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Chimeric molecules with multiple neurotrophic activities reveal structural elements determining the specificities of NGF and BDNF

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Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are two members of a family of neurotrophic factors which show both overlapping and distinct neurotrophic activities. Using site-directed mutagenesis, chimeric molecules were constructed where different combinations of sequences from BDNF replaced the corresponding sequences in NGF. The resulting molecules were transiently expressed in COS cells and conditioned media containing the chimeric proteins were assayed for biological activity in explanted chick sympathetic, spinal and nodose ganglia. Our results show that the biological specificities of the two proteins are obtained by specific combinations of a set of sequences that differ between the two molecules. Some of these combinations allowed us to engineer molecules which display multiple neurotrophic activities recruited from both the NGF and BDNF proteins.

Key words: brain-derived neurotrophic factor/dorsal root ganglion/nerve growth factor/site-directed mutagenesis/sympathetic ganglion

Introduction

Cell growth and differentiation require specific factors which in many cases belong to families of proteins with close structural and functional relationships (Goeddel *et al.*, 1986; Gospodarowicz, 1990; Roberts *et al.*, 1990). How these structurally related proteins achieve their functional specificity is, however, largely unknown. The nerve growth factor (NGF) family of neurotrophic factors presently includes three structurally related proteins that promote survival and differentiation of neurons (Leibrock *et al.*, 1989; Ernfors *et al.*, 1990a; Hohn *et al.*, 1990; Maisonpierre *et al.*, 1990b; Rosenthal *et al.*, 1990). These factors are present in limited amounts in the target fields to which the neurons project, and in the developing vertebrate nervous system they regulate both the timing and extent of neural innervation (Barde, 1989). Recently the gene for a fourth member of the family has been cloned and characterized from *Xenopus laevis* and *Vipera lebetina* (Hallböök *et al.*, 1991).

NGF, the prototype for target-derived neurotrophic factors, supports the development and maintenance of sympathetic and neural crest-derived sensory neurons (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980), as well as basal forebrain cholinergic neurons in the brain (Whittemore and Seiger, 1987; Thoenen *et al.*, 1987; Ebendal, 1989a). The second member of the NGF family, brain derived-neurotrophic factor (BDNF), was purified from the pig brain (Barde *et al.*, 1982) and subsequently shown to induce neurite outgrowth from embryonic neural crest-derived sensory ganglia cultured *in vitro* in a manner that it is indistinguishable from that of NGF (Davies *et al.*, 1986). NGF and BDNF have additive effects on neurite outgrowth (Lindsay *et al.*, 1985) and neuronal survival (Barde *et al.*, 1982) in dissociated cultures of neurons from dorsal root spinal ganglia, suggesting that they could act on different neuronal populations. Both factors are also distinguishable in their abilities to interact with their respective receptors on these neurons (Rodriguez-Tébar *et al.*, 1990). In contrast to NGF, BDNF also supports placode-derived sensory neurons from the nodose ganglia, whereas the NGF-dependent sympathetic neurons do not respond to BDNF (Lindsay *et al.*, 1985). Thus, the two factors display both overlapping and specific neurotrophic activities. Here we show that in the NGF family the biological specificity is acquired by a specific combination of a set of variable regions in these two proteins. Our results also have implications for the evolutionary process that resulted in the appearance of this family of proteins.

Results

We first defined five variable regions in which the BDNF sequence differs significantly from that of NGF (Figure 1). The amino- and carboxy-terminal ends and some scattered amino acid differences were excluded from the analysis since they represent conservative changes or positions which also vary considerably among NGF sequences from different species. Using oligonucleotide site-directed mutagenesis, the variable regions in the NGF molecule were systematically replaced by the corresponding sequences from BDNF. This procedure created a series of chimeric molecules based on a NGF skeleton which included different variable regions from the BDNF sequence. Changes in the predicted average hydrophilicity and secondary structure as a result of these replacements are summarized in Table I. The chimeric molecules were then transiently expressed in COS cells. Serial dilutions of conditioned media containing equivalent amounts of the different recombinant proteins were then compared with wild type (wt) NGF and wt BDNF by assaying their ability to promote neurite outgrowth from E9 chick sympathetic ganglia (SYMP) (characteristic of NGF activity), E9 chick nodose ganglia (NOD) (characteristic of BDNF activity) or E9 chick spinal dorsal root ganglia (SPIN) (both NGF and BDNF activities).

Initially, the five variable regions were replaced independently in the NGF molecule. The replacement of either of the three most carboxy-terminal regions (III, IV and V) did not have any effect on the activity profile of the NGF

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                23          35  40      49
Rat NGF    SSTHPVFHMGEFSVCDSVSVWV**GDKTTATDIKGKEVTVLGEVNINNSVFKQYFFETKC
Rat BDNF   **HSDPARR..L.....I.E.[TAA.[K]..V.MS.GT]...[EK.PVSKGQI]....Y....
                      Ia      Ib           II
                         \____/
                           I

           59   66        79      88   94  98
Rat NGF    RAPNPVESGCRGIDSKHWSNYCTTTHTFVKALTTDDKQ*AAWRFIRIDTACVCVLSRKAARRG
Rat BDNF   [NPMGYTKE].....KR...[Q.R..QSY.R]..M[S.KRIG].......S...T.TI.RG.**
              III                 IV           V
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Fig. 1. Alignment of the amino acid sequences (single-letter code) of rat NGF (Whittemore *et al.*, 1988) and rat BDNF (Maisonpierre *et al.*, 1990b). Boxes indicate variable regions in the BDNF sequence. In the different chimeric molecules, these regions were inserted in the same position in the NGF protein thereby replacing the corresponding NGF sequences with the different variable regions from BDNF. Numbers indicate amino acid residues in the NGF sequence.

molecule (Figure 2). Neither did the replacement of region Ia (Figure 2) which introduces two extra residues in the NGF polypeptide chain. However, substitution of region Ib, which introduces six amino acid changes (Figure 1), resulted in a weak, but consistent, activity in NOD in addition to wt NGF activity in SYMP and SPIN (Figure 2). Increasing concentrations of the Ib molecule did not, however, reach the level of stimulation in NOD observed with wt BDNF, indicating that both the specific activity and maximal stimulation of fibre outgrowth in NOD were lower than those of wt BDNF. The combination of regions Ia and Ib (chimeric molecule I) showed wt NGF activity in both SYMP and SPIN, and retained the ability to partially stimulate NOD (Figures 2, 3 and 4). Substitution of region II dramatically decreased the level of secreted recombinant protein to ~1% of that observed with wt NGF. This chimeric molecule revealed activities in SPIN and SYMP at the same levels as those obtained using equivalent amounts of wt NGF, while no activity was seen in NOD (Figure 2).

We next examined the effect of different combinations of the carboxy-terminal regions which individually did not change the specificity of NGF. The simultaneous replacement of regions III and IV resulted in a molecule that retained wt NGF activities in both SYMP and SPIN but without activity in NOD (Figure 2). In contrast, substitution of both regions III and V resulted in a significant stimulation of neurite outgrowth from the NOD, in addition to wt NGF activities in both SYMP and SPIN (Figures 2 and 4). The activity in NOD was further enhanced by adding region IV to the III + V molecule. The resulting chimera (III + IV + V) reached levels of activity in NOD comparable with those seen with wt BDNF (Figures 2 and 4). However, a quantitative comparison of the stimulation of fibre outgrowth from SYMP revealed that the specific biological activity of this chimera was reduced to approximately half compared with wt NGF (Figures 2, 3 and 4). The activity of this molecule in SPIN was intermediate between that seen with wt NGF and wt BDNF (Figures 2, 3 and 4), which may reflect the fact that this molecule is structurally half NGF and half BDNF. However, the activities in both SYMP and SPIN reached the same levels as wt NGF when higher amounts of the III + IV + V molecule were used, indicating that maximal stimulation similar to that obtained with wt NGF can be reached with this chimeric protein.

Next, the effects of combining the amino-terminal regions with some of the carboxy-terminal chimeras were investigated. Surprisingly, the activity in NOD previously detected in the region I chimera was lost by combining this region with regions III and IV in the I+III+IV molecule (Figure 2). Although this molecule contains three variable regions (out of five) replaced with the corresponding BDNF sequences, its activity profile was indistinguishable from that of wt NGF. It was therefore of interest to study whether wt NGF activities could also be retained by adding region V to the I+III+IV molecule. The resulting chimera (I+III+IV+V) displayed high levels of activity in both SYMP and SPIN as well as NOD (Figures 2 and 4). Both the dose–response curve and maximal stimulation of fibre outgrowth in NOD were similar to those seen with wt BDNF. As in the case of the III+IV+V molecule, the I+III+IV+V chimera also showed reduced levels of activity in SYMP compared with wt NGF, and intermediate levels between wt NGF and wt BDNF in SPIN (Figures 2 and 4). The fact that the I+III+IV+V molecule only retains variable region II from NGF suggested that this region was responsible for the remaining activity in SYMP associated with this chimeric molecule. This was demonstrated by adding region II to the I+III+IV+V molecule. The resulting protein showed neurotrophic specificities identical to those of wt BDNF (Figure 2), therefore indicating that region II is important for maximal stimulation of SYMP by NGF.

Table I. Predicted average hydrophilicity and secondary structure of the variable regions of NGF and BDNF.

Region	Hydrophilicity[a]		Secondary structure[b]	
	NGF	BDNF	NGF	BDNF
I	0.325	−0.034	random coil	α-helix
II	−0.094	0.274	β-sheet+ random coil	α-helix+ random coil
III	0.811	1.061	turn+ random coil	turn+ random coil
IV	0.371	1.065	β-sheet	random coil
V	0.973	1.034	β-sheet	shorter β-sheet

[a]Average hydrophilicity was calculated according to Kyte and Doolittle (1982).
[b]Secondary structure according to Chou and Fasman (1978).
All calculations were done using the computer program Plotstructure from the University of Wisconsin Genetics Computer Group (UWGCG) (Devereux *et al.*, 1984).

Discussion

In this study, a series of chimeric molecules between NGF and BDNF were engineered using oligonucleotide site-

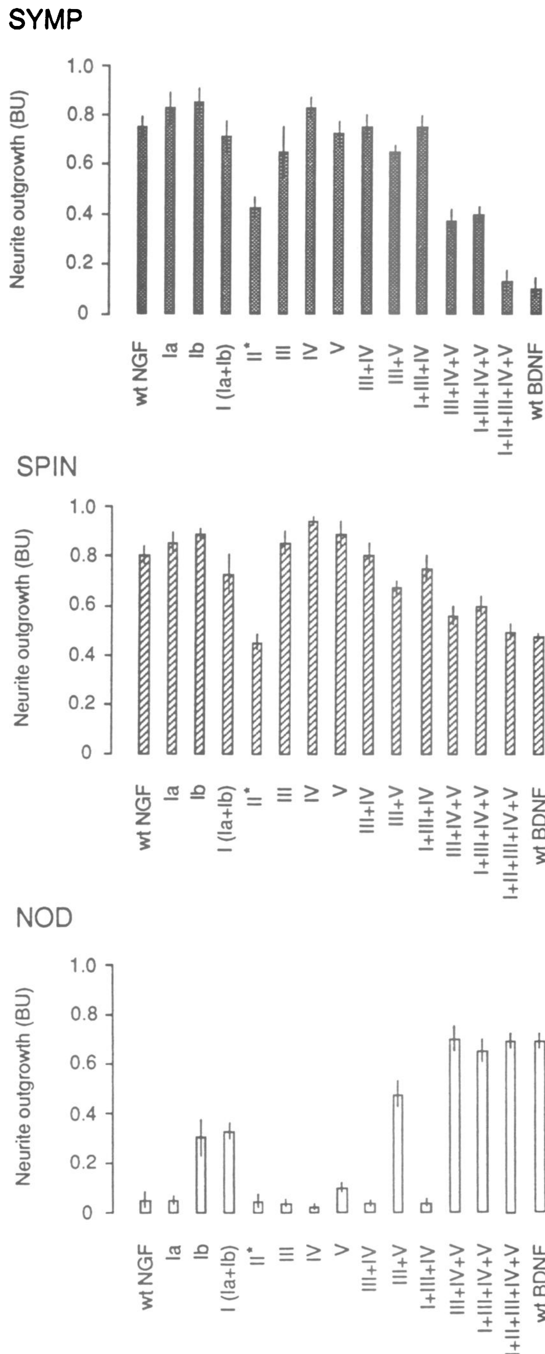

Fig. 2. Biological activities of wt NGF, wt BDNF and chimeric molecules in explanted chick embryonic day 9 SYMP (stippled bars), SPIN (hatched bars) and NOD (empty bars). Ganglia were cultured in collagen gels for 48 h in the presence of 2 ng/ml (SYMP and SPIN) or 8 ng/ml (NOD) of the indicated recombinant proteins. These concentrations of recombinant wt NGF and wt BDNF protein produced half-maximal stimulation of neurite outgrowth from the SYMP and SPIN (in the case of NGF) and from the NOD (in the case of BDNF). Star denotes that, due to its low production, chimeric molecule II was assayed at 0.5 ng/ml. The magnitude of fibre outgrowth was scored on a semi-quantitative scale in biological units (BU) (Ebendal, 1989b). Results are means of three determinations ± SEM. As previously reported by others (Barde *et al.*, 1982; Lindsay *et al.*, 1985; Davies *et al.*, 1986), more BDNF than NGF protein is necessary to reach the same level of stimulation in SPIN. Note that a complete wt BDNF activity profile (NOD + SPIN but no SYMP) was not obtained until all variable regions were replaced in the NGF molecule. In contrast, as demonstrated using the I+III+IV chimera, variable regions II and V from NGF are sufficient to maintain an entire wt NGF activity profile (SYMP + SPIN but no NOD).

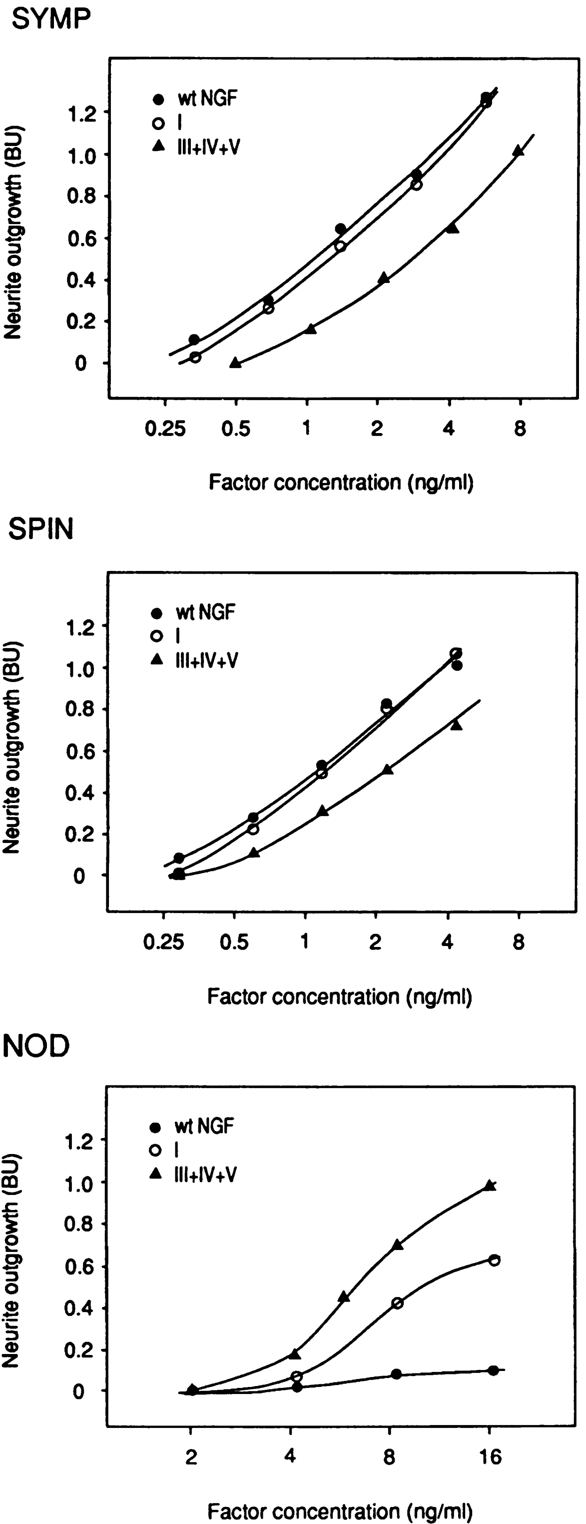

Fig. 3. Examples of dose–response curves obtained with COS cell conditioned media containing wt NGF (●), chimeric molecule I (○) and chimeric molecule III+IV+V (▲) in explanted chick SYMP, SPIN and NOD.

directed mutagenesis. Their biological activities were then assayed *in vitro* in embryonic chick peripheral ganglia. Several of these molecules displayed a broader spectrum of neurotrophic activities than the two wild type proteins and the results showed that the biological specificities of the two proteins are acquired by specific combinations of a set of variable regions in the two molecules.

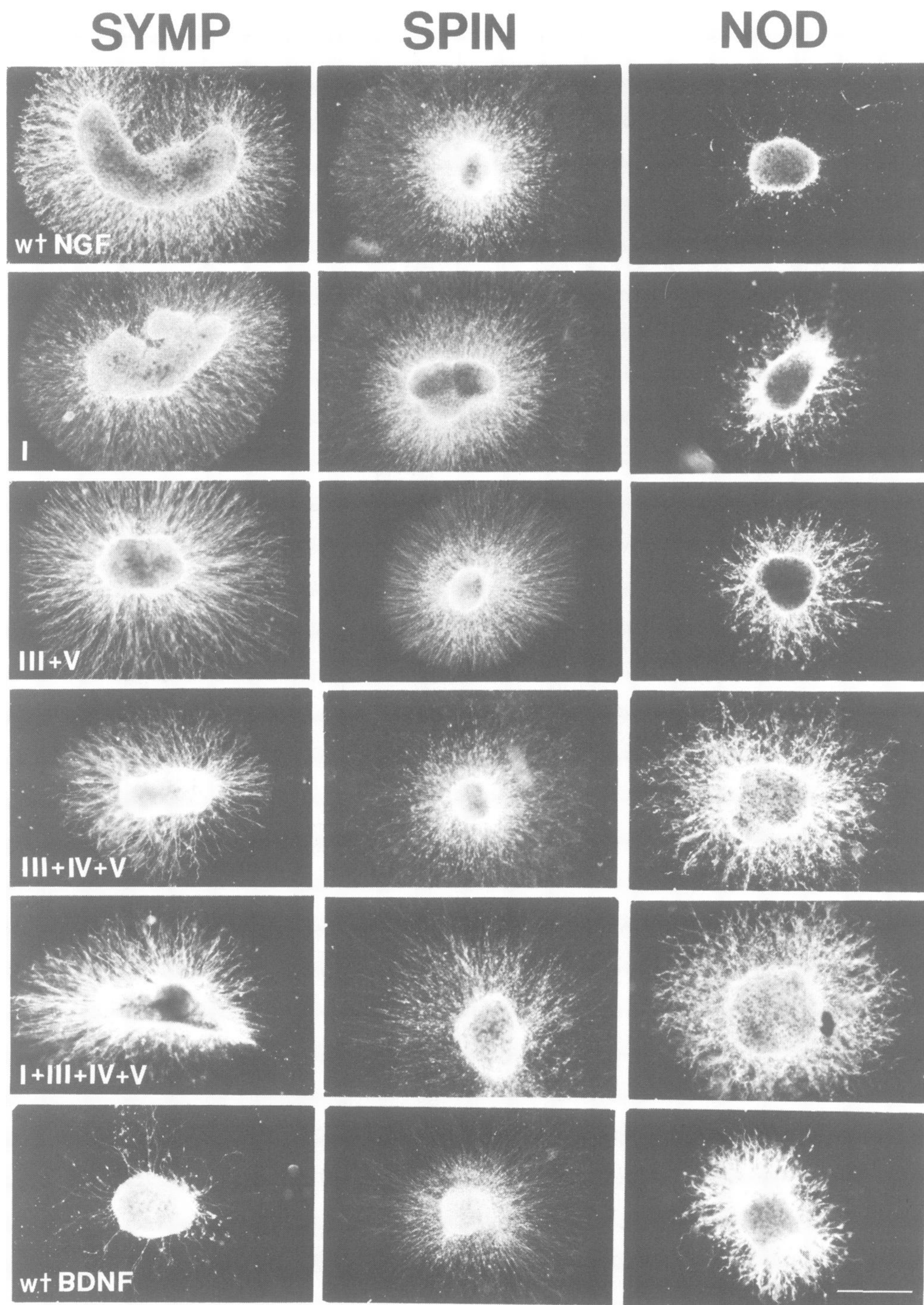

Fig. 4. Comparison of the biological activities of wt NGF, wt BDNF and chimeric molecules as assayed by neurite outgrowth stimulation of explanted chick embryonic day 9 ganglia. Dark-field photomicrographs of SYMP, SPIN and NOD cultured for 48 h in the presence of COS cell conditioned media containing 2 ng/ml SYMP and SPIN) or 8 ng/ml (NOD) of the recombinant proteins indicated in the left column. Scale bar in lower right column, 0.5 mm.

In spite of introducing a considerable number of non-conservative changes (Figure 1), the replacement of either of the three most carboxy-terminal regions (III, IV and V) or the most amino terminal region (Ia) did not affect the activity profile of the NGF molecule. In contrast, the six amino acid changes introduced by the replacement of region Ib gave to the molecule the ability to partially stimulate NOD while retaining wt NGF activities in SYMP and SPIN. Interestingly, this region has previously been suggested to have functional importance since it is one of the most conserved hydrophilic regions in the NGF molecule (Ebendal *et al.*, 1986; Meier *et al.*, 1986). This result demonstrates that some of these residues are directly involved in determining the specificity of these proteins. Chimeric molecule I (Ia+Ib) retained the ability to stimulate NOD. However, this activity was lost after the combination of region I with

regions III and IV (chimera I+III+IV), suggesting that sequences in regions III and IV may interfere with the formation of an appropriate conformation when introduced in certain structural environments. This effect could, at least in part, be caused by the marked changes in the calculated hydrophilicity and secondary structure predicted to occur by the replacement of region IV (Table I). From the broad spectrum of activities seen using regions III+V and III+IV+V, it appears that region V can compensate for this conformational interference. It is important to note that in the I+III+IV molecule, variable regions II and V from NGF are sufficient to maintain an entire wt NGF activity profile (SYMP+SPIN but no NOD). This observation, together with the results of previous structure–function studies (Ibáñez *et al.*, 1990) and evolutionary studies (Hallböök *et al.*, 1991), indicate that NGF can tolerate considerable structural changes without loss or modification of its activity profile. The dramatic reduction in the level of secreted protein observed after replacement of region II suggests that this region could be involved in determining a stable conformation of the molecule, as it has also been observed with several other point-mutated NGF molecules (Ibáñez *et al.*, 1990).

Some combinations of the carboxy-terminal regions, notably those containing regions III and V, also produced changes in the activity profile of the NGF molecule, indicating that carboxy-terminal sequences are also involved in establishing biological specificities. Interestingly, the replacements of regions III or V do not significantly change the predicted average hydrophilicity and secondary structure of the chimeric protein (Table I), suggesting that the observed effect on the activity was primarily due to changes introduced in the primary structure. The fact that the activity in NOD increased gradually from the I and III+V molecules to the I+III+IV+V, indicates that the interconversion of neurotrophic profiles in the NGF family is not an 'all or nothing' phenomenon determined by a restricted group of amino acid residues. Instead, the specificity for a defined profile of activity appears to depend more on the overall shape or conformation of the molecule. Thus, different combinations of variable regions from the two proteins are likely to generate chimeric molecules that, to a varying degree, resemble the wild type conformation. As indicated by our results, some regions (e.g. region Ib) may be more important than others which need to be combined in order to produce a change in specificity (e.g. regions III and V). As shown by the chimeric molecules III+IV+V and I+III+IV+V, some of these combinations allow a broader spectrum of neurotrophic activities than the two wild type proteins, clearly demonstrating that the activity profiles of NGF and BDNF are not mutually exclusive and can therefore be recruited in the same molecule. Chimeric proteins with a broad range of neurotrophic activities could represent molecules that combine the putative therapeutic values of both NGF and BDNF in neurodegenerative diseases.

The fact that factors with broader trophic specificities could be obtained by combining existing protein sequences poses the question of the evolutionary advantage or disadvantage of such molecules. The tissue distribution (Ernfors *et al.*, 1990a,b; Hofer *et al.*, 1990; Hohn *et al.*, 1990; Maisonpierre *et al.*, 1990a) and spectrum of activities displayed by the three members of this family suggest that the phylogenetically increased complexity of the vertebrate nervous system leads to the selection of proteins with specific sets of neurotrophic activities. The recently cloned neurotrophin-3 (NT-3), also called hippocampus-derived neurotrophic factor, (Ernfors *et al.*, 1990a; Hohn *et al.*, 1990; Maisonpierre *et al.*, 1990b; Rosenthal *et al.*, 1990) is a third member of the same gene family and it has also been shown to produce neurite outgrowth from all three ganglia. However, NT-3 is more active in NOD than BDNF, whereas SYMP respond weakly to NT-3 but strongly to NGF (Ernfors *et al.*, 1990a; Hohn *et al.*, 1990; Maisonpierre *et al.*, 1990b; Rosenthal *et al.*, 1990). This is probably explained by the fact that the NT-3 molecule contains structural features from both NGF and BDNF. Interestingly, in region II NT-3 is far more similar to NGF than to BDNF, but it is closer to BDNF in region V. This is in agreement with the data obtained from the biological activity of our chimeric molecules, showing that variable region II in NGF is important for maximal activity in SYMP while region V from BDNF, when combined with region III, induces significant activity in NOD.

The additive nature of the replacements allows one to converge on the biological property in much the same way as proteins evolve, by cycles of natural variation and selection. Our data indicate that more changes are necessary to gain a BDNF activity profile in a NGF sequence than *vice versa* (Figure 2), suggesting a direction in which the evolution of these factors may have occurred. This observation, together with the fact that BDNF sequences from different species are remarkably more conserved than those of NGF (Hallöök *et al.*, 1991), suggest that NGF may be derived from a duplicated copy of a BDNF-like ancestral sequence. The later appearance in evolution of the sympathetic system compared with the sensory system is in agreement with this speculation.

Materials and methods

DNA cloning and site-directed mutagenesis

A 770 *Eco*RI fragment containing the pre-proNGF coding sequence from the rat NGF gene (Whittemore *et al.*, 1988) was cloned into pBS KS$^+$ (Stratagene). Single stranded DNA from this plasmid was used as template for oligonucleotide based site-directed mutagenesis (Ibáñez *et al.*, 1990) as described by Kunkel (1985). The replacements were confirmed by nucleotide sequence analysis (Sanger *et al.*, 1977). For protein expression, DNA inserts containing the desired substitutions were then subcloned in pXM (Yang *et al.*, 1986). To express wt BDNF, a PCR amplified fragment containing the pre-proBDNF coding sequence from the mouse BDNF gene (Hofer *et al.*, 1990) was also subcloned in pXM. Note that the amino acid sequence of mouse BDNF is 100% identical to that of rat BDNF (Maisonpierre *et al.*, 1990b).

Production and quantitation of recombinant protein

COS cells grown to ~70% confluency were transfected with 25 μg 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 media were then analysed 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.*, 1991). In most cases, levels of protein varied between 20 and 140% of those obtained with wt NGF. The absolute amount of wt NGF protein was assessed by quantitative immunoblotting of conditioned media using standards of purified mouse NGF (Ibáñez *et al.*, 1990, 1991), and used to determine the protein concentration in the samples containing wt BDNF and chimeric proteins.

Biological assays

Serial dilutions of conditioned media containing equivalent amounts of protein (in the range of 0.2–20 ng/ml) were assayed for biological activity on explanted chick embryonic day 9 sympathetic ganglia, spinal dorsal root ganglia and nodose ganglia as previously described (Ebendal, 1984, 1989b). Conditioned medium from cells transfected with a control plasmid never gave more than 10% of the activity obtained with equivalent amounts of media containing recombinant proteins.

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