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The p75 Neurotrophin Receptor (p75NTR) Alters Tumor Necrosis Factor-mediated NF-κB Activity under Physiological Conditions, but Direct p75NTR-mediated NF-κB Activation Requires Cell Stress*

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The p75 neurotrophin receptor (p75NTR) has been linked to activation of the NF-κB transcriptional complex in oligodendrocytes, Schwann cells, and PCNA cells. In this report, tumor necrosis factor (TNF)- and neurotrophin-mediated NF (nuclear factor)-κB activation were compared in several cell lines. All cell types showed TNF-mediated activation of NF-κB, but direct neurotrophin-dependent activation of NF-κB was never observed under normal growth conditions. In PCNA cells, a modest nerve growth factor (NGF)-dependent induction of NF-κB was detected but only after cells were subjected to severe stress. Although NGF binding did not directly activate NF-κB under normal conditions, NGF consistently altered TNF-dependent NF-κB activation in each cell type examined, and extended exposure to NGF and TNF always increased NF-κB activation over that achieved with TNF alone. The increase in NF-κB activity mediated by NGF correlated with reduced levels of IκBα; NGF added alone had no effect on IκBα levels, but when added with TNF, NGF treatment significantly reduced IκBα levels. We propose that modulation of cytokine receptor signaling is a significant physiological function of the p75 neurotrophin receptor and that previous reports of direct NF-κB activation through p75NTR reflect this modulatory activity.

The neurotrophins are a well conserved family of proteins that play critical roles in the maintenance and development of the nervous system (1–7). Their cellular effects are mediated by two distinct classes of cell surface receptors. The Trk[1] receptors, a highly related family of receptor tyrosine kinases, recognize the neurotrophins with a relatively high degree of binding specificity; TrkA preferentially binds NGF, TrkB prefers BDNF and NT-4/5, and TrkC interacts only with NT-3 (8). The other class of neurotrophin receptor contains only the p75NTR. This receptor is a member of the TNF receptor superfamily that includes CD27, CD30, CD40, 4–1BB, OX40, the Fas antigen, and the tumor necrosis factor receptors TNFR1 and TNFR2 (9). Unlike for the Trk receptors, defining the precise physiological role of the p75NTR has proven difficult (10). Several studies indicate that p75NTR can functionally interact with Trk receptors to enhance or dampen intracellular signals. For example, when p75NTR is co-expressed with TrkA, it tends to dampen basal levels of TrkA activation and reduce the responses of TrkA to nonpreferred ligands (10–14). However, p75NTR also facilitates NGF binding to TrkA and thus increases TrkA responses to preferred ligands (15–18).

p75NTR also has an autonomous signaling role that in some respects is similar to other members of the TNF receptor superfamily. Binding of each of the neurotrophins to p75NTR evokes activation of cellular sphingomyelinase, which results in increased ceramide production (19, 20), and recent studies suggest that p75NTR may behave as a ligand-activated apoptotic receptor during development (21–24). Specific p75NTR interacting proteins have proven difficult to identify, but the receptor's apoptotic function may be subserved by a region of intracellular homology shared between p75NTR and other apoptotic receptors of the TNF receptor superfamily. This 80-amino acid region, termed the death domain, is required to mediate interactions of other TNF receptor superfamily members with downstream apoptotic effectors (25).

One well studied effect of TNF receptor superfamily members is activation of the transcription complex NF-κB (26). In response to ligand binding, receptors of this class bind TRAF proteins through their intracellular domains and activate a kinase cascade that culminates in activation of IKKα and IKKβ and subsequent phosphorylation of IκB subunits, which targets them for ubiquitination and proteosomal degradation (27). IκB degradation releases NF-κB subunits from their latent cytoplasmic state and allows them to translocate to the nucleus where they regulate specific gene regulatory events. There are preferential interactions of the six TRAF proteins with various members of the TNF receptor superfamily (28–31), and it is likely that differences in these TRAF protein associations play a crucial role in determining the levels of NF-κB activation that occur in response to a particular stimulus. To date, only TRAF6

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[1] The abbreviations used are: Trk, tyrosine receptor kinase; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT, neurotrophin; p75NTR, p75 neurotrophin receptor; TNF, tumor necrosis factor; DMEB, Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin; EMSA, electrophoretic mobility shift assays; NF-κB, nuclear factor κB; TIM, tetracyclin-inducible MG87-3T3 cell line; PCNA, proliferative cell, nulcear antigen; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HIV-LTR, human immunodeficiency virus-long terminal repeat; TRAF, TNF receptor-associated factor.

has been reported to interact with p75NTR (32).

NGF binding to p75NTR activates NF-κB in fibroblasts overexpressing p75NTR, in primary mouse Schwann cells (33), and in primary rat oligodendrocytes (34, 35). To extend these results, we examined p75NTR-mediated NF-κB activation in PCNA, 293HEK, 3T3, and A875 melanoma cells. Here we show that neurotrophin binding to p75NTR does not directly activate NF-κB under normal physiological conditions but instead modulates NF-κB activation induced by other stimuli.

EXPERIMENTAL PROCEDURES

Materials—NGF was purchased from Collaborative Research, and TNF was purchased from R & D Systems. BDNF was provided by Regeneron Pharmaceuticals (Tarrytown, NY), NT-3 and NT-4 were purchased from Peprotec, and the MC192 antibody (36) was prepared from ascites fluid as described (37). Antibodies against IκBα and IκBβ were purchased from Santa Cruz Biotechnology. Other reagents were purchased from either Sigma or ICN.

Cell Culture and Transfections—HeLa, 293HEK, 293T-HEK, A875, MG87-3T3, and PCNA cells were all maintained in Dulbecco's modified Eagle's medium containing 10% bovine calf serum, 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin in 5% CO_2 at 37 °C. For transient transfections, 5 μg of cytomegalovirus-driven rat p75NTR expression plasmid was introduced into cells on 100-mm plates using the calcium phosphate precipitation method. For transient transfections, 100 ng of an expression plasmid driving expression of enhanced green fluorescent protein (pEGFP-N1, CLONTECH) was included to monitor transfection efficiency. To produce cell lines in which p75NTR levels could be induced with doxycycline, MG87-3T3 fibroblasts were stably transfected with a plasmid driving expression of the rtTA chimeric transcription factor (38). Individual clones were screened for doxycycline inducible expression in transient transfection assays (data not shown), and lines showing the lowest basal expression and strong doxycycline-induced expression (termed TIM, for tetracyclin-inducible MG87-3T3) were stably transfected with an expression plasmid containing rat p75NTR under control of a doxycycline inducible promoter. A total of 30 of these clones were analyzed, and two lines (termed TIMP75-3 and TIMP75-12) that showed undetectable basal expression and strong doxycycline-inducible expression of P75NTR (data not shown) were used for detailed analyses.

Electrophoretic Mobility Shift Assays—Cultured cells were plated on 60- or 100-mm dishes, washed twice in DMEB, and then incubated in 2 or 5 ml, respectively, of DMEB supplemented as described in the figure legends. For pretreatment experiments, cells were washed twice in DMEB, preincubated in DMEB at room temperature for the times indicated in the figure legends, and then incubated in 5 ml of DMEB or DMEB supplemented with NGF for 1 h at 37 °C followed by induction with TNF for 2 h at 37 °C. After the induction period, the medium was removed, and the plates were placed on ice, rinsed with ice-cold Tris-buffered saline (20 mM Tris (pH 8.0), 137 mM NaCl) and then lyzed in 10 mM HEPES (pH 7.9), 0.1% Nonidet P-40, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Whole cell extractions were prepared by washing cells in phosphate-buffered saline with 50 nM pyrrolidine dithiocarbamate and extracted in buffer consisting of 20 mM HEPES (pH 7.9), 0.35 M NaCl, 20% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40. Nuclear extractions were prepared in 20 mM HEPES (pH 7.9), 0.42 M NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 5 μg/μl leupeptin, 5 μg/μl pepstatin, 5 μg/μl aprotinin, 0.5 mM spermidine, 0.15 mM spermine, 100 μM sodium vanadate, and 0.5 mM phenylmethylsulfonyl fluoride. Extracted lysates were analyzed for total protein content by BCA assay (Pierce), performed in triplicate. EMSAs were performed essentially as described (39) on nuclear and whole cell lysates using an NF-κB binding element from the HIV-LTR as a probe. Gels were exposed to XRP film (Kodak) and scanned using an Epson 1210 scanner. For quantitation, scanned images were analyzed using NIH Image software. Statistical comparisions of TNF and TNF + NGF conditions were performed using paired *t* tests.

Survival and Apoptosis Assays—MTT assays were used for quantitation of mitochondrial activity as per the manufacturer's instructions (Promega), with optical density quantified on a Titertek enzyme-linked immunosorbent assay plate reader and expressed as the difference between OD_{540} and OD_{690}. To quantitate the ratio of MTT-positive cells within stressed cell populations, at least four fields of 100 cells each were counted under phase contrast illumination (total cell number) and under bright field (MTT-positive cells). Data was normalized for total cells counted per field, and three separate experiments were compared. To quantify apoptosis of PCNA cells, cells were stained with propidium iodide (100 ng/ml) for 30 min prior to scoring for an apoptotic morphology. A substantial proportion of apoptotic cells were nonadherent at the time of assay, and therefore both adherent and nonadherent cells were quantified. The apoptotic index is the sum of adherent and nonadherent apoptotic figures, corrected for counting volumes.

Transcriptional Assays—NF-κB transcription was monitored using a pUC19-derived NF-κB reporter gene, which contains a tandem array of three functional κB sites derived from the HIV-LTR. These κB elements are just proximal to an SV40 minimal promoter driving expression of a LacZ open reading frame modified to include a nuclear localization signal at the amino terminus and an SV40 poly(A) sequence (plasmid p429) (40). For assays of NF-κB transcriptional activity, calcium phosphate precipitates were used to transfect plasmid p429 together with plasmid p412, a green fluorescent protein expression plasmid used to monitor expression levels and with either p288, a p75NTR expression plasmid, or parental vector. β-galactosidase activity was monitored by *o*-nitrophenyl β-D-galactopyranoside conversion using a Promega kit. Each data point was performed in quadruplicate, and experimental results were analyzed by multiple analysis of variance (ANOVA), with statistical probabilities assigned using the Tukey test for multiple comparisons.

Immunoblotting—Cytoplasmic or whole cell lysates were normalized for protein content using the BCA assay (Pierce), diluted in Laemmli sample buffer, boiled 5 min, separated on 10 or 12% SDS-polyacrylamide gels, and transferred to nitrocellulose. Immunoblots were first blocked in 10 mM Tris (pH 7.4), 150 mM NaCl, 2% bovine serum albumin, 0.2% Tween 20 and then incubated with antibodies directed against IκBα or against the p75NTR intracellular domain (23). Blocking and primary and secondary incubations for p75NTR immunoblots were performed in 10 mM Tris (pH 7.4), 150 mM NaCl, and 0.2% Tween 20 with 5% (w/v) dry skim milk. Immunoreactive bands were detected using enhanced chemiluminesence (ECL, DuPont) according to the manufacturer's instructions, and scanned images were quantified using NIH Image.

RESULTS

Previous results indicate that p75NTR activates NF-κB in fibroblasts, Schwann cells, and oligodendrocytes (33, 34). To test whether p75NTR-mediated activation of NF-κB is a general phenomenon, the activation of NF-κB by p75NTR was examined in cells that do not express endogenous p75NTR or Trk receptors. 293HEK cells were transiently transfected with a p75NTR expression vector or with the parental control vector and then treated for 2 h with either neurotrophin, TNF, or MC192, a rat p75NTR-specific monoclonal antibody. EMSA of extracted nuclear proteins revealed that TNF elicited a robust NF-κB response, yet neither the p75NTR-specific antibody nor any of the neurotrophins activated NF-κB (Fig. 1*A*). Various induction times (up to 12 h) and NGF doses (5 to 500 ng/ml) were examined, but an NGF-mediated NF-κB activation was never observed in 293HEK. Similar experiments were performed in p75NTR-transfected HeLa cells and 3T3 fibroblasts, which are commonly used cellular models for TNF signaling, but neither of these transfected cell types showed any evidence of NF-κB activation in response to NGF at any concentration or time point. TNF treatment, however, consistently produced robust NF-κB activation in these same cell lines (data not shown). To test whether neurotrophin-mediated NF-κB activation may be a feature of cell lines that express high endogenous levels of p75NTR, the A875 melanoma cell line was also examined. As with the other cell lines, EMSA revealed that TNF treatment resulted in robust NF-κB activation but NGF, BDNF, and NT3 had no effect on NF-κB activation under these conditions (Fig. 1*B*).

Previous reports showing that NGF binding to p75NTR increases NF-κB activity in EMSA in PCNA cells (33) and in primary rat oligodendrocytes (34, 35) suggest that p75NTR can activate NF-κB in primary cells and in some cell lines. We therefore examined p75NTR-mediated activation of NF-κB in

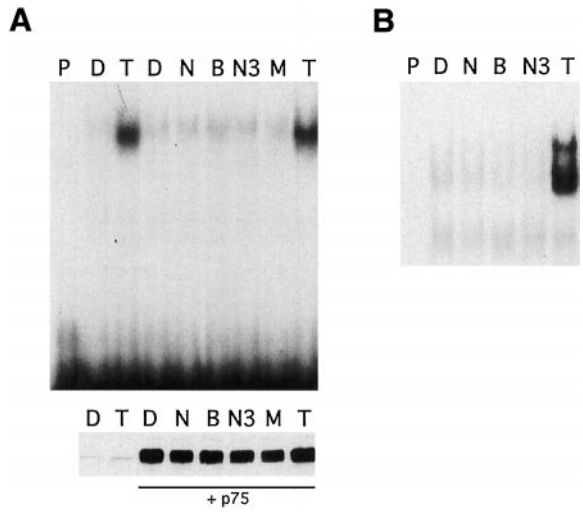

FIG. 1. **Neurotrophins do not directly activate NF-κB in 293HEK or A875 cells.** *A*, 293HEK cells were transiently transfected either with a control vector or with a p75NTR expression vector and 2 days later were incubated for 2 h in either DMEB alone or DMEB containing TNF (5 ng/ml), NGF (250 ng/ml), BDNF (250 ng/ml), NT3 (250 ng/ml), or MC192 (1 μg/ml). Nuclear extracts were analyzed by EMSA (*upper panel*) using a labeled NF-κB probe. To confirm p75NTR expression, cell lysates were analyzed for p75NTR content by immunoblotting (*lower panel*). *B*, A875 cells were treated with neurotrophin or TNF for 2 h and nuclear extracts were then analyzed by EMSA as described under "Experimental Procedures." These experiments were repeated three times. In addition, concentrations of 5–500 ng/ml neurotrophins were tested for periods of up to 10 h in both cell lines, but direct neurotrophin-mediated activation of NF-κB was never observed (data not shown). *P*, probe alone (no cellular extract added); *D*, DMEB; *T*, TNF; *N*, NGF; *B*, BDNF; *N3*, NT-3; and *M*, MC192.

PCNA cells, to determine whether our failure to detect NF-κB activation was due to cell type-specific differences in p75NTR signaling. Surprisingly, p75NTR-mediated NF-κB activation was not observed in PCNA cells in response to any of the four mammalian neurotrophins (Fig. 2*A* and data not shown). One possible reason for this is that PCNA cells might produce endogenous neurotrophins that dampen an NF-κB response to exogenous ligand. However, cells plated at low density and washed extensively to remove endogenous neurotrophin still showed no evidence of NGF-mediated activation of NF-κB. A second possibility is that p75NTR-dependent NF-κB activation depends on culture conditions. Notably, NF-κB responses can be altered under conditions of cellular stress (41), and in the first report of p75NTR-mediated NF-κB activation (33), cells were subjected to two stressful conditions: a temperature shock and a period of serum starvation.[2] We therefore tested whether these conditions might increase the ability of PCNA cells to respond to NGF. For these experiments, PCNA cells were left in room-temperature air (20 °C) for several hours in serum-free medium. This procedure reduced cellular mitochondrial activity (Fig. 2*B*) and significantly increased the incidence of apoptotic nuclei (Fig. 2*D*). Scoring of individual MTT-treated cells showed that after only 7 h of this treatment (Fig. 2*C*), cells showed strongly reduced mitochondrial activity, yet the majority still remained adherent. Virtually no cells had detectable mitochondial activity after 21 h. EMSA from PCNA cells pretreated in this manner for 7 h are shown in Fig. 2*A*. The stress treatment reduced basal NF-κB activity and strongly attenuated NF-κB activation induced by TNF. The stressed cells also revealed an NGF-dependent induction of NF-κB, an activation not observed in the unstressed cultures. Moreover, the shifted complex induced by both TNF and NGF in the stressed cells migrates more slowly than the predominant complex, activated by TNF in PCNA cells under physiological conditions. This difference presumably reflects the activation of different NF-κB components. Therefore, NGF binding to p75NTR does not directly activate NF-κB under physiological conditions but does activate an NF-κB complex in severely stressed PCNA cells.

TNF receptor superfamily members share common downstream effectors, such as the TRAF proteins, and convergent signals between various receptor types have been reported to amplify ligand-mediated NF-κB activation (42). Therefore, even though p75NTR may not directly activate NF-κB under physiological conditions, it is possible it may modulate NF-κB activation induced by other stimuli. To test this theory, 293HEK cells transiently transfected with a p75NTR expression construct were exposed to TNF, NGF, or combinations of both for 2, 6, or 10 h and then examined for NF-κB activation by EMSA. Fig. 3*A* shows that 2 h of TNF treatment causes a robust increase in NF-κB activity. This activation is attenuated when NGF is present, indicating that ligand signaling through p75NTR can affect signaling of other related cytokine receptors. Intriguingly, the modulatory effect of p75NTR changes with increasing time. After a 6-h treatment, NF-κB activation produced by TNF is equivalent to that mediated by TNF and NGF together, and by 10 h, TNF-mediated NF-κB activation is increased in the presence of NGF. Under these physiological conditions, neither increasing the time nor the concentration of NGF altered the mobility of the primary NF-κB complex induced by TNF. To confirm these results, we also examined a 293 subline (293T-HEK) using a different nuclear protein extraction protocol (33). Fig. 3*B* shows that 293T-HEK cells transfected with p75NTR are strongly modulated by NGF binding to p75NTR, with NGF initially reducing TNF-induced NF-κB activation but then increasing NF-κB activation with time, qualitatively identical to that shown in Fig. 3*A*. Our transfection efficiency in 293HEK cells is about 70%, and thus the magnitude of the NGF-induced modulation is likely an underestimate of the true magnitude of the modulatory effect of p75NTR. Densitometric quantification of scanned films revealed that the combination of NGF and TNF produced a significant average reduction of 26% at 2 h ($p < 0.01$), a 21% increase at 10 h ($p < 0.03$), but no change at 6 h ($p < 0.47$).

To determine whether p75NTR can exert modulatory effects on TNF signaling in cell lines expressing endogenous p75NTR, we turned to the A875 melanoma cell line. Primary melanocytes, originating from the neural crest, and melanoma cell lines normally co-express p75NTR and TNF receptors but have little or no TrkA expression (43, 44). Fig. 4*A* shows that NGF does not directly activate NF-κB in this cell type, but when examined 2 h after cytokine addition, NGF clearly increased TNF-mediated NF-κB activation, with maximal increases (60%) at 25 ng/ml and more moderate increases (30–40%) at higher NGF concentrations. In A875 cells, NGF increased TNF-mediated NF-κB activation at all time points examined (Fig. 4*B*). Thus, in each cell line examined, NGF ultimately results in increased TNF mediated activation of NF-κB by 10 h. To determine whether the synergistic effects of NGF and TNF are observed if cells are pretreated with NGF, A875 cells were pretreated with NGF for 1 h and then induced with TNF for an additional 2 h. Fig. 4*C* shows that the modulatory effect of NGF on TNF signaling is maintained under these pretreatment conditions.

To begin to determine the mechanism by which p75NTR modulates TNF-mediated NF-κB activation, levels of IκBα were examined in cells treated with TNF, NGF, or the two together. For these experiments, we used a 3T3-derived cell

[2] B. Carter, personal communication.

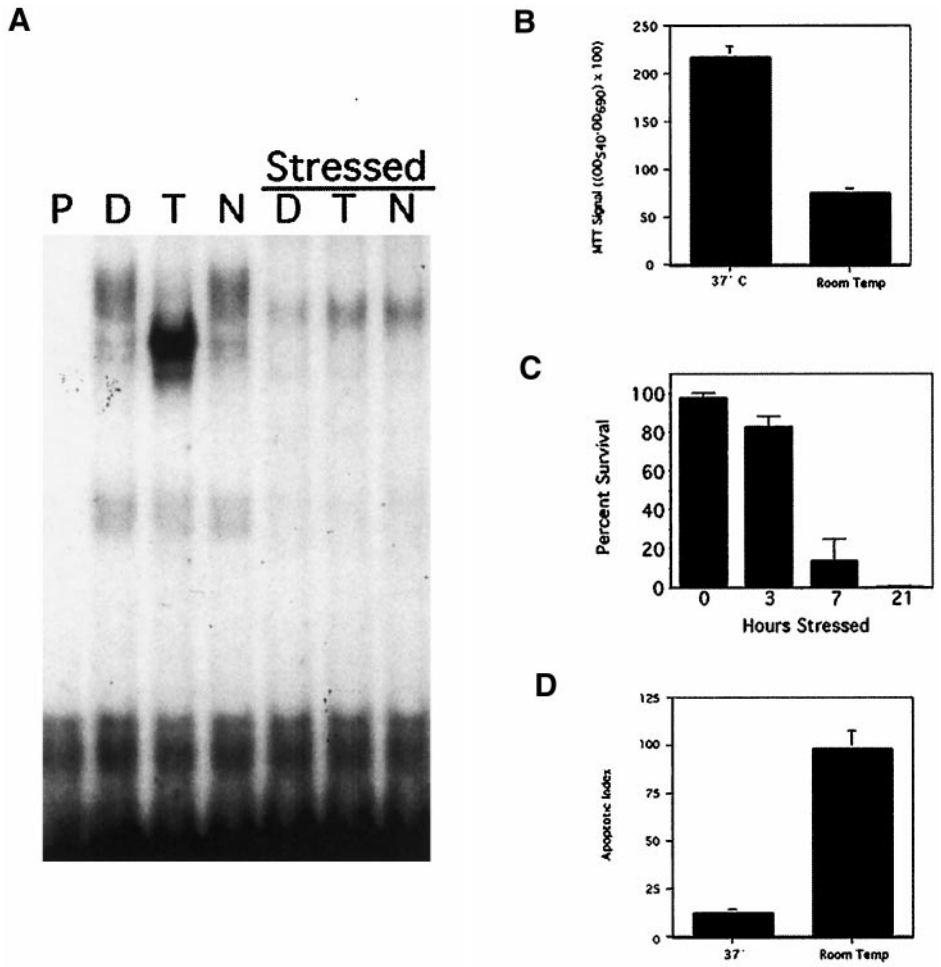

FIG. 2. **NGF mediates activation of NF-κB in PCNA cells only after severe cellular stress.** PCNA cells were maintained either in serum-containing medium at 37 °C in a 5% CO_2 atmosphere (control) or in serum-free medium in room-temperature air (20 °C) for several hours (stressed) as described under "Results." Parallel cultures were analyzed in four ways. For NF-κB activity, unstressed (*lanes 1-3*) and cells stressed for 7 h (*lanes 4-6*) cells were left untreated (DMEB) or were treated with either NGF (100 ng/ml) or TNF (20 ng/ml) for an additional 2 h at 37 °C in a 5% CO_2 atmosphere, after which nuclear proteins were extracted and analyzed by EMSA using a labeled NF-κB probe (*A*). To measure mitochondrial activity, total MTT activity was quantified after 18 h of stress (*B*) and scoring of cellular MTT production was compared after 0, 3, 7, and 21 h of stress (*C*). For cell death analysis, apoptotic bodies were determined by propidium iodide staining after 18 h of stress (*D*). Each experiment was repeated three times. *P*, probe alone (no cellular extract added); *D*, DMEB; *T*, TNF; and *N*, NGF.

line (TIMP75-3 cells) in which p75NTR levels can be regulated by the addition of doxycycline. Fig. 5*A* (*lower panel*) shows that p75NTR is undetectable in the absence of doxycycline, but receptor expression increases dramatically in response to an 18-h doxycycline treatment. In the absence of detectable p75NTR expression, long term TNF treatment led to a moderate reduction in IκBα steady-state levels, which were not affected by the addition of NGF (*lanes 3* and *4*). When p75NTR was inducibly expressed, however, the reduction in IκBα protein induced by the combination of NGF and TNF was significantly greater than by TNF alone (Fig. 5*A*, *upper panel*, *lanes 7* and *8*; $p < 0.02$).

Following TNF treatment, IκBα proteins are rapidly degraded and then resynthesized, and measurement of steady-state levels of IκBα represents the balance between these two processes. To directly test whether NGF facilitates TNF-mediated IκBα degradation, the effects of NGF and TNF on IκBα levels were determined in the presence and absence of cycloheximide, a protein synthesis inhibitor. If NGF acts to facilitate IκBα degradation, its effect on IκBα levels should still be observed in the presence of translation inhibitors. Fig. 5*B* shows that the combination of NGF and TNF produced a greater reduction in IκBα steady-state levels in A875 cells than did TNF alone (average decrease of 30%), consistent with the findings in the TIMP75-3 line. In the presence of cycloheximide, the effect of combining NGF and TNF was retained, with considerably more IκBα degradation observed compared with TNF alone. This result argues that the mechanism by which NGF acts involves, at least in part, the facilitation of TNF-mediated IκBα degradation.

EMSA revealed that the maximal increase in NF-κB activity induced by NGF is about 3–4-fold in both transfected 293HEK cells and A875 cells. To determine whether this moderate increase in activated NF-κB complexes results in increased NF-κB-dependent transcription, an NF-κB-responsive LacZ reporter construct was transfected into 293HEK cells together with a p75NTR expression plasmid or with a parental control vector. Fig. 6 shows that NGF added alone did not activate transcription from the LacZ reporter construct in 293HEK cells transfected with either control vector or p75NTR expression plasmid. NGF also had no effect on TNF-mediated NF-κB transcription in cells transfected with the parental expression vector, indicating that NGF does not exert p75NTR-independent effects on NF-κB. In cells expressing p75NTR, however, the combination of NGF and TNF significantly increases NF-κB activation compared with cells treated with TNF alone ($p < 0.0001$) and therefore suggests that the moderate increases in

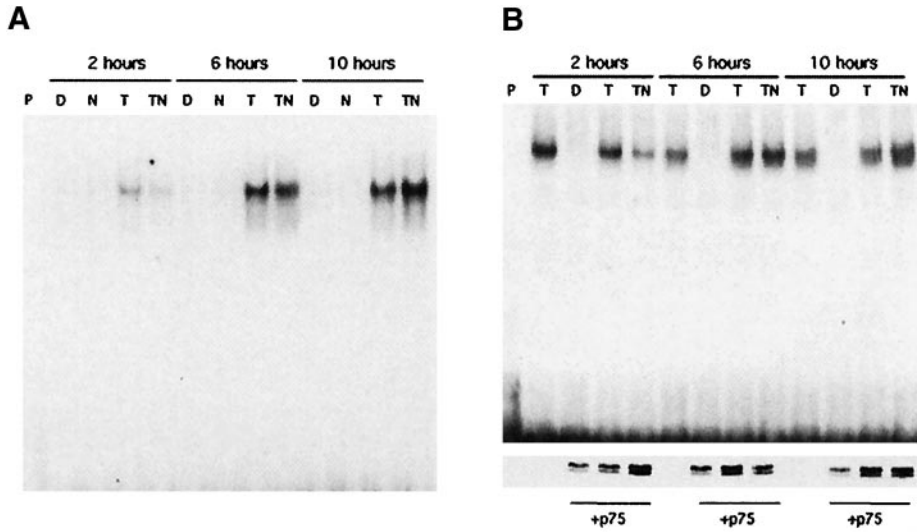

FIG. 3. **NGF modulates TNF-induced NF-κB activation in 293HEK cells expressing the p75NTR cell surface receptor in a time-dependent manner.** *A*, 293HEK cells transiently transfected with a p75NTR expression vector were incubated with TNF (5 ng/ml) with or without NGF (250 ng/ml) for 2, 6, or 10 h. Nuclear extractions were performed and analyzed for NF-κB binding activity by EMSA using a labeled NF-κB probe. Experiments were repeated four times with similar results. *B*, similar experiments were performed on a 293T-HEK subline transfected with a control vector or with a p75NTR expression vector and cellular proteins were isolated using a whole cell extraction protocol (see "Experimental Procedures"). p75NTR expression levels determined by immunoblotting of cellular lysates are shown in the *lower panel*. Experiments were repeated three times with similar results. *P*, probe alone (no cellular extract added); *D*, DMEB; *T*, TNF; *N*, NGF; and *TN*, TNF + NGF.

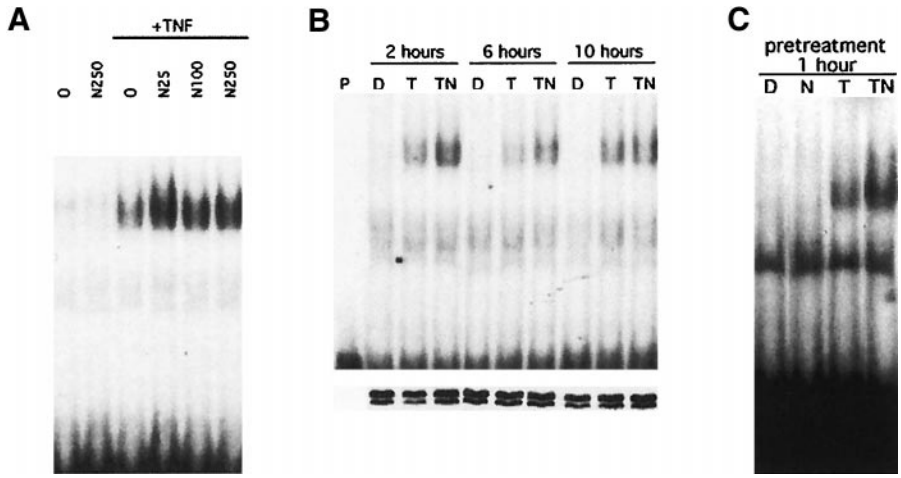

FIG. 4. **Neurotrophins do not directly activate NF-κB in A875 cells, but NGF increases TNF-induced NF-κB activation in a time- and dose-dependent manner.** *A*, A875 cells were incubated for 2 h in DMEB supplemented with either NGF (250 ng/ml) or TNF (5 ng/ml) or with TNF in the presence of increasing concentrations of NGF. Nuclear proteins were extracted and analyzed by EMSA using a labeled NF-κB probe. *B*, A875 cells ere incubated for 2, 6, or 10 h in DMEB alone or DMEB supplemented with TNF (5 ng/ml) in the absence or presence of NGF at 25 ng/ml. Nuclear proteins were extracted and analyzed by EMSA (*upper panel*) using a labeled NF-κB probe. Cell lysates were analyzed for p75NTR content by immunoblotting to confirm p75NTR expression (*lower panel*). This experiment was repeated four times with similar results. *C*, A875 cells were incubated with DMEB alone or DMEB supplemented with NGF (250 ng/ml) for 1 h and then induced with TNF for an additional 2 h. Nuclear proteins and EMSA were performed as above. This experiment was repeated three times with similar results. *P*, probe alone (no cellular extract added); *D*, DMEB; *T*, TNF; *N*, NGF; and *TN*, TNF + NGF.

NF-κB binding activity result in significant increases in the NF-κB transcriptional response.

DISCUSSION

The signaling properties of the p75NTR are not well defined. p75NTR-dependent sphingomyelinase activation and ceramide generation have been observed in a number of cell types under differing conditions, suggesting that activation of this signaling cascade may be a general property of p75NTR activation (19, 20, 45). We have previously shown that a signaling cascade involving ceramide may be the mechanism through which p75NTR regulates TrkA activity (11). In addition, binding of neurotrophin to p75NTR leads to phosphorylation of c-Jun (21, 34, 46), and p75NTR can facilitate apoptosis both in TrkA-expressing and TrkA-lacking cells (21–23, 34, 46, 47). Finally, p75NTR has been reported to activate NF-κB in oligodendrocytes, Schwann cells, and PCNA cells (33–35). In this study, we have examined the capacity of p75NTR to activate NF-κB in a variety of cell types and have asked whether p75NTR might influence activation of NF-κB mediated by TNF. Our results indicate that neurotrophin binding to p75NTR does not activate NF-κB under physiological conditions but show instead that p75NTR modulates NF-κB signaling mediated by other cytokine receptors.

Our inability to detect direct p75NTR-mediated NF-κB signaling contrasts with earlier findings (33–35). There are at least two explanations for this discrepancy. One is simply that signaling elements required for direct p75NTR-mediated

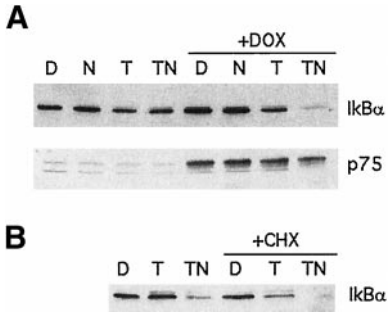

FIG. 5. **NGF does not directly reduce IκBα levels but instead facilitates IκBα degradation in the presence of TNF.** *A*, TIMP75-3 cells were treated with or without doxycycline for 18 h and then treated with TNF (10 ng/ml) with or without NGF (100 ng/ml) for 10 h as indicated. Immunoblotting of cell lysates show IκBα levels (*top panel*) and confirm p75NTR expression (*bottom panel*). *B*, A875 cells were treated with DMEB alone or supplemented with TNF (10 ng/ml) and/or NGF (100 ng/ml) in the presence or absence of cycloheximide (10 μg/ml) for 2 h as indicated. IκBα levels in cell lysates were determined by immunoblotting. The experiments shown in *A* and *B* were each repeated three times with similar results. *D*, DMEB; *T*, TNF; *N*, NGF; and *TN*, TNF + NGF.

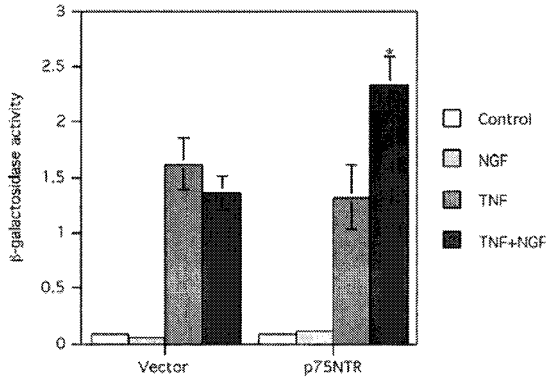

FIG. 6. **NGF has no direct effect on NF-κB transcriptional activity but increases TNF-mediated NF-κB transcriptional activity through a p75NTR-dependent pathway.** 293HEK cells transiently transfected with NF-κB dependent β-galactosidase and green fluorescent protein reporter constructs with either a control vector or a p75NTR expression vector. 24 h after transfection, cells were switched to media containing 5 ng/ml TNF, 100 ng/ml NGF, or the two combined for 16 h, and then cell lysates were prepared and analyzed for LacZ activity as described under "Experimental Procedures." Multiple analysis of variance shows that cells expressing p75NTR, TNF, and TNF + NGF treatment groups differ significantly (p value < 0.0001, indicated by an *asterisk*). This experiment was repeated three times with similar results.

NF-κB activation are absent from the cell types we have examined, leading to the somewhat pedantic conclusion that p75NTR acts in a cell-type specific manner. Indeed, our results do not rule out the possibility that some cell types may support direct p75NTR-mediated activation of NF-κB under physiological conditions. However, our observation of NGF-mediated NF-κB activation only within cells that were severely stressed prior to the NGF exposure suggests an alternative explanation. Results of our work and others have shown that NGF binding to p75NTR expressed on cultured oligodendrocytes results in nuclear translocation of the p65 NF-κB subunit and activation of NF-κB (34, 35), and in both of these studies, the oligodendrocytes analyzed were maintained in serum-free media in which death occurs continually at a low rate (21). Also, serum starvation of cultured Schwann cells is apparently a prerequisite for NGF-dependent nuclear translocation of the p65 subunit of NF-κB (32). These conditions may be analogous to the stress paradigm used in our studies, and taken together, these results are consistent with the possibility that cellular stress is necessary to observe the NGF-induced NF-κB activation reported previously by ourselves and others (32–35). The mechanism by which cellular stress may increase responsiveness to NF-κB is uncertain, but one possibility is that stress induces increases in the production of TNF or cytosolic signaling elements to "prime" the NF-κB pathway in an autocrine manner. In this scenario, NGF acting through p75NTR is not a primary inducer of the pathway but rather synergizes with a stress-induced signal to increase NF-κB activity.

Our results show that although neurotrophin binding to p75NTR does not directly activate NF-κB signaling in A875 melanoma cells, in transfected 293HEK cells, or in stably transfected 3T3 cell lines, NGF binding to p75NTR had a clear effect on levels of NF-κB activation mediated by TNF; NGF binding to p75NTR ultimately increased levels of TNF-induced NF-κB activation in each cell line analyzed. In A875 cells, NGF potentiated TNF-mediated NF-κB signaling at every time point examined, whereas 293 cells showed a more complex biphasic response to NGF. This probably reflects the fact that A875 cells are neural crest derivatives, which normally express p75NTR and are therefore a more appropriate intracellular signaling milieu for p75NTR than 293 cells. Consistent with this explanation, the concentration of NGF required for activation of the modulatory effect was considerably lower in A875 cells than in 293 cells (Fig. 4*A* and data not shown). This modulatory effect of p75NTR on NF-κB activation likely reflects a *bona fide* physiological action of the p75NTR because the NGF-mediated increases in active NF-κB complexes occur in a variety of cells grown under normal conditions using relatively low concentrations of NGF. More importantly, these NGF-mediated increases are reflected in significant changes in NF-κB driven transcription. Together, these results suggest that a major effect of p75NTR on NF-κB signaling in many cells may be to modulate NF-κB activation mediated by other stimuli. Our preliminary results indicate that this modulatory effect is not NGF-specific but is also observed with other neurotrophins (data not shown).

Alternative explanations might also account for this increased NF-κB activity. One possibility is that TNF increases p75NTR levels sufficiently to allow NGF to induce NF-κB. However, we show abundant p75NTR expression in many cells that demonstrate a complete lack of NGF-induced NF-κB activation. This finding is perhaps most clearly shown in the A875 cells, which express abundant p75NTR. Because cells that express p75NTR in abundance show no direct NF-κB activation in response to NGF, it is reasonable to conclude that NGF affects TNF signaling, not the reverse. Also, although TNF can regulate expression through NF-κB elements within the cytomegalovirus promoter present in expression constructs, in A875 cells, which show the same qualitative effect, levels of p75 remained unchanged in the 10-h time course of our experiments (see Fig. 4*B*).

Analysis of IκBα levels further supports a p75NTR-regulated modulatory ability. Using either transiently transfected cells or cells that express p75NTR endogenously, we found that NGF markedly reduces steady-state levels of IκBα and does so by enhancing TNF-mediated degradation of the protein. The effect of NGF on IκBα degradation is readily apparent and suggests that p75NTR activation is likely to impinge on the activity of IKKα or IKKβ kinases, which phosphorylate IκBα and thus target it for degradation (48, 49). The p75NTR signaling cascade that may contribute to this effect remains unknown, but the recent discovery of an interaction between p75NTR and TRAF6 raises the possibility that TRAF family members may play some role. The reductions in IκBα levels that resulted from NGF treatment were quite dramatic, and their magnitude

was clearly greater than the NGF-dependent changes observed by EMSA. It is possible that NGF may selectively affect IκBα degradation yet spare IκBβ or other IκB family members. We tested whether IκBβ family members were affected by NGF treatment, but the poor quality of the commercially available IκBβ reagents precluded definitive results.

p75NTR potentiates TNF-mediated IκBα degradation, and a key goal of future studies will be to define the precise convergence point of the NGF and TNF pathways. The precise mechanism(s) underlying the effect of p75NTR on TNF-mediated NF-κB activation remains unclear but could reflect a competition for common signaling elements that converge at or above the level of IκBα subunit phosphorylation. A similar type of transreceptor effect on NF-κB activation has been described in other systems. For example, although T cell receptor activation normally produces a very small NF-κB response, T cell receptor activation dramatically increases NF-κB activity mediated by the interleukin-1 receptor (42). This T cell receptor-mediated increase in interleukin-1 dependent NF-κB activity has recently been shown to result from increased T cell receptor-dependent IκB degradation (50). Together with our results, this suggests that transmodulatory mechanisms may be an important means for regulating cellular NF-κB activity. Therefore, p75NTR may function not only to regulate the activity of receptors with which it shares ligands, such as the Trks, but may also act to modulate signaling activity of receptors with which it shares functional or structural homology, such as the TNF receptors.

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