A Functional Interaction between the p75 Neurotrophin Receptor Interacting Factors, TRAF6 and NRIF*

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Jennifer J. Gentry, Nancy J. Rutkoski, Tara L. Burke, and Bruce D. Carter†‡

From the Department of Biochemistry and Center for Molecular Neuroscience, Vanderbilt University Medical School, Nashville, Tennessee 37232

Neurotrophin signaling through the p75 receptor regulates apoptosis within the nervous system both during development and in response to injury. Whereas a number of p75 interacting factors have been identified, how these upstream factors function in a coordinated manner to mediate receptor signaling is still unclear. Here, we report a functional interaction between TRAF6 and the neurotrophin receptor interacting factor (NRIF), two proteins known to associate with the intracellular domain of the p75 neurotrophin receptor. The association between NRIF and TRAF6 was direct and occurred with both endogenous and ectopically expressed proteins. A KrAB repressor domain of NRIF and the carboxy-terminal, receptor-binding region of TRAF6 were required for the interaction. Co-expression of TRAF6 increased the levels of NRIF protein and induced its nuclear translocation. Reciprocally, NRIF enhanced TRAF6-mediated activation of the c-Jun NH2-terminal kinase (JNK) by 3-fold, while only modestly increasing the stimulation of NF-κB. The expression of both NRIF and TRAF6 was required for reconstituting p75 activation in HEK293 cells, whereas NRIF mutants lacking the TRAF6 interaction domain were unable to substitute for the full-length protein in facilitating activation of the kinase. These results suggest that NRIF and TRAF6 functionally interact to facilitate neurotrophin signaling through the p75 receptor.

The TNF receptor superfamily is comprised of cytokine receptors that share homology in their extracellular domain and often contain an intracellular protein binding motif referred to as a death domain (1). Most receptors in this superfamily are characterized by their ability to regulate cellular survival and apoptosis, during the establishment and maintenance of the immune system as well as differentiation of neural cells and osteoclasts. Intracellular signaling pathways are activated by these receptors through recruitment of a variety of adapter proteins, including members of the TNF receptor-associated factor (TRAF) family (2–4). At present, there are six mamma-

lian TRAFs that have been identified, TRAF1–TRAF6, which contain an amino-terminal activation domain, and a carboxy-terminal TRAF-C domain, which mediates receptor binding and TRAF oligomerization (3). TRAFs 2, 5, and 6 contain RING and zinc fingers in their activation domains that function to mediate receptor stimulation of IκB kinase and c-Jun NH2-terminal kinase (JNK), and subsequently activate their respective downstream transcription factors, NF-κB and c-Jun. The activation domains of TRAFs 3 and 4 also contain RING and zinc fingers and TRAF1 has zinc fingers, but no RING finger; however, none of these TRAFs have yet been shown to initiate a signaling pathway. TRAF6 is the most divergent member of the TRAF family, exhibiting the greatest variation in its receptor binding domain through which it mediates signaling not only through members of the TNF receptor family, including CD40, RANK, and the p75 neurotrophin receptor, but also several Toll-like receptors, such as interleukin-1R and Toll-like receptor (TLR)-4 (5–8). Genetic deletion of the trafl gene has confirmed a functional interaction with many of these receptors in mice, which are deficient not only in signaling, but also in bone development, B-cell maturation, and neural tube closure (8–10).

TRAF6 has been shown to interact with the p75 neurotrophin receptor transiently in response to neurotrophin stimulation (7) and this interaction has been suggested to occur through an adaptor protein, MyD88, similar to members of the interleukin-1 receptor family (11). The association of TRAF6 with p75 has been implicated in neurotrophin-mediated activation of NF-κB and JNK in cell culture systems (7, 12). Similar to many receptors in the TNF receptor superfamily, the activation of these pathways by p75 has been shown to promote cell survival as well as induce apoptosis, depending on the cellular context (13). Activation of the stress kinase, JNK has been tightly correlated with induction of apoptosis by the receptor (14–16), whereas stimulation of NF-κB has been shown to promote survival (17–20). How this dichotomous signaling pathway is regulated by upstream signals may be critical for determining cell fate, developmentally and in response to injury within the nervous system.

A large number of p75 interacting factors have now been identified, including NRIF (21), TRAFs 2, 4 (22), and 6 (7), NRAGE (23), necdin (24), SC-1 (25), NADE (26), RhoA (27), Rac (28), ARMS (29), RIP2 (12), FAP (30), and PLAIDD (31). Whereas there is accumulating evidence for a role for many of these factors in modulation of specific aspects of p75 signaling in various cell types, how these factors function together to determine downstream effects remains to be elucidated. Here, we report on a functional interaction between two of these signaling proteins, NRIF and TRAF6.

NRIF was originally isolated in a yeast two-hybrid screen using the intracellular domain of the p75 neurotrophin receptor as bait (21). It is a C2H2-type zinc finger protein with a...
region homologous to KRAB domains, which have been shown to function as transcriptional repressors. NRIF interacts directly with the p75 receptor, and analysis of the nrif−/− mice suggested that it is required for p75-mediated apoptosis: cell death is significantly attenuated in the nrif−/− retina at embryonic day 15, a process known to be mediated by NGF stimulation of p75 (21). Exactly how NRIF functions in p75 signaling is unknown; however, given the presence of C2H2-type fingers and the KRAB domain, it has been speculated that NRIF may shuttle to the nucleus to suppress transcription.

In this study we found that TRAF6 and NRIF interact directly and this association resulted in translocation of NRIF to the nucleus and enhanced TRAF6 signaling. This interaction is important for receptor signaling because the binding of these two proteins was required for reconstituting p75 activation of JNK.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfection—COS7 and HEK293 cells were cultured in 5% CO2 at 37 °C in DMEM and 10% fetal calf serum, whereas the immortal B-cell line Wehi 231 was grown in RPMI with 10% fetal calf serum and 5% fetal bovine serum, and PC12 cells in DMEM with 10% horse serum and 5% fetal calf serum. The cells were grown on 10- or 15-cm Nunc dishes or, for immunostaining, on collagen-coated 15-mm glass coverslips. PC12 cells were differentiated by replacing the media with DMEM containing 0.5% horse serum and 50 ng/ml NGF. The cells were transfected using the standard calcium phosphate method or with LipofectAMINE reagent (Invitrogen) according to the manufacturer’s instructions. For NGF treatment, 293 cells were washed 4× in DMEM with a 1-h incubation in the third wash. DMEM with or without 100 ng/ml NGF was then added to the cells.

DNA Constructs—The plasmids containing the ATF-2 Gal-4 wild type and mutant fusion proteins, Gal-4 luciferase reporter, FLAG-tagged TRAF6 in pRKS, and FLAG-tagged dominant negative TRAF6 in pCMV4 were generous gifts of M. V. Chao (Skirball Institute, New York University). The TRAF2, 3, and 5 expression constructs were kindly provided by Dr. J. Inoue (Tokyo University, Japan) and TRAF1 was a gift from Dr. D. Ballard (Vanderbilt University, Nashville, TN).

The Flag-tagged TRAF6 mutants (Fig. 3B) were generated by PCR using pFlag-TRAF6 as template. PCR products were cloned into pFlag-CMV using EcoRI and SalI. The primer sequence used for each mutant is as follows: ΔRING forward, 5-CGCGAATTCCTGCCCAAATAAAGGC-TGTTTGC-3′ and reverse, 5′-CTAAGTCGACTACATACCCCGCAT-CAGTCT-3′; ΔSTAF and ΔCOIL forward, 5′-CGCGATTCTGAGTTCATG-CTTCTTAATACCGTGC-3′ and ΔTRAPC reverse, 5′-CTAGTCGAC-TATCAGGCTGTCCTTG-3′. ΔRING forward 5′-CTGTCGACTACATACGAGTTCGTTCTC-3′. GFP-tagged NRIF mutants (Fig. 3A) were generated by PCR and subcloned into our pEGFP-C1 with Sall and BamH1. Amino-terminal deletion mutants were generated with a common reverse primer, 5′-CGGGATCCTATCAGTACATCTCAAGGCTG-3′, and the forward primers were: 5′-CAGGTCGACCATCTCCGACACCGCTG-3′, 5′-CTGTCGACTACATACGAGTTCGTTCTC-3′. GFP-tagged NRIF mutants (Fig. 3A) were generated by PCR and subcloned into our pEGFP-C1 with Sall and BamH1. Amino-terminal deletion mutants were generated with a common reverse primer, 5′-CGGGATCCTATCAGTACATCTCAAGGCTG-3′, and the forward primers were: 5′-CAGGTCGACCATCTCCGACACCGCTG-3′, 5′-CTGTCGACTACATACGAGTTCGTTCTC-3′. GFP-tagged NRIF mutants (Fig. 3A) were generated by PCR and subcloned into our pEGFP-C1 with Sall and BamH1. The carboxyl-terminal mutants were created using the common reverse primer forward, 5′-CAGGTCGACCATCTCCGACACCGCTG-3′, and the reverse primers were: 5′-GGGATCCTATCAGTACATCTACAGGCTG-3′; and the forward primers were: 5′-CAGGTCGACCATCTCCGACACCGCTG-3′, 5′-CTGTCGACTACATACGAGTTCGTTCTC-3′.

Protein Isolation—Two proteins was required for reconstituting p75 activation of JNK.

RESULTS

NRIF Associates with TRAF6—TRAF6 binds not only to several cytokine receptors, but also to a number of intracellular signaling proteins; e.g. IRAK (32), RIP2 (33), TAB1 (34), and T6BP (35), which modify or propagate the receptor-TRAF6 signaling. This interaction is important for receptor and cell signaling because the binding of these two proteins was required for reconstituting p75 activation of JNK.
signal. We considered the possibility that TRAF6 could associate with NRIF because both proteins were shown to bind to the p75 neurotrophin receptor (7, 21). HEK293 cells were transfected with FLAG-tagged TRAF6 and pcDNA3-NRIF, or control vectors. The lysate was immunoprecipitated with antisera to TRAF6 (Fig. 1A). Alternately, cells were transfected with a GFP-tagged NRIF and FLAG-tagged TRAF6, or control vectors, and cells lysates were immunoprecipitated with antisera to the GFP epitope (Fig. 1B). Using either antibody, we observed an interaction between NRIF and TRAF6. We also observed that TRAF6 expression substantially enhanced the levels of NRIF protein (Fig. 1A). Whereas in a few experiments NRIF was expressed at higher levels, even in the absence of TRAF6, in the vast majority of experiments we observed that NRIF expression was very low or undetectable when expressed alone. The mechanism of NRIF stabilization by TRAF6 remains to be elucidated.

To determine whether the association occurred between endogenous proteins, we examined B-cells, which are dependent on TRAF6 function for proper development. TRAF6 and NRIF were immunoprecipitated from lysates of the immature B-cell line WeHi 231. The specificity of our polyclonal antisera to NRIF was verified by probing blots of HEK293 cells transfected with NRIF and TRAF6, or untransfected. The identity of the observed band as NRIF protein was confirmed by preabsorbing the antisera with GST-NRIFΔZF, the recombinant protein to which it was raised, or with control GST protein (Fig. 2A). This antibody was used to immunoprecipitate and immunoblot NRIF from WeHi 231 cells, resulting in the detection of a band at ~100 kDa. Whereas the expected molecular mass of NRIF is 95 kDa, both endogenous and untagged, ectopically expressed NRIF run at 100 kDa, indicating that the protein may be post-translationally modified. An additional slightly higher band was also frequently observed, which may represent further post-translational modification of the protein (Fig. 2A). At present, we are unable to determine whether the endogenous bands represent NRIF or its homolog, NRIF2, because we found that NRIF2 is also recognized by this antibody and coimmunoprecipitated with TRAF6 when co-expressed in HEK293 cells (data not shown). However, the predicted size of NRIF2 is 93 kDa, and when ectopically expressed in HEK293 cells it migrates below NRIF, suggesting that the higher molecular mass bands observed in the WeHi cells are not NRIF2 (data not shown). Importantly, TRAF6 was also detected in the NRIF precipitate (Fig. 2B, lower panel), providing evidence of an association between the endogenous proteins. In a reciprocal manner, when TRAF6 was immunoprecipitated from WeHi cell lysates, NRIF was also precipitated (Fig. 2B, upper panel). In addition to its role in the immune system, TRAF6 has been shown to regulate brain development with targeted deletion in TRAF6 leading to increased exencephaly in mice (9). Similar to what was seen in the WeHi cells, TRAF6 also coimmunoprecipitated with NRIF in lysates from postnatal day 4 mouse brain (Fig. 2C).

NRIF contains five zinc fingers of the C₂H₂-type in its carboxyl terminus, which often function as DNA binding domains for transcription factors. In addition to the zinc fingers, NRIF contains two amino-terminal domains found in a number of transcription factors of this subtype, a KRAB domain, and a SCAN domain. SCAN domains are thought to promote oligomerization and in vitro binding studies have shown that NRIF can form a multimeric complex (36). KRAB domains consist of KRAB A and B boxes and function as transcriptional repressor domains in a number of transcription factors (37–39). The p75-binding domain lies in between the zinc fingers and the amino-terminal domains. To further characterize the domains of NRIF required for its interaction with TRAF6, a series of NRIF deletion mutants were constructed (Fig. 3A). The carboxyl-terminal deletion mutants were much more highly expressed than the full-length or amino-terminal mutant proteins; therefore, to achieve comparable levels of expression 10-fold less NRIFΔC520 and NRIFΔC383 DNA were transfected into HEK293 cells for the co-immunoprecipitation experiments. Whereas the carboxyl-terminal deletion mutants, NRIFΔC520 and NRIFΔC383 interact more strongly with TRAF6 than the full-length protein, the ability of the amino-terminal deletion mutants, NRIFΔN161 and NRIFΔN241, to bind TRAF6 was completely abolished (Fig. 3C). These data indicate that TRAF6 interacts with the amino-terminal KRAB domain.

TRAF6, like all the TRAF family members, can be divided into an amino-terminal activation domain and a carboxyl-terminal TRAF domain (3). The activation domain, consisting of a RING finger domain and a series of five zinc fingers, function
The TRAF-C domain separates TRAF6 from other TRAF family members because of its divergence in amino acid sequence from other TRAF molecules in this region. We therefore speculated that the interaction with NRIF may be specific for TRAF6. Indeed, when TRAF1, TRAF2, TRAF3, or TRAF5 were co-expressed with NRIF, immunoprecipitation of these other TRAF molecules did not co-immunoprecipitate NRIF (Fig. 3D).

To determine whether the association between NRIF and TRAF6 was direct, or might be mediated via other adaptor proteins, a GST-TRAF6RING fusion protein was used in a pull-down assay with \textit{in vitro} translated NRIF-KRAB, which contains the KRAB and SCAN domains of NRIF. NRIF is known to oligomerize, so GST-NRIFAZF binding to \textit{in vitro} translated NRIF was used as a positive control (36). GST-TRAF6RING pulled down NRIF-KRAB, but not a luciferase control protein (Fig. 4A). In contrast, GST alone did not interact with the NRIF construct. This result indicates that NRIF interacts directly with TRAF6 and, consistent with the co-immunoprecipitation data, this interaction is mediated through the amino-terminal portion of the NRIF protein.

**NRIF Subcellular Localization Is Altered by Co-expression of TRAF6**—To further investigate the interaction between NRIF and TRAF6, we examined their subcellular localization when the two proteins were co-expressed. HEK293 cells were transfected with GFP-tagged NRIF with or without pFlag-TRAF6. In the absence of TRAF6, NRIF was expressed diffusely throughout the cell, similar to the distribution of GFP itself (Fig. 5A). Unlike GFP expression, however, GFP-NRIF was expressed in far fewer cells and at much lower levels than GFP (based on qualitative analysis of fluorescence intensity). In the presence of TRAF6, GFP-NRIF expression appeared to be higher and its localization was dramatically altered, being highly enriched in the nuclear and perinuclear region (Fig. 5, A–C). This effect was specific for TRAF6 as GFP-NRIF showed diffuse expression when TRAF1, TRAF2, TRAF3, or TRAF5 were co-expressed (Fig. 5A and data not shown). A similar result was observed when NRIF (without any tag) and TRAF6 were expressed in COS7 cells (Fig. 5B). Endogenous NRIF was also up-regulated in NGF-differentiated PC12 cells following TRAF6 transfection. Using confocal microscopy we determined that most of the NRIF was intranuclear, whereas the co-localization with TRAF6 occurred in the cytoplasm, particularly in the perinuclear region, but TRAF6 was never observed intranuclearly (Fig. 5C).

The activation domain of TRAF6 functions as a ubiquitin E3 ligase and is required for the downstream activation of both NF-κB and the stress-activated kinases. To determine whether this activation domain was required for the changes in NRIF localization observed in the presence of TRAF6, GFP-NRIF was co-transfected with the dominant negative TRAF6 (TRAF6 DN) in HEK293 cells (Fig. 5A). Co-expression of GFP-NRIF with TRAF6 DN resulted in diffuse expression, similar to GFP-NRIF with control vector, and lacked the nuclear pattern observed when co-expressed with full-length TRAF6.

Based on the localization of the NRIF deletion mutants in the presence or absence of TRAF6, it appears that the binding of the two proteins is required for changes in subcellular distribution (Fig. 6). The expression pattern of the NRIF carboxyl-terminal mutants, which bind TRAF6, was shifted upon co-expression of TRAF6, whereas the localization of the loss of association mutants (NRIFΔN161 and NRIFΔN241) was unaffected. Interestingly, the NRIF mutants lacking the amino terminus were expressed exclusively within the nucleus, even in the absence of TRAF6. Whereas localization of the carboxyl-
terminal mutants was altered by co-expression of TRAF6, these mutants never went into the nucleus but instead were found exclusively co-localized with TRAF6 in perinuclear domains. These results suggest that the carboxyl terminus of NRIF is required for its localization within the nucleus, whereas the amino terminus functions to prevent this nuclear localization.
NRIF and TRAF6 Functionally Interact

NRIF and TRAF6 were used to pull bound to GST, GST-NRIFΔZF, or GST-TRAF6ΔRING were used to pull down in vitro translated luciferase control protein, or the KRAB domain of NRIF (NRIF-KRAB). Representative of two experiments is shown. B, audioradiogram of in vitro translated luciferase and NRIF-KRAB (left panel) and Coomassie-stained recombinant GST, GST-NRIFΔZF, and GST-TRAF6ΔRING (right panel), representing the relative input for the pull-down assay.

Amino-terminal truncation, or binding of TRAF6 to the KRAB domain, may relieve this inhibition and allow the carboxyl terminus to direct NRIF localization.

TRAF6 Signaling Is Altered by Co-expression of NRIF—TRAF6 is known to mediate activation of the stress kinase, JNK, as well as the transcription factor NF-xB. We therefore examined the effects of NRIF on these signals. We used an ATF-2 Gal-4 luciferase reporter system to measure the activation of JNK, as ATF-2 is a known substrate of JNK. Overexpression of TRAF6 alone activated the reporter 25-fold and co-expression with NRIF enhanced that activity 3-fold (Fig. 7A). Whereas NRIF in the absence of TRAF6 did, in some experiments, result in a modest increase in ATF-2 reporter activity (Fig. 7A), in other experiments no measurable activation was observed by expression of NRIF alone (Fig. 7B). The variability in the effects of NRIF alone on the reporter are similar to the variability in the stability of NRIF in the absence of TRAF6, and may simply represent variable levels of NRIF protein. Indeed, we observed such a correlation between the expression level of NRIF and the slight activation of the reporter (data not shown). There was no activation with TRAF6 in the presence or absence of NRIF when a mutant reporter construct, ATF-2(T71A) Gal4, which cannot be activated by JNK, was used (data not shown). Co-expression of the NRIF mutants that do not bind TRAF6, NRIFΔN161 and NRIFΔN241, did not increase the level of TRAF6 stimulation of JNK, whereas the carboxyl-terminal mutant NRIFΔC520 retained the ability to increase activation of the reporter similar to the full-length NRIF. These results are consistent with the interaction between TRAF6 and NRIF being required for NRIF enhancement of TRAF6 activation of JNK. The NRIFΔC383 mutant did not enhance TRAF6 activation, suggesting that while amino acids 383–520 are nonessential for its interaction with TRAF6, these amino acids are required for enhancement of JNK activation. Similar to the specificity of NRIF-TRAF6 binding, the amplification of TRAF6-mediated JNK activation by NRIF was specific to TRAF6, as no enhancement was observed with any of the other TRAF family members (Fig. 7B).

In addition to stimulating JNK, TRAF6 expression also leads to the activation of the transcription factor NF-xB. NRIF co-expression with TRAF6 increased the activation of NF-xB, although the increase was far less dramatic than that observed with JNK activation. There was no observed effect of NRIF alone on the activity of the transcription factor and unlike the effects of NRIF on TRAF6-mediated JNK activation, the augmentation of the NF-xB response did not correlate with NRIF binding to TRAF6 (Fig. 8).

Association between NRIF and TRAF6 Is Required for p75-mediated Activation of JNK—Given the ability of NRIF to enhance TRAF6-mediated JNK activity, we considered the possibility that the interaction of these proteins may affect receptor activation of this pathway. Therefore, HEK293 cells stably expressing the p75 neurotrophin receptor were transiently transfected with TRAF6, NRIF, or both, then stimulated with NGF. Western blots of the lysates were probed with an antibody directed against the phosphorylated form of the JNK substrate, c-Jun (Fig. 9). NGF treatment of cells transfected with control vectors, TRAF6 or NRIF alone, did not result in a detectable increase in JNK activity. Expression of TRAF6 alone did result in an increased basal activation of JNK, but there was no further increase in the level of phosphorylated c-Jun following NGF addition. In contrast, cells expressing both NRIF and TRAF6 responded to NGF with a marked increase in phosphorylation of c-Jun, indicating that both receptor binding proteins are required for p75-mediated activation of the kinase.

To analyze the importance of the interaction between NRIF and TRAF6 in p75-mediated activation of JNK, we utilized the NRIF amino-terminal deletion mutants that do not interact with TRAF6, NRIFΔN161 and NRIFΔN241. Both NRIF mutants retain the p75 binding domain and were still able to co-immunoprecipitate with the receptor (Fig. 10A); however, cells co-expressing TRAF6 and NRIFΔN161 or NRIFΔN241, showed no increase in JNK activity in response to NGF stimulation (Fig. 10B). This result suggests that the interaction between NRIF and TRAF6 is required for p75-mediated activation of JNK.

The interaction between TRAF6 and p75 has been suggested to occur through adaptor proteins (11), whereas NRIF was shown to bind directly to the receptor (36). Hence, we considered the possibility that NRIF was serving to recruit TRAF6 to p75, thereby facilitating activation of the JNK pathway. However, expression of NRIF with p75 and TRAF6 appeared to decrease the co-immunoprecipitation of the receptor and TRAF6 (Fig. 11). Similarly, the interaction of NRIF and TRAF6 was reduced in the presence of p75. These results suggest that the interaction of TRAF6 and NRIF is competitive with binding to p75. Thus, the ability of TRAF6 and NRIF to mediate receptor signaling to JNK is not simply a matter of facilitating p75-TRAF6 interaction, rather it is dependent on the separate association of the two signaling proteins.

DISCUSSION

In this report, we describe a functional interaction between two p75 receptor-binding proteins, TRAF6 and NRIF. These two proteins could be co-immunoprecipitated when exogenously expressed in HEK293 cells as well as from B-cells and brain lysate. The proteins could directly bind and the association resulted in a change in the subcellular distribution of the putative transcription factor, NRIF, to the nucleus. The interaction with NRIF also enhanced TRAF6-mediated activation of JNK. Moreover, this modulation of TRAF6 function by NRIF
was required for reconstituting neurotrophin signaling through p75 to JNK, because NGF was only able to activate the kinase in the presence of both NRIF and TRAF6. Collectively, these results reveal a novel interaction between two cytokine receptor signaling proteins that appears to play a role in signaling through the p75 neurotrophin receptor.

TRAF proteins are defined by a region of homology in the carboxyl terminus that mediates both receptor binding and TRAF oligomerization (40, 41). The TRAF-C portion of the TRAF domain determines the specificity of interaction with various receptors. TRAF6 is unique in its receptor binding properties, as expected, because the receptor binding region of TRAF6 is the most divergent of the TRAF family members. Whereas all other TRAF family members modulate signaling of members of the TNF receptor superfamily exclusively, TRAF6 also transduces signals from receptors of the TLR family (32). The interaction between TRAF6 and members of the TLR family is mediated through the adaptor proteins MyD88 and IRAK, whereas binding between all TRAFs to receptors of the TNF receptor family is thought to occur through a direct interaction. Recent crystallographic studies of TRAF6 and several of its binding partners, both receptors and adaptor proteins, revealed a consensus TRAF6 binding sequence (42). This amino acid motif (PXEXXAr/Ac) is found in many TNF receptor family members as well as the TRAF6 binding sequence, with a single lysine insertion (PHESVKF). Whereas it remains to be determined whether or not this lysine affects the interaction between NRIF and TRAF6, the co-immunoprecipitation results (Fig. 3C) and the direct binding studies (Fig. 4) demon-
strated that the association occurs within this amino-terminal KRAB domain of NRIF.

Our deletion analysis revealed that the TRAF6-NRIF association requires the TRAF-C, receptor binding domain (Fig. 3D), which suggested that NRIF either competes with receptors for TRAF6 binding or serves as an adaptor to facilitate receptor association. The effects of NRIF expression on the p75-TRAF6 interaction demonstrated that NRIF antagonizes TRAF6 binding to the receptor (Fig. 11). Thus, recruitment of NRIF and TRAF6 to p75 may be followed by a higher affinity interaction between the two cytoplasmic proteins, which could be the mechanism by which TRAF6 is released from p75, thereby facilitating activation of downstream signals. Although we have not observed any significant change in the interaction of NRIF, TRAF6, and p75 upon ligand binding in these ectopic expression studies (data not shown), it is likely that such changes in association will require analysis of endogenous protein complexes. It is noteworthy that TNF-mediated apoptosis was recently shown to involve the formation of a protein complex that dissociates from the receptor, although how the complex is released from the receptor is not known (43).

NRIF was originally identified as a p75 neurotrophin receptor-binding protein and analysis of the nrif−/− mice suggested that this protein is required for p75-mediated apoptosis (21). Nevertheless, the molecular mechanism through which NRIF...
participates in mediating the cell death signal remains undefined. A number of amino acid motifs within the protein indicate that it may function as a transcription factor. NRIF contains a series of five zinc fingers of the C2H2-type, which often function as DNA binding domains, and in the amino terminus there are two motifs found in many such zinc finger proteins, a SCAN and KRAB domain. SCAN domains allow oligomerization of transcription factors, whereas KRAB boxes function as potent transcriptional repressor modules (44–48). KRAB box-mediated repression has been suggested to occur through recruitment of co-repressors such as KAP-1 (49, 50). More direct evidence that NRIF may function as a transcription factor is its ability to bind specifically to a predicted target DNA sequence, created based on the amino acid composition of the zinc fingers (51).

If NRIF functions as a transcription factor, then it must translocate to the nucleus. Ectopic expression of NRIF resulted in very low levels of protein expression and a diffuse pattern of expression throughout the cytoplasm and nucleus (Figs. 1 and 5); however, when TRAF6 was co-expressed, the level of NRIF protein was dramatically increased and it was found exclusively within and adjacent to the nucleus (Fig. 5). A similar localization of endogenous NRIF was observed in PC12 cells transfected with TRAF6. This localization is especially interesting in terms of the potential function of NRIF as a transcriptional repressor. Other KRAB box containing proteins have been shown to localize to intranuclear regions referred to as centromeric heterochromatin transcriptional repressor domains (52), where they function to down-regulate expression of target genes.

The nuclear localization of NRIF was dependent on its association with TRAF6 and the NRIF zinc finger domain (Fig. 6). Carboxyl-terminal mutants of NRIF, which were able to associate with TRAF6, were never observed within the nucleus, but instead accumulated in perinuclear regions in the presence of TRAF6. This indicates that the zinc fingers of NRIF are required for its nuclear localization. In contrast, the amino-terminal mutants of NRIF, which were unable to associate with TRAF6, were found exclusively within the nucleus, regardless of TRAF6 expression. Because deletion of the KRAB domain caused NRIF to translocate into the nucleus, similar to the effect of TRAF6 expression. Because deletion of the KRAB domain caused NRIF to translocate into the nucleus, similar to the effect of TRAF6 interaction, yet no NRIF cleavage was observed in the presence of TRAF6, we speculate that the KRAB domain prevents NRIF entry into the nucleus and that this block is released by binding to TRAF6 or post-translational modification by TRAF6. How TRAF6 allows nuclear targeting of NRIF is not clear; however, TRAF6 was recently shown to be a non-canonical E3 ubiquitin ligase, which can polyubiquitinate itself, leading to activation of downstream pathways but not proteasome degradation (53). Although such a modification would fit with the inability of the dominant negative TRAF6 to translocate NRIF (Fig. 5A) or to increase NRIF protein level,
we have not been able to detect ubiquitination of NRIF by TRAF6 (data not shown), thus there may be another component involved or an alternate mechanism.

The ability of TRAF6 to ubiquitinate itself leads to activation of the transcription factor, NF-κB (53). The oligomerization of TRAF6 is thought to be essential for propagation of downstream signals (54). The ability of NRIF to interact with TRAF6 may lead to the formation of a multimeric signaling complex. Indeed, by co-immunoprecipitating FLAG-tagged versions of both proteins and quantitatively immunoblotting for FLAG, we estimate that ~15 TRAF6 molecules associate with each NRIF. The formation of such a complex could explain the ability of NRIF to enhance TRAF6-mediated activation of JNK by 3-fold (Fig. 7). In addition to enhancement of JNK activity, NRIF slightly, although significantly, increased TRAF6 activation of the transcription factor NF-κB (Fig. 8). Surprisingly, however, the enhancement was much weaker than that observed with JNK activity and the activation did not appear to be dependent on the interaction. This would imply that the moderate increase in NF-κB signaling by NRIF is indirect and that the formation of the NRIF-TRAF6 complex is sufficient to increase the activation of the JNK pathway, whereas stimulation of NF-κB may require additional components, for example, RIP2, which also associates with TRAF6 (12).

Both NF-κB and JNK are downstream effectors of neurotrophin signaling through the p75 receptor; however, activation of these pathways appears to be specific to particular cellular contexts. One explanation for the cell type specificity is restricted expression of signaling components of the p75 pathway. Indeed, we observed that NGF binding to p75 was unable to activate JNK in HEK293 cells, yet both NRIF and TRAF6 were expressed the cells responded. No NGF response was ever observed with expression of either protein alone. Moreover, the interaction between NRIF and TRAF6 was required for p75-mediated activation of JNK because p75-induced phosphorylation of c-Jun was not reconstituted by the amino-terminal mutants of NRIF, which could not associate with TRAF6 but could bind p75. Thus, the NRIF-TRAF6 interaction not only affects the function of these two proteins independently, but also serves an important role in mediating receptor signaling. Although the expression of TRAF6 and NRIF was sufficient to allow p75 signaling to JNK, given the myriad of p75 interacting proteins, it is likely that additional components endogenous to the HEK293 cells, are present in the signaling complex. It will be interesting in future studies to determine the composition of p75-activated signalosomes under various conditions.

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