Recent evidence indicates that nerve growth factor (NGF) produces its effects through signaling contributions from both TrkA and the p75 receptor. In contrast to its trophic actions through TrkA, NGF binding to p75 has been shown to activate programmed cell death through a mechanism involving the stress kinase JNK. However, this receptor also activates nuclear factor κB (NF-κB), the role of which has yet to be determined. We investigated the function of p75-mediated NF-κB stimulation in regulating cell survival in the rat schwannoma cell line RN22, which expresses p75, but not TrkA. Gel shift assays demonstrated activation of NF-κB in response to NGF within 30 min and lasting at least 4 h. NGF also stimulated JNK in the cells (detected by in vitro kinase assays) with a similar time course. Preventing activation of NF-κB with the specific inhibitor SN50 resulted in NGF-induced cell loss. Similarly, transfection of the cells with a mutant form of the endogenous NF-κB inhibitor (IκBαΔN), which cannot be degraded and therefore remains bound to NF-κB, preventing its activation, resulted in a significant increase in the number of apoptotic cells following NGF treatment. These results suggest that NGF activation of NF-κB through the p75 receptor promotes survival, counterbalancing the pro-apoptotic signal.

Neuronal survival during vertebrate development is dependent on a family of trophic factors referred to as the neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor, and neurotrophin-3-6 (1). However, in contrast to their well-established pro-survival activity, a number of recent studies have suggested that NGF and other members of the neurotrophin family can promote programmed cell death in specific cell types.

Although the survival activity of the neurotrophins has been shown to be mediated primarily by the Trk proteins, a family of tyrosine kinase receptors, the p75 receptor has been linked to neurotrophin-induced apoptosis. Sequence homology between p75 and members of the TNF receptor superfamily has led to speculation that this NGF receptor may signal analogously, including stimulation of cell death. Recent findings by several groups have demonstrated that neurotrophin binding to p75 can induce programmed cell death in cultured rat oligodendrocytes (2, 3), certain neuroblastoma cells (4), rat sympathetic neurons (5), chick trigeminal neurons (6), and Schwann cells (7). Recent in vivo data also support a role for p75-mediated apoptosis during the development of embryonic chick (8) and mouse (9) retinal cells as well as mouse spinal cord (9) and cholinergic forebrain neurons (10). The cell death induced by p75 in oligodendrocytes (2) and sympathetic neurons (5) was found to correlate with activation of c-Jun N-terminal kinase (JNK), a pathway implicated in neuronal apoptosis (11). Furthermore, a specific inhibitor of this kinase protected the oligodendrocytes from the effects of NGF (3). This is in contrast to the TNF receptor and Fas, which stimulate apoptosis independent of JNK via the adapter protein Fas-associated protein with death domain (FADD) and subsequent recruitment of caspase-8 (12).

In addition to promoting apoptosis, several members of the TNF receptor family have been shown to activate the transcription factor NF-κB. This led to the finding that in cultured Schwann cells expressing p75, but not TrkA, NGF treatment activated NF-κB (13, 14). Similar findings have been reported for cultured oligodendrocytes (3, 15) and neuroblastoma cells (4). Currently, the role of NF-κB activation in the nervous system is not known; it has been suggested to have both pro-apoptotic (16) and anti-apoptotic (17, 18) properties, depending on the cell type. Interestingly, it was recently demonstrated that activation of NF-κB by the TNF receptor promotes survival in lymphoid cells and fibroblasts (19–22). Thus, the TNF receptor activates a bifurcating pathway, with one component being anti-apoptotic through the activation of NF-κB and the other leading to induction of apoptosis. A similar dichotomous signal may originate from the p75 neurotrophin receptor. This may explain how p75 can facilitate a TrkA pro-survival signal when coexpressed in neurons (23), but also induce apoptosis when activated independently.

Here we have investigated the role of NGF activation of NF-κB in the balance between cell survival and death in response to p75 signaling. To delineate p75 signaling independent of TrkA, we used a rat schwannoma cell line (RN22) expressing p75, but not TrkA. Inhibition of NF-κB activity resulted in apoptosis in response to NGF, suggesting that this
transcription factor provides a survival signal counterbalancing the cell death signal activated by p75.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The rat schwannoma cell line RN22 was cultured in 5% CO₂ at 37 °C in DMEM with 10% fetal calf serum. The cells were grown on 60- and 100-mm tissue culture dishes (Becton Dickinson) or four-well glass chamber slides (Nalgene Nunc International) for staining.

**Polymerase Chain Reaction Analysis—Oligonucleotides** specific for p75 (5′-GTGAGTTCACACTGGGG) and TrkA (5′-GGAGAGATTCAG-9) were used to reverse-transcribe 5 μg of total RNA isolated from RN22 or PC12 cells. The cDNA was first amplified using primers specific for p75 (sense, 5′-GTGAGTTCACACTGGGG; and antisense, 5′-GGAGAGATTCAG-9) with 45 amplification cycles. Each cycle consisted of 30 s of denaturation at 94 °C, followed by 30 s of annealing at 52 °C and 1 min of extension at 72 °C. Nested primers for p75 (sense, as above; and antisense, 5′-GGAGAGATTCAG-9) were used to further amplify the DNA. Using 10% of the initial reaction, 30 cycles were performed for each nested primer reaction, with each cycle consisting of 30 s of denaturation at 94 °C, followed by 30 s of annealing at 55 °C and 1 min of extension at 72 °C. Nested primers for TrkA (sense, as above; and antisense, 5′-GGAGAGATTCAG-9) with 45 amplification cycles. Each cycle consisted of 30 s of denaturation at 94 °C, followed by 30 s of annealing at 55 °C and 1 min of extension at 72 °C. Nested primers for TrkA (sense, as above; and antisense, 5′-GGAGAGATTCAG-9) were used to further amplify the DNA. Using 10% of the initial reaction, 30 cycles were performed for each nested primer reaction, with each cycle consisting of 30 s of denaturation at 94 °C, followed by 30 s of annealing at 55 °C and 1 min of extension at 72 °C.

**Western Blot Analysis**—To determine the levels of IκB protein, RN22 cells were rinsed three times with medium and treated with 100 ng/ml NGF (all experiments were carried out using recombinant human NGF generously provided by Regeneron Pharmaceutical, Inc.) for the indicated times or were left untreated in culture medium. The cells were then rinsed and harvested in PBS on ice, centrifuged at 3000 × g for 5 min, and lysed in Nonidet P-40 lysis buffer (1% Nonidet P-40, 20 mM Tris, pH 7.5, 137 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin). The lysates were cleared by centrifugation at 14,000 × g for 10 min at 4 °C, and equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis on a 10% gel. Following transfer of the proteins to nitrocellulose, the membrane was blocked in 20 mM Tris, pH 7.5, 137 mM NaCl, and 0.1% Tween 20 with 5% milk and 1% bovine serum albumin and blotted with an antiserum to IκBα (used at 0.5 μg/ml; Santa Cruz Biotechnology). For detecting p75 expression by Western blotting, total protein from untreated RN22 cells was subjected to SDS-polyacrylamide gel electrophoresis and blotted with an antiserum raised against the intracellular portion of p75 (9992 antiserum, kindly provided by Moses Chao). Immunoreactive proteins were detected using peroxidase-conjugated goat anti-rabbit secondary antibodies (Roche Molecular Biochemicals) and chemiluminescence (Pierce). TrkA expression was similarly assessed using a pan-Trk antiserum raised against the carboxyl terminus of TrkA (generously provided by David Kaplan). As a positive control for TrkA, lysates from HEK 293 cells transiently transfected with pCMV-TrkA (kindly provided by SungOk Yoon) were used.

**Electrophoretic Mobility Shift Assay (EMSA)**—The activation of NF-κB was assayed as described previously (3). Briefly, schwannoma cells were washed three times in culture medium and treated with 100 ng/ml NGF or 25 ng/ml TNF (R&D Systems) in culture medium. In some experiments, the cells were rinsed and treated in serum-free medium; however, the results were not different from those with cells treated in serum-containing medium. Following treatment, the cells were placed on ice, washed in ice-cold PBS, harvested by scraping, and pelleted by centrifugation at 1000 × g for 5 min at 4 °C. The cells were lysed in high salt lysis buffer (20 mM HEPES, pH 7.9, 0.35 M NaCl, 20% glycerol, 1% Nonidet P-40, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin), which extracts nuclear proteins, and the insoluble material was pelleted at 14,000 × g for 10 min at 4 °C. The supernatants were stored frozen at −80 °C until used in the binding reaction. The binding of activated NF-κB in the lysates to an oligonucleotide corresponding to the κ light...
chain enhancer (Promega) was assessed by incubating the lysate (10–40 μg, equal amounts of protein were used for each assay) with 10 μl of binding buffer (44 mM HEPES, pH 7.9, 4% glycerin, 140 mM KCl, 0.1 mM EDTA, 0.05% Nonidet P-40, 8% Ficoll 400, 2 mg/ml bovine serum albumin, 0.2 mg/ml poly(dI-dC), 4 mM dithiothreitol, and 1 μM phenylmethylsulfonyl fluoride) and 5,000–100,000 cpm 32P-labeled oligonucleotide in a final reaction volume of 20 μl. After a 30-min incubation on ice, the samples were separated on a 4% nondenaturing polyacrylamide gel and visualized by autoradiography. For antibody supershift analysis, 2 μg of the indicated antibody (Santa Cruz Biotechnology) were added into the binding reaction.

**Luciferase Activity—**NF-κB in RN22 cells was also assessed by transcription of the luciferase reporter 6×κB-Luc (a gift from Larry Kerr). Approximately 0.5–1×10⁶ cells were transfected with 5 μg of pFLAG or pFLAG-IκBΔN (kindly provided by Dean Ballard), 2 μg of 6×κB-Luc reporter, and 0.5 μg of pCMV-locZ using Tfx-50 (Promega) as described below. In some experiments, only the reporter construct and locZ were transfected (in a 6:1 ratio). After 24 h, the cells were treated with NGF or TNF, as indicated, for 4–24 h and lysed in 100 μl of reporter lysis buffer (Promega). After centrifugation for 10 min at 14,000×g to clear the lysate, 10 μl were used to measure luciferase activity according to the manufacturer’s protocol in a luminometer (Monolight 2010, Analytical Luminescence Laboratory). β-Galactosidase activity was assayed using 30 μl of the sample in 600 μl of Z buffer (57.2 mM NaH₂PO₄, 42.3 mM Na₂HPO₄, pH 7.0, 2 mM KCl, 1 mM MgSO₄, and 50 mM β-mercaptoethanol) and 120 μl of 4 mg/ml o-nitrophenyl-β-D-galactopyranoside in 0.1 M Tris HCl, pH 7.8. After incubating at 37 °C for 12–24 h (the time was constant within each experiment), the reaction was stopped with 300 μl of 1 M Na₂CO₃, and absorbance was measured at 420 nm. The luminescence values were normalized by the individual β-galactosidase activity. All readings were done in triplicate for each experiment.

**In Vitro Kinase Assay—**Cells were rinsed and treated as for EMSA and lysed in Triton lysis buffer (20 mM Tris, pH 7.5, 137 mM NaCl, 2 mM EDTA, pH 7.4, 1% Triton X-100, 25 mM β-glycerophosphate, 1 mM sodium vanadate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin), and the lysates were centrifuged at 14,000×g for 10 min at 4 °C. The supernatants were normalized for protein concentration (100–200 μg) with the Bradford assay in a final volume of 300 μl in Triton lysis buffer, and JNK was immunoprecipitated with anti-JNK polyclonal antibody (Santa Cruz Biotechnology) and protein A-Sepharose (Sigma). The immunoprecipitates were washed in Triton lysis buffer and then kinase buffer (25 mM HEPES, pH 7.4, 25 mM β-glycerophosphate, 25 mM MgCl₂, 0.1 mM sodium vanadate, and 2 mM dithiothreitol) and resuspended in kinase buffer with 3 μg of JNK substrate, recombinant GST-c-Jun-(1–79), 18 μg of GST, and 0.1 μCi of [32P]ATP in a final reaction volume of 50 μl. Reactions were incubated at 37 °C for 15 min and stopped by the addition of Laemmli loading buffer. The reaction mixture was then separated by SDS-polyacrylamide gel electrophoresis using a 10% gel, and the phosphorylated substrate was visualized by autoradiography or a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

**MTT Assay—**RN22 cells were plated at 3000 cells/well on a 96-well plate in DMEM alone; and after allowing the cells to adhere for 1 day, either Sn50 (a cell-permeable peptide inhibitor of NF-κB) from BIOMOL Research Labs Inc.; 100 μg/ml PMA was used with and without NGF (100 ng/ml). After 4 h, 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added; and following 2–3 h of incubation, the cells were lysed with 10% SDS in 0.1 M HCl, and the absorbance was read at 570 nm with 690 nm as a reference. The data shown are the means ± S.E. of six experiments, each done in triplicate, with 100% set to the value for untreated cells.

**Transfection and TUNEL Staining—**The RN22 schwannoma cells, passaged onto four-well Nunc slide chambers the day before transfection (10⁶ cells/well), were transfected with 0.25 μg of pCMV-locZ and 1.6 μg of pFLAG or pFLAG-IκBΔN using Tfx-50 reagent according to the manufacturer’s protocols for 1 h in serum-free medium. Cells were then washed gently and incubated overnight in DMEM with 10% fetal calf serum to determine the effects of NGF, the cells were rinsed twice and incubated for 4 h at 37 °C in the absence or presence of 100 ng/ml NGF in DMEM with 5% FBS serum.

Apoptotic cells were identified by TUNEL staining (Roche Molecular Biochemicals) following the manufacturer’s recommended protocol. Following the TUNEL labeling, the cells were incubated for 30 min at room temperature in PBS with 0.1 mM glycine, pH 7.3, and then washed twice in PBS containing 0.1% Tween 20 (PT buffer) and blocked in PT buffer with 10% goat serum. The cells were incubated overnight with monoclonal antibody directed against β-galactosidase (1:100; Life Technologies), washed in PT buffer, and then incubated with a polyclonal antibody to the p55 subunit of NF-κB (1:100; Santa Cruz Biotechnology) or to the phospho-
activity of NF-\(\kappa\)B for 4–24 h, and the transcriptional
expression was undetectable using both Western blotting and
level of the p75 receptor message and protein, whereas TrkA
activate NF-\(\kappa\)B was assessed by measuring the luciferase activity. In place of the NF-\(\kappa\)B reporter plasmid, some cells
were transfected with a mutant promoter lacking the NF-\(\kappa\)B element (*mutant reporter*). All values are expressed relative
to that for untreated cells and have been normalized to \(\beta\)-galactosidase activity. Depicted are the means \(\pm\) S.E. of at least
seven experiments. The asterisk denotes statistically significant differences from the value for NGF activation as determined by ANOVA analysis with a Newman-Keuls multiple comparison test \((p < 0.05)\).

**RESULTS**

Characterization of NGF Receptor Expression—To investigate the role of NGF-activated NF-\(\kappa\)B, we used a cell line derived from a rat sciatic nerve tumor, RN22 schwannoma (24). NGF binding to p75 on Schwann cells was previously shown to activate NF-\(\kappa\)B (13, 14) and was recently shown to induce apoptosis under conditions of Bcl-2 overexpression (7). However, these cells were found only to be responsive to NGF treatment for the first few days in culture, making biochemical analysis difficult (13, 14). The schwannoma cell line RN22 is phenotypically similar to primary Schwann cells in morphology and the expression of markers such as S100 (24). Important for our studies, the schwannoma cells were found to express high levels of the p75 receptor message and protein, whereas TrkA expression was undetectable using both Western blotting and reverse transcription-polymerase chain reaction (Fig. 1).

**NGF Activation of NF-\(\kappa\)B**—To assess NF-\(\kappa\)B activation in RN22 cells in response to NGF, EMSAs were carried out using total cell lysates and the light chain \(\kappa\)B sequence as the DNA-binding probe. Although there was significant basal level activation, sequence-specific NF-\(\kappa\)B binding complex was further activated within 30 min of treatment with NGF, and this activation increased with stimulation time up to 4 h, the longest time investigated (Fig. 2A). Similarly, both phorbol ester (phorbol 12-myristate 13-acetate) and TNF, potent activators of NF-\(\kappa\)B, were able to activate this transcription factor in these schwannoma cells.

NF-\(\kappa\)B is functionally active as a transcriptional regulator in a dimeric form consisting of homo- or heterodimers, the prototypic NF-\(\kappa\)B dimer consisting of the p65 and p50 subunits. In addition to p65 and p50, other subunits of NF-\(\kappa\)B include p52, c-Rel, and RelB. Supershift assays were used to elucidate the composition of the NGF-activated complexes. The specific bands in the EMSA were partially reduced by antisemur to either p65 or p50. In contrast, the antisemur to c-Rel and the control (anti-rabbit IgG) had no effect. This would suggest that NGF activates primarily dimers composed of p65 and/or p50 (Fig. 2B).

To determine if p75-activated NF-\(\kappa\)B functions as a transcriptional activator, the cells were transfected with a reporter construct containing NF-\(\kappa\)B consensus binding sequences linked to the luciferase gene. Treatment of transfected cells with 100 ng/ml NGF for 4 h led to a 1.5 \(\pm\) 0.1-fold increase in luciferase activity (no further increase above control was observed up to 24 h after treatment) (Fig. 3). A similar level of induction was seen with 25 ng/ml TNF (Fig. 3) and phorbol ester (data not shown). No induction of luciferase was observed when a reporter construct lacking the NF-\(\kappa\)B consensus sequence was used, indicating that luciferase activity was due to transcriptional activation at the NF-\(\kappa\)B-binding site. Although significant \((p < 0.01, \text{relative to the effects of NGF on the mutant reporter by ANOVA analysis})\), the induction of luciferase was surprisingly small, especially for TNF and phorbol ester, given the strong signal observed in the EMSA. As a control, HEK 293 cells were similarly tested for their response to TNF. In these cells, a 13-fold activation of luciferase was observed \((n = 2; \text{data not shown})\). Thus, additional mechanisms regulating NF-\(\kappa\)B-dependent transcription must exist in the schwannoma cells, the nature of which remains to be deter-
Fig. 5. Activation of JNK in response to NGF. JNK activity was evaluated by immunoprecipitating JNK from whole cell lysates of RN22 cells treated with 100 ng/ml NGF for the indicated times or left untreated (CONTROL). Samples were then incubated with the JNK substrate GST-c-Jun (1–79) and radiolabeled ATP and then separated by SDS-polyacrylamide gel electrophoresis and visualized with a PhosphorImager.

mined. Similar disparate results between EMSAs and reporter assays have been observed in other systems (e.g. Ref. 38).

The activation of NF-κB occurs primarily through the degradation of the IκB proteins, a family of inhibitory proteins bound to the NF-κB dimers. In response to activating stimuli, the inhibitory proteins are phosphorylated, which targets them for ubiquitination and subsequent degradation. One member of this inhibitor family, IκBa, is degraded in response to the majority of NF-κB activators (25). However, the reduction in IκBa is only transient because the IκBa gene contains NF-κB-binding sequences in its promoter; therefore, transcription of the inhibitor is up-regulated in response to active NF-κB. To examine the mechanism of NF-κB activation in response to NGF, Western blots of total cell extracts from RN22 cells treated with NGF for varying times were probed with a poly-

Fig. 6. Effects of the NF-κB inhibitor SN50 on cell viability. A, RN22 schwannoma cells were treated with or without (control [CON]) 100 μg/ml SN50 for 30 min prior to the addition of NGF. After a 1-h incubation with or without 100 ng/ml NGF, the cells were harvested, and NF-κB activity was assessed in the cell lysates by EMSA as described in the legend to Fig. 2. Excess unlabeled oligonucleotide was added to the binding reaction to identify NF-κB-specific binding components (NGF + COLD). B, RN22 schwannoma cells in serum-free medium were treated with or without 100 μg/ml SN50 in the presence or absence of 100 ng/ml NGF, and cell viability was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay 24 h later. Depicted are the means ± S.E. of six experiments. The asterisk indicates a statistically significant difference from the other columns based on ANOVA with a Newman-Keuls multiple comparison test (p < 0.05).

required for phosphorylation-dependent degradation of the inhibitor. IκBAN has been shown to function as a dominant repressor of NF-κB in response to a variety of stimuli, including TNF and phorbol 12-myristate 13-acetate (26). RN22 schwannoma cells cotransfected with a NF-κB-luciferase reporter and pFLAG-IκBAN were insensitive to NGF activation of NF-κB-dependent transcription (Fig. 3).

Although the reporter assays demonstrated that IκBΔN pre-
vanented NF-κB activation of NF-κB, the assay requires transcription and translation of the luciferase gene, which take several hours. It is possible that during that time, many of the NF-κB-treated cells are dying. Therefore, we examined the functional effects of expressing IκBα on p75-induced activation of NF-κB in a shorter time frame by immunostaining. The cells were cotransfected with lacZ as a marker for transfection and a 5-fold excess of pFLAG-IκBαN or pFLAG vector and treated with NGF for 1 h (no cell death induced by any of the aforementioned conditions was observed in <1 h; data not shown). By immunostaining the cells with an antiserum to the p65 subunit of NF-κB, we observed that NGF-stimulated nuclear localization of p65 was blocked in cells expressing IκBαN, confirming the ability of this repressor to prevent p75 activation of NF-κB (Fig. 7, a–c). Expression of IκBαN did not affect activation of JNK in response to NGF, as shown by immunostaining with an antiserum directed against the phosphorylated active form of c-Jun (Fig. 7, d–f).

To examine the relationship between p75-mediated activation of NF-κB and the regulation of cell survival, the RN22 cells were cotransfected with pFLAG-IκBαN or pFLAG vector and lacZ. After 24 h, the cells were exposed to 100 ng/ml NGF in serum-free medium and then fixed and co-stained for β-galactosidase and TUNEL reactivity to identify cells committed to an apoptotic signal. It has been demonstrated that TNF activation of NF-κB can up-regulate the anti-apoptotic genes c-IAP1 and c-IAP2 (26, 32); however, the mechanism by which p75 activation of NF-κB promotes survival remains to be determined.

**Discussion**

Recently, several reports have suggested that the p75 neurotrophin receptor can induce programmed cell death in a variety of neuronal and glial cell types under specific conditions (see above and reviewed in Ref. 23). Nevertheless, the majority of cells expressing this receptor do not undergo apoptosis in response to NGF. p75 is a member of the TNF receptor superfamily that was initially classified based on sequence homology in its extracellular domain and more recently by a region in its intracellular domain with both sequence homology as well as structural characteristics similar to the death domain motif described for other members of this family (27). The death domain in the TNF receptor acquires its nomenclature based on its necessity for the well established ability of TNF to induce apoptosis. However, the TNF receptor also activates NF-κB, and this signal was demonstrated to functionally oppose the apoptotic signal since inhibiting its activation facilitated or enhanced TNF-induced death (19–22). Hence, the TNF receptor activates a bifurcating signal pathway, resulting in both initiation of caspase-dependent cell death and transcriptional activation of pro-survival genes. NGF has also been shown to activate the transcription factor NF-κB in both neuronal cells (4, 18, 28) and glia (3, 13–15), although the biological role of this signal is not known. Therefore, we hypothesized that the activation of NF-κB by NGF binding to p75 could be anti-apoptotic, in opposition to the p75 pro-death signal.

The physiological function of NF-κB in the nervous system is not well understood. In specific regions of the cortex and hippocampus, constitutive NF-κB activity has been observed; and in several cell culture systems, the activation of NF-κB has been shown to promote neuronal survival (17, 18, 29). In contrast, this transcription factor has also been suggested to be pro-apoptotic. It was shown to be induced following seizure (30) or ischemic injury (16), and the neuronal loss following a focal ischemic injury was significantly reduced in mice lacking the p50 subunit of NF-κB (16). However, it is possible that the effects observed in vivo are a result of activation of NF-κB in microglia, which can release neurotoxins (31). Thus, it remains to be determined whether activation of this transcription factor is neuroprotective or lethal.

Here we have shown that NGF binding to p75 activates a dual signaling pathway in Schwannoma cells, stimulating both NF-κB (Fig. 2) and JNK (Fig. 5). Similar to what has been reported for the TNF receptor, the subunits of NF-κB activated by p75 include p65 and p50. Treatment of the cells with the specific NF-κB inhibitors SN50 and IκBαN had no significant effect on cell survival alone and did not activate JNK, but did render the cells sensitive to NGF killing. These findings suggest that NF-κB activation of NF-κB can serve as an anti-apoptotic signal. It has been demonstrated that TNF activation of NF-κB can up-regulate the anti-apoptotic genes c-IAP1 and c-IAP2 (26, 32); however, the mechanism by which p75 activation of NF-κB promotes survival remains to be determined.
In neurons, the p75 receptor is usually expressed with one of the Trk proteins, the tyrosine kinase neurotrophin receptors, which are well established as transducers of survival signals. Expression of p75 has been shown to facilitate Trk signaling (23), suggesting that these receptors can collaborate to promote survival. Supporting this notion, it was demonstrated in terminally differentiated oligodendrocytes that although activation of p75 alone promoted apoptosis, when TrkA was coex-

**FIG. 8. NGF induces apoptosis in cells expressing IκBΔN.** A, RN22 cells were transfected with either pFLAG-IκBΔN (c–f) or pFLAG (a and b) and pCMV-lacZ. 24 h after transfection, the cells were treated with 100 ng/ml NGF for 4 h (a, b, e, and f) or were left untreated (c and d). The cells were then fixed and co-stained for TUNEL to label cells undergoing apoptosis and for anti-β-galactosidase to label transfected cells and were visualized by fluorescence microscopy. TUNEL staining is depicted in b, d, and f, and staining for β-galactosidase in a, c, and e. B, quantitation of NGF-induced apoptosis in cells expressing either pFLAG-IκBΔN or pFLAG and lacZ was done by counting β-galactosidase (β-gal)-positive cells and scoring for TUNEL reactivity. At least 100 cells were counted for each condition. Depicted are the means ± S.E. of six experiments. The asterisk indicates that the value for the NGF-treated cells transfected with pFLAG-IκBΔN was the only one significantly different from the other conditions based on ANOVA (p < 0.05).
pressed, the death signal was suppressed. The presence of TrkA in the oligodendrocytes suppressed the activation of JNK by p75, but NGF remained capable of activating NF-κB (3). In cultured sympathetic neurons and the neuronal cell line PC12, which both coexpress Trk and p75, NGF has been shown to stimulate NF-κB (18, 28). Moreover, inhibiting the activation of NF-κB in PC12 cells (29) or in sympathetic neurons (18) resulted in apoptosis, suggesting NF-κB activity is anti-apoptotic. However, since these cells express both NGF receptors, the mechanism by which NGF stimulates NF-κB in neural cells remains to be determined.

Although NGF binding to Trk activates neuronal survival pathways such as the kinase Akt, the activation of NF-κB may work in concert under normal conditions and become more essential during stress conditions. It is interesting to consider the observation of Cheng and Mattson (33) in this context. These authors observed that NGF could protect hippocampal neurons from death induced by glucose deprivation. These neurons do not express TrkA, the NGF tyrosine kinase receptor, but do express high levels of p75 under conditions of stress such as when dissociated in culture. Thus, one may speculate that NGF is protecting these neurons from glucose deprivation by activating NF-κB, although how the death signal of p75 is repressed is not clear.

A physiological role for p75 activation of NF-κB in stress or injury situations is particularly relevant in Schwann cells. Under normal conditions in adult animals, Schwann cells do not express appreciable levels of p75. However, in culture or following nerve injury, p75 expression is strongly increased in the distal Schwann cells (34, 35). Interestingly, not only does p75 and NGF expression increase in peripheral nerves following an insult (35), but recent findings suggest that NF-κB is activated in distal Schwann cells following sciatic nerve crush. Following such an insult in adult animals, there is no Schwann cell apoptosis in the distal nerve stump (36). Indeed, it has been suggested that mature Schwann cells possess an autocrine survival loop that prevents massive apoptosis following loss of axonal contact (37). It will be interesting to determine if NGF activation of NF-κB could, in part, account for this survival. In contrast to adult nerves, during development, there is extensive apoptosis of promyelinating Schwann cells (36). These cells require contact with an axon for trophic support, and failure to make contact is the most likely explanation for the cell death. However, the promyelinating cells also express p75, which may play a role in facilitating their death. In support of this, Soilu-Hanninen et al. (7) measured cell death induced by trophic factor withdrawal in isolated Schwann cells from postnatal day 2 mouse pups, a time when many of the Schwann cells would be in the promyelinating stage. They observed that when these early Schwann cells were exposed to NGF, there was a significant increase in apoptosis, which did not occur in p75−/− mice. Therefore, one can envision a role for the dual signaling of p75 in regulating the number of Schwann cells such that cells in the adult distal nerve stump, deprived of trophic support from the axon, will survive via activation of NF-κB through NGF binding to p75. In contrast, during development, the promyelinating cells that do not contact their axon may be susceptible to NGF-induced apoptosis via p75. How the NF-κB and cell death pathways are regulated under such conditions remains to be determined.

In summary, our results provide evidence that NGF activation of p75 in RN22 cells leads to a bifurcating signal pathway, resulting in activation of both pro-survival and pro-apoptotic branches. Blocking NF-κB activation resulted in the cells becoming susceptible to p75-induced cell death in response to NGF. Thus, p75-activated NF-κB may act as a survival factor in Schwann cells, and this may serve a physiological role in response to nerve injury.

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Note Added in Proof—While this manuscript was under review, similar findings were reported in sensory neurons (39).

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3 S. Kanwal and B. D. Carter, unpublished observations.
Nerve Growth Factor Activation of Nuclear Factor κB through Its p75 Receptor Is an Anti-apoptotic Signal in RN22 Schwannoma Cells
Jennifer J. Gentry, Patrizia Casaccia-Bonnefil and Bruce D. Carter

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