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Genetic Dissection of Neurotrophin Signaling through the p75 Neurotrophin Receptor

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SUMMARY

Structural determinants underlying signaling specificity in the tumor necrosis factor receptor superfamily (TNFRSF) are poorly characterized, and it is unclear whether different signaling outputs can be genetically dissociated. The p75 neurotrophin receptor (p75^{NTR}), also known as TNFRSF16, is a key regulator of trophic and injury responses in the nervous system. Here, we describe a genetic approach for dissecting p75^{NTR} signaling and deciphering its underlying logic. Structural determinants important for regulation of cell death, NF-κB, and RhoA pathways were identified in the p75^{NTR} death domain (DD). Proapoptotic and prosurvival pathways mapped onto nonoverlapping epitopes, demonstrating that different signaling outputs can be genetically separated in p75^{NTR}. Dissociation of c-Jun kinase (JNK) and caspase-3 activities indicated that JNK is necessary but not sufficient for p75^{NTR}-mediated cell death. RIP2 recruitment and RhoGDI release were mechanistically linked, indicating that competition for DD binding underlies crosstalk between NF-κB and RhoA pathways in p75^{NTR} signaling. These results provide insights into the logic of p75^{NTR} signaling and pave the way for a genetic dissection of p75^{NTR} function and physiology.

INTRODUCTION

Plasma membrane receptors relay extracellular signals by altering the activities of multiple intracellular effectors and signaling pathways. Understanding how different receptor signaling outputs interact with each other and contribute to changes in cell and animal physiology has been one of the main challenges in signal transduction research. In receptor tyrosine kinases, individual phosphotyrosine residues govern signaling output and specificity. Substitutions in specific intracellular tyrosines can generate receptor mutants that become uncoupled from individual signaling effectors, such as PI3 kinase, Grb2, or PLCγ, providing unparalleled understanding of the physiological relevance of individual receptor signaling outputs. Aside from a handful of receptor tyrosine kinases, however, such level of understanding is either very limited or inexistent for other types of receptors.

Members of the tumor necrosis factor receptor superfamily (TNFRSF) engage different signaling pathways, including NF-κB, c-Jun kinase (JNK), and caspase cascades, through protein-protein interactions mediated by intracellular death domains (DDs), a six-helix bundle globular domain that is essential for TNFRSF signaling (Park et al., 2007a; Haase et al., 2008). However, structural determinants underlying signaling specificity in the TNFRSF are poorly characterized, and it is unclear whether different signaling outputs can be genetically dissociated. p75 neurotrophin receptor (p75^{NTR}), also known as TNFRSF16, is a transmembrane receptor for neurotrophic factors of the neurotrophin family, which includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4 (Dechant and Barde, 2002; Roux and Barker, 2002; Chao, 2003; Gentry et al., 2004; Underwood and Coulson, 2008). Neurotrophin binding to p75^{NTR} can lead to activation of NF-κB and cell survival (Carter et al., 1996; Khursigara et al., 2001), activation of JNK, caspases, and cell death (Yoon et al., 1998; Friedman, 2000), and inhibition of the small GTPase RhoA and axonal growth (Yamashita et al., 1999; Yamashita and Tohyama, 2003). In addition, activation of p75^{NTR} by unprocessed neurotrophins (proneurotrophins) together with the coreceptor sortilin is thought to preferentially result in cell death (Lee et al., 2001; Nykjaer et al., 2004). Like other members of the TNRSF, p75^{NTR} lacks catalytic activity, and signaling proceeds via ligand-induced recruitment and release of cytoplasmic effectors to and from its intracellular domain. Numerous intracellular proteins have been identified by their ability to interact with p75^{NTR} (Dechant and Barde, 2002; Roux and Barker, 2002; Gentry et al., 2004), but, with a few exceptions, their identification has not clarified our understanding of p75^{NTR} function and physiology. How p75^{NTR} connects to different signaling pathways and how these contribute to p75^{NTR} function remain key challenges in the field.

In order to address these questions, we have undertaken a genetic approach to dissect p75^{NTR} signaling and decipher its underlying logic. A comprehensive structure-function analysis was performed on the p75^{NTR} DD (Liepinsh et al., 1997), thereby linking specific structural determinants to each of the three major signaling outputs of p75^{NTR}.

RESULTS AND DISCUSSION

Alanine-Scanning Mutagenesis of the p75^{NTR} DD

An arbitrary cutoff of 50% relative solvent accessibility identified 30 highly exposed amino acid residues in the p75^{NTR} DD (Figures S1A and S1B). Exposed residues are less likely to play structural roles and represent candidate sites for interaction with downstream effector proteins. Three Gly residues playing important structural roles in loops connecting α helices were excluded from the analysis. The remaining 27 residues, as well as Glu^{363} (30% relative solvent accessibility), were targeted by alanine (Ala)-scanning mutagenesis (Cunningham and Wells, 1989). The four Alas in the native sequence were substituted by Asp. With the exception of L342, T343, and A390, all other highly exposed residues are conserved between the DDs of rat and human p75^{NTR}. A total of 22 p75^{NTR} mutants were generated carrying either individual substitutions or combinations. A deletion construct lacking the entire DD (ΔDD), but retaining the juxtamembrane region and the C-terminal tail, was also generated for comparison. All p75^{NTR} mutants were expressed at comparable levels in transfected cells and retained wild-type (WT) activity in at least one, but often more, signaling pathways (see below), indicating that the mutations preserved the structural integrity of the domain.

Functional Epitopes Distributed throughout the p75^{NTR} DD Mediate Activation of Caspase-3 and Cell Death by Proneurotrophins

The ability of WT p75^{NTR} and DD mutants to induce cleavage and activation of caspase-3 in response to pro-BDNF was first tested in transfected HEK293 cells by fluorescence-activated cell-sorting (FACS) analysis. pro-BDNF induced a robust increase in cleaved caspase-3 in cells expressing WT p75^{NTR} after 12 hr treatment but had no effect in vector-transfected cells (Figure 1A). No caspase-3 activation was observed in cells expressing the ΔDD construct. Multiple DD mutants showed an impaired response to pro-BDNF in HEK293 cells (labeled red in Figure 1A). Of the 28 residues probed by Ala-scanning mutagenesis, 13 residues distributed throughout most of the p75^{NTR} DD were found to be involved in the ability of p75^{NTR} to activate caspase-3 in response to pro-BDNF. The complete set of p75^{NTR} mutants was then tested for induction of apoptotic cell death in response to pro-BDNF by TUNEL/FACS assay. There was a good correspondence between activation of caspase-3 and induction of apoptotic death in the functional profiles of the mutants (Figure 1B). The cell death functional map of the p75^{NTR} DD was verified in hippocampal neurons isolated from p75^{NTR} knockout (KO) embryos. Endogenous p75^{NTR} has been shown to mediate cell death in response to neurotrophins in these neurons (Friedman, 2000). Hippocampal neurons transfected with a green fluorescent protein (GFP) reporter along with either empty vector or a subset of mutant p75^{NTR} constructs were stimulated with pro-BDNF and assayed for activation of caspase-3 by immunohistochemistry 12 hr later (Figure 1C). Treatment with pro-BDNF induced robust activation of caspase-3 in KO neurons reconstituted with WT p75^{NTR} but not in neurons receiving empty vector (Figure 1D), indicating that the effect of pro-BDNF was mediated by p75^{NTR}. The ΔDD mutant was unable to activate caspase-3 in response to pro-BDNF. There was a good correspondence between the functional profiles of p75^{NTR} mutants in hippocampal neurons and HEK293 cells. Mutants 341, 341/342/343, 350/353, 378, 392/393, and 404/405 were significantly impaired in their ability to mediate caspase-3 activation in hippocampal neurons in response to pro-BDNF (Figure 1D). Together, these data indicated that functional epitopes distributed throughout the DD are required for induction of neuronal death by p75^{NTR} in response to proneurotrophin stimulation (Figure 1E). Because it is unlikely that a single downstream effector is capable of interacting with all those residues simultaneously, these data suggest that cell death induced by p75^{NTR} requires the interaction of its DD with multiple intracellular proteins. Whether such proteins bind simultaneously to the p75^{NTR} DD or assemble sequentially onto receptor complexes that mature during ligand-mediated activation is unclear at present. p75^{NTR} forms disulphide-linked dimers at the plasma membrane through Cys^{257} in its transmembrane domain (Vilar et al., 2009). It is therefore possible that the two DDs in the activated p75^{NTR} dimer associate with different interactors in the complex that leads to activation of caspase-3 and cell death.

Activation of JNK Is Required, but Not Sufficient, for p75^{NTR}-Mediated Cell Death

Although the requirement of JNK activation for p75^{NTR}-mediated cell death is well established (Casaccia-Bonnefil et al., 1996; Yoon et al., 1998; Friedman, 2000; Bhakar et al., 2003), its sufficiency has not yet been determined. To investigate the activation of JNK downstream of mutant p75^{NTR} molecules, we assessed JNK phosphorylation in response to pro-NGF treatment in HEK293 cells and hippocampal astrocytes transfected with a subset of p75^{NTR} DD mutants. pro-NGF increased JNK phosphorylation via WT p75^{NTR} and triple-mutant D355A/H359A/E363A (abbreviated 355/359/363) (Figures 1F and 1G). In contrast, double-mutant D372A/S373A (abbreviated 372/373) and single-mutant A378D were unable to mediate JNK phosphorylation (Figures 1F and 1G). These data correlated with the profile of these mutants in caspase-3 and cell death assays and support the requirement of JNK activation in p75^{NTR}-mediated cell death. Interestingly, although the P341A mutant was also unable to activate caspase-3 and induce cell death, it retained the ability to induce JNK phosphorylation in response to pro-NGF (Figures 1F and 1G), indicating that JNK activation is not sufficient for p75^{NTR}-mediated cell death and can be genetically dissociated from it. There may be a JNK activation threshold that needs to be exceeded in order to trigger cell death by p75^{NTR}, and the P341A mutant may signal below that threshold. Alternatively, neurotrophin binding to p75^{NTR} may result in the activation of different cellular pools of JNK, not all of which lead to cell death. The P341A mutant may thus be defective in the activation of only the subpool of JNK that couples to caspase-3 activation and cell death. Interestingly, it has been shown that death of cerebellar granule neurons after withdrawal of trophic support requires nuclear, but not cytosolic, JNK activity (Björkblom et al., 2008). Using a pharmacological approach, an earlier study demonstrated that the JNK pathway is essential for p75-mediated death of hippocampal neurons (Friedman, 2000). We used compartment-specific JNK inhibitors

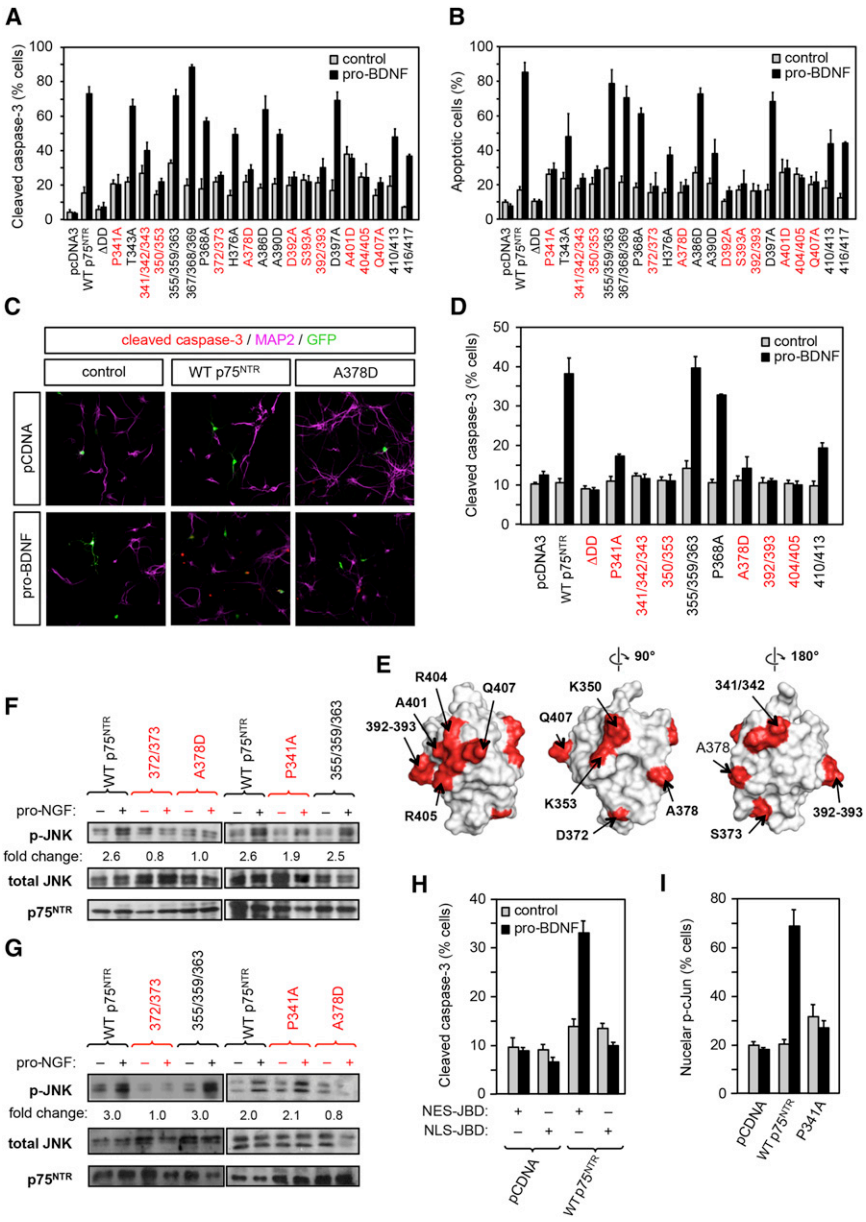

Figure 1. Functional Epitopes in the p75^{NTR} DD that Mediate Activation of Caspase-3, Cell Death, and JNK

(A) Caspase-3 activation by p75^{NTR} DD mutants in HEKs93 cells treated with pro-BDNF as analyzed by FACS. Mutants deficient in pro-BDNF-mediated caspase-3 activation are highlighted in red. Results show the percentage of cells displaying cleaved caspase-3 (from 10,000 cells) as average ± SD of triplicate measurements.

(B) Induction of cell death by p75^{NTR} DD mutants in HEK293 cells treated with pro-BDNF as analyzed by TUNEL/FACS. Mutants deficient in pro-BDNF-mediated cell death are highlighted in red. Results show the percentage of cells displaying TUNEL signal (from 10,000 cells) as average ± SD of three independent experiments.

(C) Hippocampal neurons from p75^{NTR} KO mouse embryos were transfected with the indicated constructs along with a GFP reporter (green), stimulated for 12 hr with pro-BDNF, and stained for activated caspase-3 (red) and MAP-2 (purple).

(D) The proportion of caspase-3 activation among transfected hippocampal neurons was assessed in the presence and absence of pro-BDNF. Mutants deficient in pro-BDNF-mediated caspase-3 activation are highlighted in red. Results are presented as average ± SD of at least three independent experiments each performed in duplicate.

(E) Surface representation of the p75^{NTR} DD with residues involved in caspase-3 activation and cell death highlighted in red. Views on the center and right were rotated 90° and 180°, respectively, compared to that on the left.

(F) Induction of JNK phosphorylation by p75^{NTR} DD mutants in HEK293 cells. Mutants deficient in neurotrophin-dependent caspase-3 activation and cell death are highlighted in red. Fold change was calculated by densitometric scanning of phospho-JNK signals normalized to total JNK levels. Results are representative of three experiments.

(G) Induction of JNK phosphorylation by p75^{NTR} DD mutants in hippocampal astrocytes. Fold change in response to pro-NGF is indicated. Results are representative of three experiments.

(H) Activation of caspase-3 in p75^{NTR} by pro-BDNF in KO hippocampal neurons reconstituted with WT p75^{NTR} or empty vector in the presence of cytoplasmic (NES-JBD) or nuclear (NLS-JBD) JNK inhibitors. Only the nuclear inhibitor was capable of inhibiting activation of caspase-3. Results show the percentage of cells displaying cleaved caspase-3 (average ± SD of triplicate measurements).

(I) Nuclear c-Jun phosphorylation in response to pro-BDNF treatment in KO hippocampal neurons reconstituted with WT p75^{NTR}, P341A mutant, or empty vector. Only WT p75^{NTR}, but not the P341A mutant, was able to induce nuclear c-Jun phosphorylation in response to pro-BDNF. Results show the percentage of cells displaying nuclear phospho-c-Jun staining (average ± SD of triplicate measurements).

(Björkblom et al., 2005) to investigate whether p75^{NTR}-mediated cell death of hippocampal neurons in response to neurotrophins requires cytoplasmic or nuclear JNK activity. Cytoplasmic (NES-JBD) or nuclear (NLS-JBD) constructs capable of inhibiting JNK activity were transfected in hippocampal neurons derived from p75^{NTR} KO mice along with WT p75^{NTR} or empty vector plasmids. Treatment with pro-BDNF resulted in robust increase of activated caspase-3 in p75^{NTR}-transfected neurons that received NES-JBD, but neurons that received NLS-JBD were protected from pro-BDNF-induced apoptosis (Figure 1H), indicating that, similar to cerebellar granule neurons, neurotrophin-induced death of hippocampal neurons through p75^{NTR} also requires nuclear JNK activity. We hypothesized that the P341A mutant may thus be deficient in the activation of the nuclear subpool of JNK. We used phosphorylation of c-Jun (a nuclear target of JNK) as a reporter of nuclear JNK activity and tested the ability of WT p75^{NTR} and P341A mutant to induce nuclear c-Jun phosphorylation in hippocampal neurons derived from p75^{NTR} KO

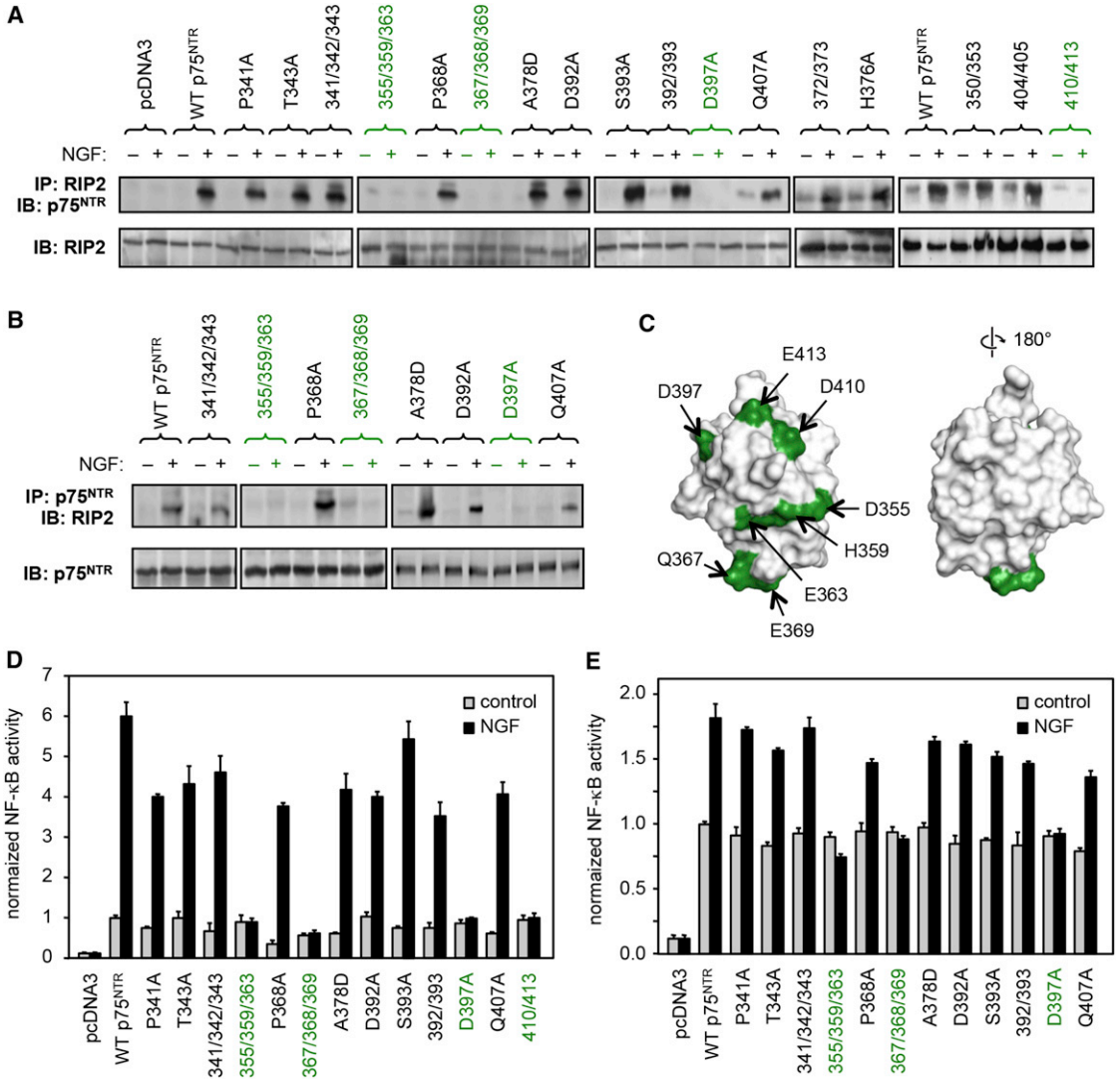

Figure 2. Residues Required for Recruitment of RIP2 and Activation of NF-κB by the p75^{NTR} DD

(A) Coimmunoprecipitation of p75^{NTR} DD mutants and RIP2 in HEK293 cells induced by NGF stimulation. Following immunoprecipitation (IP) with RIP2 antibodies, membranes were probed with p75^{NTR} antibodies (immunoblotting [IB]). Mutants deficient in RIP2 binding are highlighted in green. Similar results were obtained in three independent experiments.

(B) Ligand stimulated coimmunoprecipitation of p75^{NTR} DD mutants and endogenous RIP2 in hippocampal astrocytes. Mutants deficient in RIP2 binding are highlighted in green.

(C) Surface representation of the p75^{NTR} DD with residues involved in RIP2 binding highlighted in green.

(D) NF-κB activity in M23 fibroblasts. Mutants deficient in NGF-dependent activation of NF-κB are highlighted in green. Results are expressed as average of triplicate measurements ± SD normalized to unstimulated WT p75^{NTR}. Similar results were obtained in two independent experiments.

(E) NF-κB activity in hippocampal astrocytes. Mutants deficient in NGF-dependent activation of NF-κB are highlighted in green. Results are expressed as average of triplicate measurements ± SD normalized to unstimulated WT p75^{NTR}.

mice. pro-BDNF treatment induced a marked increase in nuclear c-Jun phosphorylation in KO neurons reconstituted with WT p75^{NTR}, but it had no effect in neurons that received the P341A mutant (Figure 1I), indicating that the mutant is unable to induce nuclear JNK activity. Together, these results suggest that p75^{NTR} signaling leads to the activation of a nuclear subpool of JNK that is required for neuronal cell death and that different JNK-mediated activities can be genetically dissociated in p75^{NTR} signaling.

Residues Required for Recruitment of RIP2 and Activation of NF-κB Concentrate on One Face of the p75^{NTR} DD

Receptor-interacting protein-2 (RIP2) has been shown to interact with the p75^{NTR} DD and mediate the ability of p75^{NTR} to activate NF-κB-dependent transcription and Schwann cell survival (Khursigara et al., 2001). NGF treatment greatly stimulated the interaction between WT p75^{NTR} and RIP2 in transfected HEK293 cells (Figure 2A). Although the majority of p75^{NTR}

mutants behaved as WT, four mutants (labeled green in Figure 2A) showed a dramatic impairment in NGF-dependent recruitment of RIP2. Similar results were obtained for a subset of the mutants in hippocampal astrocytes by assessing the recovery of endogenously expressed RIP2 after immunoprecipitation of transfected $p75^{NTR}$ constructs (Figure 2B). A subset of $p75^{NTR}$ DD mutants was also tested for their ability to enhance NF-κB-mediated transcription after NGF stimulation in M23 fibroblasts and hippocampal astrocytes. There was total correspondence between RIP2 binding and NF-κB activity profiles in both cell types (Figures 2D and 2E), indicating that RIP2 is the main effector linking $p75^{NTR}$ to the NF-κB pathway. In contrast to the caspase-3/cell death pathway, DD residues that mediated RIP2 recruitment and NF-κB activation showed a more restricted distribution, mapping onto one face of the domain (Figure 2C). The caspase-recruitment domain (CARD) at the C terminus of RIP2 has previously been shown to mediate RIP2 binding to the $p75^{NTR}$ DD (Khursigara et al., 2001). DDs, CARDs, and death-effector domains constitute a superfamily of structurally related domains that mediate the formation of large protein complexes between TNFRSF receptors, caspases and a host of adaptor proteins (Park et al., 2007a). These domains interact with each other through six different types of topologically homologous interfaces related to those observed in the crystal structures of the complexes between Tube and Pelle DDs (Xiao et al., 1999), Apaf1 and caspase-9 CARDs (Qin et al., 1999), and PIDD and RAIDD DDs (Park et al., 2007b), respectively. The mutations that disrupted RIP2 binding mapped on the type Ib "Tube-like" (residues 410 and 413) and type IIb "Apaf1-like" (residues 355, 359, 363, 367, and 369) interfaces of the $p75^{NTR}$ DD (Weber and Vincenz, 2001; Park et al., 2007b), suggesting that complex formation with RIP2 and perhaps a third CARD- or DD-containing protein is required for activation of the NF-κB pathway by $p75^{NTR}$.

Different Structural Determinants in the $p75^{NTR}$ DD Mediate RhoGDI Binding and Release

The third major signaling pathway regulated by $p75^{NTR}$ involves activation of the small GTPase RhoA by constitutive binding of the $p75^{NTR}$ DD to Rho GDP dissociation inhibitor (RhoGDI), thereby preventing RhoGDI from inhibiting RhoA (Yamashita et al., 1999; Yamashita and Tohyama, 2003). NGF binding to $p75^{NTR}$ releases RhoGDI resulting in RhoA inhibition (Yamashita and Tohyama, 2003). As expected, WT $p75^{NTR}$ interacted with RhoGDI and activated RhoA in HEK293 cells, whereas NGF treatment decreased RhoGDI binding and RhoA activity (Figures 3A and 3B). Two types of phenotypes were identified in our collection of $p75^{NTR}$ DD mutants. Double mutants K350A/N353A (abbreviated 350/353) and D410A/E413A (abbreviated 410/413) were unable to bind RhoGDI and elevate RhoA activity (Figures 3A and 3B, yellow). Neither these mutants nor ΔDD was affected by NGF treatment. Residues 350/353 and 410/413 cluster close to each other forming a tight functional epitope near the N and C termini of the domain (Figure 3C), suggesting that they represent a RhoGDI binding site in the $p75^{NTR}$ DD. This RhoGDI epitope showed good correspondence with one of the two regions targeted by Pep5, a small peptide isolated in a phage display screen for its ability to bind to the $p75^{NTR}$ DD (Ilag et al., 1999). Pep5 has been shown to inhibit RhoGDI binding to $p75^{NTR}$ (Yamashita and Tohyama, 2003) and to block the ability of $p75^{NTR}$ to activate RhoA (Park et al., 2010). These observations are consistent with the idea that RhoGDI and Pep5 bind to the same epitope on the $p75^{NTR}$ DD.

RIP2 Is Required for the Release of RhoGDI from $p75^{NTR}$ in Response to NGF

A second set of mutants bound RhoGDI and enhanced RhoA activity similar to WT $p75^{NTR}$ but failed to release RhoGDI and decrease RhoA activity in response to NGF (Figures 3A and 3B, green). This was unexpected because loss-of-function mutations normally result in weaker, not tighter, binding. Remarkably, this was the same set of mutants that showed deficient RIP2 binding (Figures 2A–2C). The fact that both RhoGDI and RIP2 required residues 410/413 for binding to the $p75^{NTR}$ DD suggested a competitive interaction between the two effectors, such that recruitment of RIP2 mediates the release of RhoGDI. This notion was investigated in mouse embryo fibroblasts (MEF) derived from RIP2 KO mice (Kobayashi et al., 2002). MEF cells express RIP2 and RhoGDI endogenously but lack $p75^{NTR}$. As expected, endogenous RhoGDI was found associated with $p75^{NTR}$ in transfected WT MEF cells, and this interaction was decreased by NGF treatment (Figure 3D, left). RhoGDI was also coimmunoprecipitated with $p75^{NTR}$ in RIP2 KO MEF cells, but in contrast to WT cells, NGF treatment had no effect on RhoGDI binding to $p75^{NTR}$ (Figure 3D, right). Importantly, the ability of NGF treatment to release RhoGDI from the receptor was restored after transfection of RIP2 in KO MEF cells (Figure 3E). These data support the idea that RIP2 and RhoGDI compete for binding to the $p75^{NTR}$ DD. They also bring a note of caution on the use of Pep5 to displace RhoGDI from $p75^{NTR}$ because this peptide could also be interfering with the regulation of the NF-κB pathway. Together, our results reveal an unexpected mechanistic link between the NF-κB and RhoA pathways in $p75^{NTR}$ signaling.

Conclusions

The three major pathways activated by neurotrophin binding to $p75^{NTR}$ have so far been studied in isolation, and it has been unclear whether—or how—they are mechanistically linked at the level of the receptor. The mechanisms by which $p75^{NTR}$ couples to these pathways and how they contribute to $p75^{NTR}$ function have been outstanding questions in the field. We have elucidated a structure-function map of the $p75^{NTR}$ DD linking individual residues to distinct interactors and downstream signaling pathways (Figures 4A and 4B). This study demonstrates that the major signaling outputs of $p75^{NTR}$ are genetically separable at the level of the receptor and that, in a way analogous to receptor tyrosine kinases, it is possible to generate $p75^{NTR}$ mutants that are selectively deficient in one pathway but not others. We present examples of how this knowledge can be used to reveal the underlying logic of $p75^{NTR}$ signaling. This structure-function map can now serve as a conceptual and technical framework for clarifying the physiological relevance of each of the major signaling pathways regulated by $p75^{NTR}$ and other TNFRSF receptors and for aiding in the discovery of new strategies for inhibiting $p75^{NTR}$ signaling in nervous system injury and degeneration.

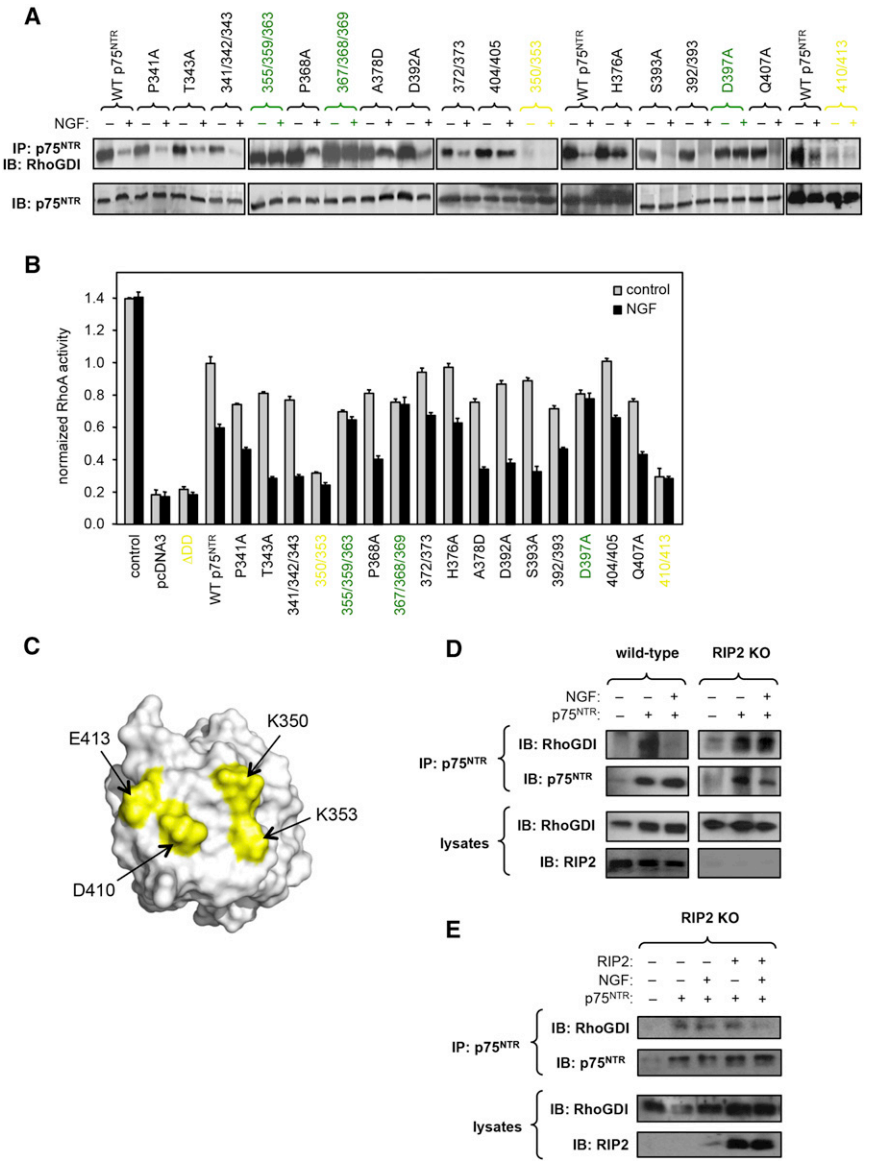

Figure 3. Different Structural Determinants in the p75^{NTR} DD Mediate RhoGDI Binding and Release

(A) Coimmunoprecipitation of p75^{NTR} DD mutants and RhoGDI in HEK293 cells. NGF induces the release of RhoGDI from WT p75^{NTR}. Mutants deficient in RhoGDI binding are highlighted in yellow. Mutants deficient in NGF-dependent RhoGDI release are highlighted in green. Similar results were obtained in three independent experiments.

(B) Regulation of RhoA activity by p75^{NTR} DD mutants in HEK293 cells. Expression of WT p75^{NTR} increases RhoA activity, and this is decreased by NGF. Mutants that fail to increase RhoA activity are highlighted in yellow. Mutants that fail to respond to NGF are highlighted in green. Constitutively active RhoA protein (provided by the kit manufacturer) was used as positive control. Results are expressed as average of triplicate measurements ± SD normalized to unstimulated WT p75^{NTR}. Similar results were obtained in two independent experiments.

(C) Surface representation of the p75^{NTR} DD with residues involved in RhoGDI binding highlighted in yellow.

(D) Coimmunoprecipitation of RhoGDI with WT p75^{NTR} in MEFs isolated from WT or RIP2 KO mice before and after NGF stimulation. Endogenous RhoGDI coimmunoprecipitates with p75^{NTR} in both cells, but NGF treatment can only release RhoGDI from the receptor in WT MEF cells.

(E) Transfection of RIP2 in RIP2 KO MEF cells rescues the ability of NGF to displace RhoGDI from p75^{NTR}.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, and Proteins

Full-length rat p75^{NTR} was expressed from a pCDNA3 vector backbone (Invitrogen). Mutations were introduced using QuikChange Site-Directed Mutagenesis Kit (Stratagene) and verified by DNA sequencing. Normal expression of all p75^{NTR} constructs was verified by immunoblotting and FACS. Plasmids to express RIP2 and RhoGDI were previously described by Vilar et al. (2009). EGFP plasmid was from Clontech. Luciferase reporter plasmid for NF-κB was from Promega. NES-JBD and NLS-JBD constructs were previously described by Björkblom et al. (2005, 2008). The origin of antibodies was as follows: MC192 anti-p75^{NTR} (for immunoprecipitation) from Millipore; 9992 anti-p75^{NTR} (for immunoblotting) from Promega; anti-phospho-JNK, anti-JNK, cleavage-specific anti-caspase-3 from Cell Signaling; anti-RIP2 from Enzo Life Sciences; anti-RhoGDI, anti-tubulin, and anti-MAP2 from Sigma-Aldrich; and anti-myc from Santa Cruz Biotechnology. NGF, pro-NGF, and pro-BDNF were purchased from Alomone Labs. Both proneurotrophins were mouse mutant-uncleavable forms. NGF was used at 100 ng/ml; pro-NGF and pro-BDNF were used at 20 ng/ml.

Tissue Culture and Cell Transfection

HEK293 and M23 cells were cultured under standard conditions in DMEM supplemented with 10% fetal calf serum. The HEK293 cells used in this study were found to express endogenous sortilin. M23 is a clonal derivative of MG87 cells, originally derived from mouse NIH 3T3 fibroblasts. WT and RIP2 KO MEFs were obtained from Koichi Kobayashi and Richard Flavell (Kobayashi et al., 2002) and cultured under standard conditions. Cell lines were transfected with Lipofectamine 2000 (GIBCO). Primary hippocampal neurons were prepared from E16–E17 p75 KO mice obtained from Jackson Labs and originally described by Lee et al. (1992). Neurons were maintained in Neurobasal supplemented with 2% B27 (Invitrogen), 1 mM glutamine, and pen/strep mix (GIBCO). Transfection was performed after 3 days in vitro with Lipofectamine LTX (Invitrogen). Neurons were treated with vehicle or 20 ng/ml pro-BDNF for 12 hr before fixation. Hippocampal astrocytes were isolated from newborn (P0–P1) rat pups and cultured in DMEM (GIBCO) supplemented with 10% fetal bovine serum (FBS) and pen/strep. After 7–10 days in culture, cells were dissociated and replated. Astrocytes showed no detectable p75^{NTR} expression after two passages. Second-passage astrocytes were transfected with Lipofectamine 2000. Animal protocols were approved by Stockholms Norra djurförsöksetiska nämnd and are in accordance with the ethical guidelines of the Karolinska Institute.

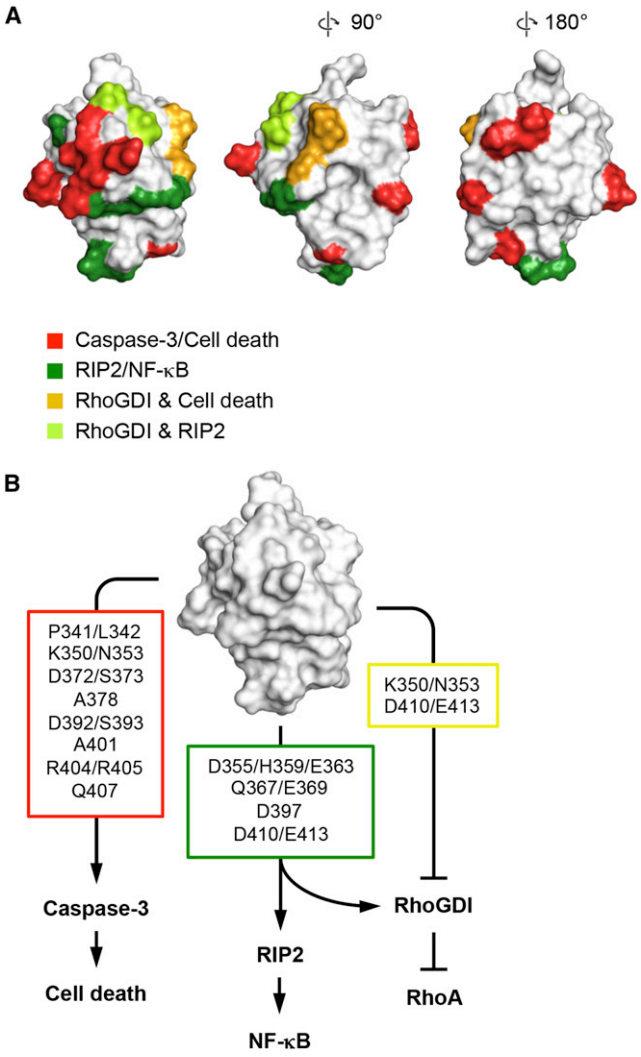

Figure 4. A Structure-Function Map of the p75^{NTR} DD

(A) Surface representation of the p75^{NTR} DD with residues involved in caspase-3 activation/cell death, RIP2 binding/NF-κB activity, and RhoGDI binding/RhoA activity highlighted as indicated. Views on the center and right were rotated 90° and 180°, respectively, compared to that on the left. Views are similar to those shown in Figures S1B, 1E, and 2C.

(B) Summary of DD residues involved in p75^{NTR} signaling and their function. Arrowheads denote a positive effect on the corresponding effector or pathway. Residues in the yellow rectangle are required for RhoGDI binding, thereby preventing it from inhibiting RhoA. Residues in the green rectangle are required for RhoGDI release, thereby allowing it to inhibit RhoA. Neurotrophin-dependent binding of RIP2 and activation of NF-κB are thus mechanistically linked to release of RhoGDI and downregulation of RhoA activity.

Immunoprecipitation and Immunoblotting

After 48 hr transfection, cells were starved from serum for a few hours and stimulated with neurotrophins for 15–30 min as indicated. Lysates were immunoprecipitated with the appropriate antibody overnight at 4°C with gentle shaking. Immunoblots were developed using the ECL Western Blotting Kit (Thermo Scientific) and exposed to Kodak X-Omat AR films. Image analysis and quantification of band intensities were done with ImageQuant (GE Healthcare).

Immunocytochemistry

For immunostaining, fixed cells were incubated at 4°C overnight with anti-cleaved caspase-3 (1:500) and monoclonal anti-MAP2 (1:400) antibodies, followed by incubation with anti-rabbit-555 and anti-mouse-647 Alexa-conjugated secondary antibodies (Molecular Probes; 1:2,000). GFP/MAP2-positive cells were analyzed with a LSM Imager Z2 confocal microscope (Zeiss) to detect cleaved caspase-3, and normalized to the total number of GFP/MAP2-positive neurons to estimate the extent of cell death among transfected neurons.

Flow Cytometry

HEK293 cells were stained using the FITC Active Caspase-3 Apoptosis Kit (BD Biosciences) or assessed by TUNEL using kit from Roche. Flow cytometry analysis was performed on a Becton-Dickinson FACSArray apparatus using CELLQuest software (BD Biosciences).

Assays of NF-κB and RhoA Activity

NF-κB activity was assayed in M23 cells using the Dual-Luciferase Reporter Assay System kit (Promega). NGF was added 24 hr after transfection and left overnight prior to cell lysis. RhoA activity was evaluated after 30 min neurotrophin stimulation using the RhoA G-Lisa kit from Cytoskeleton.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.11.009.

LICENSING INFORMATION

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