TRAF Family Proteins Interact with the Common Neurotrophin Receptor and Modulate Apoptosis Induction*

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The common neurotrophin receptor, p75 NTR, has been shown to signal in the absence of Trk tyrosine kinase receptors, including induction of neural apoptosis and activation of NF-κB. However, the mechanisms by which p75 NTR initiates these intracellular signal transduction pathways are unknown. Here we report interactions between p75 NTR and the six members of TRAF (tumor necrosis factor receptor-associated factors) family proteins. The binding of different TRAF proteins to p75 NTR was mapped to distinct regions in p75 NTR. Furthermore, TRAF4 interacted with dimeric p75 NTR, whereas TRAF2 interacted preferentially with monomeric p75 NTR. TRAF2-p75 NTR, TRAF4-p75 NTR, and TRAF6-p75 NTR interactions modulated p75 NTR-induced cell death and NF-κB activation with contrasting effects. Coexpression of TRAF2 with p75 NTR enhanced cell death, whereas coexpression of TRAF6 was cytoprotective. Furthermore, overexpression of TRAF4 abrogated the ability of dimerization to prevent the induction of apoptosis normally mediated by monomeric p75 NTR. TRAF4 also inhibited the NF-κB response, whereas TRAF2 and TRAF6 enhanced p75 NTR-induced NF-κB activation. These results demonstrate that TRAF family proteins interact with p75 NTR and differentially modulate its NF-κB activation and cell death induction.

The neurotrophins, including NGF,1 brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4/5, are critical for differentiation and survival of specific neuronal populations during development. The cellular responses to neurotrophins are elicited via two specific surface receptors: Trk (tropomyosin-related kinase) tyrosine kinase receptors and the common neurotrophin receptor, p75 NTR (1). Trk proteins have intrinsic tyrosine kinase activities. Binding of neurotrophins to Trks activates their kinase domains, triggering downstream Ras signaling pathways, which are responsible for a series of trophic factor responses, e.g. neuronal survival, neurite outgrowth from cultured neurons, and differentiation of PC12 cells (2).

In comparison to Trk family members, p75 NTR functions are less well established and remain controversial (3–5). p75 NTR has been shown to facilitate and regulate the function of Trk receptors (6, 7). However, p75 NTR is also capable of triggering cellular responses independent of Trk receptors (7). p75 NTR confers neurotrophin dependence in neural cells (8, 9); in the absence of neurotrophins, overexpression of p75 NTR induces apoptosis. Furthermore, studies of p75-null mice revealed increased numbers and somal sizes of medial septal and diagonal band cholinergic neurons compared with control animals (10, 11). These findings indicate that p75 NTR is likely to be involved in the programmed cell death of this population of neurons. In addition, p75 NTR has been reported to mediate NGF-induced NF-κB (nuclear factor κB) activation in Schwann cells (12) and SAPK/JNK (stress-activated protein kinase or c-Jun N-terminal kinase) activation in oligodendrocytes (13).

p75 NTR belongs to the TNFR/NGFR cell surface receptor superfamily (14). Based on their intracellular domains, the TNFR/NGFR receptor superfamily members are divided into two major subgroups: one group that includes the so-called “death domain” and another that does not (4). Members of the former group, including Fas and TNFR 1, typically mediate apoptosis. In contrast, members of the latter group often differentially deliver signals for either induction or suppression of apoptosis, depending upon the cell contexts. Remarkably, compared with the extensive literature describing signal transduction of TNFR, FAS/APO-1, and other members of this cytokine receptor family, little is known about the p75 NTR signal transduction pathways. Ligand binding to TNFR and FAS/APO-1 induces receptor aggregation, recruiting cytoplasmic signaling proteins such as FADD and pro-caspase-8 to the receptor complex (15). However, this has not been shown to be the case for p75 NTR.

One family of signaling proteins in the TNF receptor complex is composed of the TRAF family proteins, originally described by Goeddel and colleagues (16). TRAF proteins were isolated based on their ability to interact with the cytosolic domains of specific TNFR family members. To date, six members of the TRAF family have been identified. These TRAF proteins have...
two main characteristics: first, all contain conserved C-terminal TRAF domains, which are responsible for homo- or hetero-
ligomerization and for the interaction with the cytoplasmic regions of specific TNFR superfamily receptors (16–19). Sec-
ond, all TRAF proteins other than TRAF1 contain a N-termi-
nal RING finger structure and multiple zinc fingers, which appear to be critical for their effector functions (17, 20).

TRAF proteins are signal transduction adapter proteins. TRAF2, -5, and -6 have been shown to be mediators of both NF-kB activation (20–22) and SAPK/JNK activation (23, 24). The activation processes involve successes of protein-protein interactions and phosphorylation of protein kinases. TRAF2, TRAF5, and TRAF6 interact with the downstream kinase NF-kB inducing kinase (24, 25), which in turn interacts with the kinases within the IκB kinase complex (26, 27). In addition, the death domain kinase (reporter-interacting protein) (28, 29) and the serine-threonine kinase IRAK (21) have also been shown to interact with TRAF proteins and mediate NF-kB activation. On the other hand, apoptosis signal-regulating kinase ASK1, a TRAF interacting kinase, was recently demonstrated to be a downstream target of TRAF2, TRAF5, and TRAF6 in the JNK signaling pathway (30).

To investigate signal transduction mediated by p75NTR, we analyzed the interaction between p75NTR and the TRAF family members. Here we show that all six TRAF proteins interact with p75NTR. These proteins constitute the first group of adapter molecules to be identified for p75NTR. We also demonstrate that TRAF2 and TRAF4 bind to distinct domains of p75NTR, and TRAF2, TRAF4, and TRAF6 differentially modulate the ability of this receptor to induce cell death and NF-kB activation.

MATERIALS AND METHODS

Cell Culture and Transfection Procedures—Immortalized 293T cells, derived from human embryonic kidney, were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma). Transient transfections of HEK 293T cells were performed using a modified calcium-phosphate procedure as described previously (31). Efficiency of transfection was estimated in parallel experiments using eukaryotic assay vector pCH110 (Amersham Pharmacia Biotech). pCH110 contains the gene encoding β-galactosidase under control of the SV40 early promoter. Cells that express β-galac-
tosidase were monitored by 5-bromo-chloro-3-indolyl-
DNS-phosphate staining.

Plasmid Constructs—The coding region of human TRAF1, mouse TRAF5, and human TRAF6 were amplified by polymerase chain reaction from pSG5-TRAF1 (gift of G. Mosialos), pMKITNeo-HA-mTRAF5, and pGEM-TRAF6 (gift of C. Ware), respectively. The primers used for polymerase chain reaction are:

- 5′-CGCGGATCCACCTGGCGACATCTGGCAGGC-3′ (for p75IC- N82).
- 5′-CGCGGATCCACCTGGCGACATCTGGCAGGC-3′ (for p75IC-
N82).
- 5′-ACGTCGACTACAG-GGGGATG-3′ (for p75IC- C83).
- 5′-ACGTCGACTACAG-GGGGATG-3′ (for p75IC- C83).
- 5′-ACGTCGACTACAG-GGGGATG-3′ (for p75IC- C83).

GST:p75IC was cloned into the pGEX-4T-1 vector and expressed in E. coli. Anti-GST antibody (Amersham Pharmacia Biotech) was used for immunoblotting.

Association of TRAF Family Members with p75NTR—In light of the signal transduction mediated by the interactions between TNFR family members and the TRAF proteins, we inves-
tigated the potential interactions between p75NTR and TRAF family members. Flag-tagged p75NTR and HA- or Myc-
tagged TRAF1, -2, -3, -4, -5, and -6 were coexpressed in HEK 293T cells and immunoprecipitated with anti-Flag antibody. After the immune complexes were subjected to immunoblotting analysis, the association of the TRAF proteins with p75NTR was determined using the Boehringer Luciferase Reporter Gene Assay kit according to the manufacturer's instruction.

Immunoblotting—One-dimensional immunoblotting experiments using antibodies raised against p75NTR (Promega), Myc epitope (Babco), or HA epitope (Roche Molecular Biochemicals) were performed as described previously (36). The results were detected with the ECL system (Amersham) and autoradiographs were recorded onto X-Omat AR films (Kodak). The analysis was performed within the range of linearity of the film.

Cell Death Assay—Cell death was analyzed using the trypan blue staining procedure as described previously (37). 48 h after transfection, cells were treated with 17 μM tamoxifen (Sigma) for 2 h before being collected, washed in serum-free media, and resuspended in serum-free medium. Cells were harvested and counted. The percentage of cell death was determined as the percent-
age of trypan blue positive cells in each sample.

Generation of GST Fusion Protein and in Vitro Binding Assays—pGEX-4T-1, p75IC-N82, p75IC-C83, p75IC- C83, and p75IC-
C83 were polymerase chain reaction amplified and cloned into GST (glutathione S-transferase) fusion expression vectors (Amersham Pharmacia Biotech). The 5′ primers were: 1) 5′-CGCGGATCCCGAAT-
GAGTGTTGACAGCCTG3′ (for p75IC and p75IC-C83); 2) 5′-CGCGGATCCACCTGGCGACATCTGGCAGGC-3′ (for p75IC-
N82). The 3′ primers were: 1) 5′-ACGTCGACTCACTACAGGAG-
TTCGACCTGAC-3′ (for GST:p75IC and C83, 2) 5′-ACGTCGACTCACTAG-
GGACCTGAGCTAG-3′ (for GST:p75IC-C83); and 3) 5′-CGGAAATC-
TGGTACCATCCAAATGTCACG3′ (for GST:p75IC-N82). GST:

RESULTS

Association of TRAF Family Members with p75NTR—In light of the signal transduction mediated by the interactions between TNFR family members and the TRAF proteins, we inves-
tigated the potential interactions between p75NTR and TRAF family members. Flag-tagged p75NTR and HA- or Myc-
tagged TRAF1, -2, -3, -4, -5, and -6 were coexpressed in HEK 293T cells and immunoprecipitated with anti-Flag antibody. After the immune complexes were subjected to immunoblotting with anti-TRAF2, anti-HA or anti-Myc antibodies, p75NTR was found to associate with all of the TRAF proteins (Fig. 1). These findings suggested that p75NTR bound to TRAF proteins via the conserved C-terminal TRAF domain. In support of this idea, N-terminal deletion mutants of TRAF2 and 4, which retained the TRAF domains, were found to associate with p75NTR (Fig. 1, B and C).

The intracellular domain of p75NTR contains two distinct regions: a highly conserved and unstructured juxtamembrane region and a six-helical bundle type II death domain-like sequence in the C terminus (38). To map the domains required for

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TRAF interaction within p75NTR, we utilized a GST pull-down assay. Various deletion mutants of GST-p75IC fusion proteins were incubated with TRAF2 or TRAF4 in HEK 293T cell extracts. Immunoblotting of the proteins precipitated by the glutathione-Sepharose showed that TRAF2 interacted with the C-terminal helical region of the p75IC (Thr327-Leu386) (Fig. 2B). In contrast, TRAF4 associated with the N-terminal juxtamembrane region of p75IC (Lys245-Gly313) (Fig. 2B). In a recent report, it was shown that TRAF6 also bound to the juxtamembrane region of p75NTR (39).

Activation of NF-κB by p75NTR Is Modulated by TRAF Proteins—TRAF2, -5, and -6 have been demonstrated to be mediators of NF-κB activation initiated by multiple TNFR family members (20–22). Previous studies indicated that p75NTR may induce the nuclear translocation of the p65 subunit of NF-κB in Schwann cells following NGF binding (12). To determine whether TRAF proteins have any effect on p75NTR-mediated NF-κB activation, we utilized a luciferase reporter system in HEK 293T cells. Overexpression of p75NTR in HEK 293T cells led to a modest activation of NF-κB, measured by NF-κB dependent luciferase activity (data not shown).

Both TRAF2-p75NTR and TRAF6-p75NTR coexpression had synergistic effects on NF-κB activation. Overexpression of TRAF2 by itself led to an approximately 5-fold induction of NF-κB dependent luciferase activity. While expression of p75NTR alone led to only a modest NF-κB activation of 3-fold, coexpression of TRAF2 with p75NTR led to an approximately 12-fold increase (Fig. 3A). In contrast to the positive effect on NF-κB activation conferred by TRAF2 and TRAF6, TRAF4 had an inhibitory effect on NF-κB activation induced by TRAF2 or TRAF6 (Fig. 3B), suggesting the possible competition between TRAF4 and TRAF6 for NF-κB activation.

We further analyzed whether TRAF4 could affect NF-κB activation induced by TRAF2/p75NTR or TRAF6/p75NTR. Interestingly, TRAF4 selectively blocked NF-κB activation induced by co-transfection of TRAF6 and p75NTR (Fig. 3D), suggesting the possibility of competition between TRAF4 and TRAF6 for p75NTR. TRAF4 expression did not affect NF-κB activation by TRAF6 alone, nor did it affect NF-κB activation by TRAF2 or TRAF2/p75NTR (Fig. 3D).

p75NTR-induced Apoptosis Is Modulated by TRAFs in a Multimerization-dependent Fashion—One of the important functions of p75NTR is apoptosis induction, which has been described both in the absence of neurotrophins (8, 9, 11) and following neurotrophin binding (43, 44). In cases in which p75NTR was shown to sensitize cells to apoptosis in the absence...
of ligand binding, the pro-apoptotic effect of p75NTR overexpression could be reversed by dimeric peptides derived from NGF (45), FKBP based dimerizing drugs,2 or (dimeric) neurotrophins (8). Therefore, we evaluated the effects of TRAF family members on p75NTR-induced apoptosis, with and without enforced dimerization.

We utilized the fusion construct of p75IC and the FK506-binding protein (FKBP), pFKBP-p75IC, described by Wang et al.2 This construct allows enforced dimerization of p75IC upon addition of the divalent ligand AP1510 2 (46). As described,2 expression of monomeric p75IC in the absence of AP1510-induced cell death in HEK 293T cells. Addition of AP1510 led to the dimerization of p75IC and inhibition of cell death, demonstrating that p75IC in the monomeric form, but not multimeric form, induces cell death.

Coexpression of TRAF2, TRAF4, or TRAF6 with pFKBP-
p75IC had prominent yet distinct effects on p75NTR-induced apoptosis in HEK 293T cells. Coexpression of TRAF2 with p75NTR led to a marked increase of p75NTR-induced cell death (Fig. 5A). Expression of TRAF2 in the absence of p75NTR was not pro-apoptotic, arguing that TRAF2 enhances the pro-apoptotic effect of p75NTR but is not, in and of itself, pro-apoptotic (Fig. 5A). Furthermore, expression of a TRAF2 dominant negative mutant lacking the N-terminal 272 amino acids (T2ΔN) significantly decreased p75NTR-mediated apoptosis (Fig. 5B). Apoptosis induced by the coexpression of TRAF2 and pFKBP-p75IC was completely suppressed by enforced dimerization with AP1510. Thus although TRAF2 markedly enhanced p75NTR-induced apoptosis, it did not affect the ability of dimerization to suppress the pro-apoptotic effect of p75NTR.

In contrast, TRAF4 expression did not enhance monomeric p75NTR-induced apoptosis (Fig. 5C). However, TRAF4 completely suppressed the ability of p75NTR dimerization to block cell death induction by p75NTR (Fig. 5C).

TRAF6, in contrast, protected HEK 293T cells from p75NTR-induced apoptosis. Coexpression of TRAF6 suppressed cell death induced by monomeric p75NTR. The suppression of cell death was as efficient as that resulting from enforced dimerization of pFKBP-p75IC with AP1510 (Fig. 5D). As with TRAF2, TRAF6 did not affect the suppression of p75NTR-induced cell death upon dimerization with AP1510 (Fig. 5D).

**TRAF4 Associates with Dimeric p75NTR Whereas TRAF2 Interacts Preferentially with Monomeric p75NTR.** Because of the multimerization-dependent effects of TRAF2 and TRAF4 on p75NTR-induced apoptosis, TRAF2 affecting p75NTR-induced apoptosis, which is a function of p75NTR monomer, and TRAF4 affecting the inhibition of apoptosis induction that occurs with p75NTR dimerization, we investigated the interactions between TRAF2, TRAF4, and p75NTR as a function of p75NTR dimerization. pFKBP-p75IC was coexpressed with TRAF2 or TRAF4 in HEK 293T cells, and AP1510 was administered to induce dimerization of p75NTR. Fig. 6B shows that TRAF4 coimmunoprecipitated with dimeric, but not monomeric, p75NTR. In contrast, TRAF2 interacted preferentially with monomeric p75NTR (Fig. 6A). Some p75NTR did coimmunoprecipitate with TRAF2 in the presence of AP1510. This may either represent incomplete dimerization of pFKBP-p75IC by AP1510 (in which case, residual monomeric p75NTR may be coimmunoprecipitated with TRAF2) or binding of TRAF2 to dimeric p75NTR with reduced affinity. In either case, however, TRAF2 interacted preferentially with monomeric p75NTR, whereas TRAF4 interacted solely with dimeric p75NTR.

**DISCUSSION**

Although accumulated evidence has demonstrated the ability of the common neurotrophin receptor p75NTR to signal in the absence of Trk proteins in certain cellular contexts (4, 47), the nature of p75NTR signaling pathways has been elusive. In the current study, we observed that multiple TRAF family members interacted with the common neurotrophin receptor, p75NTR, as detected by coimmunoprecipitation after overexpression in HEK 293T cells. Considering that TRAF proteins are capable of forming hetero- and homodimers through the common TRAF domain (or the unique isoleucine zipper regions in certain TRAF family members (48), the interactions we detected between TRAF proteins and p75NTR may be direct or indirect.

NMR structural analysis has disclosed two distinct structural domains in the intracytoplasmic region of p75NTR, an unstructured N-terminal region and a C-terminal region with six α-helices. The latter region is reminiscent of the structure of the death domains of Fas and RAIDD (38), with the exception that Fas and RAIDD display a type I death domain, whereas p75NTR displays a type II death domain. In the current study,
we found that TRAF2, TRAF4, and TRAF6 selectively interact with these two different regions of p75NTR. Specifically, the TRAF2-p75NTR interaction required the helical C-terminal region (residues Thr327-Leu386) and the TRAF4-p75NTR and TRAF6-p75NTR interactions required the juxtamembrane region (residues Lys245-Gly313) (39). A similar scenario has been reported for CD40-TRAF interactions: TRAF1, -2, -3, and -5 bind to a different region of CD40 than the site required for TRAF6 interaction (48, 49).

TRAF2 and TRAF6, when coexpressed with p75NTR, enhanced the modest NF-κB activation induced by p75NTR expression alone, consistent with their roles as NF-κB activators in the signaling pathways triggered by other members of the TNFR/NGFR superfamily. TRAF6 was a much stronger inducer of NF-κB activation than TRAF2. No previous role has been described for TRAF4, although it demonstrates structural similarity to other TRAF proteins. Our current results indicate that TRAF4 is a negative regulator of NF-κB activation by p75NTR. TRAF4 inhibited NF-κB activation by both p75NTR and p75NTR/TRAF6 while it did not affect NF-κB activation induced by TRAF2-p75NTR coexpression. These data suggest that signals derived from TRAF2 and TRAF6 may be differentially regulated, which would be compatible with the observation that TRAF4 and TRAF6 bound to the juxtamembrane region of p75NTR, while TRAF2 interacted with the C-terminal helical region of p75NTR.

We also found that the interactions between TRAF proteins and p75NTR could modulate p75NTR-induced apoptosis in HEK 293T cells with markedly differing results: TRAF2 enhanced monomeric p75NTR-mediated apoptosis, but had no effect on apoptosis in the absence of p75NTR. Furthermore, a dominant-negative mutant of TRAF2, TRAF2 (272–501), inhibited p75NTR-induced cell death, arguing that TRAF2 plays a role in the endogenous pathway of p75NTR-mediated apoptosis. TRAF2, however, did not affect the dimerization-induced inhibition of p75NTR-mediated apoptosis. This combination of effects is compatible with the finding that TRAF2 interacted preferentially with monomeric p75NTR.

In contrast, TRAF4 interacted with dimeric, but not monomeric, p75NTR, and its functional effects on p75NTR-induced apoptosis were found to be compatible with that interaction: TRAF4 had no effect on p75NTR monomer-induced apoptosis, but TRAF4 completely suppressed the dimerization-induced inhibition of p75NTR-mediated apoptosis.

In the case of TRAF6, it protected HEK 293T cells from p75NTR monomer-induced cell death, but did not have a significant effect on the inhibition of apoptosis upon dimerization of p75NTR. According to the recent report by Khursigara et al. (39), TRAF6 interacts with multimerized p75NTR since interaction between TRAF6 and p75NTR is dependent on NGF binding.

The role of NF-κB activation during apoptosis induced by various stimuli is still in debate. In our current studies, NF-κB activation did not correlate well with apoptosis induction, implying that other variables (e.g., SAPK/JNK activation) may turn out to be more important. On one hand, NF-κB activation appeared to protect HEK 293T cells from p75NTR-induced cell death, since the expression of the transdominant inhibitor for NF-κB activation, IkBeM, led to an elevated level of apoptosis when cells were challenged by overexpression of p75NTR. Also, the strong NF-κB inducer TRAF6 was cell protective, and the NF-κB inhibitor TRAF4 blocked cell rescue upon dimerization of p75NTR.

On the other hand, TRAF2 promoted cell death yet increased NF-κB activation. It has been reported that targeted disruption of the TRAF2 gene in mice results in increased sensitivity to TNF-induced apoptosis, implying an antiapoptotic function for TRAF2 (50). However, there may be distinct regulation of the signals derived from the interactions between p75NTR and TRAF2 or TRAF6, since these interactions were mapped to different regions in the intracytoplasmic region of p75NTR. In addition, the fact that TRAF2 interacts with reporter-interacting protein, a death-domain-containing protein kinase may possibly explain why, as a NF-κB activator, TRAF2 is able to enhance p75NTR-induced cell death, given that overexpression of reporter-interacting protein has been shown to induce apoptosis, mimicking TNFα effect on cells (51).

The effect of TRAF2 and TRAF4 on p75NTR-induced cell death and NF-κB activation is consistent with our unpublished observations3 that TRAF2 and TRAF4 are expressed endogenously in PC12 cells. TRAF4 is reported to be expressed in post-mitotic undifferentiated neurons (52), indicating its function in neural development and neurogenesis.

Our results suggest a model for the effects of TRAF2, TRAF4, and TRAF6 on p75NTR-mediated NF-κB activation and apoptosis (Fig. 7). They do not exclude the possibility that these TRAF family members have effects on other cellular parameters. Furthermore, other cells other than HEK 293T cells may respond differently. HEK 293T cells were utilized because the pro-apoptotic effects of p75NTR in neural cells have been reproduced in HEK 293T cells,2 and thus HEK 293T cells serve as a useful model for studies of p75NTR-mediated apoptosis. It is important to analyze the functions of the various TRAF proteins in vivo using mice with targeted disruption of the TRAF genes. Future in vivo studies should provide additional insight into the functions of these signal transducing molecules and the signaling pathways triggered by the common neurotrophin receptor p75NTR.

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