated TrkB (C345S) that is efficiently activated by BDNF (Klein *et al.*, 1992; Ip *et al.*, 1993), suggesting that BDNF and NT-4 may be utilising partially distinct domains within the TrkB receptor. Thus, NT-4, in contrast to BDNF, may require presentation by $p75^{LNGFR}$ for efficient binding to TrkB in neuronal cells. Our data do not, however, offer any clues as to why sensory neurons from $p75^{LNGFR}$ knock-out mice appear to have a normal responsiveness to NT-4 (Davies *et al.*, 1993). The presence of $p75^{LNGFR}$ on the membrane of normal neurons may somehow determine the requirement for binding to this receptor for maximal NT-4 action, perhaps by inducing a particular spatial arrangement of TrkB receptors or by modulating TrkB signalling. It should also be noted that the lack of $p75^{LNGFR}$ may have led to changes in other properties of knock-out mice neurons, perhaps related to membrane or signalling components. In this respect, it is interesting to note that neurons from $p75^{LNGFR}$ knock-out mice show reduced retrograde transport of lectins, suggesting that these cells may have other more general deficits secondary to the loss of $p75^{LNGFR}$. Finally, we cannot rule out the possibility that part of the effects reported here may be due to direct signalling through $p75^{LNGFR}$ (Dobrowsky *et al.*, 1994).

In summary, we have mapped functional epitopes in the neurotrophins responsible for binding to their common receptor $p75^{LNGFR}$ and have generated neurotrophin mutants deficient in binding to this receptor. Three-dimensional models of the charge distribution on the surface of each neurotrophin revealed differences that may underlie selective ligand–receptor interactions. Finally, we have shown that neuronal cells co-expressing TrkB and $p75^{LNGFR}$, but not cells expressing TrkB alone, display altered responses to a mutant NT-4 deficient in binding to $p75^{LNGFR}$. Our findings provide evidence for a selective role of $p75^{LNGFR}$ in regulating neurotrophin responsiveness.

Materials and methods

Cell lines, antisera, proteins, plasmids and site-directed mutagenesis

NIH3T3 fibroblasts expressing rat TrkB (NIH3T3 TrkB) used in phosphorylation assays were kindly provided by Drs Dan Soppet and Luis Parada, University of Texas, TX. A875 is a human melanoma cell line expressing high levels (~10^5 molecules per cell) of $p75^{LNGFR}$ (Buxser *et al.*, 1983). The generation of Trk-expressing MG87-NIH3T3 fibroblasts has been described previously (Glass *et al.*, 1991). nnr5 PC12-TrkB cells are NGF non-responding mutant PC12 cells stably transfected with a TrkB cDNA, which only respond to BDNF and NT-4, and were kindly provided by Dr Lloyd Greene, Columbia University, NY. Anti-panTrk rabbit polyclonal antiserum 203 (which recognizes TrkA, TrkB and TrkC), anti-NT-3 and anti-NT-4 rabbit polyclonal antisera were kindly provided by Dr David Kaplan, National Cancer Institute, Frederick, MD. Anti-BDNF rabbit polyclonal antiserum was kindly provided by Dr Ravinder Sehgal, University of California, San Francisco, CA. Anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology Inc. (New York). Purified mouse NGF was from Promega (WI). Recombinant BDNF, NT-3 and NT-4 were produced in *Escherichia coli* and subsequently purified as described previously (Ip *et al.*, 1993; Fandl *et al.*, 1994). Fragments containing the pre-pro-coding sequences of rat NGF (Whittemore *et al.*, 1988), mouse BDNF (Hofer *et al.*, 1990), rat NT-3 (Ernfors *et al.*, 1990) and rat NT-4 (Ip *et al.*, 1992) were cloned into pBS KS^+. Single-stranded DNA from these plasmids was used as template for oligonucleotide-based site-directed mutagenesis as described by Kunkel (1985) and the replacements were confirmed by nucleotide sequence analysis. For recombinant protein production in COS cells or Sf-21 insect cells, DNA inserts containing the desired replacements were subcloned into pXM (Yang *et al.*, 1986) or into pBacPAK1 (Clontech, CA), respectively.

Production and purification of recombinant mutant and chimeric proteins

Wild-type (wt) NGF, wt BDNF, NGF/BDNF chimeric molecules and the NT-3 mutants K95A and R31A+H33A+K95A used in binding assays were produced in transiently transfected COS cells as previously described (Ibáñez *et al.*, 1992, 1993; Ilag *et al.*, 1994). The amounts of recombinant protein produced by the different constructs were determined as previously described (Ibáñez *et al.*, 1992, 1993; Ilag *et al.*, 1994). All assays were performed using equivalent amounts of recombinant wild-type and chimeric neurotrophins.

Recombinant baculovirus clones used for production of mutant neurotrophins from insect cells were produced using a kit of reagents from Clontech (CA) according to manufacturer's instructions. Low-multiplicity, low-passage virus stocks were used to infect Sf-21 cells cultured in serum-free medium (Sf-900-II, GIBCO-BRL, MD) in 1 l spinner flasks with an agitation rate of 30 r.p.m.. Conditioned medium was harvested at 72 h post-infection, adjusted to pH 8.0 with 5 M NaOH, clarified of debris and readjusted to pH 6.5 with concentrated HCl. The medium was then subjected to chromatography on High Performance S-Sepharose (Pharmacia, Sweden) in 50 mM phosphate pH 6.5, 5 mM EDTA and eluted with a 0 to 1 M NaCl linear gradient. Selected fractions, identified by Western blotting, were concentrated by ultrafiltration (Amicon, MA), pooled and chromatographed on Superdex 75 (Pharmacia, Sweden) in 50 mM phosphate pH 7.4, 5 mM EDTA, 0.5 M NaCl, 10% acetonitrile. Selected fractions, identified by Western blotting, were concentrated by ultrafiltration (Amicon, MA), pooled and adjusted to pH 3.5 with concentrated acetic acid. This sample was then applied to a reverse-phase C8 HPLC column (Vydac) and eluted with a 0 to 60% acetonitrile linear gradient in 0.1% TFA. Selected fractions, identified by Western blotting, were vacuum dried, reconstituted in water, and aliquoted. SDS–PAGE and silver staining showed all mutant proteins had a purity of ≥90%.

Binding assays

Purified neurotrophins were labelled with ^{125}I by the lactoperoxidase method to an average specific activity of 1×10^8 c.p.m./μg. A875 cells were used at $2–10\times10^6$ cells/ml. Steady-state binding was measured in competition assays performed at 4°C using 1.5×10^{-9} M iodinated factor and serial dilutions of wt or mutated proteins. All components were added at the same time and the cells were collected by centrifugation after equilibrium was reached (90–120 min incubation). Cell pellets were then counted in a gamma counter. 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 the iodinated factors. Non-specific binding was measured in a parallel incubation to which 300- to 1000-fold molar excess of unlabelled purified factor was added. All results were corrected for this non-specific binding. The concentration of each chimeric, mutant and wild type molecule that gave 50% binding (IC_{50}) was determined, and relative binding was calculated using the relationship: (mutant IC_{50}/wild-type IC_{50}) ×100.

Phosphorylation assays

A confluent 15 cm plate containing ~2.5×10^7 cells was treated for 5 min at 37°C with wt or mutant factors and subsequently lysed with 1 ml of ice-cold buffer containing 1% NP-40, 20 mM Tris pH 8.0, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1 mM PMSF, 0.15 U/ml aprotinin, 20 μM leupeptine and 1 mM Na orthovanadate. Plates were incubated 15 min at 4°C after which insoluble material was removed by centrifugation. Cell lysates were normalized for protein content before immunoprecipitation. Trk immunoprecipitation was performed by incubating lysates with 1 μl of anti-panTrk polyclonal antiserum 203. After 2 h at 4°C, immunocomplexes were collected with protein A–Sepharose (Pharmacia, Sweden), washed in lysis buffer and boiled for 5 min before SDS–PAGE. After electrophoresis, gels were blotted to nitrocellulose membranes, reacted with anti-phosphotyrosine monoclonal antibody

4G10 (UBI, New York) and developed with the ECL Western Detection System (Amersham, UK).

Bioassays

Biological activities in Trk-expressing MG87-NIH3T3 fibroblasts were assayed as previously described (Glass *et al.*, 1991; Ip *et al.*, 1993). Briefly, cells were incubated with different neurotrophin molecules. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added at 48 h after plating and culture was continued for another 4 h. Labelled cells were then lysed with dimethyl sulphoxide and optical density was measured as an indicator of cell survival and proliferation.

Dissociated neurons from chick E8 or rat E16 lumbar DRG were plated on plastic for 2 h and then cultured in DMEM:F12 (1:1) supplemented with 1 mg/ml BSA, glutamine, antibiotics. 24-well plates coated with poly-L-ornithine and laminin were seeded at a density of 10^5 cells/well. Serial dilutions of purified wild-type or mutant factors were added at the time of plating in DMEM:F12 serum-free medium and neuronal survival was determined after 48–72 h by phase contrast microscopy, scoring the number of surviving neurons in a defined area of triplicate wells.

nnr5 PC12-TrkB cells plated in 24-well plates coated with rat tail collagen were incubated in RPMI supplemented with 10% horse serum and 5% fetal calf serum together with serial dilutions of purified wild type or mutant factors. 72 h after plating, the percentage of cells bearing fibres longer than two cell diameters was determined microscopically in a defined area of triplicate wells.

Modelling of neurotrophins

Models of BDNF, NT-3 and NT-4 (rat sequences) were built using the programs O (Jones *et al.*, 1991) and SYBYL (Tripos Inc., USA) based on the crystal structure of murine NGF (PDB code 1BET). The rationale behind the models was to preserve the main chain conformation as well as conserved side-chain–side-chain and main-chain–main-chain hydrogen bonds. It was also necessary to avoid clashes at the dimer interface, and the 2-fold symmetry of the dimer was preserved.

Conformations of individual variable regions are:

30–35: this loop has conserved residues Asp30 and Gly33, which are assumed to define its conformation in all neurotrophins.

40–49: this loop is poorly defined in the crystal structure, and there are large sequence variations between members of the family. Due to its proximity to the 2-fold axis, it was modelled to avoid clashes. There is little chance that only one conformation of this loop will be present in any member of the family.

59–66: the conformation of this loop depends largely on the presence of Pro63 in NGF. This is also present in NT-3, thus NT-3 can be modelled closely on NGF. In BDNF, there is a proline two residues earlier in the sequence, and the loop has been remodelled to accommodate this and allow the side chain of Tyr63 to be directed outwards. In NT-4 there is an eight-residue insertion in this region, and no attempt has been made to model it; the model here contains the NGF sequence.

70–79: this is a rather extended structure in NGF and there is no difficulty in making side chain substitutions.

92–98: this contains a one-residue insertion in all neurotrophins compared with NGF. The conformation of the loop is limited by the requirement of main chain hydrogen bonds not only within the loop but also between atoms O(90) to N(39), N(92) to O(37) and O(92) to N(37), and further, by the preservation of side-chain hydrogen bonds 95 to 100 and 100 to 91. These limitations serve to keep the loop in a similar orientation with respect to the rest of the molecule.

In BDNF a two-residue insertion is present after position 22. This is difficult to insert, while preserving all the main-chain hydrogen bonds. The conformation suggested here has no disallowed main-chain conformational angles, and does not lead to clashes at the dimer interface. It is, however, possible that the BDNF structure is significantly different from the NGF structure at this point.

Pictures of the electrostatic surface potential were made with GRASP (Nicholls *et al.*, 1991) at the level of the solvent accessible surface with a probe radius of 1.4 Å.

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