The in vitro biological effect of nerve growth factor is inhibited by synthetic peptides

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Nerve growth factor (NGF)¹ is a neurotrophic polypeptide that acts via specific receptors to promote the survival and growth of neurons. To delineate the NGF domain(s) responsible for eliciting biological activity, we synthesized small peptides corresponding to three regions in NGF that are hydrophilic and highly conserved. Several peptides from mouse NGF region 26–40 inhibited the neurite-promoting effect of NGF on sensory neurons in vitro. Inhibition was sequence-specific and could be overcome by increasing the concentration of NGF. Moreover, peptide actions were specific for NGF-mediated events in that they failed to block the neurotrophic activity of ciliary neuronotrophic factor (CNTF) or phorbol 12-myristate 13-acetate (PMA). In spite of the inhibition of NGF activity, peptides did not affect the binding of radiolabeled NGF. These studies define one region of NGF that may be required for neurotrophic activity.

Introduction

Nerve growth factor (NGF) is required for survival, differentiation, and neurite outgrowth from certain neurons (Levi-Montalcini, 1987). Like other polypeptide hormones, NGF exerts its actions through binding to specific cell-surface receptors (James and Bradshaw, 1984; Levi-Montalcini, 1987; Misko *et al.*, 1987). In spite of considerable effort to define NGF actions at its receptor, the molecular domain(s) of NGF that triggers biological activity is as yet undefined. Delineating the receptor-activating site of NGF would make it possible to define structural fea-

tures required for activity, identify receptor regions that interact with NGF, elucidate NGF-receptor interactions, and synthesize NGF agonists and antagonists to examine its physiological role in vivo.

One strategy for identifying polypeptide domains that interact with their receptors entails synthesizing short peptides with sequences corresponding to the potential active site. At relatively high concentrations (micro- or millimolar), peptides containing an active site may block or mimic biological activity of the native polypeptide (Pierschbacher and Ruoslahti, 1984; Baird *et al.*, 1988). In one example, a cell-binding domain of fibronectin was identified by antagonist and agonist activities of small peptides corresponding to one region (Pierschbacher and Ruoslahti, 1984). We adopted a similar approach. Anticipating the possibility that NGF-receptor interactions may be complex, we reasoned that inhibition of NGF effects may be more readily achieved than NGF agonist responses. Therefore we initiated our studies by looking for NGF antagonist peptides. A relatively high degree of hydrophilicity and cross-species conservation suggested that sequences within three regions of the NGF molecule (Figure 1, regions A, B, and C) may interact with the NGF receptor (Hopp and Woods, 1981; Ebendal *et al.*, 1986; Meier *et al.*, 1986; Selby *et al.*, 1987; Fahnestock and Bell, 1988). Regions A and C also had a high probability of containing a beta-turn (CHOFAS program, National Biomedical Research Foundation, Washington, D.C.), a structural feature that may favor receptor interaction (Pierschbacher and Ruoslahti, 1984). Through their antagonism of NGF activity, a subset of these sequences has defined one domain of NGF that may be required for neurotrophic activity.

Results

Peptides shown in Figure 1 were synthesized and assayed for their ability to block NGF-induced neurite outgrowth from dorsal root ganglion neurons. The N-terminal octapeptide of

Abbreviations used: CNTF, ciliary neuronotrophic factor; HPLC, high-performance liquid chromatography; NGF, nerve growth factor; PMA, 12-myristate 13-acetate.

1	26	64	99	118
NGF: SSTHPVFHMG	TTATDIKGKEVTVLA	VESGCRGIDSKHW	WRFIRIDTA	ATR
O1: SSTHPVFH (ND)	C 1: ATDIKGKEVTV (1.1)	A1: SGCRGIDSKH (ND)	B2: RIDT (ND)	
	C 2: DIKGKEVTV (1.2)	A2: RGIDSKH (ND)	B3: RFIRIDT (ND)	
	[ran.] C 2: DVEITVKKG (ND)	A3: RGIDS (ND)	B4: RFIRID (ND)	
	C 10: ATDIKG (ND)	A4: RGIDSK (ND)		
	C 6: DIKG (ND)			
	C 5: KGKE (1.0)			
	[³³ V] C 5: KVKE (ND)			
	C 7: KGK (ND)			
	C 12: GKE (ND)			
	C 14: VTV (2.7)			

Figure 1. Inhibition of NGF-induced neurite outgrowth by synthetic NGF peptides. The sequence of mouse NGF is illustrated on the first line; the sequence of synthetic peptides derived from four regions (O, C, A, and B) are listed below. Peptides were synthesized and purified as indicated in Methods. Neurons were cultured in the presence of NGF (260 pg/ml) and peptides at concentrations from 0 to 4.0 mM. Each concentration was tested in duplicate or triplicate wells in at least three separate bioassays. The concentration of each peptide (in millimoles) necessary to achieve 50% inhibition (IC_{50}) of neurite growth is listed in parentheses. ND, no inhibition detected.

NGF can be cleaved without eliminating trophic activity (Mobley *et al.*, 1976). Because this region was unlikely to be a part of a receptor-interacting site, the octapeptide served as a control. In initial experiments, the dose-response curve for NGF promotion of neurite outgrowth was carefully established. Antagonist activity of peptides was assayed with a concentration of NGF that supported 60–90% of maximum neurite outgrowth.

None of the A- or B-region peptides inhibited neurite growth at concentrations up to 1.0 mM. Nor was inhibition seen with the N-terminal octapeptide. It was only in region C that inhibition was detected. Peptides C1 and C2 both inhibited neurite outgrowth (Figure 2A). To establish whether a smaller domain within C1 and C2 may be effective, several additional peptides were assayed. In the N-terminal portion of C1, peptides C6 and C10 had no effect at 1.0 mM. Near the center, peptide C5 retained the inhibitory effect of C1. Peptides lacking the N-terminal K (C12) or the C-terminal E (C7) of C5 were inactive. At the C-terminus of C1, C14 produced two- to threefold less inhibition than C1. The appearance of neurons cultured in the presence of NGF, with or without C5, is shown in Figure 3 (A–C). In the presence of C5, fewer neurons had neurites, and the remaining neurites were often shorter. Inhibition due either to C5 or C2 was sequence-specific. The sterically nonconservative substitution of V for G in C5 eliminated the neurite blocking effect (Figure 2A). A peptide containing the residues of C2, but in random order, was also inactive. Inhibition of neurite outgrowth was prevented by culturing in the presence of increased NGF; recovery of neurite outgrowth was NGF concentration dependent,

and in the presence of high NGF concentrations (≥ 3200 pg/ml), inhibition by C5 was no longer detected (Figure 2B).

Neurons must attach to the culture dish before extending neurites. To establish whether the neurite blocking effect of synthetic peptides may be due to inhibition of cell attachment, the effect of C5 on this property was assessed. The number of attached cells/well at 2 h was determined. NGF had no influence on the extent of initial cell attachment. In the absence of NGF, an average of 1685 ± 190 ($n = 3$ determinations in one bioassay; $\pm$ SE) cells were present. At the highest concentration used (3200 pg/ml), 1680 ± 70 ($n = 3$) cells were counted. Adding C5 to NGF produced no change (1645 ± 45 , $n = 3$). These data suggest that C5 acted to inhibit the initiation or rate of neurite extension.

To address the specificity of peptide actions for NGF-mediated neurite outgrowth, neurons were cultured with other agents with neurotrophic activity for sensory neurons: phorbol 12-myristate 13-acetate (PMA) (Montz *et al.*, 1985) and ciliary neuronotrophic factor (CNTF) (Manthorpe *et al.*, 1986; Skaper and Varon, 1986). Optimal concentrations of NGF, PMA, and CNTF supported the same number of neurite-bearing cells/well (NGF = 310 ± 16 ; PMA = 311 ± 14 ; CNTF = 286 ± 16 ; $n = 4$ determinations, $\pm$ SE). No additive effects on neurite growth were noted when NGF was combined with PMA (320 ± 17) or CNTF (304 ± 13). These observations suggested that NGF, PMA, and CNTF acted on the same neurons. The inhibition of neurite outgrowth by C5 was specific to NGF, as illustrated in Figure 4. C5 (2.0 mM) inhibited NGF-induced neurite outgrowth from both embryonic day (ED) 8 neurons (Figure 4A) and ED

Figure 2. Inhibition of neurite outgrowth by synthetic NGF peptides. Panel A: Inhibition assays. Neurons were cultured in the presence of NGF at 260 pg/ml with increasing concentrations of each peptide. Data are expressed as mean percent $\pm$ SEM of the NGF response. Peptides were assayed in duplicate or triplicate wells for each concentration in the following number (n) of assays: C1, n = 6 (□); C2, n = 6 (▲); C5, n = 7 (●); C14, n = 3 (■); [ran.]C2, n = 3 (Δ); [V^{33}]C5, n = 4 (○). Panel B: NGF dose-response curves in the presence and absence of C5. Neurons were cultured in the presence of increasing concentrations of NGF (duplicate or triplicate wells for each concentration) without C5 (●), n = 6 bioassays; or with C5 at 0.25 mM (○), n = 4 or 0.5 mM (□), n = 5. For each bioassay, the maximum number of neurite-bearing cells/well (200–300) was normalized to 100% after subtracting background values (0–5% of total). The absence of inhibition at concentrations of NGF below 200 pg/ml suggests that C5 did not act simply as a competitive antagonist.

10 neurons (Figure 4C). In contrast, C5 had no effect on neurite outgrowth supported by PMA or CNTF, even though these agents were present at concentrations that would favor demonstration of inhibition (Figure 4, B and D). Peptides C2 and C14 also had no effect on CNTF-induced neurite outgrowth (data not shown).

Our data for inhibition of NGF-mediated neurite outgrowth suggested that peptides may act

at the NGF receptor. To determine whether C2 or C5 influenced receptor binding of NGF, binding studies were carried out under conditions similar to those used for the bioassays. With radiolabeled NGF at 10 pM, binding is predominantly to high-affinity receptors, $K_d = 10^{-11}$ M, (Sutter *et al.*, 1979). Steady-state specific binding of NGF (which ranged from 3.8% to 6.9% of NGF added) was not altered by C2 at 2.0 mM

Figure 3. In vitro activity of NGF peptides. The inhibitory effect of C5 was demonstrated by culturing embryologic day 8 dorsal root ganglion neurons on polyornithine treated wells for 24 h under the following conditions: A, no NGF; B, NGF at 260 pg/ml; C, NGF at 260 pg/ml and C5 at 2.0 mM.

Figure 4. C5 specifically inhibited NGF-mediated neurite outgrowth. C5 (2.0 mM) was tested for its ability to inhibit the neurite-promoting actions of NGF, PMA, and CNTF. ED-8 neurons were used to compare NGF and PMA; ED-10 neurons were used to compare NGF and CNTF because the latter has been shown to have a more marked effect at this stage (Skaper and Varon, 1986). The sensitivity of detecting any inhibitory effect was optimized by adding C5 to concentrations of each trophic factor that were in or near the mid-range of their dose-response curves. A and C demonstrate the neurite outgrowth response to NGF (●, $n = 8$ determinations) and NGF plus C5 (○, $n = 3$) for ED-8 and ED-10 neurons. For both ED-8 and ED-10 neurons, C5 significantly inhibited neurite outgrowth ($p < 0.001$, 1-tailed Student t test). B shows the response to PMA (●, $n = 8$) and PMA plus C5 (○, $n = 3$); D demonstrates the response for CNTF (●, $n = 8$) and CNTF plus C5 (○, $n = 3$). For each assay, the maximum number of neurite-bearing cells/well (250–350) was normalized to 100% after subtracting background values. Each point is the mean $\pm$ SEM.

(mean $\pm$ SD = $108 \pm 14\%$ [$n = 3$ cultures]) or C5 at 1.0 mM ($104 \pm 13\%$ [$n = 4$]). In studies with 800 pM NGF binding to both high- and low-affinity receptors, $K_d = 10^{-9}$ M (Sutter *et al.*, 1979) was examined. Again, specific NGF binding (0.21–0.52% of NGF added) was unaffected by the presence of C2 ($119 \pm 31\%$ [$n = 3$]) or C5 ($89 \pm 22\%$ [$n = 4$]).

Discussion

This study demonstrates that synthetic peptides corresponding to one of several hydrophilic, conserved domains of NGF can inhibit an *in vitro* biological effect of NGF. It was shown that: 1) NGF-induced neurite outgrowth was inhibited by peptide C1 (ATDIKGKEVTV) and C2 (DIKGKEVTV); 2) this inhibitory activity was fully retained in peptide C5 (KGKE); 3) inhibition was

reversed by increasing the NGF concentration; and 4) inhibition was specific for the sequence of active peptides and for NGF-mediated events. These data and the correspondence of the sequence of these peptides with NGF are evidence that C-region peptides acted via the NGF receptor and that our studies have defined one region of NGF that may be required for neurotrophic activity.

The absence of a change in NGF binding in the presence of C2 or C5 raises two possibilities. One is that these peptides do not act at the NGF receptor; however, it is difficult to reconcile this possibility with the data outlined above. A second possibility is that C2 and C5 primarily interfere with receptor transduction rather than receptor binding (Ariens *et al.*, 1979). Studies of synthetic glucagon peptides give evidence that receptor binding and trans-

duction are primarily mediated by different amino acid residues (Krstenansky *et al.*, 1986). In a recent evaluation of small peptides modeled after fibroblast growth factor, several peptides (fibroblast growth factor peptides [Tyr⁵⁰] 30–50, 32–53, 121–146, [Tyr¹³⁹] 139–146) inhibited or mimicked the biological activity of the native polypeptide without significantly altering its binding (Baird *et al.*, 1988). Therefore it is possible that C2 and C5 interfere at the receptor with the function of a transducing domain of NGF.

NGF structure-function relationships have also been studied with the use of chemically modified derivatives of NGF. Tryptophan-21 (Frazier *et al.*, 1973; Cohen *et al.*, 1980) and perhaps tryptophan-99 (Cohen *et al.*, 1980), histidine-75 and -84 (Dunbar *et al.*, 1984) and amino groups (Rosenberg *et al.*, 1986) may be important for receptor binding and biological activity. However, such studies may be difficult to interpret; a modified residue not normally directly involved in receptor interaction may nevertheless affect binding by disrupting relevant tertiary structure (Knowles, 1987). A positive aspect of the approach taken herein is that specific domains of NGF have been investigated. Although active C-region peptides contain neither tryptophan nor histidine, our studies do not rule out a contribution to NGF-receptor interactions by residues implicated in chemical modification studies.

The concentrations of C2 and C5 used to demonstrate activity were considerably higher than for NGF, but were similar to those used to demonstrate inhibition of fibronectin actions by peptides containing the arg-gly-asp sequence (Pierschbacher and Ruoslahti, 1984). The discovery that arg-gly-asp constitutes a cell-binding domain of fibronectin led to important insights regarding fibronectin and its receptor and provided a novel means of modulating its function *in vitro* and *in vivo* (Boucaut *et al.*, 1984; Gehlsen *et al.*, 1988). C-region peptides, or potent synthetic analogues thereof, may help to elucidate NGF-receptor interactions and to modulate NGF actions *in vivo*.

Methods

Peptide synthesis and purification

Peptides were synthesized in the C-terminal amide form on an Applied Biosystems 430A peptide synthesizer by solid-phase methods (Merrifield, 1986). Peptides were isolated by reversed-phase high-performance liquid chromatography (HPLC) (A1 through A4, B2, B3, B4, C1, C2, C5, [V³³] C5, C10, C14), by cation-exchange HPLC (C1, C2, C5, C6, C7,

C10, C12), or by reversed-phase followed by cation-exchange HPLC (C2, [ran.]C2 (residues of C2 randomized), C5). For reversed-phase, peptides were eluted from a preparative C18 μ Bondapak column at a flow rate of 3 ml/min (19 $\times$ 150 mm; Waters, Milford, MA); the column was developed in 0.1% trifluoroacetic acid for 10 min, followed by a linear gradient of acetonitrile (0–60%) in 0.1% trifluoroacetic acid over 1 h. For cation exchange, peptides were eluted from a semipreparative cation-exchange column (PolySULFOETHYL-A; 9.4 $\times$ 200 mm; Poly LC, Columbia, MD) at a flow rate of 3 ml/min; the column was developed in 20 mM ammonium acetate (Aldrich, Gold Label grade), pH 6.6, in 25% acetonitrile for 10 min, followed by a linear gradient of ammonium acetate from 20 to 500 mM at pH 6.6 in 25% acetonitrile over 60 min. Peptide fractions were lyophilized, resuspended in sterile water, aliquoted, and stored at -70°C . All peptide preparations underwent quantitative amino acid analysis to confirm peptide composition and to determine concentration. Two separate syntheses of C2 and C5 were also sequenced by automated Edman degradation (Beckman 890M liquid phase). The molecular weights of C2, [ran.]C2, and C5 were confirmed by fast atom bombardment mass spectrometry (performed by M-Scan Inc., West Chester, PA on a VG analytical ZAB-2SE high field mass spectrometer).

Early attempts to purify peptides by the use of different mobile phases and gradients resulted in preparations that were toxic to cultured cells. Toxic cultures frequently contained a diffuse precipitate and many vesicle-filled and fragmented cells. Toxicity was defined by poor cell attachment and survival and decreased neurite growth in the presence of non-NGF neurotrophic factors (PMA and CNTF) (Montz *et al.*, 1985; Manthorpe *et al.*, 1986). Toxic preparations also inhibited the survival of N1E 115 cells, a neuronal cell line that survives in the absence of added trophic factors. To avoid toxicity, peptides used in this study were first screened by adding them to cultures of CNTF- or PMA-supported dorsal root ganglion neurons or N1E 115 cells. Preparations of C2 and C5 were least likely to be toxic when they were purified by reversed-phase followed by cation-exchange HPLC. Only when peptides had no effect on cell appearance or neurite growth in the concentration range to be used were they accepted for use in NGF-inhibition assays. Highlighting the problems associated with toxicity, four peptides from the C region were obtained from a standard commercial source. Although described as >99% pure after reversed-phase HPLC, all four were markedly toxic, and an extensive precipitate was present in these cultures.

Bioassays

NGF was purified by ion-exchange chromatography from male mouse submaxillary glands and characterized as described (Mobley *et al.*, 1986). Dorsal root ganglia were dissected from ED 8 or ED 10 chick embryos and placed in calcium and magnesium-free Dulbecco's balanced salt solution on ice. They were incubated for 10 min in 2.5 ml 0.01% trypsin at 37°C after which 500 μl of fetal calf serum was added. Ganglia were centrifuged at $500 \times g$ for 4 min, washed once with 4 ml of culture medium (Dulbecco's Modified Eagle's Medium H-16 with glutamine, 1 mM pyruvate, 3.0 g/L glucose, 3.7 g/L HCO_3 , and 10% fetal calf serum) and dissociated in 1.5 ml culture medium by gentle trituration (10–15 times) through a 1-mm-diameter flamed glass pipet. The resulting cell suspension was diluted to 7 ml in culture medium, added to a 100-mm-diameter plastic culture dish (Falcon), and incubated for 2 h at 37°C in 5% CO_2 -95% air. During this incubation, nonneuronal cells more readily attached to the substratum; therefore the resulting supernatant con-

tained an enriched neuronal population (80–85% neurons). After differential attachment, each ganglion yielded 8000–10 000 cells, >95% of which excluded trypan blue. Tissue culture wells (Costar, 0.16 cm²/well) were precoated with 50 µl/well of polyornithine (Sigma; 2 µg/ml in phosphate buffered saline) for 1 h. After aspiration, each well received 1) 12.5 µl of peptide diluted in culture medium, 2) 12.5 µl of NGF in culture medium to reach a final concentration in the assay of 260 pg/ml (10 pM), and 3) 25 µl of cell suspension diluted with culture medium (100 000 cells/ml, ~2000 neurons/well). Low cell densities were used to minimize the effect of any endogenous trophic activity (Longo *et al.*, 1982). After 24 h in culture, cells were fixed with glutaraldehyde (2% in phosphate buffered saline) and assessed under phase-contrast microscopy. Horizontal and vertical strips of each well (corresponding to ~30% of the well area) were examined, and the number of cells bearing a neurite greater than one cell diameter were counted. In the absence of NGF, 0–10 neurite-bearing cells/well were counted. In the presence of optimal levels of NGF, there were 250–350 neurite-bearing cells. The dose-response curve of NGF under these conditions was carefully established so that an NGF concentration near the midrange of its curve could be used for peptide inhibition assays.

The resistance of C5 to proteolytic cleavage during cell culture was also assessed. Culture medium collected from 24-h cultures containing C5, fresh culture medium to which C5 was added, and culture media alone were each filtered through a Centricon-10 membrane (Amicon). The peptide content of the filtrate was assessed by analytical reversed-phase HPLC. Comparison of C5 peaks showed that 81% of C5 was present after 24 h in culture.

Binding assays

NGF was radiolabeled with Na ¹²⁵I to a specific radioactivity of 50–70 cpm/pg and binding studies were conducted essentially as described (Sutter *et al.*, 1979). Cells were prepared as for bioassays and seeded to a final density of 50 000–150 000 cells/ml in polyornithine-treated 16-mm culture plates (Costar) containing ¹²⁵I-NGF, with or without peptide (total assay volume was 500 µl). To assess NGF binding to its high affinity receptor (K_d = 10⁻¹¹ M) (Sutter *et al.*, 1979), ¹²⁵I-NGF was present at 10 pM; for assessment of binding to high- plus low-affinity receptors (K_d = 10⁻⁹ M), the ¹²⁵I-NGF concentration was 800 pM. Cultures were incubated at 4°C to minimize uptake and accumulation of ¹²⁵I-NGF (Sutter *et al.*, 1979). Initial studies showed that ¹²⁵I-NGF binding reached equilibrium by 5 h in the presence or absence of peptide. At 5 h, cultured cells (the majority of which were attached to the substratum) were resuspended within the culture plate by repetitive gentle aspiration, and duplicate aliquots of 15 µl of cell suspension were collected to determine total NGF content in the assay. A second set of duplicate aliquots of 200 µl were collected and centrifuged for 2 min at 10 000 × g, and the supernatant was discarded. The tubes were cut to include the pellet and radioactivity was measured to determine the amount of total ¹²⁵I-NGF bound. Nonspecific binding (10% of total bound at 10 pM; 30% at 800 pM) was determined in the presence of a large excess of unlabeled NGF (400 nM). Specific binding (bound radioactivity minus nonspecifically bound) was determined and expressed as percent of total NGF content.

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