RESUBMITTED – JUNE 8, 2000

NGF loop 4 dimeric mimetics activate ERK and AKT and promote NGF-like neurotrophic effects

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Running Title: Neurotrophic Activity of NGF Loop 4 Mimetics

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Key words: Neurotrophins, NGF, Nerve Growth Factor, Synthetic Peptides, Peptidomimetics, Trk, p75, ERK, AKT
Summary

Previous work indicating that NGF protein loops 2 and 4 interact with TrkA receptors raise the possibility that small molecule mimetics corresponding to TrkA-interacting domains that have NGF agonist activity can be developed. We applied our previously developed strategy of dimeric peptidomimetics to address the hypothesis that loop 4 small molecule dimeric mimetics would activate TrkA-related signal transduction and mimic NGF neurotrophic effects in a structure-specific manner. A loop 4 cyclized peptide dimer demonstrated NGF-like neurotrophic activity while peptides with scrambled sequence, added or substituted residues, or cyclized in monomeric form were inactive. Activity was blocked by the TrkA inhibitors K252a and AG879 but not by NGF p75 receptor blocking antibody. Dimeric, but not monomeric, peptides partially blocked NGF activity. This profile was consistent with that of a NGF partial agonist. ERK and AKT phosphorylation was stimulated only by biologically active peptides and was blocked by K252a. The ERK inhibitor U0126 blocked the neurite- but not the survival-promoting activity of both NGF and active peptide. These studies support the proof-of-concept that small molecule NGF loop 4 mimetics can activate NGF signaling pathways and can mimic death-preventing and neurite-promoting effects of NGF. This finding will guide the rational design of NGF single-domain mimetics and contribute to elucidating NGF signal transduction mechanisms.
**Introduction**

Nerve growth factor (NGF) acts via TrkA and p75 receptors to regulate neuronal survival, promote neurite outgrowth and upregulate certain neuronal functions such as mediation of pain and inflammation (1, 2, 3, 4, 5). These actions suggest that NGF agonists or antagonists might be useful in regulating these processes (6, 7, 8). Factors limiting therapeutic applications of the NGF protein include restricted penetration of the CNS and the poor medicinal properties characteristic of most proteins (9, 10). The development of small molecule mimetics with favorable chemical properties that function as agonists or antagonists that mimic or inhibit NGF functions in the appropriate biological context will be critical in advancing potential *in vivo* applications of NGF. Moreover, in settings in which NGF might contribute to neuronal death, pain or inflammatory mechanisms, NGF antagonists may be particularly relevant. Creation of single domain NGF mimetics will also constitute a powerful approach for linking specific NGF domains with specific patterns of intracellular signal transduction.

A NGF mimetic (agonist or antagonist) would be expected to contain structural determinants of one or more NGF active sites that interact with NGF receptors. Multiple techniques have been used to deduce which domains of the NGF protein interact with NGF receptors (11). A peptide mapping approach in which synthetic peptides with sequences corresponding to specific NGF regions were tested for their ability to inhibit NGF activity pointed to residues 29-35 as a key active site (12). Subsequent NGF crystallography and molecular modeling studies revealed that NGF contains three surface hydrophilic β-hairpin loops (loops 1, 2 and 4) that are likely candidates for receptor interaction sites (13, 14, 15). Loop 1 consists of the previously mapped residues 29-35 and subsequent studies confirmed that region 29-35 synthetic peptides inhibited NGF activity and NGF p75 receptor binding (16). Recombinant substitution studies indicated
that residues Lys\textsuperscript{32} and Lys\textsuperscript{34} (17) and other residues (18) are likely to interact with p75 receptors. The NGF sites interacting with TrkA have also been derived via chemical modification (11, 19, 20), recombinant protein (18, 20, 21, 22, 23) and NGF-TrkA co-crystalization approaches (24). Taken together, these studies indicate that TrkA binding sites consist of residues in NGF loop 2 (residues 40-49), loop 4 (residues 91-97), the N-terminus (residues 1-8) and the C-terminus (residues 111-115).

We have established that synthesis of peptide derivatives mimicking single NGF domains provides an approach for deriving the proof-of-concept that mimetics of a given single-site domain can function as NGF antagonists or agonists (12, 25). This approach has demonstrated that a cyclized dimer, but not linear or cyclized monomer peptides, corresponding to NGF loop 1 can function as a NGF agonist to prevent neuronal death via a p75-dependent, Trk kinase-independent signaling mechanism (25). The establishment of this proof-of-concept for NGF loop 1 along with the emerging role of peptidomimetics as a key intermediate-stage strategy in small molecule drug design (26, 27, 28, 29) will contribute to rational screening and design programs for producing potent NGF small molecule mimetics with agonist activity. Our NGF dimeric peptidomimetic strategy has been further encouraged by the synthesis of small peptides mimicking the 34 kD dimeric protein erythropoietin (EPO) and the finding that EPO receptor activation by peptidomimetics also requires the dimeric form (30). These EPO peptidomimetics have served as starting points for the design of EPO small molecule compounds (31). Using strategies derived from our development of a NGF loop 1 dimeric peptidomimetic with agonist activity, we tested the hypothesis that dimeric peptidomimetics corresponding to NGF loop 4 can function as NGF agonists to mimic NGF biological activity and activate well-established NGF signaling intermediates. Studies describing the biological activity of the present loop 4 mimetics have been published in abstract form (Xie and Longo, Society for Neuroscience, 1997).
Experimental Procedures

Peptide synthesis, purification and characterization. Methods for peptide synthesis, purification and characterization were conducted as previously described (25). Peptides were synthesized in the C-terminal amide form by Peninsula Laboratories (Belmont, CA) following our specifications. The amide form was chosen in order to reduce susceptibility to proteolytic cleavage. Peptide conformation was stabilized by cyclization (32, 33). Cysteine or penicillamine (β,β-dimethylcysteine) residues were added to each end of the linear sequence to provide sulfhydryl groups for oxidative peptide cyclization. Penicillamine has been found to facilitate cyclization and to provide greater conformational constraint relative to cysteine-cysteine cyclization (33, 34). Peptides were cyclized into monomeric or dimeric forms via oxidative formation of disulfide bonds by the addition of K3[Fe(CN)6] to peptides solubilized in water at either low (monomeric cyclization) or high (dimeric cyclization) concentrations (34). Following cyclization, peptides were lyophilized and then purified by reversed-phase HPLC (12, 25). Analytical HPLC was used to confirm purity of >98%.

One aliquot from each purified HPLC fraction was used for quantitative amino acid analysis to confirm peptide composition and to determine concentration. The composition and cyclized state of each peptide was further confirmed using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry to determine molecular mass (AnaSpec Inc., San Jose, CA). Prior to each bioassay, peptide aliquots were lyophilized and resuspended to known concentrations in culture media.

Bioassays for neurotrophic activity. Dorsal root ganglia were dissected from embryonic day 8 chick embryos and placed in calcium and magnesium-free Dulbecco's balanced salt solution on ice. Ganglia were then resuspended and incubated for 20 min in 2.5 ml 0.05%
trypsin at 37°C, washed once with 4 ml of culture medium (Dulbecco’s Modified Eagle’s Medium H21 with glutamine, 1 mM pyruvate, 3.0 g/L glucose, 3.7 g/L HCO3 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 pipette. The resulting cell suspension was diluted to 7 ml in culture medium and added to a 100 mm diameter plastic tissue culture dish (Falcon) and incubated for 2.5 hrs at 37°C in 5% CO2-95% air resulting in a supernatant containing ~80% neurons. For ninety-six well plate studies (Costar A/2, 0.16 cm²/well), wells were pre-coated with 50 µl/well of polyornithine (Sigma; 0.1 mg/ml in phosphate buffered saline, PBS) for 1 hr followed by 50 µl/well laminin (Gibco, 10 mg/ml in PBS) for 3 hrs to provide a substrate permissive for neurite outgrowth. Following laminin coating each well received 25 µl of peptide serially diluted in culture medium and 25 µl of cell suspension (60,000 cells/ml, 1500 neurons/well). Control cultures performed with each assay included serial dilutions of NGF to generate NGF dose-response curves and cultures without NGF or peptides to determine background survival. Background survival was typically 5-15% of that seen with optimal concentrations of NGF. Mouse submaxillary βNGF was purified via 7S NGF and generously provided by Dr. William Mobley.

For six well plate studies (Costar 9.5 cm²/well) wells were pre-coated with 1.0 ml/well of polyornithine and 1.0 ml/well of laminin at the concentrations listed above. Highly purified neuronal preparations were obtained by incubating DRG cell mixtures in 100 mm diameter plastic tissue culture dishes coated with type I rat tail collagen (Collaborative Biomedical Products) (35). Cells were incubated for 2.5 hrs at 37°C in 5% CO2-95% air and shaken for 30 seconds every 10-15 min resulting in a supernatant containing > 95% neurons. Each well in six well plates then received 1.0 ml of cell suspension (60,000 cells/ml) and a final concentration of 10 ng/ml NGF or 125 µM peptide in a total final volume of 2.0 ml. Neuronal survival and neurite outgrowth were
assessed by a combination of morphological criteria and the MTT colorimetric assay (26). MTT was added to cultures 20 hrs or 68 hrs following cell plating and cells were fixed at 24 hrs or 72 hrs with glutaraldehyde (4% in H2O), respectively. Under phase contrast microscopy, the number of surviving neurons per area was determined using a combination of MTT staining and morphological criteria (25).

For ninety-six well plate assays, horizontal and vertical strips of each well were examined and the number of surviving cells (defined as large intact cells without fragmented cell membrane or accumulations of vesicles and containing blue MTT product) were counted. For each peptide or NGF concentration, duplicate wells were counted in each bioassay and four values (two strips counted/well) were averaged. For six well plate assays, a vertical strip encompassing twelve microscopic fields was assessed. The number of surviving neurons in each field was counted and the average of the twelve counts was determined for each well. In the presence of NGF, 25-45 surviving neurons per field were counted.

Bioassays with p75 antiserum. Antiserum (antibody 9561) directed against the third and fourth cysteine rich repeats of the extracellular domain of mouse p75 was generously provided by Dr. Moses Chao. This antibody inhibits NGF neurotrophic activity by interfering with NGF-p75 interaction (36). Antiserum and control non-immune sera were tested at final dilutions of 1:100 in the presence of NGF or peptide.

Bioassays with Trk and ERK inhibitors. The Trk inhibitors K252a (Calbiochem, San Diego, CA) or AG879 (Calbiochem) were added to cultures at final concentrations of 100 nM and 10 µM, respectively. K252a or AG879 were added concomitantly with NGF or P92. ERK inhibitor UO126 and its inactive control UO124 (Calbiochem) were prepared
as a 500 μM solution in culture medium with 2.5% DMSO and added to cultures to a final concentration of 50 μM. DRG cultures were placed in serum-free medium and pretreated for 1 hr with 50 μM of the ERK inhibitor U0126 or its control prior to addition of NGF or P92.

**Measurement of ERK or AKT activation.** Neuron-enriched cell suspensions were plated in six-well plates under the same conditions as described for bioassays except that the number of cells plated per well was increased by approximately two-fold. Two hours after plating, NGF or peptides were added. For K252a experiments, neurons were pre-incubated for 10 min with K252a at a final concentration of 100 nM prior to the addition of NGF or peptide. Following 10 min, 30 min or 3 hrs of culture, cells were lysed at 4°C in lysis buffer (20 mM Tris-HCl pH 7.5, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 500 μM sodium orthovanadate, 1 mM phenylmethylsulfonfonyl fluoride, 10 μg/ml aprotinin) for 30 minutes. Insoluble material was removed by centrifugation at 4°C (10,000 g for 10 minutes). Supernatant protein concentrations were determined by the BCA method (Pierce, Rockford, IL). Equal amounts of protein were mixed with electrophoresis sample buffer (100 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue containing 100 mM DTT) boiled for 5 minutes and applied to 7.5% acrylamide gels with a 5% stacking gel. Proteins were electrophoretically transferred to nitrocellulose filters (Pharmacia, Piscataway, NJ) for 1 hr at 100 V. Filters were blocked in 5% nonfat dry milk (Biorad, Hercules, CA) in TBST (20 mM Tris-HCl pH 7.5, 137 mM, 0.2% Tween 20) for 1 hr at room temperature followed by incubation overnight at 4°C with 0.5 μg/ml of monoclonal phospho-p44/42 MAPK antibody or 1:500 dilution of rabbit polyclonal phospho-AKT (Ser473) (New England Biolabs, Beverly, MA). Blots were washed five times with TBST (5 minutes per wash), incubated for 1 h with 1:1000 dilution of peroxidase-conjugated anti-mouse IgG antibody or anti-rabbit IgG (Amersham, Arlington Heights, IL), washed again with TBST, and then processed with the Amersham ECL
detection kit. After exposure to X-ray film, blots were stripped and processed again using a 1:1000 dilution of polyclonal p44/p42 MAPK antibody or 1:500 dilution of polyclonal AKT antibody (New England Biolabs, Beverly, MA). Chemiluminescence autoradiographs in the linear range were densitometrically scanned.
Results

A dimeric peptide containing NGF residues 92-96 functions as a partial agonist. In previous studies we determined that dimerization via cysteine and penicillamine residues was required for activity of NGF loop 1 peptidomimetics (24). Therefore, an analogous approach was applied to NGF loop 4 peptides (Figure 1a). In a first series of assays the effects of peptide P92 and various control peptides on neuronal survival were determined using a standard twenty-four hour assay of NGF survival promoting activity (25). P92 contains NGF loop 4 residues Thr^{92}, Asp^{93}, Glu^{94}, Lys^{95} and Gln^{96} (TDEKQ). Residues Glu^{94}, Lys^{95} and Gln^{96} have been shown to mediate TrkA binding and activation (21, 22). Dimeric peptide P92 promoted neuronal survival at a level three-fold higher than baseline survival levels and 10-15% of the maximum survival promoted by NGF (Figure 1b). This effect was dose-dependent over a range of 8-125 µM. In separate studies, doses up to 250 µM showed no further increase in survival promoting effect, suggesting that P92 was functioning as a NGF partial agonist rather than full agonist. The term partial agonist is used in the present study to describe a ligand producing a maximum response lower than the maximum response produced by the full or native (i.e. NGF) agonist (37).

Structure-function studies based on P92 variants were conducted (Figure 1 b-d). The monomeric form of P92 had no activity, indicating that the survival promoting effect of P92 was dimer-dependent. The activity of P92 was lost entirely if residues were scrambled (P92S) or if the amide terminus cysteine was substituted with a penicillamine residue (P92P). For peptide P90, amino acid residues were added at either end in order to flank the cysteine and penicillamine moieties and further restrain peptide conformation. This approach for additional structural constraint led to increased potency for cyclic RGD-containing fibronectin peptides (34). In the case of the P92 peptide, activity was
lost entirely when flanking residues were added. These structure-function studies demonstrated that the activity of P92 was highly sequence- and structure-dependent.

**P92 displays the activity profile of a classic partial agonist.** In order to further characterize the partial agonist nature of P92 and further establish its interaction with NGF receptors, P92 in either monomeric or dimeric form was added to cultures in the presence of a fixed concentration of NGF. By definition, a partial agonist functioning at the same receptor as the native ligand would be expected to compete with and thus partially inhibit the effect of the native ligand (37, 38). As shown in Figure 1e, P92 dimer but not P92 monomer inhibited NGF activity by approximately 40% in a dose-dependent manner. This inhibitory effect began to be clearly detectable over the concentration range of 32 to 64 µM. This pattern of inhibition of NGF by P92 dimer was consistent with P92 functioning as a NGF partial agonist and also consistent with previous work showing that cyclized peptides corresponding to loop 4 block NGF binding to TrkA over a similar concentration range (16). The absence of inhibitory activity by the P92 monomer further indicated that the P92 dimer activity profile was structure-dependent.

**P92 demonstrates neurotrophic activity in long-term cultures.** In long-term DRG cultures (greater than twenty-four hours), NGF promotes both neuronal survival and neurite outgrowth (39). In order to determine whether P92 activity also resembled that of NGF beyond the twenty-four hour period, assays underwent two key modifications. Highly purified preparations of neurons were obtained so that the presence of proliferating non-neurons would be minimized. In addition, six well plates were used allowing neurons to be plated at very low densities allowing greater area for neurite outgrowth. Under these conditions, assessment of the number of surviving neurons per area showed that P92 supported survival at levels two-fold greater than background and at 38% of the NGF maximum survival activity in both one-day and three-day cultures.
(Figure 2). This level of activity, measured as a percentage of the NGF effect, was more than two-fold higher than the 10-15% range observed using the standard ninety-six well assays. Monomeric and scrambled forms of P92 had no activity, again demonstrating that P92 NGF mimetic activity was structure- and sequence-dependent. The morphological features of DRG cultures supported by NGF and peptides are shown in Figure 3. Gross cellular morphology and overall neurite outgrowth patterns appeared similar in NGF and P92 treated cultures. In six well plate cultures, essentially all NGF- and P92-supported surviving neurons were associated with neurites. This pattern was in marked contrast to NGF loop 1 peptidomimetics which primarily promoted neuronal survival with relatively less effect on neurite outgrowth (25).

**The survival-promoting activity of P92 is blocked by TrkA inhibitors but not by p75 antibody.** Inhibitors of the TrkA and p75 receptors known to block NGF activity were used to further test the hypothesis that P92 acts via NGF signaling mechanisms. Although no inhibitor entirely specific for TrkA is available, K252a is relatively specific for TrkA (40, 41, 42) and would be expected to block activity of P92 if its action were TrkA-dependent. Similarly, the TrkA inhibitor AG879 (43) would also be expected to block P92. Since the tyrosine kinase targets of K252a and AG879 are partially non-overlapping (41, 43), inhibition of P92 by both compounds would further support the hypothesis that P92 acts via TrkA. As shown in Figure 4, the TrkA inhibitor K252a blocked NGF activity by 66% and P92 activity by 49%. AG879 blocked NGF by 85% and P92 activity by 66%. In contrast, p75 receptor antibody partially blocked NGF as previously established (25, 36) but had no effect on P92 activity. These findings supported the hypothesis that P92 prevented neuronal death primarily via TrkA receptors.

**P92 induces ERK and AKT activation.** Activation of TrkA receptors by NGF triggers multiple signaling cascades including the ERK and AKT pathways (1, 2, 44, 45, 46, 47,
As established by these previous studies, ERK and AKT activation were assessed using the ERK and AKT phosphorylation assays. As shown in Figure 5A and 5B, P92 induced ERK activation. At the 10 and 30 minute time points, P92 induced ERK activation to 17% and 16% of the levels induced by NGF, respectively. At the 3 hour time point, P92-induced ERK activation remained above baseline at 9% of NGF levels. The relative level of P92-induced ERK signaling was proportional to its relative neurotrophic efficacy as compared to NGF. The hypothesis that P92 acts via TrkA receptors also predicted that the TrkA inhibitor K252a would block P92 induced ERK activation. As shown in Figure 5C, K252a blocked ERK activation induced by both NGF and P92.

P92 also induced AKT activation (Figure 6A and 6B). At the 10 and 30 minute time points, P92 induced AKT activation to 26% and 66% of the levels induced by NGF, respectively. At the 3 hour time point, P92-induced ERK activation remained above baseline at 14% of NGF levels. Consistent with the partial agonist effect of P92, the degree of AKT activation was less than that induced by NGF. The hypothesis that P92 acts via TrkA receptors also predicted that the TrkA inhibitor K252a would block P92 induced AKT activation. As shown in Figure 6C, K252a blocked AKT activation induced by both NGF and P92.

**ERK Inhibitor U0126 blocks NGF- and P92-induced neurite outgrowth but not neuronal survival.** The hypothesis that the P92 mimics NGF via ERK signaling was further assessed using the ERK inhibitor U0126 and its inactive chemical control U0124 (49). As shown in Figure 7, U0126, but not U0124 inhibited neurite outgrowth induced by both NGF and P92. Quantitative analysis of neuronal survival showed that U0126 had no effect on cell survival supported by NGF. The survival level in the presence of U0126 was 99.5% of that measured without U0126. Similarly, U0126 had no effect on cell
survival supported by P92 with survival in the presence of U0126 at 104% of that measured without U0126. These findings are consistent with previous studies demonstrating that inhibition of growth factor-induced ERK pathway signaling results in a loss of neurite outgrowth without affecting cell survival (50, 51). The matching profile of the inhibitory effects of U126 on NGF and P92 further support the hypothesis that P92 acts, in part, via NGF-like activation of ERK signal transduction.
Discussion

These studies demonstrate that P92 dimeric peptidomimetics can activate TrkA-related signaling intermediates and mimic neurotrophic actions of NGF in primary neurons. Together with previous work showing that NGF loop 4 interacts with TrkA (21, 22, 23) and that loop 4 monomeric peptidomimetics block the binding of NGF to TrkA (16), the present study supports the possibility that NGF loop 4 mimetics can serve as a basis for development of NGF small molecule therapeutics.

Eight lines of evidence support the hypothesis that P92 acts, in part, via TrkA-related signal transduction. First, P92 corresponds to NGF loop 4, one of the two primary NGF loop domains interacting with TrkA receptors (22, 23). Deviations from loop 4 sequence eliminated activity. Second, P92 activity is highly structure-dependent; alterations in cyclization or flanking residues eliminated activity. While conformational states of P92 and P90 were not directly measured, the loss of activity associated with the likely increased conformational constraint of P90 is consistent with studies suggesting that NGF β-loop conformational flexibility is required for induced-fit ligand recognition of TrkA (14). Third, P92 partially inhibits NGF bioactivity in a structure-dependent manner. Moreover, P92 inhibited NGF over the same concentration range that a cyclized peptide containing NGF residues 92-96 was shown to block $^{125}$I[NGF] binding (16). Fourth, the biological profile elicited by P92 of both preventing neuronal death and stimulating neurite outgrowth parallels that triggered by NGF interaction with TrkA. Fifth, the absolute requirement of dimerization for P92 activity is consistent with models in which the NGF protein dimer induces receptor dimerization. Sixth, P92 activity was blocked by the TrkA inhibitors K252a and AG879. While inhibitors entirely specific for TrkA are not available, the largely non-overlapping tyrosine kinase target profiles of these Trk inhibitors suggests that P92 activity is TrkA-dependent. Seventh, P92
stimulates ERK and AKT activation. In addition, the lower amplitudes of ERK and AKT activation compared to that of NGF are consistent with the partial efficacy of P92. As found with NGF, P92-induced ERK and AKT activation was blocked by K252a. Eighth, the neurite outgrowth activity but not the survival promoting activity of both NGF and P92 was blocked by the ERK inhibitor U0126.

Given the limited quantities of cells and TrkA protein present in primary neuron based assays, evidence suggesting that P92 acts directly with TrkA receptors is largely indirect. Studies of P92 direct actions at the TrkA receptor including phosphorylation of specific TrkA activating tyrosine residues, receptor cross-linking, ligand cross-linking and binding studies will require application of P92 to TrkA expressing cell lines where cell numbers and TrkA protein levels are sufficient. The finding that P92 promotes both TrkA-related signaling and neurotrophic effects in the biological context of primary neurons will encourage direct receptor mechanism studies for P92. While the present studies were underway, Maliartchouk et al (52) demonstrated that a loop 4 dimeric peptide, C(92-97)$_{dim}$ supported survival at 19% of the NGF maximum in assays using the 4-3.6 cell line (rat neuroblastoma cells expressing human TrkA). In the same assays, the previously reported loop 4 monomer, C(92-96) had no survival effect. Interestingly, C(92-97)$_{dim}$ but not C(92-96) induced TrkA tyrosine phosphorylation in the same cell line. Signaling studies were not conducted in primary neurons. Taken together, these and the present studies support the possibility that loop 4 mimetics can be developed that act via TrkA and its downstream signaling pathways.

The partial agonist nature of the P92 activity is of particular interest. In general, partial agonism can result from either ‘incorrect’ fit between the partial agonist and receptor or ‘correct’ but incomplete receptor binding (38). NGF interaction with TrkA induces both dimerization and conformational changes of TrkA (53); therefore, absence
of full agonist activity by a NGF small molecule mimetic could result from the absence of either of these binding properties. Parallel NGF/TrkA and P92/TrkA co-crystalization and physical-chemical studies will play an important role in elucidating binding mechanisms and designing P92 derivatives more closely mimicking NGF-TrkA binding. The NGF two-receptor system raises other potential mechanisms contributing to the partial agonist profile of P92. Several studies suggest that unbound p75 negatively regulates and bound p75 positively regulates TrkA function (54, 55, 56). Thus, the absence of p75 ligand interaction by a TrkA agonist might result in reduced TrkA signaling. A similar mechanism was suggested by the finding that TrkA-directed antibodies achieve only partial NGF responses (55, 57). It is also important to note that a given partial agonist can be associated with a wide range of efficacies for different signal transduction and biological endpoints (58). Thus, it will be important to assess the effects of P92 and subsequent partial agonist derivatives on additional signaling intermediates and to include additional biological endpoints.

Another key feature of the partial agonist profile is the well established precedent that the partial agonist nature of some compounds makes possible their application in vivo as antagonists while maintaining low levels of receptor stimulation. For example drugs such as clonidine, oxymetazoline and tamoxafin are partial agonists with efficacies well under 50% of their full agonist counterparts (58, 59, 60). In pathological states such as neural regeneration NGF agonists might be relevant. However, given the important functions of endogenous NGF, it is likely that NGF partial agonists rather than pure antagonists will be useful for downregulation of the contribution of NGF to states of chronic pain or inflammation (61). The proof of concept demonstrated here that NGF loop 4 small molecule mimetics can function as partial agonists to activate TrkA-related signaling intermediates in primary neurons points to important novel and physiologically relevant signal transduction and biological endpoints that should be included in the
screening and design of NGF small molecule mimetics. This proof-of-concept is also likely to be important for other neurotrophins such as brain-derived neurotrophic factor (BDNF). For example, the finding that cyclized monomers corresponding to loop 2 of BDNF function as BDNF antagonists (62) raises the possibility that dimeric peptidomimetics of BDNF might demonstrate agonist or partial agonist activity.

The distinct profiles in receptor dependency and biological profiles of the P92 NGF loop 4 mimetic described here and the previously described NGF loop 1 mimetic P7 (25) constitute a first reduction-to-practice of the theoretical possibility that neurotrophin small molecules mimicking different domains might act via differential mechanisms to trigger differential responses. While P92 is likely to act via TrkA-related signaling, P7 was found to act via a p75-dependent, TrkA-independent mechanism. P92 promoted both survival and neurite outgrowth while P7 primarily promoted survival rather than neurite outgrowth. This distinction in biological activity profile is consistent with studies demonstrating that TrkA regulates both survival and neurite outgrowth while p75 primarily regulates cell death (3, 4, 17, 63). These distinct profiles in TrkA versus p75 mediation and resulting biological activity by the P92 and P7 peptidomimetics indicate that it may be possible to design small molecule NGF mimetics acting via partly non-overlapping signaling networks to promote (or prevent) different sets of biological endpoints. It is clear that peptidomimetics do not have the potency or chemical properties necessary for medicinal compounds. Nevertheless, the identification of a NGF loop 4 partial agonist peptidomimetic and the structure-function relationships demonstrated here will serve as an important guide for rational NGF small molecule screening and design programs.

Acknowledgments. This work was supported by the Alzheimer’s Association (F.L.), the John Douglas French Foundation (F.L.), the Veterans Administration (F.L.) and NIH
grant NS37309 (T.Y.). We thank Dr. William Mobley for many helpful discussions and critical assessment of this work.
References


Figure Legends

Figure 1. NGF loop 4 peptides prevent neuronal death. (A) Peptides containing the indicated residues where cyclized to form cyclized dimers via disulfide bond formation between cysteine or penicillamine (Pen) residues. Variations used to assess structure-activity relationships included cyclization between Pen residues (P92P), scrambling of residues (P92S), addition of residues flanking Pen and cysteine residues (P90) and cyclization to the monomeric form (P92M). (B) Chick embryo DRG neurons were cultured and survival was measured at 24 hrs as described in methods. P92 prevented cell death over a concentration range of 8-125 µM while P92M had no activity. At concentrations greater than 125 µM efficacy remained in the range of 15% of that of the NGF maximum (not shown) (C, D) Scrambling of residues, cyclization via Pen-Pen groups and addition of flanking residues resulted in complete loss of activity. (E) Starting in the dose range of 32-64 µM, P92D but not P92M inhibited NGF activity. For each peptide dose, 6-16 pairs (n) of culture wells were assessed. Mean ± SE is shown.

Figure 2. P92 promotes neuronal survival in long-term cultures. NGF (400 pg/ml), and the peptides P92 dimer (P92D), P92 monomer (P92M) or P92 with scrambled sequence (P92S) were added to cultures (125 µM final concentration) followed by fixation after one (black bars) or three days (stippled bars). Using phase contrast microscopy the number of surviving neurons were counted. Mean ± SE are shown. At both one and three days, P92 stimulated a significant degree of survival compared to that found in control (CM; culture medium) cultures (p < 0.0001, n = 24 fields counted, Mann-Whitney test).
Figure 3. **P92 promotes survival and neurite outgrowth of DRG neurons.** DRG neurons were cultured in the presence of: (A) control culture medium, (B) NGF at 400 pg/ml, (C) P92 Scrambled at 125 µM, and (D) P92 at 125 µM. MTT was added to cultures four hours prior to fixation and cultures were fixed after three days. Addition of P92, but not P92Scrambled was associated with surviving, intact neurons containing MTT product and extensive neurite outgrowth.

Figure 4. **Effect of TrkA and p75 inhibitors on P92 activity.** NGF or P92 was added to cultures in the presence of the TrkA inhibitors K252a or AG879 or p75 blocking antibody 9561. K252a significantly inhibited both NGF (p < 0.0001, n = 5 assays, Mann-Whitney test) and P92 (p < 0.05, n = 5 assays) death-preventing activity. AG879 also significantly inhibited both NGF (p < 0.0001, n = 5 assays) and P92 (p < 0.0001, n = 5 assays) death-preventing activity. Antibody directed against the p75 receptor inhibited NGF (p < 0.0001, n = 6 assays) but not P92 activity. CM, culture medium baseline survival.

Figure 5. **P92 activates ERK tyrosine phosphorylation.** (A and B) DRG cells were incubated in culture medium (CM) without additive or with NGF (10 ng/ml) or P92 (250 µM) for 10 min, 30 min or 3 hours. Following incubation, cells were collected and protein extracts subjected to polyacrylamide gel electrophoresis and transferred for Western blotting. Blots were first probed with anti-phosphorylated ERK (ERK-p) antibody and then reprobed with anti ERK antibody. Blots were exposed to x-ray film and film was processed by scanning densitometry. The ratio of signal derived from ERK-p over ERK bands was calculated (n = 3 separate Western blots for 10 min and 3 hr time points and n = 7 blots for 30 min time points; mean ± SE is shown). (C) DRG cells in culture medium (CM) without additive or with NGF (10 ng/ml) or P92 (250 µM) were
also incubated with or without K252a (200 nM). After 10 minutes, cells were processed as described for A and B and ERK signal assessed.

**Figure 6. P92 activates AKT serine phosphorylation.** (A and B) DRG cells were incubated in culture medium (CM) without additive or with NGF (10 ng/ml) or P92 (250 µM) for 10 min, 30 min or 3 hours. Following incubation, cells were collected and protein extracts subjected to polyacrylamide gel electrophoresis and transferred for Western blotting. Blots were first probed with anti-phosphorylated (Ser473) AKT (AKT-p) antibody and then reprobed with anti AKT antibody. Blots were exposed to x-ray film and film was processed by scanning densitometry. The ratio of signal derived from AKT-p over AKT bands was calculated (n = 4 separate Western analyses for 10 min and 3 hr time points and n = 6 for 30 min time points; mean ± SE is shown). (C) DRG cells in culture medium (CM) without additive or with NGF (10 ng/ml), P92 scrambled control (P92S; 250 µM) or P92 (250 µM) were also incubated with or without K252a (200 nM). After 10 minutes, cells were processed as described for A and B and AKT signal assessed.

**Figure 7. ERK inhibitor U126 blocks the neurite outgrowth promoting effects of NGF and P92.** DRG neurons were cultured in the presence of: (A) culture medium; (B) NGF without inhibitor; (C) NGF with inactive UO124 control; or (D) NGF with UO126 ERK inhibitor. In the same experiments, neurons were also cultured in the presence of: (E) P92 without inhibitor; (F) P92 with inactive UO124 control; or (G) P92 with UO126. Cultures were fixed after 12 hours. NGF and P92 concentrations were 10ng/ml and 250 µM, respectively. UO126 and UO124 were added to reach a final concentration of 50 µM. UO126, but not UO124, led to a decrease in NGF-induced neurite outgrowth (D vs. C). Similarly, UO126, but not UO124, led to a decrease in P92-induced neurite outgrowth (G vs. F).
Figure 1

A. **P92**: Pen-T-D-E-K-Q-C-NH₂

---

**P92S**: Pen-D-Q-K-T-E-C-NH₂

---

**P92M**: Pen-T-D-E-K-Q-C-NH₂

---

**P92P**: Pen-T-D-E-K-Q-Pen-NH₂

---

**P90**: L-Pen-T-D-E-K-Q-C-A-NH₂

---

**P92**: Pen-T-D-E-K-Q-C-NH₂

---

**B.**

Neuronal Survival (% NGF Maximum)

---

**C.**

Neuronal Survival (% NGF Maximum)

---

**D.**

Neuronal Survival (% NGF Maximum)

---

**E.**

Neuronal Survival (% NGF Maximum)

---

Peptide (μM)
Neuronal survival (% NGF maximum)

- NGF
- P92D
- P92M
- P92S
- CM

Neuronal survival in different conditions with NGF maximum as a reference.
Figure 6

A

AKT-p

AKT

B

AKT-p

AKT

C

<table>
<thead>
<tr>
<th>Control</th>
<th>10 m</th>
<th>30 m</th>
<th>3 hr</th>
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<tbody>
<tr>
<td>NGF</td>
<td>5</td>
<td>4</td>
<td>3</td>
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<tr>
<td>P92</td>
<td>2.5</td>
<td>2</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Duration of treatment

C

(-) K252a

CM P92S NGF P92D

AKT-p

AKT

(+ ) K252a

CM P92S NGF P92D

AKT-p

AKT
NGF loop 4 dimeric mimetics activate ERK, prevent neuronal death and promote neurite outgrowth
Youmei Xie, Michelle A. Tisi, Tracy T. Yeo and Frank M. Longo

J. Biol. Chem. published online July 13, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M005071200

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