Research Article

Ligand-independent signaling by disulfide-crosslinked dimers of the p75 neurotrophin receptor

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Summary

Dimerization is recognized as a crucial step in the activation of many plasma membrane receptors. However, a growing number of receptors pre-exist as dimers in the absence of ligand, indicating that, although necessary, dimerization is not always sufficient for signaling. The p75 neurotrophin receptor (p75NTR) forms disulfide-linked dimers at the cell surface independently of ligand binding through Cys257 in its transmembrane domain. Here, we show that crosslinking of p75NTR dimers by cysteine-scanning mutagenesis results in constitutive, ligand-independent activity in several pathways that are normally engaged upon neurotrophin stimulation of native receptors. The activity profiles of different disulfide-crosslinked p75NTR mutants were similar but not identical, suggesting that different configurations of p75NTR dimers might be endowed with different functions. Interestingly, crosslinked p75NTR mutants did not mimic the effects of the myelin inhibitors Nogo or MAG, suggesting the existence of ligand-specific activation mechanisms. Together, these results support a conformational model of p75NTR activation by neurotrophins, and reveal a genetic approach to generate gain-of-function receptor variants with distinct functional profiles.

Key words: Intracellular signaling, Nerve growth factor, Receptor activation, Receptor dimerization

Introduction

Nervous system development and function is regulated by the action of both cell intrinsic determinants and secreted factors. The neurotrophins nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) are among some of the most important regulators of neuronal differentiation and survival (Bibel and Barde, 2000). The mature form of the neurotrophins is released by proteolytic cleavage from a pre-pro-peptide and can interact with cell surface receptors pre-exist as dimers in the absence of ligand, indicating that, although necessary, dimerization is not always sufficient for signaling. The p75 neurotrophin receptor (p75NTR) forms disulfide-linked dimers at the cell surface independently of ligand binding through Cys257 in its transmembrane domain. Here, we show that crosslinking of p75NTR dimers by cysteine-scanning mutagenesis results in constitutive, ligand-independent activity in several pathways that are normally engaged upon neurotrophin stimulation of native receptors. The activity profiles of different disulfide-crosslinked p75NTR mutants were similar but not identical, suggesting that different configurations of p75NTR dimers might be endowed with different functions. Interestingly, crosslinked p75NTR mutants did not mimic the effects of the myelin inhibitors Nogo or MAG, suggesting the existence of ligand-specific activation mechanisms. Together, these results support a conformational model of p75NTR activation by neurotrophins, and reveal a genetic approach to generate gain-of-function receptor variants with distinct functional profiles.

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we set out to test the possibility that p75NTR can be activated by altering its dimeric conformation in the absence of ligand, and to investigate the impact that different dimer configurations might have on receptor signaling.

**Results**

Disulfide-mediated crosslinking of p75NTR dimers

The role of dimer conformation in p75NTR activation and signaling was studied using a cysteine-scanning mutagenesis strategy. A series of consecutive cysteine substitutions were introduced in the extracellular juxtamembrane region of p75NTR to constrain p75NTR dimers at different distances from their native dimerization contact at Cys257 (Fig. 1A). A similar approach has been used to produce disulfide-linked variants of receptor kinases (Burke and Stern, 1998). Surface expression of disulfide-linked p75NTR dimers and monomers was assessed in transfected cells following biotinylation of surface proteins, immunoprecipitation and gel electrophoresis of monomers was assessed in transfected cells following biotinylation under nonreducing and reducing conditions, respectively. As previously observed, wild-type p75NTR generated disulfide-linked dimers and monomers at approximately equal levels, and mutation of Cys257 to alanine abolished dimer formation (Fig. 1B). All cysteine mutants were expressed at levels comparable with wild-type p75NTR at the cell membrane, but generated higher amounts of disulfide-linked dimers (Fig. 1B).

None of the cysteine substitutions prevented NGF binding to p75NTR (Fig. 2A). Moreover, the capacity of the mutants to undergo γ-secretase-dependent intramembrane cleavage was neither augmented or diminished compared with wild-type p75NTR under basal conditions (Fig. 2B). The phorbol ester PMA stimulated cleavage to a similar extent in wild-type and mutant p75NTR constructs (Fig. 2B).

Neurotrophin-independent signaling by disulfide-crosslinked p75NTR dimers

We tested the signaling activities of different disulfide-crosslinked p75NTR mutants and compared them with wild-type p75NTR following ligand activation. The recruitment of the downstream signaling effector RIP2 to wild-type and T248C and T249C mutant receptors was investigated using coimmunoprecipitation experiments in transfected cells. Both T248C and T249C mutants bound constitutively to RIP2 at levels comparable with those observed after stimulation of wild-type p75NTR with NGF (Fig. 3A).

Recruitment of RIP2 to p75NTR has been shown to result in activation of NF-κB (Khursigara et al., 2001). Cerebellar granule cells express endogenous p75NTR and their stimulation with NGF induced NF-κB activity in a time-dependent manner (Fig. 3B). Although overexpression of wild-type p75NTR had no effect on NF-κB activity, transfection of either T248C or T249C mutants induced activation of NF-κB at levels comparable with or above those observed following NGF stimulation of untransfected neurons (Fig. 3B). In fibroblasts lacking endogenous p75NTR, NGF was able to induce NF-κB activity only in cells transfected with wild-type p75NTR (Fig. 3C). Expression of the T248C or T249C mutants could again induce robust NF-κB activity, even in the absence of NGF, and ligand treatment did not increase this effect any further (Fig. 3C). A similar effect was observed with the P242C and D250C mutants (Fig. 3C).

Next, the recruitment of NRIF (Casademunt et al., 1999) and TRAF6 (Khursigara et al., 1999) to wild-type and mutant receptors was investigated in coimmunoprecipitation experiments. All four mutants tested bound NRIF independently of ligand to levels that were similar to those observed with wild-type p75NTR after NGF treatment (Fig. 4A,B). Stimulation with NGF had only a modest effect on the activity of the mutants. T249C was less efficient than the other mutants at recruiting NRIF. Three of the mutants, P242C, T248C and D250C, were also able to interact constitutively with TRAF6 (Fig. 4C,D). Intriguingly, however, T249C did not behave any differently from wild-type p75NTR in TRAF6 recruitment (Fig. 4C), indicating the existence of functional differences between disulfide-crosslinked p75NTR mutants.

We also studied activation of the downstream effectors JNK and caspase-3 in HEK293 cells expressing wild-type or mutant receptors after stimulation with the pro-neurotrophin proBDNF, a potent activator of these signaling pathways (Lu et al., 2005). All four mutants investigated displayed strong ligand-independent activity,
Disulfide-crosslinked p75NTR dimers undergo constitutive axonal retrograde transport and promote cell death independently of ligand binding

Disulfide-crosslinked p75NTR dimers triggered constitutive axonal retrograde transport and cell death, regardless of ligand binding. This suggests that p75NTR dimers can induce these effects independently of ligand engagement.

One of the best characterized biological effects of p75NTR is its role in mediating cell death by caspase-mediated apoptosis (Troy et al., 2002). The ability of crosslinked p75NTR dimers to promote cell death was first observed in HEK293 cells. We observed robust ligand-dependent apoptosis upon proBDNF treatment of cells transfected with wild-type p75NTR and sortilin.

Plasmids encoding wild-type and mutant p75NTR were microinjected into embryonic spinal motoneurons, and axonal trafficking of the expressed receptors was visualized by time-lapse confocal microscopy using antibodies against their N-terminal hemagglutinin (HA) epitope tag. As previously reported (Deinhardt et al., 2007), very few retrograde carriers containing wild-type p75NTR were detected in the absence of NGF, but these were greatly increased upon NGF addition (Fig. 7A,B). By contrast, three mutant receptors tested, P242C, T249C and D250C, displayed constitutive recruitment to axonal retrograde transport carriers in the absence of ligand, albeit at somewhat lower levels than those observed with wild-type p75NTR after NGF stimulation (Fig. 7B,C). NGF treatment of cells carrying these mutant receptors had only a very modest stimulatory effect on transport. Retrograde carriers containing mutant receptors displayed average speeds comparable with those carrying NGF-stimulated wild-type p75NTR, indicating the existence of functional differences between disulfide-crosslinked p75NTR dimers.
as assessed by TUNEL assay (Fig. 8A). Under these conditions, all four dimeric p75NTR mutants tested induced comparable levels of cell death either in the absence or presence of proBDNF (Fig. 8A), indicating constitutive, ligand-independent activity. We then extended this analysis to a more physiological setting and tested the effects of wild-type p75NTR and the T249C mutant after in ovo electroporation in the neural tube of chicken embryos. Mutant and wild-type p75NTR constructs were expressed at comparable levels in electroporated chick neural tube as judged by immunohistochemistry (Fig. 8B; and data not shown).

Overexpression of wild-type p75NTR had virtually no effect on the number of cells showing active caspase-3 compared with the control nonelectroporated side (Fig. 8B,C). However, electroporation of T249C induced a robust increase in caspase-3 activity (Fig. 8B,C). Together, these data support the notion that disulfide-mediated crosslinking of p75NTR dimers can trigger downstream signaling events and biological effects that mimic those elicited by binding of neurotrophins to p75NTR. In addition, the functional differences observed between p75NTR mutants suggest that the relative arrangement of receptor subunits in the p75NTR dimer might be important, and that variations in their configuration might determine significant functional outcomes.

Discussion

Using cysteine-scanning mutagenesis, we have generated a collection of p75NTR dimers constrained at different positions in the extracellular juxtamembrane stalk region of the receptor. This resulted in constitutive, ligand-independent receptor activation with a profile that mimicked signaling events elicited by neurotrophins, but not by other p75NTR ligands such as MAG. In a previous study, we reported that p75NTR forms constitutive dimers at the plasma membrane that are stabilized by both covalent (i.e. through Cys257) and non-covalent intramembrane interactions (Vilar et al., 2009). This suggests that the mechanism by which cysteine substitution

Fig. 4. Neurotrophin-independent signaling to NRIF and TRAF6 by disulfide-crosslinked p75NTR mutants. (A,B) Binding of NRIF to wild-type and mutant p75NTR in transfected HEK293 cells. (C,D) Binding of TRAF6 to wild-type and mutant p75NTR in transfected HEK293 cells.

Fig. 5. Neurotrophin-independent signaling to JNK and caspase-3 by disulfide-crosslinked p75NTR mutants. (A) Phosphorylation of JNK in HEK293 cells transfected with vector or p75NTR constructs in the presence of sortilin. Results are expressed as average of triplicate measurements ± s.d. *P<0.05 vs wild type without proBDNF; n=3. (B) Activation of caspase-3 in HEK293 cells transfected with vector or p75NTR constructs in the presence of sortilin. Results are expressed as average of triplicate measurements ± s.d. *P<0.05 vs wild type without proBDNF; n=3. (C) Phosphorylation of JNK in HEK293 cells transfected with vector or p75NTR constructs in the presence of sortilin. (D) Activation of caspase-3 in HEK293 cells transfected with vector or p75NTR constructs in the presence of sortilin. Arrows denote cleaved caspase-3.

Fig. 6. Disulfide-crosslinked p75NTR mutants do not mimic the activities of myelin ligands MAG and Nogo. (A) Binding of RhoGDI to wild-type p75NTR and T249C mutant in COS cells cotransfected with NgR and Lingo-1 and stimulated with MAG-Fc (top) or Nogo-66 peptide (bottom). Similar results were observed in two additional experiments. (B) RhoA activity in COS cells transfected with wild-type p75NTR and the indicated mutants in the presence of NgR and Lingo-1 following stimulation with MAG-Fc. Results are expressed as mean ± s.d. relative to wt without MAG treatment. *P<0.05 vs control; n=3.
Constructive activation of p75NTR mutants activate p75NTR does not involve receptor dimerization but rather conformational rearrangements of preformed p75NTR dimers. Since the signaling profile of disulfide-crosslinked p75NTR mutants resembled that normally induced by neurotrophin binding to native p75NTR, we suggest that cysteine crosslinking induces similar conformational changes to those elicited by neurotrophin binding. Based on fluorescence resonance energy transfer (FRET) studies of the C257A mutant, we have recently proposed a ‘snail-tong’ model for the conformational changes that underlie the activation of p75NTR in response to neurotrophins (Vilar et al., 2009). In this model, neurotrophin binding is proposed to elicit a scissor-like movement of p75NTR subunits with Cys257 acting as the fulcrum, resulting in the relative separation of intracellular domains. However, it remained unclear in that model whether such conformational rearrangement was brought about by opening or closure of extracellular domains upon ligand binding (Barker, 2009). Our present results show that crosslinking of p75NTR dimers by disulfide bonding of the juxtamembrane region of the receptor extracellular domains mimics activation elicited by neurotrophin binding. This suggests that separation of intracellular domains upon ligand binding is elicited by closure, not opening, of extracellular domains onto the ligand.

The activity profiles of different cysteine substitution mutants were found to be similar but not identical. By constraining the p75NTR dimer at different distances from the Cys257 axis, different mutations would be predicted to cause somewhat different configurations and hence result in different functional profiles, which is in agreement with what we observed. Although the precise molecular bases of those functional differences are unknown at present, it is possible that different cysteine bridges induce different degrees of separation between p75NTR intracellular domains that may favor the recruitment or activation of different subsets of intracellular effectors or downstream pathways. At the molecular level, these differences are likely to entail very small distances and may therefore be difficult to assess experimentally. We speculate that neurotrophin binding to native p75NTR might induce an array...
of receptor configurations, each with its own particular functional bias, but which as a whole encompass the full repertoire of p75NTR functions. It is also possible that different configurations are visited by the same receptor complex during the course of its activation by ligand.

It has been previously shown that p75NTR can be cleaved by γ-secretase in sympathetic neurons in response to proapoptotic ligands, and that inhibition of this cleavage blocked NRIF nuclear entry and prevented apoptosis (Kenchappa et al., 2006). In another study, it was shown that proteolytic cleavage of p75NTR induced by MAG binding to cerebellar neurons was necessary for activation of Rho and inhibition of neurite outgrowth (Domeniconi et al., 2005). Except for these two studies, however, the significance of intramembrane proteolysis for the signaling and biological activities elicited by p75NTR remains by and large poorly understood. Despite displaying elevated, constitutive activity across several signaling pathways, the disulfide-crosslinked p75NTR mutants described in this study did not show increased levels of intramembrane cleavage compared with the wild-type receptor, either under basal conditions or after phorbol ester stimulation. This suggests that intramembrane cleavage might not itself be essential for p75NTR activation, but it could instead have a permissive function in downstream signaling. In other words, although required for activation of some pathways, p75NTR cleavage might not be quantitatively related to the overall level of signaling, as long as some cleavage occurs. It should be noted that several of the signaling events analyzed here, in particular recruitment of downstream effectors, involved the full-length receptor, indicating that downstream signaling is clearly initiated before receptor cleavage and that at least some signaling events can in principle occur independently of it. A full understanding of the importance of intramembrane cleavage in p75NTR signaling will require a systematic survey of its role in all known pathways activated by this receptor in different cell types.

Interestingly, disulfide crosslinking of p75NTR subunits mimicked the effects of neurotrophins and pro-neurotrophins, but not the myelin inhibitors Nogo and MAG. In agreement with this, we previously showed that dimerization of native receptors through Cys257 was required for p75NTR signaling in response to NGF but not to MAG (Vilar et al., 2009). Together, these data suggest that myelin-derived ligands activate p75NTR via a different mechanism from that used by neurotrophins. Although p75NTR dimerization is important for activation by mature and unprocessed neurotrophins, the stoichiometries of the complexes formed by p75NTR with sortilin on the one hand, and NgR and Lingo-1 on the other, need to be further investigated.

In conclusion, the results of this study support a conformational model of p75NTR activation by neurotrophins. Disulfide-crosslinked p75NTR mutants offer a new genetic tool to explore the functions of p75NTR. The ability of these molecules to specifically mimic the activities of neurotrophins, but not of other p75NTR ligands, make them particularly useful for dissecting ligand-specific p75NTR effects in gain-of-function experiments.

Materials and Methods

Plasmids, antibodies and reagents

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 a QuickChange kit (Stratagene) and verified by DNA sequencing. Plasmids to express RIP2, TRAF6, NRIF, sortilin, Lingo-1 and RhoGDI were previously described (Kurshigara et al., 2001; Kurshigara et al., 1999; Nykjaer et al., 2004; Mi et al., 2004; Yamashita et al., 2003). GFP plasmid was from Clontech. Luciferase reporter plasmid for NF-kB was from Promega. The origin of antibodies was as follows: MC192 anti-p75NTR, Phil Barker (Montreal Neurological Institute, McGill University, Montreal, Canada); anti-IA, Roche; anti-Myc, anti-phospho and anti-total JNK, anti-activated caspase-3, anti-RhoA and anti-RhoGDI; Cell Signaling; anti-tubulin, Sigma; anti-RIP2, Santa Cruz. MAG-Fc was purchased from Alomone Labs (Jerusalem, Israel), MAG-Fc and Nogo peptide from R&D, proBDNF was obtained from Masami Kojima (AIST, Tokyo, Japan) and Phyllis Dan (Alomone Labs). Neurotophins were purchased from Alomone Labs, MAG-Fc and Nogo peptide from R&D. Neurotophins were typically applied at 100 ng/ml for 30 minutes unless otherwise indicated. MAG-Fc was used at 25 μg/ml for 30 minutes. PMA was used at 200 nM for 1 hour. Epoxomicin (1 μM) and DAPT (2 μM) were applied 1.5 hours before PMA. All compounds were from Sigma.

Cell transfection and tissue culture

COS-7 cells were transfected with polyethylenimine (PEI), HEK293, PC12, M23 [a derivative of MG87 fibroblasts (Paratcha et al., 2001)] and cerebellar granule neurons were transfected with Lipofectamine 2000 (Gibco). 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 RhoGDI in COS-7, NF-kB in M23 and P-JNK and caspase-3 in HEK293 cells. This might be related to the specific complement of downstream effectors expressed by each cell type. Granule neurons were isolated from postnatal day 4 rat cerebellum and motoneurons from E14.5 rat spinal cord (Deinhardt et al., 2007). Cell lines were cultured under standard conditions and primary neurons in serum-free, N2-supplemented DMEM:F12 medium (Gibco).

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 octylglucoside, 10 mM iodoacetamide and protease inhibitors (Roche). For immunoprecipitation, lysates were precleared for 1 hour with protein-G-Sepharose beads (Amersham) and immunoprecipitated with the appropriate antibody overnight at 4°C. Protein-G-Sepharose beads were incubated with the lysates for 4 hours at 4°C with gentle shaking. Beads were collected by centrifugation, washed four times with lysis buffer, and
resuspended in SDS loading buffer. Proteins were separated by SDS-PAGE, blotted to PVDF membranes (Amer sham), followed by immunoreaction with specific antibodies. For reducing conditions, immunoreagents were boiled in sample buffer containing 1 M DTT. Biotinylated proteins were detected using Neutravidin conjugated to alkaline phosphatase (Sigma). Filters were developed by chemiluminescence (A mer sham) and scanned on a STORM840 fluorimeter (Mol Dynamics). Autoradiography was done on a STORM840 phosphorimager. Quanti fications of immunoblots and autoradiograms were done with ImageQuant software (Mol Dynamics). Statistical analyses were performed with the Student’s t-test.

Assays of NF-κB, RhoA and cell death
NF-κB activity was assayed using the Dual-Luciferase Reporter Assay System kit (Promega). NFκB was added 2 days after transfection and left for 24 hours before cell lysis. RhoA activity was evaluated using the RhoA-G-Lisa kit from Cytoskeleton. Cell death was assessed by the TUNEL method using kits from Roche (In Situ Cell Death Detection) and Biocolor (APOPercentage). NGF was added 2 days after transfection and left for another day in serum-free medium before assay of cell death. Statistical analyses were performed with the Student’s t-test.

Axonal retrograde transport and in ovo electro poration
Assays of axonal retrograde transport in primary motoneurons involved microinjection of p75NTR constructs, cell labeling with fluorescently tagged antibodies and time lapse confocal microscopy as previously described (Deinhardt et al., 2007). For in ovo electro porations, pCAGGs-p75NTR-HA constructs (wt and T249A) and pCAGGs- eGFP were electroporated into spinal cord of Hamburger-Hamilton stage 10-12 chick embryos. After 24 hours, the embryos were fixed in 4% PFA and processed for immunohistochemistry. The number of activated caspase-3-positive cells per section was counted and electro porated and control sides were compared. The counts from 20-22 sections (4-6 embryos) were collected.

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