Activation of the p75 Neurotrophin Receptor through Conformational Rearrangement of Disulphide-Linked Receptor Dimers

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DOI 10.1016/j.neuron.2009.02.020

SUMMARY

Ligand-mediated dimerization has emerged as a universal mechanism of growth factor receptor activation. Neurotrophins interact with dimers of the p75 neurotrophin receptor (p75NTR), but the mechanism of receptor activation has remained elusive. Here, we show that p75NTR forms disulphide-linked dimers independently of neurotrophin binding through the highly conserved Cys257 in its transmembrane domain. Mutation of Cys257 abolished neurotrophin-dependent receptor activity but did not affect downstream signaling by the p75NTR/ NgR/Lingo-1 complex in response to MAG, indicating the existence of distinct, ligand-specific activation mechanisms for p75NTR. FRET experiments revealed a close association of p75NTR intracellular domains that was transiently disrupted by conformational changes induced upon NGF binding. Although mutation of Cys257 did not alter the oligomeric state of p75NTR, the mutant receptor was no longer able to propagate conformational changes to the cytoplasmic domain upon ligand binding. We propose that neurotrophins activate p75NTR by a mechanism involving rearrangement of disulphide-linked receptor subunits.

INTRODUCTION

The neurotrophins are a family of neurotrophic factors that control multiple aspects of nervous system development and function, including neurogenesis, neuronal differentiation, cell survival, neurite outgrowth, target innervation, and synaptic plasticity (Bibel and Barde, 2000). Four neurotrophins are present in mammals: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). The neurotrophins are synthesized as a prepro-peptide from which the mature portion is released by proteolytic cleavage. The mature forms of neurotrophins interact with two distinct receptors, a cognate member of the Trk receptor tyrosine kinase family and the common p75 neurotrophin receptor (p75NTR), a member of the tumor necrosis factor receptor (TNFR) superfamily of death receptors (Friedman and Greene, 1999; Kaplan and Miller, 2000; Lee et al., 2001a; Patapoutian and Reichardt, 2001). On the other hand, unprocessed neurotrophins (proneurotrophins) are thought to display selectivity for p75NTR over Trk receptors (Lee et al., 2001b) with the aid of the coreceptor sortilin (Nykjaer et al., 2004). While this interaction is thought to preferentially lead to cell death (Lee et al., 2001b), cell survival is mediated by Trk signaling (Patapoutian and Reichardt, 2001). A key issue in neurotrophin research is the elucidation of the molecular mechanisms underlying each of the physiological actions of the neurotrophins in different cell types, throughout development, and in pathological situations.

Unlike Trk receptors, p75NTR lacks catalytic activity. The intracellular region of p75NTR contains a flexible juxtamembrane segment followed by a globular domain known as the death domain (Liepinsh et al., 1997). Signal transduction by p75NTR is thought to proceed via ligand-dependent recruitment and release of cytoplasmic effectors to and from the receptor. Over 20 different intracellular interactors of p75NTR have been identified to date (Barker, 2004; Bronfman and Fainzilber, 2004; Dechant and Barde, 2002; Roux and Barker, 2002), but this wealth of interactions has not translated in a comparable understanding of receptor function. Some of the major downstream signaling events triggered by p75NTR in response to
RESULTS

Transmembrane Cys\textsuperscript{257} Mediates Formation of Disulphide-Linked p75\textsuperscript{NTR} Dimers

Surface expression of p75\textsuperscript{NTR} was assessed in transfected cells following biotinylation of cell surface proteins, immunoprecipitation, and gel electrophoresis under nonreducing and reducing conditions. This analysis revealed that wild-type p75\textsuperscript{NTR} forms disulphide-linked dimers in the plasma membrane of transfected cells (Figure 1A). At high levels of expression in COS-7 cells, comparable amounts of cell surface p75\textsuperscript{NTR} monomers and disulphide-linked dimers could be detected under nonreducing conditions (Figure 1A). However, the proportion of disulphide-linked p75\textsuperscript{NTR} dimers in transfected cells depended upon the level of expression of the receptor. At moderate overexpression levels, less than 10% of wild-type p75\textsuperscript{NTR} was disulphide linked (Figure 1B). NGF treatment had no effect on the proportion of disulphide-linked p75\textsuperscript{NTR} dimers (data not shown). Since neither the extracellular (Gong et al., 2008; He and Garcia, 2004) nor intracellular (Liepins et al., 1997) domains of p75\textsuperscript{NTR} have been reported to form disulphide-linked dimers, we turned our attention to the transmembrane domain of the receptor, which contains a Cys residue (Cys\textsuperscript{257}) that is absolutely conserved in all p75\textsuperscript{NTR} orthologs known to date, from echinoderms to mammals (Bothwell, 2006) (Figure 1C). Mutation of Cys\textsuperscript{257} to alanine (C257A) abolished the ability of p75\textsuperscript{NTR} to form disulphide-linked dimers (Figure 1A), indicating that such dimers are generated by disulphide bonding of two p75\textsuperscript{NTR} molecules through Cys\textsuperscript{257} in the transmembrane domain. Disulphide-linked dimers could also be detected to varying degrees on the surface of cells endogenously expressing p75\textsuperscript{NTR}, such as PC12 pheochromocytoma, RN22 Schwannoma, and sympathetic neurons from the rat superior cervical ganglion (SCG) (Figure 1D), as well as in total extracts of newborn cortex, hippocampus, and cerebellum (Figure 1E). Interestingly, in the latter cases the majority of p75\textsuperscript{NTR} was disulphide linked. Of note, a previous study found high levels of disulphide-linked p75\textsuperscript{NTR} dimers in one melanoma cell line (A875) but not in another (HS294) (Grob et al., 1985). Importantly, the C257A mutation did not affect the ability of p75\textsuperscript{NTR} to bind NGF (Figure 1F), nor its capacity to undergo \gammasecretase-dependent intramembrane cleavage upon stimulation with the phorbol ester PMA (Figure 1G).

\textbf{Cys\textsuperscript{257} Is Essential for p75\textsuperscript{NTR} Signaling in Response to Neurotrophins}

The ability of native p75\textsuperscript{NTR} to form disulphide-linked dimers prompted the question of its possible significance to p75\textsuperscript{NTR} activation and downstream signaling. We therefore investigated the effects of the C257A mutation on the ability of the receptor to recruit intracellular effectors and activate downstream pathways in response to NGF. Unlike wild-type p75\textsuperscript{NTR}, the C257A mutant was unable to interact with NRIF, either in the presence or absence of NGF (Figure 2A). Although a weak interaction could be observed between the C257A mutant and TRAF6, this was not increased by NGF treatment, which on the other hand readily promoted TRAF6 recruitment to wild-type receptors (Figure 2B). These data suggested that, although wild-type p75\textsuperscript{NTR} is present as both monomers and disulphide-linked dimers at the cell...
membrane, only the latter appear to be capable of recruiting downstream effectors such as NRIF and TRAF6 in a ligand-dependent manner. In order to address this directly, we tested the ability of NRIF and TRAF6 to differentially interact with monomers and disulphide-linked dimers of wild-type p75NTR by analyzing pull-down products under reducing and nonreducing conditions. We found that NRIF interacted exclusively with disulphide-linked p75NTR dimers, but not at all with monomers, and NGF treatment could readily increase this interaction (Figure 2C). On the other hand, although TRAF6 could pull down both p75NTR monomers and dimers, NGF treatment only stimulated TRAF6 binding to p75NTR dimers, but not monomers (Figure 2D). Also in sympathetic neurons from rat SCG, NRIF interacted only with p75NTR disulphide-linked dimers, not with monomers, and BDNF treatment strongly stimulated NRIF recruitment to the receptor (Figure 2E).

We also examined three different downstream readouts of p75NTR activity in transfected fibroblasts and 293 cells, including activation of NF-κB (Figure 3A), caspase-3 (Figure 3B), and induction of cell death (Figure 3C) in response to NGF. In all cases, the C257A mutant was unresponsive to stimulation, indicating that Cys257 is required for neurotrophin-dependent p75NTR signaling and downstream biological effects.

In order to probe the functional importance of Cys257 for p75NTR signaling in a more physiological context, we developed two shRNA constructs directed to 3' UTR sequences of the rat p75NTR mRNA to knock down endogenous p75NTR expression in neurons. When introduced together into primary cultures of
rat SCG neurons, these shRNAs effectively suppressed endogenous p75NTR expression, while a control shRNA had no effect (Figure 4A). As expected, control shRNA did not affect the ability of BDNF to induce JNK phosphorylation (Figure 4B) and cell death (Figure 4C) in SCG neurons, which express p75NTR and TrkA but not TrkB. In contrast, shRNAs directed against p75NTR abolished both responses, indicating their dependence on p75NTR signaling (Figures 4B and 4C). Under those conditions, BDNF-mediated JNK phosphorylation and cell death could be restored by introduction of a wild-type p75NTR expression construct that is insensitive to our p75NTR shRNAs (Figures 4B and 4C). In contrast to the wild-type construct, the C257A mutant was unable to restore either JNK phosphorylation or cell death in response to BDNF in SCG neurons (Figures 4B and 4C). Together, these results demonstrate that Cys 257 is essential for p75NTR signaling in response to neurotrophins and support the notion that disulphide-linked p75NTR dimers are the active, neurotrophin-sensitive receptor species in neurons.

**Cys^{257} Is Not Required for Downstream Signaling by the p75NTR/NgR/Lingo-1 Complex in Response to MAG**

The importance of Cys^{257} in neurotrophin-dependent p75NTR signaling prompted the question of its role in the activation of p75NTR by other types of ligands. We therefore tested the ability of wild-type and mutant p75NTR to recruit RhoGDI and to increase RhoA activity in response to the myelin-derived ligand MAG. These activities require the presence of two additional receptor subunits, NgR and Lingo-1. Interestingly, cells expressing p75NTR, NgR, and Lingo-1 responded equally well to stimulation with MAG in both RhoGDI recruitment to p75NTR (Figure 5A) and RhoA activity (Figure 5B), regardless of whether they received wild-type or the C257A mutant. Thus, and in contrast to activation by neurotrophins, Cys^{257} is not required for the ability of p75NTR to signal in response to the non-neurotrophin ligand MAG, suggesting the existence of mechanistic differences in the activation of p75NTR by different ligands.
Cys²⁵⁷ Allows Disulphide-Linked p75NTR Dimers to Propagate Conformational Changes to Intracellular Domains following NGF Binding

How does Cys²⁵⁷ contribute to p75NTR signaling in response to neurotrophins? Its localization in the transmembrane domain suggested that it may play a role in the mechanism of receptor activation, either by regulating p75NTR oligomerization or by allowing the propagation of conformational changes induced by neurotrophin binding. Although Cys²⁵⁷ clearly contributes to the formation of disulphide-linked p75NTR dimers, it remained unclear whether this was the primary determinant of receptor oligomerization. In particular, the ability of the C257A mutant to bind NGF at normal levels (Figure 1E) suggested that the mutation may not have disrupted the oligomeric state of p75NTR at the cell surface, as this would have been expected to affect ligand binding affinity. In order to examine the possible role of noncovalent interactions in p75NTR oligomerization, we performed chemical crosslinking studies of wild-type and mutant cell surface receptors in living COS-7 cells. Following chemical crosslinking of cell surface proteins, p75NTR was immunoprecipitated from cell lysates and then subjected to SDS/PAGE under reducing conditions, so that only oligomeric complexes

Figure 3. Cys²⁵⁷ Is Essential for p75NTR Signaling to NF-κB, Caspase-3, and Cell Death in Response to NGF
(A) NF-κB activity in transfected M23 fibroblasts in the presence and absence of NGF.
(B) Activation of caspase-3 visualized with a cleavage-specific antibody in HEK293 cells transfected with p75NTR constructs in response to NGF. Reprobing controls for p75NTR and GAPDH are shown.
(C) Cell death assay in HEK293 cells transfected with p75NTR constructs in response to NGF. Results are expressed as mean ± SD of three independent experiments, each performed in duplicate.

Cys²⁵⁷ Allows Disulphide-Linked p75NTR Dimers to Propagate Conformational Changes to Intracellular Domains following NGF Binding

Figure 4. Cys²⁵⁷ Is Essential for p75NTR Signaling in SCG Neurons
(A) Downregulation of p75NTR expression in rat SCG neurons following transfection of p75NTR shRNAs.
(B) Assay of JNK phosphorylation in SCG neurons in response to BDNF. SCG neurons were transfected with the indicated shRNA constructs. In rescue experiments, wild-type p75NTR or C257A p75NTR constructs insensitive to p75NTR shRNAs were also introduced by DNA transfection. JNK phosphorylation was assessed by immunoblotting of total cell lysates.
(C) Cell death in SCG neurons transfected with p75NTR shRNAs in response to BDNF. Rescue experiments with wild-type and C257A p75NTR constructs were performed as above. *p < 0.05 versus wild-type, n = 3.
stabilized by chemical crosslinking would be visualized. As expected, chemical crosslinking allowed the detection of wild-type receptor dimers (Figure 6A), demonstrating that p75NTR indeed forms dimers at the surface of living cells in the absence of ligand. As chemical crosslinkers have only a limited efficiency, the actual proportion of receptor dimers and monomers at the cell surface cannot be determined with this method. NGF binding did not alter the proportion of p75NTR dimers detected by chemical crosslinking in transfected cells (Figure 6C). When introduced in full-length p75NTR together with C257A, the G266I mutation diminished the formation of p75NTR dimers at the plasma membrane (Figure 6D). Together, these data indicate that p75NTR homodimers can be stabilized at the cell surface by both covalent and noncovalent interactions between transmembrane domains and that the primary function of Cys257 is not in the formation of receptor dimers.

We then considered the possibility that Cys257 may instead contribute to p75NTR signaling by mediating the propagation of conformational changes induced by neurotrophin binding. We reasoned that conformational changes involving alterations in the relative positions of p75NTR intracellular domains may be detected by fluorescence resonance energy transfer (FRET). Unlike methods based on enzyme complementation, in FRET, the transfer of energy between two chromophores depends both on the distance between them and their relative orientation, and it is therefore more suitable to quantitatively assess conformational rearrangements of receptor subunits in a complex. We chose homo-FRET over other more conventional FRET techniques because of its greater sensitivity and the advantage of using a single spectral variant to detect interactions between identical molecules (Squire et al., 2004). Homo-FRET can be detected as a decrease in steady-state fluorescence anisotropy that results from energy transfer between identical fluorophores. Monomeric enhanced green fluorescent protein (EGFP1) displays high anisotropy (i.e., low FRET), while a concatenated EGFP trimer (EGFP3) shows very low anisotropy (i.e., very high FRET), and they were used as negative and positive controls, respectively (Figures 7A, 7B, and 7E). Wild-type and C257A p75NTR were tagged at the C terminus with monomeric EGFP (see Experimental Procedures) and transfected in COS-7 cells. Anisotropy and total fluorescence were measured at different locations in the plasma membrane of transfected cells. At basal conditions, wild-type p75NTR-EGFP displayed lower anisotropy than EGFP1 (i.e., higher FRET) at the plasma membrane (Figures 7C and 7E), in agreement with its oligomeric state in living cells. Using the anisotropy value of EGFP1 as baseline, the FRET level of wild-type p75NTR-EGFP could be estimated to be about 25% that of EGFP3 (Figure 7E), which gives a very strong FRET signal. Mutant C257A p75NTR-EGFP also showed lower anisotropy than EGFP1 at the cell surface, albeit not as much as wild-type.

(Figure 6B), which is characteristic of self-associating transmembrane domains, including those from integrins and glycoporphin A (GpA) (Kubatzky et al., 2001). Interactions between isolated transmembrane domains can be studied in a biological membrane using the bacterial ToxCAT system (Russ and Engelman, 1999) (see Experimental Procedures for further details). Dimization of wild-type and mutant p75NTR transmembrane domains was assessed along with wild-type GpA transmembrane domain, which is known for its strong self-association, and a GpA point mutant (G83I) that disrupts homodimerization, as positive and negative controls, respectively. The transmembrane domain of p75NTR was found to homodimerize in this system to about 60% of the level shown by GpA (Figure 6C). Mutation of Cys257 had only a small effect on transmembrane homodimerization (Figure 6C). In contrast, mutation of Gly266 to Ile (G266I), which is analogous to the G83I in GpA, abolished self-association of the p75NTR transmembrane domain (Figure 6C).

Figure 5. Cys257 Is Not Required for Downstream Signaling by the p75NTR/NgR/Lingo-1 Complex in Response to MAG
(A) Binding of RhoGDI to wild-type and C257A p75NTR in COS-7 cells cotransfected with NgR and Lingo-1 and stimulated with MAG-Fc.
(B) RhoA activity in COS-7 cells transfected with wild-type and mutant p75NTR in the presence of NgR and Lingo-1 after stimulation with MAG-Fc for 30 min. Results are expressed as mean ± SD relative to wild-type without MAG treatment. *p < 0.05 versus control, n = 3.
p75NTR (Figures 7D and 7E), which is in accordance with our crosslinking studies showing that this mutant retains the ability to form dimers at the plasma membrane. Interestingly, both p75NTR-EGFP constructs appeared mainly monomeric at intracellular locations (Figures 7C and 7D), suggesting that oligomerization occurs upon transit to the plasma membrane.

NGF was added to cells expressing wild-type and C257A p75NTR-EGFP, and changes in anisotropy were evaluated by time-lapse microscopy. Acute addition of NGF produced an increase in anisotropy (i.e., decrease in FRET) at the plasma membrane of cells expressing wild-type p75 NTR-EGFP (Figure 7E). This anisotropy rise was observed in all cells examined (n = 30), showed a consistent peak 2–3 min after NGF addition, and lasted for 3–10 min, waning toward the end of the recording (15 min) (Figures 7F and 7G). In contrast, NGF had no effect on cell surface anisotropy in cells expressing the mutant C257A p75NTR-EGFP (n = 22 cells examined) (Figures 7F and 7G). These results indicate that NGF induces a decrease in FRET in wild-type p75NTR-EGFP at the plasma membrane, indicating that receptor activation by neurotrophins involves conformational changes in the relative position or orientation of p75NTR intracellular domains. The inability of NGF to induce any change in FRET in cells expressing C257A p75NTR-EGFP correlates with the loss of neurotrophin-dependent p75NTR signaling in this mutant and supports a role for Cys257 in the propagation of conformational changes induced by neurotrophin binding.

DISCUSSION

In this study, we have investigated the mechanism of activation of the p75 neurotrophin receptor. The first major finding of this work is the ability of native p75NTR to form disulphide-linked dimers through the conserved Cys 257 in the transmembrane domain. The second is the unexpected requirement of this cysteine residue for the recruitment of intracellular effectors and downstream signaling by p75NTR in response to neurotrophins, but not in response to other ligands, such as MAG. The third is the ability of the p75NTR dimer to undergo a conformational change in response to NGF and the essential role of Cys257 in this process. Based on these observations, we propose a previously unknown mechanism of receptor activation involving ligand-induced rearrangement of disulphide-linked receptor subunits.

While it is relatively straightforward to visualize how receptor kinases may become activated upon ligand binding (Schlesinger, 2002), this is less obvious for noncatalytic receptors. Since these signal by selective interactions with intracellular effectors, the question that arises is how ligand binding regulates those interactions. Clearly, something ought to change in the receptor after ligand binding that makes its intracellular domain more or less prone to interact with downstream effectors. Ligand binding may change the oligomerization state of the receptor—by, for example, inducing dimers—leading to cooperative binding or release of downstream effectors. Although this has been the prevalent model for p75NTR activation to date, our results show that the active p75NTR species pre-exists as a disulphide-linked dimer, which is essential for downstream signaling in response to neurotrophins, obviating a classical ligand-mediated dimerization mechanism. Although an earlier study using artificially deglycosylated p75NTR extracellular domain had suggested that NGF may activate a preformed receptor dimer by inducing its dissociation into monomers (He and Garcia, 2004),
more recent work has clearly shown that glycosylated p75NTR extracellular domain is monomeric in the absence of ligand and that NGF interacts with p75NTR dimers, not monomers (Gong et al., 2008). Another possible mechanism for receptor activation is that ligand binding changes the relative orientation of receptor subunits in a dimeric or multimeric receptor complex. In some cytokine and growth factor receptors, this is achieved through the relative rotation of their transmembrane domains within the plane of the membrane (Moriki et al., 2001; Seubert et al., 2003). In the case of p75NTR, however, the disulphide link formed by Cys257 in the transmembrane domain clearly restricts the possibilities for relative movement of receptor subunits.

Our FRET experiments indicate that the two intracellular domains of the p75NTR dimer are in close proximity under basal conditions. This is in agreement with data from a recent study using a β-gal complementation strategy (Wehrman et al., 2007). Unlike FRET, however, enzyme complementation gives an all-or-none response at the single-molecule level and is hence less suited to reveal subtle conformational changes that do not alter the oligomeric state of the receptor. In our experiments, we observed a consistent decrease in FRET upon NGF binding to wild-type p75NTR. This could either result from a separation of p75NTR intracellular domains or changes in their relative orientation due to rotation of receptor subunits. Although these two possibilities are not mutually exclusive, it is difficult to envision how changes in the relative orientation of intracellular domains can be brought about by ligand binding to the extracellular region of a dimeric receptor that is covalently crosslinked at the plasma membrane. As explained above, a disulphide bridge linking the transmembrane regions of two receptor subunits would clearly prevent the propagation of rotational movements from the extracellular to the intracellular domains. Thus, we believe that the decrease in FRET observed after NGF binding most likely reflects the separation of p75NTR extracellular domains. Importantly, and in contrast to the wild-type receptor, we found no evidence of NGF-mediated conformational changes in the intracellular domain of the C257A p75NTR mutant. Based on these observations, we propose that neurotrophin binding produces a scissors-like movement of disulphide-linked p75NTR subunits with the Cys257-Cys257 disulphide link acting as the fulcrum, thereby altering the relative proximity of intracellular domains (Figure 8). In this model, Cys257 would function as the pin in the

Figure 7. Analysis of Conformational Changes in p75NTR Intracellular Domains by Anisotropy Microscopy
(A–D) Steady-state anisotropy in transfected cells. Examples of areas used for anisotropy measurements are boxed and shown as high-magnification insets. Monomeric EGFP (EGFP1, high anisotropy, low FRET) and a concatenated EGFP trimer (EGFP3, low anisotropy, high FRET) were used as controls. The calibration bar of the look-up table is shown below.
(E) Steady-state anisotropy of wild-type and C257A p75NTR-EGFP in COS-7 cells. The anisotropy value of EGFP1 was arbitrarily set to zero and used as baseline for the histogram. Bars show average ± SD (n = 8–11 cells for EGFP and 22–30 cells for p75NTR).
(F) Representative examples of anisotropy traces after addition of NGF or medium in cells expressing wild-type or C257A p75NTR-EGFP. A peak in anisotropy was observed after NGF addition in all cells expressing wild-type p75NTR that were examined (n = 30) regardless of their initial baseline anisotropy level.
(G) Anisotropy change after addition of NGF or medium. The difference in anisotropy before and after addition of NGF (i.e., peak minus baseline value) or medium was calculated for wild-type and C257A p75NTR-EGFP. Results are expressed as average ± SD (n = 15–17 cells examined). *p < 0.0001 versus C257A.
scissors: in its absence, relative movements at one end cannot be propagated to the other. Unlike normal scissors, however, our results suggest that as the extracellular domains come closer together on binding to the neurotrophin dimer, the intracellular domains separate, not unlike a “snail-tong” mechanism (Figure 8). This type of movement could be achieved if the p75NTR protein were to be kinked, as opposed to straight, relative to the plane of the plasma membrane, in the vicinity of Cys257. Proline residues are known to introduce kinks that may vary from 5°C14° to 50°C14° and are common in transmembrane helices (Yohannan et al., 2004). Thus, the highly conserved Pro254, three residues upstream of Cys257 (see Figure 1C), seems like a good candidate for such function. Although not as well conserved, an unusual Pro triad is also present in the intracellular juxtamembrane region of p75NTR, 20 residues away from the plasma membrane (Pro295-Pro296-Pro297). This portion of p75NTR is not known to be helical in vivo and has been characterized by nuclear magnetic resonance as unstructured in solution (Liepinsh et al., 1997). The conformational changes inferred from our FRET experiments require a relatively rigid structure connecting the extra- and intracellular domains and suggest that the juxtamembrane region of p75NTR may be stabilized in vivo by interaction with other intracellular components.

The conformational changes observed upon NGF binding showed a characteristic maximum at 2–3 min after NGF addition and lasted for an additional 3–10 min. This kinetics is consistent with the onset of p75NTR internalization and downstream signaling, which requires at least 15 min after ligand stimulation (Bronfman et al., 2003). The separation of intracellular domains induced by NGF binding may facilitate the recruitment of downstream effectors by exposing determinants important for their interaction with the receptor. This may be particularly important for receptors with relatively small intracellular domains, such as p75NTR. Weak homotypic interactions between these domains may keep the receptor in a closed, inactive state and could potentially explain the residual amount of dimers observed after simultaneous mutation of Cys257 and Gly266. Although the p75NTR death domain was found to be monomeric in solution up to 2.5 mM (Liepinsh et al., 1997), such interactions might be detected in crystallization experiments. Even though it is unclear at present why the conformational change observed is not sustained for longer periods of time, the variation observed in its duration could reflect interactions with different effector molecules or activation of different downstream signaling events.

That p75NTR activation involves separation of intracellular domains is consistent with a previous study using chimeric intracellular domains artificially tethered to the plasma membrane that were induced to dimerize by addition of a dimeric drug (Wang et al., 2000). In this work, dimerization inhibited the pro-apoptotic effect of the constructs, from which the authors inferred...
that neurotrophin-mediated receptor dimerization should likewise silence p75NTR activity, a conclusion that was at odds with the bulk of studies in the field. Our results indicating that p75NTR is already dimeric before ligand binding and that p75NTR activation by NGF involves the separation of intracellular domains reconcile those results and suggest that this is in fact the way in which neurotrophins activate this receptor.

Previous work has shown that p75NTR can enhance Trk responses to neurotrophins, particularly when these are present at limiting concentrations. For example, sensory and sympathetic neurons from p75NTR knockout mice show reduced responsiveness to NGF (Lee et al., 1994), and a mutant NGF unable to bind p75NTR displays lower binding affinity and reduced biological activity in PC12 cells and sensory and sympathetic neurons (Rydén et al., 1997). This positive modulatory role of p75NTR on neurotrophin activities mediated by Trk receptors has been attributed to receptor-receptor interactions and/or ligand concentration or presentation effects. It is unclear at present whether these activities require disulphide-linked dimerization of p75NTR. Given the proposed role for the TrkA and p75NTR transmembrane domains in their functional interaction (Esposito et al., 2001), it is possible that formation of high-affinity NGF-binding sites may be sensitive to conformational changes mediated by Cys257.

The fact that p75NTR signaling in response to MAG does not require Cys257 suggests that myelin-derived ligands activate p75NTR by a different mechanism. Although our results indicate that disulphide-linked dimerization is important for p75NTR activation by neurotrophins, the stoichiometry of the complex formed by p75NTR, NgR, and Lingo-1 needs to be further investigated. The lack of involvement of Cys257 in signaling by MAG opens an opportunity to specifically disrupt neurotrophin-specific p75NTR effects in loss-of-function studies. Intriguingly, we found that the proportion of cell surface disulphide-linked p75NTR dimers varied in different cell types and with different levels of receptor expression. As only such dimers are able to respond to neurotrophins, changes in their proportion or abundance at the cell surface, e.g., between different cell types or in response to external stimuli or redox states, could determine the relative degree of p75NTR responsiveness to different ligands. Cys257 represents a critical element in the activation mechanism of p75NTR by neurotrophins and is absolutely conserved in all vertebrate and invertebrate p75NTR molecules isolated so far (Bothwell, 2006). We propose that neurotrophins neither activate p75NTR by inducing receptor dimers or disassembly into monomers, as previously suggested, but through a rearrangement of disulphide-linked receptor subunits. Many receptors in the TNFR superfamily—to which p75NTR belongs—bear intramembrane cysteines, including Fas, DR6, RANK, RELT, BCM, TACI, CD30, CD40, and receptors for TNF, lymphotoxin beta, and TRAIL. The “snail-tong” mechanism described here could therefore represent a general way by which receptors bearing intramembrane cysteines are activated.

**EXPERIMENTAL PROCEDURES**

Plasmids, Antibodies, Proteins, and Chemicals

Rat p75NTR was expressed from the pCDNA3 vector backbone (Invitrogen) using a full-length coding sequence flanked by an N-terminal hemagglutinin (HA) epitope tag. Mutations were introduced using QuickChange (Stratagene) and verified by DNA sequencing. Plasmids to express RIP2, TRAF6, NRIIF, sortilin, Lingo-1, and RhodGDI were previously described. EGF plasmid was from Clontech. Luciferase reporter plasmid for NF-κB was from Promega. The origin of antibodies was as follows: MC192 anti-p75NTR from Phil Barker; anti-HA from Roche; anti-myc, anti-phospho, and -total JNK, cleavage-specific anti-caspase-3, anti-RhoA, and anti-RhodGDI from Cell Signaling; anti-tubulin from Sigma. NGF was purchased from Alomone Labs; MAG-Fc from R&D. NGF was typically applied at 100 ng/ml for 30 min unless otherwise indicated. MAG-Fc was used at 25 μg/ml for 30 min. PMA was used at 200 nM for 1 hr. Epoxomycin (1 μM) and DAPT (2 μM) were applied 1.5 hr prior to PMA. All compounds were from Sigma.

**Cell Transfection and Tissue Culture**

COS-7 cells were transfected with polyethyleneimine (PEI), HEK293 and M23 cells were transfected with Lipofectamine 2000 ( Gibco ). M23 is a clonal derivative of MG87 cells (Ekström et al., 1999), originally derived from mouse NIH3T3 fibroblasts. Cells were typically used on the second day after transfection for short-term signaling assays, at which point cell death was still low or undetectable. We found that different signaling assays worked best in different cell lines: RhoA and RhodGDI in COS-7, NF-κB in M23 and P-JNK, and caspase-3 in HEK293 cells. This may be related to the specific complement of downstream effectors expressed by each cell type. Cell lines were cultured under standard conditions and primary neurons in serum-free, N2-supplemented DMEM:F12 medium ( Gibco ).

**Sympathetic Neuron Culture and p75NTR shRNAs**

Sympathetic neurons from P4 rat SCGs were cultured and transfected with control or p75NTR shRNAs alone or cotransfected with wild-type or mutant p75NTR as previously described (Kenchappa et al., 2006; Palmada et al., 2002). The target sequence of the first p75NTR shRNA was AGGCGACTTACTCTGACGTGAAA, the second was ATGCGGTGACTTCTTACGGAAA. Control shRNA was targeted against EGF sequences. Three days after transfection, neurons were treated with 200 ng/ml of BDNF for 1 hr and lysed in NP-40 buffer (10% glycerol, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mg/ml leupeptin and aprotinin) to assess JNK activation. For cell survival assay, the cells were fixed in 4% paraformaldehyde 48 hr after BDNF treatment and scored for apoptosis as previously described (Kenchappa et al., 2006).

**Biotinylation, Immunoprecipitation, Immunoblotting, and Chemical Crosslinking**

Cell surface proteins were biotinylated with Sulfo-NHS-LC-Biotin (Pierce). Cells were lysed in buffer containing 1% Triton X-100, 60 mM octyl-glucoside, 10 mM iodoacetamide, and protease inhibitors (Roche). For reducing conditions, immunoprecipitates were boiled in sample buffer containing 1 M DTT. Cells were lysed in NP-40 buffer (10% glycerol, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mg/ml leupeptin and aprotinin) to assess JNK activation. For cell survival assay, the cells were fixed in 4% paraformaldehyde 48 hr after BDNF treatment and scored for apoptosis as previously described (Kenchappa et al., 2006).

**Assays of NF-κB, RhoA, and Cell Death**

NF-κB activity was assayed using a luciferase reporter kit (Promega). NF-GF was added 2 days after transfection and left for 24 hr prior to cell lysis. RhoA activity was evaluated using a kit from Cytoskeleton. Cell death was assessed by the TUNEL method using kits from Roche and Biocolor. NF-GF was added 2 days after transfection and left for another day in serum free-medium prior to assay of cell death.

**ToxCAT Assay of Self-Association of Transmembrane Domains**

The reporter system exploits the ability of the ToxR transcription activator of the Vibrio Cholerae pathogen to bind the cholera toxin (ctx) gene promoter only when dimerized. A transmembrane segment of interest fused to ToxR is
delivered to the bacterial inner membrane by fusion to maltose-binding protein (MBP). Varying amounts of ToxR dimers will be formed in the cytosol in direct proportion to the oligomerization ability of the TM domain. Binding of dimerized ToxR to the ctx DNA element triggers expression of a chloramphenicol transferase (cat) gene reporter and production of CAT protein, which can then be quantified by a CAT-ELISA kit (Roche Diagnostics) as described by McClain et al. (McClain et al., 2003). Expression levels of different ToxR-TM-MBP chimeras were determined by western blotting. The ToxR and ToxCAT systems have been previously applied to demonstrate self-association of a range of TM domains from glycoporphin A (GpA) (Kubatzky et al., 2001), integrin 2a2 (Li et al., 2004), ErBß1 to –4 (Mendrola et al., 2002), and the Epo receptor (Kubatzky et al., 2001).

Anisotropy Microscopy and FRET

Anisotropy microscopy was done as described by Squire et al. (Squire et al., 2004) in transiently transfected COS-7 cells. Images were acquired 15–24 hr posttransfection, using a Olympus IX81 inverted microscope (Olympus, Germany) equipped with a MT20 illumination system. A linear dichroic polarizer (Meadowlark Optics, Frederick, Colorado, US) was placed in the illumination path of the microscope, and two identical polarizers were placed in an external filter wheel at orientations parallel and perpendicular to the polarization of the excitation light. The fluorescence was collected via a 20X 0.7 NA air objective, and parallel and polarized emission images were acquired sequentially on an Orca CCD camera (Hamamatsu Photonics, Japan). Data acquisition was controlled by the CellR software supplied by the microscope manufacturer. NGR (or vehicle) was added 1 min after the start of the time lapse at a concentration of 100 ng/ml. Anisotropy values were extracted from image stacks of 30 images acquired in both parallel and perpendicular emission modes every 30 s for a time period of 15 min after NGR addition. For each stack of 30 images acquired in both parallel and perpendicular emission at a concentration of 100 ng/ml. Anisotropy values were extracted from image stacks of 30 images acquired in both parallel and perpendicular emission modes every 30 s for a time period of 15 min after NGR addition. For each stack of 30 images acquired in both parallel and perpendicular emission at a concentration of 100 ng/ml. Anisotropy values were extracted from image stacks of 30 images acquired in both parallel and perpendicular emission modes every 30 s for a time period of 15 min after NGR addition.

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