Heterodimeric Neurotrophins Induce Phosphorylation of Trk Receptors and Promote Neuronal Differentiation in PC12 Cells*

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Neurotrophins are a family of highly conserved proteins that affect the development and maintenance of distinct neuronal populations. Neurotrophins exist in vivo as homodimers, but we show that neurotrophins can exist as heterodimers in vitro and are pluripotent, being able to bind to and to activate different Trk tyrosine kinase receptors as well as promote neuronal differentiation in PC12 cells as effectively as wild type homodimers. These asymmetric neurotrophin dimers allow unique characterization of neurotrophin structure-function relationships with Trk receptors. The chimeric Trk activities of these heterodimers suggest an alternative model of neurotrophin-Trk receptor activation in which the critical Trk-interacting elements may be attributed to a single protomer.

The neurotrophins represent a family of structurally conserved, basic proteins including nerve growth factor (NGF),1 brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4/5 (NT4/5). These proteins are critical for the development and maintenance of a wide range of neuronal populations in both the peripheral and central nervous system (Purves, 1988; Thoenen, 1991). Neurotrophins are believed to exist in solution as non-covalent dimers (Angeletti et al., 1971; McDonald et al., 1991; Narhi et al., 1993) at physiologically relevant concentrations (Bothwell and Shooter, 1997). The protomers of the dimer are held together through non-polar interactions of a flat interface formed structurally by three anti-parallel pairs of β-strands, which are highly conserved in terms of primary sequence, between all known neurotrophins (McDonald et al., 1991). The neurotrophins exert their biological effects through selective interaction with and proposed dimerization (Jing et al., 1992) of a family of high affinity tyrosine kinase receptors (Trks) such that NGF binds primarily to TrkA (Kaplan et al., 1991a, 1991b; Klein et al., 1991a), BDNF (Klein et al., 1991b; Soppe et al., 1991; Squinto et al., 1991), and NT4/5 (Berkemeier et al., 1991; Escandon et al., 1993) to TrkB and NT3 to TrkC (Lamballe et al., 1991; Tsoufas et al., 1993). It has been argued that a single neurotrophin dimer interacts with two Trk receptors to form a functional receptor homodimer (Jing et al., 1992; Meakin and Shooter, 1992; Ibnez et al., 1993) and that the high affinity interaction between the receptor subunits and the neurotrophin is the driving force for receptor dimerization.

We have previously shown that chimeric neurotrophin dimers can be formed from human and mouse NGF and that these act identically to either human or mouse NGF homodimers in bioassays (Moore and Shooter, 1975; Burton et al., 1992; Luo and Neet, 1992; Schmelzer et al., 1992). We and others have also shown that three different COOH-terminal forms of recombinant human NGF are capable of forming chimeric dimers that exhibit biological activity (Moore and Shooter, 1975; Burton et al., 1992; Luo et al., 1992).

Analysis of residues with high surface accessibility, at the interface of the mouse NGF homodimer, indicates a strict conservation of this structure between the different members of the neurotrophin family.2 The high degree of homology between interface residues led us to hypothesize that it might be possible to form heterodimeric neurotrophins. Furthermore, since these molecules would contain the variable domains from two different neurotrophins with similar tertiary structure, we were interested to see how active these molecules would be in terms of receptor binding.

Evidence from proteolyzed neurotrophins and mutagenesis has shown that the amino terminus is the most important binding determinant for interaction of NGF with the TrkA receptor (Moore and Shooter, 1975; Burton et al., 1992; Ibnez et al., 1992; Kahle et al., 1992; Luo and Neet, 1992; Shih et al., 1994). Since neurotrophin heterodimers would have two distinct amino termini, it was also necessary to determine whether both amino termini were implicit in a functional interaction with the receptor. To this end, asymmetric neurotrophin heterodimers were generated to act as specific probes to investigate some of the structural elements important for neurotrophin-receptor interactions.

EXPERIMENTAL PROCEDURES

Heterodimer Production, Purification, and Stability—Neurotrophins used for the production of heterodimer neurotrophins were generated as described by Schmelzer et al. (1992) and Burton et al. (1992). Acid-
driven rearrangement has been used to generate a series of potential neurotrophin heterodimers, which are at present being assayed. All proteins were analyzed for their amino acid compositions using a Beckman 6300 amino acid analyzer and quantitated relative to a norleucine internal standard before use. Neurophins were stored in 20 mM sodium/potassium phosphate buffer, 200 mM KCl, pH 7.2. For the stability studies, samples were stored at four temperatures (37, 25, 4, and 0°C) for up to 300 h, and 10-µg aliquots were monitored during this time course by HPIEC. Percent composition was calculated using peak integration.

Receptor Phosphorylation Assays—Cell suspension binding assay is essentially that described by Vale and Shooter (1985). Briefly, 1 x 10^6 cells, resuspended in 200 µl of Leibovitz's L15 media without NaHCO₃ (Life Technologies, Inc.) (with 10 mM HEPES, pH 7.4, and 1 mg/ml bovine serum albumin), were vigorously agitated at room temperature for 1 h with the iodinated neurotrophin (30–50 pM, specific activity ~250 cpm/pg) and competed with a range of concentrations of unlabeled neurotrophin; each point was carried out in triplicate. The iodinated ligand concentration could be decreased 10-fold with no apparent change in Cᵢₒ. Neurotrophins were iodinated using a modified enzymatic method described by Escandon et al. (1993), and the concentration was estimated by trichloroacetic acid precipitation. Free and bound counts were separated by centrifugation through a phosphate-buffered saline 0.15 M sucrose cushion. Examples of displacement binding curves are presented as the percentage of total 125I-neurotrophin bound such that both specific displaceable counts and nonspecific binding can easily be seen. Each point was determined in triplicate, and the mean Cᵢₒ values shown in Table 1 are averaged from over four separate determinations.

Receptor Phosphorylation Assays—Neurotrophin heterodimers were assayed for their ability to induce phosphorylation using a cell suspension assay. Cells were harvested and resuspended in Leibovitz’s L15 media without NaHCO₃ (Life Technologies, Inc.) (with 10 mM HEPES, pH 7.4, and 1 mg/ml bovine serum albumin) and stored on ice before use. 1 x 10⁵ TrkA- or TrkB receptor-expressing cells (a gift from Dr. L. Parada) or 5 x 10⁵ PC12 cells were stimulated with a range of concentrations of neurotrophin for 10 min at 37°C in a total volume of 200 µl. Cells were immediately centrifuged, washed, and placed in 500 µl of ice-cold lysis buffer (20 mM HEPES, pH 7.4, 100 mM NaF, 4 mM EDTA, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 1% Nonidet P40, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM N-ethylmaleimide, 8.5 µg/ml aprotinin) on ice. Proteins that were tyrosine phosphorylated were immunoprecipitated with agarose-linked anti-phosphotyrosine antibodies (Oncogene Science) and solubilized, and Western blots of these samples were probed with a pan-anti-Trk antibody (a gift from Dave Kaplan).

NGF:NT4/5 was also compared to NT4/5 (NGF:NT4/5) as well as a NGF dimer of full-length NGF and an amino-terminal truncated des-1–9 NGF (NGF:Δ9NGF), which are now reported. Heterodimeric neurotrophins were prepared by the acidification and subsequent neutralization of combined pools of homogeneous, intact, or proteolytically altered recombinant human neurotrophins, as has been used previously for the purification of chimeric NGF dimers (Moore and Shooter, 1975; Burton et al., 1992; Luo and Neet, 1992; Schmelzer et al., 1992). This procedure resulted in a mixture of species including homodimeric and heterodimeric neurotrophin molecules, which were purified to homogeneity by HPIEC (Moore and Shooter, 1975; Burton et al., 1992; Luo et al., 1992; Schmelzer et al., 1992) (Fig. 1A). SDS-polyacrylamide gel electrophoresis and reversed phase high performance liquid chromatography (HPLC) showed that the purified heterodimer could be separated into two equimolar bands (Fig. 1B) or peaks (Fig. 1C), which corresponded to both component neurotrophin proteins. Reversed phase HPLC peaks, when recombined and neutralized, formed the same ratio of peak fraction sizes and retention times seen during the initial purification by HPIEC (data not shown). This technique has now been used successfully to generate a large number of different neurotrophin heterodimers. Radziejewski and Robinson (1993) have shown that heterodimer formation can be accelerated using not only acidic conditions but also with solvents and urea. Stability studies were carried out on samples stored under physiological salt and pH conditions at four temperatures. Aliquots removed at selected times were analyzed by HPIEC, and the rates of breakdown of NGF:Δ9NGF and NGF:NT4/5 were estimated (Fig. 1D). The stability of the heterodimers was temperature dependent. The rate of breakdown was very slow at ~37, 4, and 25°C but faster at 37°C. By this criteria, we were able to exclude the possibility that homodimer neurotrophins were reformed from dissociated heterodimer in quantities able to significantly affect the assays carried out subsequently. To further control for any possible variations, matched series of dilutions were used for both the receptor binding and phosphorylation experiments.

HPIEC peak ratios during initial production of heterodimers match the equilibrium peak ratios observed during rearrangement of heterodimers at 37°C in the stability studies carried out. These peak ratios and the rate at which they rearrange most likely reflect the relative affinities of the different neurotrophin protomers for each other. It is difficult to compare rearrangement rates in homodimers due to difficulties in tagging the individual protomer subunits of the dimer accurately. Stability studies were carried out on human NGF (Knusel et al., 1994); PC12 Neurite Outgrowth Assays—PC12 cells were tested using an accelerated response paradigm (Greene et al., 1987) to decrease the possibility of neurotrophin rearrangement affecting the neurite outgrowth response. PC12 cells were first “primed” by growth in the presence of 50 ng/ml NGF for 10 days. The cells were washed and replated at low seeding density in the presence of a range of concentrations of neurotrophin and left for 24 h before neurite-bearing cells were counted. Shorter periods of stimulation were tested using a wash-out protocol in which neurotrophins were left on the cells for 1.5, 3, or 6 h or for the full assay period of 24 h. Each point was analyzed in quadruplicate and in separate wells. Approximately 150–200 cells were counted per well.

RESULTS

Production of Heterodimer Neurotrophins—Two neurotrophin heterodimers, that of full-length NGF and NT4/5 (NGF:NT4/5) as well as a NGF dimer of full-length NGF and an amino-terminal truncated des-1–9 NGF (NGF:Δ9NGF), are most likely reflect the relative affinities of the different neurotrophin protomers for each other. It is difficult to compare rearrangement rates in homodimers due to difficulties in tagging the individual protomer subunits of the dimer accurately.
using a Vydac C4 reversed phase HPLC.

The formation of a mixture of homo- and heterodimers in the culture medium.

Furthermore, heterodimers could be formed in any situation where two or more neurotrophin homodimers exist together. Furthermore, Shooter, 1977), they undergo rearrangement even in their intact homodimeric state. This is important since it suggests that heterodimers could be formed in any situation where two or more neurotrophin homodimers exist together. Furthermore, J ungbeth et al. (1994) showed that co-expression of neurotrophins (NT-3 and BDNF) in a single cell line can result in the formation of a mixture of homo- and heterodimers in the culture medium.

Radioligand Receptor Binding of Heterodimers—Radioligand-receptor binding assays were carried out on a number of different cell lines expressing specific neurotrophin receptors. IC50 values obtained by competition binding isotherms were averaged and are summarized in Table I. Both NGF:Δ9NGF and NGF:NT4/5 were as effective as either homodimeric NGF or NT4/5 at displacing 125I-NGF from the low affinity p75 receptor of A875 cells (for example, see Fig. 2A), suggesting that the hybrid molecules had a structural conformation comparable to homodimeric neurotrophins at least with regard to the p75 binding region(s). A putative p75 binding site is structurally close to residues involved in Trk receptor binding (Ibanez et al., 1992) such that p75 receptor interactions act as a reasonable control for the overall conformational structure of the heterodimeric proteins.

In PC12 cells, a rat pheochromocytoma cell line, which expresses p75 and a smaller number of TrkA receptors, NGF:Δ9NGF and NGF:NT4/5, were able to displace all specifically bound 125I-NGF, as efficiently as NGF, while homodimeric NT4/5 displaced a maximum of 80% (probably corresponding to the p75 component) (for example, see Fig. 2B). These results suggested that both NGF:Δ9NGF and NGF:NT4/5 were able to displace not only the p75 component of PC12 cell binding but also the TrkA specific component. Radioligand-receptor binding was then studied on NIH 3T3 cells selectively expressing rat TrkA (Kaplan et al., 1991a, 1991b) or rat TrkB (Soppet et al., 1991) using matched dilution curves of the homo- and heterodimeric neurotrophins. Both NGF:NT4/5 (for example, see Fig. 2C) and NGF:Δ9NGF (for example, see Fig. 2E) were able to displace 125I-NGF from TrkA-expressing NIH 3T3 cells showing an average 2-fold increase in IC50 compared to homodimeric NGF (Table I), whereas NT4/5 was only able to displace 125I-NGF from the TrkA receptor if taken to micromolar concentrations (for example, see Fig. 2C). NGF:NT4/5 displayed a 20-fold increase in IC50 compared to NT4/5 for displacement of 125I-labeled NT4/5 from the rat TrkB receptor, while no displacement was detectable for NGF (for example, see Fig. 2D). These results indicate that binding interactions of NGF:Δ9NGF and NGF:NT4/5 with the rat TrkB receptor are very similar to homodimeric NGF. Although NGF:NT4/5 binds rat TrkB 20-fold less efficiently than NT4/5, the binding is clearly significant compared to homodimeric NGF with the TrkB receptor. Importantly, preliminary results analyzing the displacement binding properties of NGF:NT4/5 from cells expressing the human TrkA or human TrkB receptors indicate only 2–3-fold increases in IC50 when compared to cognate homodimeric neurotrophins. These observations suggest that the differences seen with NGF:NT4/5 on the rat TrkB-expressing cell line could be due to species variations in the receptor (data not shown). Small variations seen in IC50 values may be due to slight variations in conformation of the receptor binding domain of the heterodimer or perhaps differential involvement of amino termini in binding to their cognate Trk receptor.

To further investigate the promiscuity of the amino termini between neurotrophins with regard to their potential involvement with binding domains on the other protomer of the dimer, a small quantity of heterodimeric Δ9NGF:NT4/5 was generated, and some preliminary binding studies were carried out. Δ9NGF:NT4/5 displaces 125I-labeled NT4/5 bound to human TrkB similar to homodimeric NT4/5 and 125I-NGF from human TrkA similar to Δ9NGF (300-fold less than homodimeric NGF). The loss of an amino terminus from NGF does not appear to alter the interaction of NT4/5 with TrkB, and the presence of an amino terminus of NT4/5 in the dimeric structure of Δ9NGF:NT4/5 does not facilitate Δ9NGF binding to the TrkA receptor, consistent with the data generated with NGF:NT4/5.
eral tyrosine residues found within the conserved tyrosine kinase domain of the Trk family of receptors are phosphorylated in a dose-dependent manner upon exposure to their cognate neurotrophin. This signal transduction event is rapid with peak phosphorylation seen within 5 min, such that the breakdown of NGF:Δ9NGF or NGF:NT4/5 would be less than 0.1% during the time course of this assay. NGF:NT4/5 and NGF:Δ9NGF were able to induce phosphorylation of the TrkA receptor as efficiently as NGF (Fig. 3, A and B), while homodimeric NT4/5 and fully truncated NGF induced significantly lower phosphorylation. The increased IC50 of NGF:NT4/5 in competition binding studies on the rat TrkB receptor was paralleled by an approximately 20-fold decrease in the phosphorylation of the TrkB receptor when compared to NT4/5 (Fig. 3, A), while homodimeric NGF induced no detectable phosphorylation. Similar to the results with NGF:NT4/5 stimulation of TrkB in fibroblasts, primary cultures of rat cortex showed an approximate 10-fold decrease in phosphorylation of Trk receptors compared to NT4/5 (Fig. 3, C). These results indicate that both NGF:Δ9NGF and NGF:NT4/5 are capable of binding and initiating a signal transduction event through phosphorylation of Trk receptors.

Biological Analysis of Heterodimer Activities—Neurotrophins were tested in a "primed" PC12 cell bioassay (Greene et al., 1987) (Fig. 3, D and E). These cells respond to NGF, changing from a chromaffin cell to a neuronal-like phenotype, with increased size and neuritic projections. In an accelerated paradigm, these cells respond to NGF within 24 h (Greene et al., 1987). Analysis of neurite outgrowth in PC12 cells stimulated for varying time periods (1.5, 3, 6, and 24 h with wash out) showed almost equivalent dose-dependent response in the percentage of cells bearing neurites (Fig. 3D), although qualitatively it appeared that the cells stimulated for longer periods were more robust in terms of cell size. This suggests that the mechanism that controls neurite outgrowth in “primed” PC12 cells is induced relatively quickly and that perhaps the steady-state level of neurotrophin/receptor binding reached in the first few hours of the assay is critical to the response seen after 24 h. Both NGF:NT4/5 and NGF:Δ9NGF were able to induce neurite outgrowth as efficiently and with similar potency as NGF on PC12 cells (Fig. 3E), whereas Δ9NGF and NT4/5 showed a greatly decreased or non-detectable EC50, respectively. Together with the stability data (Fig. 1D), these results suggest that the bioactivity observed is due to the heterodimer and not as a consequence of reassociated NGF. It appears, from these experiments that the ability of a neurotrophin to displace ligand from the Trk receptor correlates positively with the ability of these molecules to induce both signal transduction and biological response. In addition, these results suggest that only one intact NGF amino terminus attached to the NGF dimer structure is sufficient for the functional activity of the neurotrophin, consistent with the results seen with NGF:NT4/5.

Proteolytic cleavage of the amino termini from other neurotrophin family members leads to equivalent increases in IC50 of neurotrophin displacement from the cognate Trk receptor, as seen with NGF and Δ9NGF interactions with the TrkA receptor, suggesting a general requirement for this structural feature (data not shown). At present, it is unclear whether both amino termini of the neurotrophins act solely as a site of Trk interaction or whether they might also contribute to the stabilizing interaction with the TrkA-expressing NIH 3T3 cells. A, B, C, and E represent A875 (p75 only), PC12 (p75 and TrkA), and TrkA (only) expressing cell binding of 125I-NGF and competition with unlabeled NGF, NGF:NT4/5, NT4/5, NGF:Δ9NGF, and homodimeric Δ9 NGF. D represents TrkB-expressing cell binding of 125I-labeled NT4/5 and competition with unlabeled NGF, NGF:NT4/5, and NT4/5.
ity of neurotrophin dimeric structure. Amino-terminal-deleted neurotrophins remain competent in terms of p75 binding, which probably indicates little effect on overall structure.

**DISCUSSION**

In this report, we demonstrate the capability of neurotrophin family members to form heterodimers that appear to be relatively stable. These molecules are asymmetric neurotrophin dimers, in contrast to symmetric dimers of neurotrophin produced by mutagenesis, and as such can be used as unique structural probes for ligand-receptor binding interactions and activation. Surprisingly, their demonstrated specificity for receptor interactions and biological activity appears to be largely predictable from the homodimer equivalents of their subunit parts. Furthermore, analysis of the amino terminus of NGF suggests that only one intact amino terminus is necessary for NGF to elicit significant actions through the TrkA receptor. These findings suggest that the binding surface(s) sufficient for NGF receptor-ligand interactions can be largely carried on each protomer of the dimeric molecule.

A structural model of NGF-Trk receptor interactions has been proposed based upon extensive mutagenesis of variable sequence regions identified within the neurotrophin family (Ibanez et al., 1991, 1992, 1993; Suter et al., 1992; Shih et al., 1994). In our present work and in previous studies (Moore and Shooter, 1975; Burton et al., 1992; Kahle et al., 1992; Luo and Neet, 1992; Shih et al., 1994), we have observed that the amino-terminal region of NGF is critical for high affinity binding and biological activity. In agreement with this, Ibanez et al. (1993) suggest that the amino terminus may be the most significant contributor to the binding energy of NGF with TrkA. Other sites of receptor interaction, i.e., between variable regions I, II, IV, and V, although helping to stabilize the ligand-receptor complex, appear to be secondary. In contrast to the proposed nature of the amino terminus-receptor interaction, the other variable regions, particularly region II, may be involved in receptor activation more than receptor binding (Ibanez et al., 1993). The proposed models of NGF-Trk interaction suggest that multiple specific contacts within the variable regions of NGF, described above, form a continuous binding surface on both sides of the molecule (Ibanez et al., 1993). The bivalent nature of this proposed binding surface is consistent with the 2-fold symmetry of NGF suggested to be responsible for Trk dimerization and signal transduction (Fig. 4B, model 1) (Jing et al., 1992). Structural elements from both protomers have been implied to contribute to the binding surface. For example, part of the Trk binding/activation surface of NGF has been suggested to be a patch of residues that contains variable regions I, IV, and V from one protomer and region II and possibly the amino terminus from the other protomer. NGF:Δ9NGF has similar biological activity to homodimeric NGF, suggesting that this proposed domain on NGF involving the amino termini and variable regions does not have to be bilateral for receptor binding, signal transduction, and thus receptor dimerization to
NGF from the human TrkA receptor effectively. This is consistent with the data from NGF:NT4/5 and further indicates that the amino termini are not promiscuous in their interactions between protomers to form binding domains. The amino termini do appear to be a very mobile domain in solution as the lack of electron density indicates in the murine NGF crystallographic structure (McDonald et al., 1991; Holland et al., 1994). However, the exact role(s) of the amino termini in NGF dimer stability and in binding domains remains unclear.

The current data suggest an alternative mechanism for NGF-Trk receptor signal transduction in which the NGF binding/activation domains are contained primarily upon a single protomer (Fig. 4B, model 2). Co-crystallization of the soluble human growth hormone receptor-ligand complex reveals that a single protomeric hormone can dimerize two identical receptors through interactions with two distinct binding domains (Cunningham et al., 1991). Our preliminary results with other heterodimeric neurotrophin combinations indicate that they display dual activities consistent with their subunit parts and this model. The potential involvement of conserved residues upon binding interactions with Trk receptors is under further study. It will be interesting to produce heterodimers containing mutated protomers to analyze whether dimerization of Trk receptors is induced by cross-bridging with the neurotrophin (Fig. 4B, model 1 and 2) or perhaps by other receptor interactions which in turn induce receptor dimerization and transphosphorylation (Fig. 4B, models 3 and 4).

In addition to providing an approach to investigating Trk receptor signal transduction, the relative stability and biological activity of heterodimeric neurotrophins suggests that they may represent novel pharmacological agents in vivo. For example, in models of peripheral neuropathy, certain neuronal populations, such as dorsal rat ganglia sensory neurons (McMahon et al., 1994), express multiple Trk receptors and may be responsive to pluripotent heterodimeric neurotrophins. Interestingly, multiple neurotrophins can be co-expressed in some neurons (Philips et al., 1990) and glioma cell lines (Hamel et al., 1993). Jungbluth et al. (1994), using the co-expression of NT-3 and BDNF in a rabbit kidney cell line (RK13) by vaccinia virus, have shown that heterodimers can be purified from serum-free conditioned medium and show some biological activity. Our stability data, generated in vitro under physiological conditions, indicate that heterodimers rearrange to an equilibrium containing significant amounts of heterodimer (40–50%), which suggests the possibility that neurotrophin heterodimers may exist in vivo. Whether or not neurotrophin heterodimers exist in a neuronal context remains an intriguing question.

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