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 (p75^{NTR}) forms disulfide-linked dimers at the cell surface independently of ligand binding through Cys257 in its transmembrane domain. Here, we show that crosslinking of p75^{NTR} 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 p75^{NTR} mutants were similar but not identical, suggesting that different configurations of p75^{NTR} dimers might be endowed with different functions. Interestingly, crosslinked p75^{NTR} 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 p75^{NTR} 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 two distinct receptors, a member of the Trk receptor tyrosine kinase family and the p75 neurotrophin receptor (p75^{NTR} or NGFR) (Friedman and Greene, 1999; Kaplan and Miller, 2000; Lee et al., 2001a; Patapoutian and Reichardt, 2001). Unprocessed neurotrophins (pro-neurotrophins) are thought to display selectivity for p75^{NTR} over Trk receptors (Lee et al., 2001b) together with the coreceptor sortilin (Nykjaer et al., 2004), an interaction that has been shown to preferentially result in cell death (Lee et al., 2001b).

p75^{NTR} signals via ligand-mediated recruitment and release of cytoplasmic effectors such as receptor-interacting protein 2 (RIP2) (Khursigara et al., 2001), neurotrophin receptor-interacting factor (NRIF) (Casademunt et al., 1999) and TNF-receptor-associated factor 6 (TRAF6) (Khursigara et al., 1999). Some of the major downstream signaling events triggered by p75^{NTR} in response to neurotrophins include activation of NF-κB (Carter et al., 1996), Jun kinase (JNK) (Friedman, 2000; Yoon et al., 1998) and caspases (Troy et al., 2002). p75^{NTR} can also activate the small GTPase RhoA (Yamashita et al., 1999) in response to myelin-associated glycoprotein (MAG) and Nogo (Wang et al., 2002; Wong et al., 2002; Yamashita et al., 2002), but this requires the association of p75^{NTR} with a lipid-anchored ligand-binding subunit known as the Nogo receptor (NgR) (Fournier et al., 2001) and the transmembrane protein Lingo-1 (Mi et al., 2004). p75^{NTR} is also known to undergo proteolytic cleavage upon activation by several of its ligands or induction of membrane metalloproteases with phorbol esters (Jung et al., 2003; Kanning et al., 2003). Although the functional significance of p75^{NTR} cleavage remains to be fully elucidated, some signaling activities, such as NRIF-mediated neuronal death of sympathetic neurons and inhibition of neurite outgrowth by MAG, have been shown to require intramembrane proteolysis of p75^{NTR} by γ-secretase (Domeniconi et al., 2005; Kenchappa et al., 2006).

We have recently shown that p75^{NTR} forms disulfide-linked dimers at the cell surface in the absence of neurotrophins through the highly conserved Cys257 in its transmembrane domain (Vilar et al., 2009). This cysteine residue was found to be required for the recruitment of intracellular effectors and downstream signaling by p75^{NTR} in response to neurotrophins, but not in response to other ligands such as MAG. Cys257 contributes to p75^{NTR} signaling by allowing the propagation of conformational changes from extracellular to intracellular domains following neurotrophin binding. These findings indicated that the mechanism of p75^{NTR} activation by neurotrophins involves the rearrangement of disulfide-linked receptor subunits (Vilar et al., 2009). In the present study,

we set out to test the possibility that p75^{NTR} 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 p75^{NTR} dimers

The role of dimer conformation in p75^{NTR} 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 p75^{NTR} to constrain p75^{NTR} 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 p75^{NTR} dimers and monomers was assessed in transfected cells following biotinylation of surface proteins, immunoprecipitation and gel electrophoresis under nonreducing and reducing conditions, respectively. As previously observed, wild-type p75^{NTR} 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 p75^{NTR} at the cell membrane, but generated higher amounts of disulfide-linked dimers (Fig. 1B).

None of the cysteine substitutions prevented NGF binding to p75^{NTR} (Fig. 2A). Moreover, the capacity of the mutants to undergo γ-secretase-dependent intramembrane cleavage was neither augmented or diminished compared with wild-type p75^{NTR} under basal conditions (Fig. 2B). The phorbol ester PMA stimulated cleavage to a similar extent in wild-type and mutant p75^{NTR} constructs (Fig. 2B).

Neurotrophin-independent signaling by disulfide-crosslinked p75^{NTR} dimers

We tested the signaling activities of different disulfide-crosslinked p75^{NTR} mutants and compared them with wild-type p75^{NTR} 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 p75^{NTR} with NGF (Fig. 3A).

Recruitment of RIP2 to p75^{NTR} has been shown to result in activation of NF-κB (Khursigara et al., 2001). Cerebellar granule cells express endogenous p75^{NTR} and their stimulation with NGF induced NF-κB activity in a time-dependent manner (Fig. 3B). Although overexpression of wild-type p75^{NTR} 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 p75^{NTR}, NGF was able to induce NF-κB activity only in cells transfected with wild-type p75^{NTR} (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 p75^{NTR} 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 p75^{NTR} in TRAF6 recruitment (Fig. 4C), indicating the existence of functional differences between disulfide-crosslinked p75^{NTR} 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,

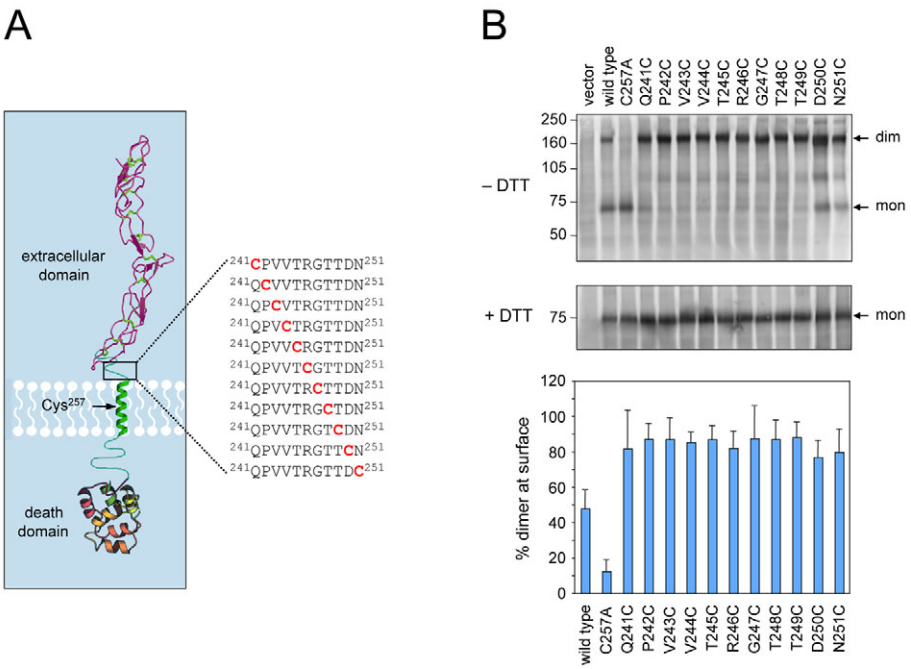

Fig. 1. Disulfide-linked dimers of p75^{NTR}. (A) Cysteine-scanning mutagenesis in the juxtamembrane region of the p75^{NTR} extracellular domain. (B) Cell-surface expression of disulfide-linked p75^{NTR} dimers (dim) and monomers (mon) in transfected COS cells visualized by Neutravidin probing of p75^{NTR} immunoprecipitates under nonreducing (–DTT) and reducing (+DTT) conditions. Note that, in nonreducing conditions, p75^{NTR} dimers run somewhat higher, and monomers lower, than their predicted molecular masses. Results are expressed as average percentage of surface receptor present in the disulfide-linked dimer form ± s.d. of three independent determinations.

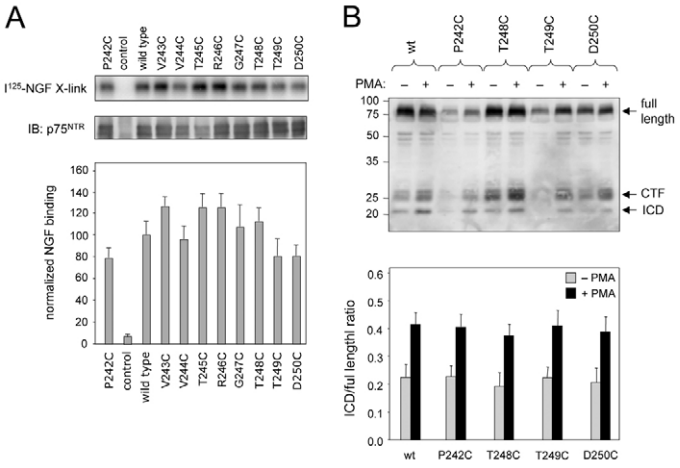

Fig. 2. Disulfide-crosslinked p75^{NTR} mutants display normal NGF binding and intramembrane proteolysis. (A) Binding of ^{125}I-NGF to wild-type and mutant p75^{NTR} analyzed by chemical crosslinking. Samples were run under reducing conditions. For each construct, binding counts were normalized to levels of expression as assessed by immunoblotting (IB). Results are expressed as average ± s.d. of three independent determinations. (B) Intramembrane cleavage of wild-type and and mutant p75^{NTR} following stimulation with PMA in transfected COS cells. The proteasome inhibitor epoxomycin was used to prevent degradation of CTF and ICD fragments. Mutant and wild-type (wt) p75^{NTR} molecules were visualized by immunoblotting. CTF, C-terminal fragment; ICD, intracellular domain. The histogram plots the ratio between ICD and full-length p75^{NTR} constructs expressed as average ± s.d. of three independent experiments.

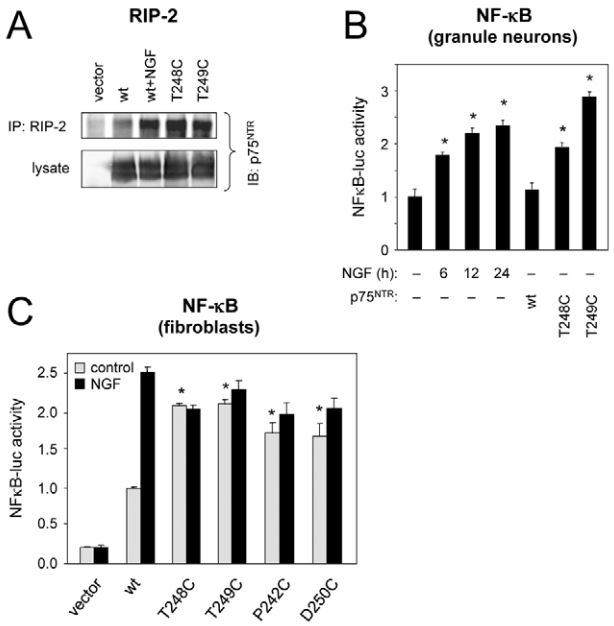

Fig. 3. Neurotrophin-independent signaling to RIP2 and NF-κB by disulfide-crosslinked p75^{NTR} mutants. (A) Binding of RIP2 to wild-type and mutant p75^{NTR} in transfected COS cells assayed by immunoprecipitation (IP) and immunoblotting (IB). (B) NF-κB activity in native and transfected cerebellar granule cells. Results are expressed as average of triplicate measurements ± s.d. *P<0.05 vs control (i.e. without NGF or exogenous p75^{NTR}); n=3. (C) NF-κB activity in transfected M23 fibroblast cells. Results are expressed as average of triplicate measurements ± s.d. *P<0.05 vs wt without NGF; n=3.

inducing JNK phosphorylation and caspase-3 cleavage to levels comparable with those obtained after stimulation of wild-type p75^{NTR} with proBDNF (Fig. 5A,B,C,D).

Finally, we investigated the effects of disulfide-crosslinking mutations in the association of p75^{NTR} with RhoGDI and activation of RhoA. For these experiments, COS cells were transfected with p75^{NTR} along with coreceptors NgR and Lingo-1, and subsequently stimulated with either MAG or a Nogo peptide. Both treatments stimulated the interaction of RhoGDI with wild-type p75^{NTR}, but unlike neurotrophin-mediated activities, no constitutive recruitment was observed with the T249C mutant, which behaved similarly to the wild-type receptor (Fig. 6A). Likewise, neither T248C nor T249C mimicked the effects of MAG stimulation of wild-type p75^{NTR} in a RhoA-activation assay (Fig. 6B). We conclude from these experiments that disulfide-crosslinked p75^{NTR} mutants display constitutive, ligand-independent activity in several pathways that are normally engaged upon neurotrophin binding to native receptors, but do not mimic the effects of non-neurotrophin ligands such as MAG or Nogo.

Disulfide-crosslinked p75^{NTR} dimers undergo constitutive axonal retrograde transport and promote cell death independently of ligand binding

Next, we investigated whether the signaling activities of dimeric p75^{NTR} mutants translated into relevant biological effects in neuronal cells. First, we looked at axonal retrograde transport of p75^{NTR} complexes, which is a prerequisite for cell body-mediated responses to p75^{NTR} signaling in neurons. Although the specific signaling steps leading to retrograde transport of p75^{NTR} are still unknown, this process has recently been shown to be ligand mediated (Deinhardt et al., 2007). Plasmids encoding wild-type and mutant p75^{NTR} 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 p75^{NTR} 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 p75^{NTR} 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 p75^{NTR} (Fig. 7B; and data not shown). Intriguingly, however, the fourth p75^{NTR} mutant tested, T248C, did not behave differently to the wild type in this assay: it showed negligible transport under basal conditions, and was robustly stimulated by NGF treatment (Fig. 7B,C). We conclude that disulfide-mediated crosslinking of p75^{NTR} dimers can trigger constitutive axonal transport in the absence of ligand. The fact that the T248C mutant failed to do this underscores the existence of functional differences between disulfide-crosslinked p75^{NTR} mutants.

One of the best characterized biological effects of p75^{NTR} signaling is the induction of cell death by caspase-mediated apoptosis (Troy et al., 2002). The ability of crosslinked p75^{NTR} mutants to promote cell death was first tested in HEK293 cells. We observed robust ligand-dependent apoptosis upon proBDNF treatment of cells transfected with wild-type p75^{NTR} and sortilin,

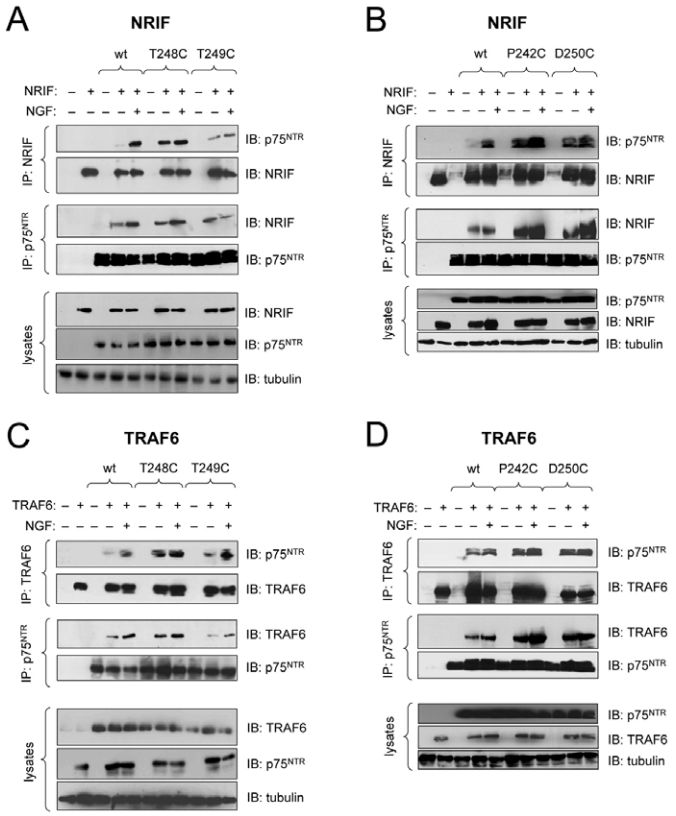

Fig. 4. Neurotrophin-independent signaling to NRIF and TRAF6 by disulfide-crosslinked p75^{NTR} mutants. (A,B) Binding of NRIF to wild-type and mutant p75^{NTR} in transfected HEK293 cells. (C,D) Binding of TRAF6 to wild-type and mutant p75^{NTR} in transfected HEK293 cells.

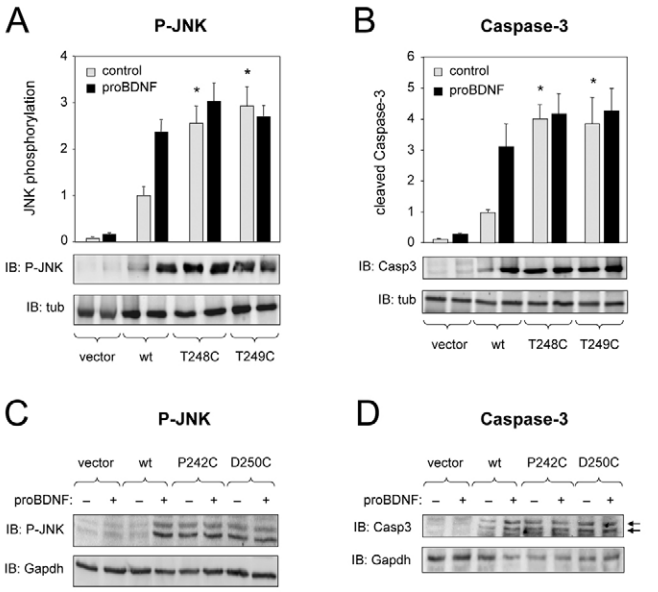

Fig. 5. Neurotrophin-independent signaling to JNK and caspase-3 by disulfide-crosslinked p75^{NTR} mutants. (A) Phosphorylation of JNK in HEK293 cells transfected with vector or p75^{NTR} constructs in the presence of sortilin. Results are expressed as average of triplicate measurements ± s.d. *P<0.05 vs wild type without proBDNF; n=3. (B) Activation of caspase-3 in HEK293 cells transfected with vector or p75^{NTR} 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 p75^{NTR} constructs in the presence of sortilin. (D) Activation of caspase-3 in HEK293 cells transfected with vector or p75^{NTR} constructs in the presence of sortilin. Arrows denote cleaved caspase-3.

as assessed by TUNEL assay (Fig. 8A). Under these conditions, all four dimeric p75^{NTR} 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 p75^{NTR} and the T249C mutant after in ovo electroporation in the neural tube of chicken embryos. Mutant and wild-type p75^{NTR} 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 p75^{NTR} 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 p75^{NTR} dimers can trigger downstream signaling events and biological effects that mimic those elicited by binding of neurotrophins to p75^{NTR}. In addition, the functional differences observed between p75^{NTR} mutants suggest that the relative arrangement of receptor subunits in the p75^{NTR} 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 p75^{NTR} 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 p75^{NTR} ligands such as MAG. In a previous study, we reported that p75^{NTR} 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

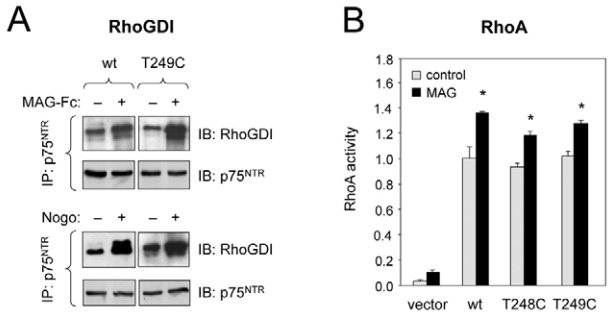

Fig. 6. Disulfide-crosslinked p75^{NTR} mutants do not mimic the activities of myelin ligands MAG and Nogo. (A) Binding of RhoGDI to wild-type p75^{NTR} and T249C mutant in COS cells cotransfected with NgR and Lingo-1 and stimulated with MAG-Fc (top) or Nogo-66 peptide (bottom). Similar results were observed in two additional experiments. (B) RhoA activity in COS cells transfected with wild-type p75^{NTR} 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.

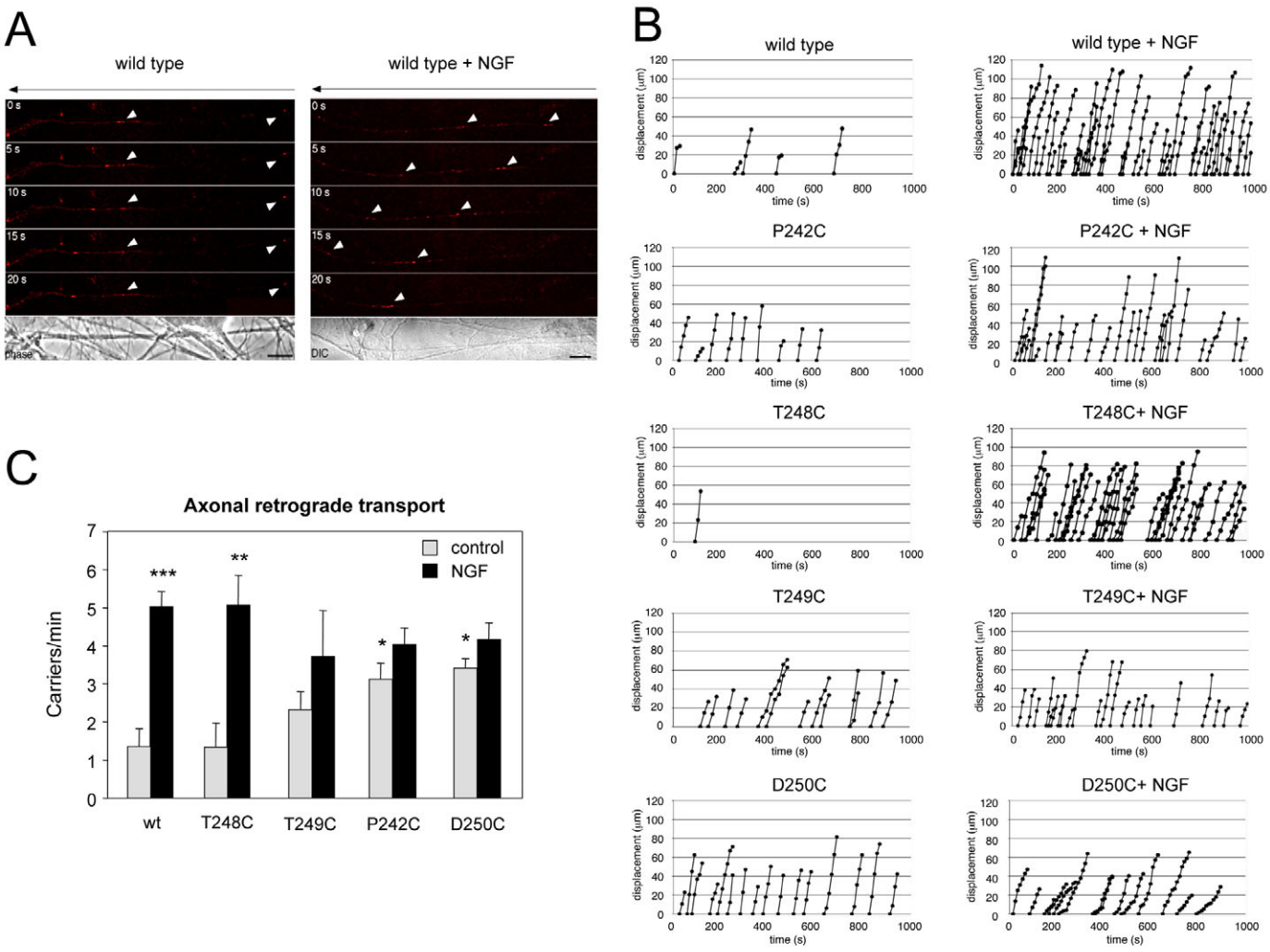

Fig. 7. Constitutive axonal retrograde transport of disulfide-linked p75^{NTR} mutants. (A) Five individual frames from a confocal time series of a motoneuron axon displaying the kinetics of transport of HA-tagged wild-type p75^{NTR} before and after NGF treatment. Arrowheads indicate p75^{NTR}-containing vesicles. Corresponding phase or DIC images are shown below. The retrograde direction is indicated by the arrow on top. Scale bar: 10 µm. (B) Representative displacement graphs of axonal retrograde carriers containing wild-type and p75^{NTR} mutants in the presence and absence of NGF. (C) Quantitative analysis of axonal retrograde transport (carriers per minute) for wild-type and mutant p75^{NTR} constructs. Results are expressed as mean ± s.e.m. of 3-5 independent experiments. Each independent experiment consisted in the analysis of at least three axons. ***P<0.001 vs control; **P<0.01 vs control; *P<0.05 vs wt.

mutants activate p75^{NTR} does not involve receptor dimerization but rather conformational rearrangements of preformed p75^{NTR} dimers. Since the signaling profile of disulfide-crosslinked p75^{NTR} mutants resembled that normally induced by neurotrophin binding to native p75^{NTR}, 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 p75^{NTR} in response to neurotrophins (Vilar et al., 2009). In this model, neurotrophin binding is proposed to elicit a scissor-like movement of p75^{NTR} 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 p75^{NTR} 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 p75^{NTR} 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 p75^{NTR} intracellular domains that may favor the recruitment or activation of different subsets of intracellular effectors or downstream pathways. At the molecular level, those differences are likely to entail very small distances and may therefore be difficult to assess experimentally. We speculate that neurotrophin binding to native p75^{NTR} might induce an array

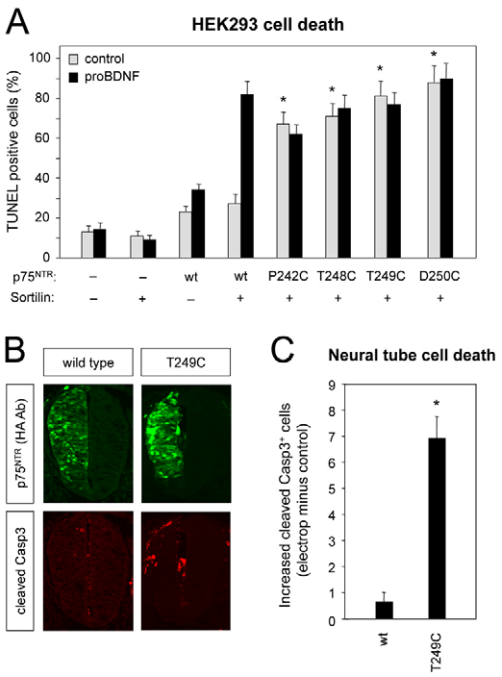

Fig. 8. Disulfide-linked p75^{NTR} mutants induce cell death in vitro and in vivo. (A) Cell death assayed by TUNEL 24 hours after addition of proBDNF in HEK293 cells transfected with p75^{NTR} constructs and sortilin as indicated. Results are expressed as mean ± s.d. of three independent experiments, each performed in duplicate. *P<0.05 vs wt. (B) Expression of p75^{NTR} constructs (HA immunohistochemistry, green) and activated caspase-3 (red) in electroporated chick neural tubes. (C) Analysis of cell death by activated caspase-3 staining in electroporated chick neural tubes. Results are expressed as the difference between electroporated and control sides in the number of active caspase-3-positive cells per section ± s.e.m. (n=4-6 embryos, 20-22 sections were analyzed per embryo).

of receptor configurations, each with its own particular functional bias, but which as a whole encompass the full repertoire of p75^{NTR} 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 p75^{NTR} 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 p75^{NTR} 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 p75^{NTR} remains by and large poorly understood. Despite displaying elevated, constitutive activity across several signaling pathways, the disulfide-crosslinked p75^{NTR} 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 p75^{NTR} activation, but it could instead have a permissive function in downstream signaling. In other words, although required for activation of some pathways, p75^{NTR} 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 p75^{NTR} 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 p75^{NTR} 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 p75^{NTR} signaling in response to NGF but not to MAG (Vilar et al., 2009). Together, these data suggest that myelin-derived ligands activate p75^{NTR} via a different mechanism from that used by neurotrophins. Although p75^{NTR} dimerization is important for activation by mature and unprocessed neurotrophins, the stoichiometries of the complexes formed by p75^{NTR} 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 p75^{NTR} activation by neurotrophins. Disulfide-crosslinked p75^{NTR} mutants offer a new genetic tool to explore the functions of p75^{NTR}. The ability of these molecules to specifically mimic the activities of neurotrophins, but not of other p75^{NTR} ligands, make them particularly useful for dissecting ligand-specific p75^{NTR} effects in gain-of-function experiments.

Materials and Methods

Plasmids, antibodies and proteins

Rat p75^{NTR} 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 QuikChange 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-κB was from Promega. The origin of antibodies were as follows: MC192 anti-p75^{NTR}, Phil Barker (Montreal Neurological Institute, McGill University, Montreal, Canada); anti-HA, 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. NGF 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. Epoxomycin (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-κB 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 (Amersham), followed by immunoreaction with specific antibodies. For reducing conditions, immunoprecipitates were boiled in sample buffer containing 1 M DTT. Biotinylated proteins were detected using Neutravidin conjugated to alkaline phosphatase (Sigma). Filters were developed by chemifluorescence (Amersham) and scanned on a STORM840 fluorimager (MolDynamics). Radioiodination of NGF was done with lactoperoxidase (Sigma), and a mixture of EDAC (5 mM) and SulfoNHS (0.25 mM) (Pierce) was used as chemical crosslinker. Autoradiography was done on a STORM840 phosphorimager. Quantifications of immunoblots and autoradiograms were done with ImageQuant software (MolDynamics). 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). NGF 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 electroporation

Assays of axonal retrograde transport in primary motoneurons involved microinjection of p75^{NTR} constructs, cell labeling with fluorescently tagged antibodies and time-lapse confocal microscopy as previously described (Deinhardt et al., 2007). For in ovo electroporations, pCAGGs-p75^{NTR}-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 electroporated and control sides were compared. The counts from 20-22 sections (4-6 embryos) were collected.

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