Cytokine-induced Nuclear Factor Kappa B Activation Promotes the Survival of Developing Neurons

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Abstract. Ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), and interleukin 6 (IL-6) comprise a group of structurally related cytokines that promote the survival of subsets of neurons in the developing peripheral nervous system, but the signaling pathways activated by these cytokines that prevent neuronal apoptosis are unclear. Here, we show that these cytokines activate NF-κB in cytokine-dependent developing sensory neurons. Preventing NF-κB activation with a super-repressor IκB-α protein markedly reduces the number of neurons that survive in the presence of cytokines, but has no effect on the survival response of the same neurons to brain-derived neurotrophic factors (BDNF), an unrelated neurotrophic factor that binds to a different class of receptors. Cytokine-dependent sensory neurons cultured from embryos that lack p65, a transcriptionally active subunit of NF-κB, have a markedly impaired ability to survive in response to cytokines, but respond normally to BDNF. There is increased apoptosis of cytokine-dependent neurons in *p65*$^{-/-}$ embryos in vivo, resulting in a reduction in the total number of these neurons compared with their numbers in wild-type embryos. These results demonstrate that NF-κB plays a key role in mediating the survival response of developing neurons to cytokines.

Key words: leukemia inhibitory factor • ciliary neurotrophic factor • cardiotrophin-1 • apoptosis • neuron

Introduction

Nuclear factor kappa B (NF-κB)[1] is a ubiquitously expressed transcription factor that consists of homodimers or heterodimers of a family of structurally related proteins (Baldwin, 1996; Ghosh et al., 1998). In most cell types, it is present as a heterodimer comprising p65 (RelA) and p50 subunits, which is held in an inactive form in the cytosol by interaction with a member of the IκB family of inhibitory proteins (Verma and Stevenson, 1997; Karin, 1998). NF-κB is activated by the phosphorylation and subsequent degradation of IκB, which results in translocation of the liberated NF-κB to the nucleus where it induces transcription of target genes. A wide variety of noxious stimuli, such as viral and bacterial infection, UV light, ionizing radiation, and free radicals, as well as a variety of lymphokines and cytokines, activate NF-κB which in turn positively regulates the expression of genes that mediate an acute inflammatory response (Baldwin, 1996; Baeuerle and Baichwal, 1997). In addition, NF-κB activation suppresses apoptosis induced by tumor necrosis factor (TNF) and various genotoxic agents in lymphoid and fibroblast cell lines (Beg and Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996) and protects hippocampal neurons against oxidative stress-induced apoptosis (Mattson et al., 1997). These data and the finding that there is massive hepatocyte death in *p65*$^{-/-}$ mouse embryos (Beg et al., 1995) indicate that NF-κB also plays a role in regulating cell survival.

In the nervous system, NF-κB is not only involved in mediating inflammatory responses (O'Neill and Kaltschmidt, 1997), but is induced by several molecules that play key roles in neural function and development. For

example, NF-κB is induced by glutamate in cerebellar granule cells (Guerrini et al., 1995; Kaltschmidt et al., 1995), by nerve growth factor (NGF) in Schwann cells (Carter et al., 1996), and NGF-dependent sympathetic and sensory neurons (Maggirwar et al., 1998; Hamanoue et al., 1999). In the case of Schwann cells and sensory neurons, it has been shown that NGF activates NF-κB by binding to the p75 neurotrophin receptor (Carter et al., 1996; Hamanoue et al., 1999). Although p75 is not essential for the survival response of neurons to NGF, binding of NGF to this receptor in neurons coexpressing the NGF receptor tyrosine kinase, TrkA, significantly enhances the number of neurons that survive (Davies et al., 1993; Lee et al., 1994; Horton et al., 1997; Ryden et al., 1997). Preventing NF-κB activation in NGF-treated sensory neurons causes a modest reduction in survival, suggesting that the p75-mediated enhancement of the NGF survival response is at least partly due to NF-κB activation (Hamanoue et al., 1999). Likewise, preventing NF-κB activation in NGF-treated sympathetic neurons reduces the survival of these neurons (Maggirwar et al., 1998).

In addition to the neurotrophins (NGF, brain-derived neurotrophic factor [BDNF], neurotrophin 3 [NT-3], and NT-4), several other families of neurotrophic factors play key roles in promoting and regulating neuronal survival. Ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), and interleukin-6 (IL-6) comprise a family of cytokines that have multiple actions on cells of the nervous system and promote the survival of various kinds of neurons during development (Sendtner et al., 1994; Stahl and Yancopoulos, 1994). Although there is <15% amino acid sequence identity between these factors, they share several characteristic structural features (Bazan, 1991; Robinson et al., 1994; McDonald et al., 1995) and signal via oligomeric receptor complexes that have one or more components in common (Davis et al., 1993; Stahl et al., 1994; Wollert et al., 1996). The transmembrane glycoproteins gp130 and LIFRβ are common components of the receptor complexes for CNTF, LIF, oncostatin M (OSM), and CT-1. The CNTF receptor complex has an additional GPI-linked CNTFRα subunit, and the IL-6 receptor consists of two gp130 subunits and an IL-6Rα subunit. Binding of these cytokines to their receptor complexes results in the direct or indirect activation of several signaling pathways including the JAK-Stat, PI-3 kinase, Ras/MAP kinase, and PLC-γ pathways (Boulton et al., 1994; Stahl et al., 1994; Frank and Greenberg, 1996).

To further investigate the role of NF-κB in regulating neuronal survival, we measured and experimentally manipulated NF-κB activation in populations of sensory neurons that survive in response to cytokines. Neurons from the trigeminal and nodose ganglia of embryonic day 18 (E18) mouse embryos were used for these studies because their large size facilitates microinjection of constructs used for measuring and manipulating NF-κB activation. These neurons are supported by neurotrophic cytokines in culture and die by apoptosis after neurotrophic factor deprivation (Horton et al., 1998). We show that NF-κB activation plays a major role in mediating the survival response of these neurons to neurotrophic cytokines.

Materials and Methods

Neuron Culture, Microinjection, and Survival Assays

For microinjection studies, dissociated cultures of trigeminal and nodose ganglion neurons were established from E18 CD1 embryos. The dissected ganglia were trypsinized and dissociated by trituration, and the neurons were purified free of nonneuronal cells by differential sedimentation (Davies, 1986). The neurons (>95% pure) were grown in defined, serum-free medium on a poly-ornithine/laminin substratum in 60-mm diam tissue culture petri dishes (Davies et al., 1993). After an initial 12–24-h incubation period with a neurotrophic factor that promotes survival, the neurons were washed extensively to remove this factor and were injected intranuclearly (Allsopp et al., 1993) with pcDNAIII expression plasmids. The pcDNAIII plasmid without a cDNA insert was used to control for nonspecific effects of the injection procedure, and pcDNAIII plasmids containing cDNAs encoding the human p65 NF-κB subunit or a mutated IκB-α that is defective in signal-induced degradation (Rodriguez et al., 1996) were used to investigate the role of NF-κB activation in promoting neuronal survival. The expression plasmids were diluted in 100 mM potassium phosphate buffer, pH 7, to a concentration of 100 μg/ml and filtered through a 0.22-μm filter before injection. Some cultures were resupplemented with neurotrophic factor 30 min after injection. The number of surviving neurons was counted at 24 hourly intervals after injection and is expressed as a percentage of the number injected. About 150 neurons were injected for each experimental condition.

For studies of the endogenous p65 on neuronal survival, cultures were established from mice that have a null mutation in the *p65* gene (a gift of Amer Beg and David Baltimore (Beg et al., 1995)). Heterozygous mice were crossed to obtain *p65*⁻/⁻, *p65*⁺/⁻ and *p65*⁺/⁺ embryos. After 12- or 14-d gestation, the pregnant females were killed and nodose ganglia were dissected from each embryo separately. After genotyping the embryos by a PCR based method, the ganglia of each genotype were pooled and low-density, dissociated cultures were established. The neurons were grown in defined medium in laminin/poly-ornithine coated petri dishes with a range of concentrations of CNTF, LIF, CT-1, IL-6, or BDNF as described above. The number of neurons surviving after 48-h incubation is expressed as a percentage of the number of attached neurons counted 6 h after plating.

Measurement of NF-κB Activation

E18 trigeminal neurons were grown for 24 h with 2 ng/ml NGF, washed thoroughly to remove the NGF, and incubated in F14 medium for a further 2 h before being injected with two DNA constructs: an NF-κB–dependent luciferase reporter gene that consists of three synthetic copies of the NF-κB–consensus sequence from the Ig-κ chain promoter upstream of the luciferase gene (Rodriguez et al., 1996) and a lacZ gene that is driven by an RSV promoter. In some experiments, neurons were also coinjected with the mutated IκB-α expression plasmid. Injected neurons were harvested using a rubber policeman 6 h later. Several hundred neurons were injected for each assay. The cultures were treated with 50 ng/ml CNTF 30 min after injection; untreated cultures served as controls. Luciferase activity was measured by using a luminometer as described previously (Rodriguez et al., 1996) and β-galactosidase activity was measured by using the Galactolight kit (Tropix) as recommended by the manufacturer. The relative level of NF-κB activation in each experiment was calculated by dividing the luciferase activity by β-galactosidase activity.

Quantification of the Number of Neurons in the Nodose Ganglia

E14 mouse embryos in litters resulting from overnight matings of *p65*⁺/⁻ mice were fixed in Carnoy's fluid (60% ethanol, 30% chloroform, 10% acetic acid) for 20 min before dehydration and wax-embedding. Serial sections of the heads were cut at 8 μm, mounted on poly lysine-coated slides, and cleared in xylene and dehydrated before quenching (10% methanol, 3% hydrogen peroxide in PBS). To identify all neurons in these preparations, the sections were stained for neurofilament protein as described previously (Middleton et al., 1998). In addition, the sections were counterstained with cresyl fast violet to permit identification of pyknotic nuclei. Estimation of neuronal number was carried out as described previously (Piñón et al., 1996) using the Abercrombie method to correct for split nuclei (Abercrombie, 1946). Pyknotic nuclei were recognized as one or more

darkly stained spherical structures contained within a clearly visible membrane (Piñón et al., 1996).

Immunocytochemical Detection of Cytokine Receptors on Nodose Neurons

To ascertain if the *p65* null mutation affects the expression of cytokine receptors on nodose neurons, immunocytochemistry was used to assess the proportion of neurons that express cytokine receptor components in cultures established from the nodose ganglia of E12 and E14 wild-type $p65^{+/-}$ and $p65^{-/-}$ embryos. Dissociated cultures of nodose neurons were cultured with a cocktail of neurotrophic factors (50 ng/ml CNTF, 50 ng/ml LIF, 50 ng/ml CT-1, and 10 ng/ml BDNF) to sustain the survival of the maximum number of neurons. 6 h after plating, the cultures were fixed for 15 min in neutral buffered formalin. After washing with PBS, endogenous peroxidase activity was quenched with 1% hydrogen peroxidase in methanol. After 20 min incubation in PBS containing 0.5% Triton X-100 and 10% serum (rabbit serum for goat primary antibody and goat serum for rabbit primary antibody), the cultures were incubated for 1 h at room temperature in 1:300 dilution of one of the following antibodies: rabbit anti-gp130, rabbit anti-LIFRβ, goat anti-CNTFRα, and rabbit anti-IL-6Rα (all from Santa Cruz). After washing with PBS, bound primary antibodies were labeled using biotinylated secondary antibody (1:200), avidin, and biotinylated HRP macromolecular complex (Vectastain ABC Kit, Vector Laboratories). The substrate used for the peroxidase reaction was 1 mg/ml diaminobenzidine tetrachloride (Sigma Chemical Co.). The number of labeled neurons was assessed by bright-field illumination on an inverted microscope.

Results

CNTF Activates NF-κB

To ascertain whether neurotrophic cytokines activate NF-κB in neurons, we microinjected neurotrophic factor-deprived neurons with an NF-κB–dependent luciferase reporter construct (Rodriguez et al., 1996) and assayed luciferase activity after cytokine treatment. Because relatively large numbers of neurons have to be injected to produce a clearly quantifiable signal, we used trigeminal neurons for these studies because the trigeminal ganglia are by far the largest ganglia in the peripheral nervous system of the mouse embryo. These studies were carried out at E18 because trigeminal neurons display a clear survival response to CNTF at this stage (Horton et al., 1998) and have grown to a sufficiently large size to facilitate microinjection. To standardize luciferase activity measurements between experiments, we coinjected a lacZ gene driven by a constitutive RSV promoter and additionally assayed for β-galactosidase activity. Separate experiments showed that β-galactosidase activity was unaffected by CNTF (data not shown). Fig. 1 shows that five hours after CNTF treatment, the relative luciferase activity was fourfold higher than in untreated control cultures, indicating that CNTF activates NF-κB in these neurons. The activity of a control luciferase reporter lacking NF-κB binding sites was unchanged by treatment with CNTF (data not shown).

To determine if the rise in luciferase activity after CNTF treatment was a direct consequence of NF-κB–dependent transcription, the NF-κB–dependent luciferase reporter was coinjected with a plasmid that expresses a super-repressor form of the NF-κB inhibitor, IκB-α. This IκB-α protein associates normally with NF-κB, but carries serine-to-alanine mutations at residues 32 and 36 that prevents signal modification and proteosome-mediated degradation, thereby preventing release and translocation of NF-κB to the nucleus (Rodriguez et al., 1996; Roff et al.,
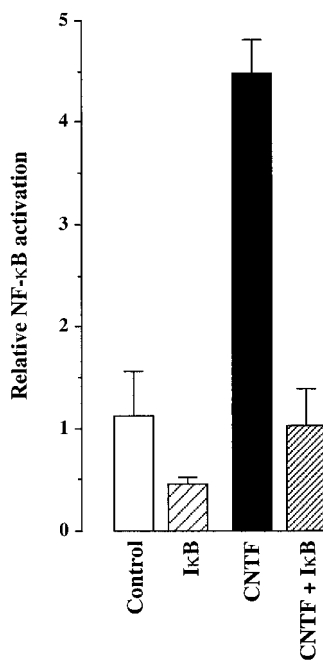

Figure 1. Bar chart of relative NF-κB activation (luciferase activity divided by β-galactosidase activity) in E18 trigeminal neurons under different experimental conditions. The neurons were initially cultured with NGF for 24 h, washed, and incubated in NGF-free medium for 2 h, then coinjected with the NF-κB–dependent luciferase reporter construct and the RSV lacZ expression plasmid (plus the super-repressor IκB-α expression plasmid in the cultures indicated), and maintained for a further 6 h before assaying for luciferase and β-galactosidase activities. CNTF (50 ng/ml) was added to the cultures indicated immediately after injection. Relative NF-κB activity at each point is calculated from the ratio of luciferase to lacZ activity compared with the untreated control. The mean and SEM are shown for three separate experiments.

1996). This super-repressor IκB-α completely abolished the rise in luciferase activity after treatment with CNTF (Fig. 1), indicating that this rise was due to NF-κB–dependent transcription.

NF-κB Activation Plays a Role in Mediating the Cytokine Survival Response

Having ascertained that CNTF activates NF-κB, we investigated whether preventing NF-κB activation would interfere with the CNTF survival responses. Injection of the super-repressor IκB-α expression plasmid into E18 trigeminal neurons caused a highly significant reduction in the number of neurons surviving with CNTF, compared with neurons injected with an empty expression plasmid ($P < 0.0001$, *t* test; Fig. 2). These results suggest that the survival response of trigeminal neurons to CNTF is mediated to a large extent by the activation of NF-κB.

Because nodose ganglion neurons are more responsive to cytokines than trigeminal neurons (Horton et al., 1998), we extended our analysis of the antiapoptotic function of NF-κB to this population of sensory neurons. Fig. 3 shows that injection of E18 nodose neurons with the super-repressor IκB-α expression plasmid markedly reduced the number of neurons surviving with CNTF, CT-1, and LIF, compared with neurons injected with an empty expression plasmid ($P < 0.0001$ in all cases, *t* tests). The percentage reduction in survival ranged from 45% with LIF to 62% with CNTF. In contrast, injection of nodose neurons with the super-repressor IκB-α expression plasmid had no effect on the survival response of these neurons to BDNF (Fig. 3). This not only clearly indicates that expression of super-repressor IκB-α does not exert a nonspecific detrimental effect on neuronal survival, but shows that NF-κB
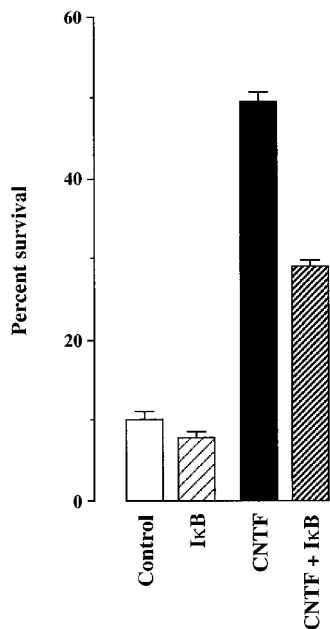
Figure 2. Bar chart of the number of E18 trigeminal ganglion neurons surviving 24 h after injection with either and empty pcDNAIII plasmid (control and CNTF) or the super-repressor IκB-α plasmid (IκB and CNTF + IκB) expressed as a percentage of the number of injected neurons. The mean and SEM are shown for three separate experiments.

activation plays a highly selective role in mediating neurotrophic factor responses.

To confirm that NF-κB activation independent of cytokine treatment could sustain the survival of neurotrophic factor-deprived nodose neurons, we microinjected a plasmid expressing the transcriptionally active p65 subunit of NF-κB into neurotrophic factor-deprived nodose neurons. In these experiments, cultures of purified nodose neurons were first incubated for 24 h in LIF-supplemented medium. The neurons were then deprived of LIF by extensive washing and injected with the p65 expression plasmid or an empty plasmid to control for the injection procedure. Counts of surviving neurons made at 24 hourly intervals after injection revealed that virtually all neurotrophic factor-deprived neurons injected with the empty expression plasmid had died after 72 h, whereas a large proportion of p65 overexpressing neurons survived at all time points (Fig. 4). Coinjection of the super-repressor IκB-α plasmid with the p65 plasmid completely prevented the antiapoptotic function of overexpressed p65 (data not shown), indicating that exogenous p65 prevented apoptosis as a direct consequence of NF-κB activation. Neurons that were resupplemented with LIF or CNTF survived better than neurotrophic factor-deprived neurons overexpressing p65 (Fig. 4), suggesting that NF-κB activation may not be sufficient to account for the full survival-promoting effects of these cytokines, as suggested in the data shown in Figs. 2 and 3.

p65-deficient Sensory Neurons Are Less Responsive to Cytokines

To further investigate the role of NF-κB activation in mediating the response of neurons to cytokines, we compared the dose responses of nodose neurons obtained from wild-type embryos and embryos that are homozygous for a null mutation in the p65 gene (Beg et al., 1995). The latest stage at which these experiments could be carried out was E14 because p65$^{-/-}$ embryos die in utero
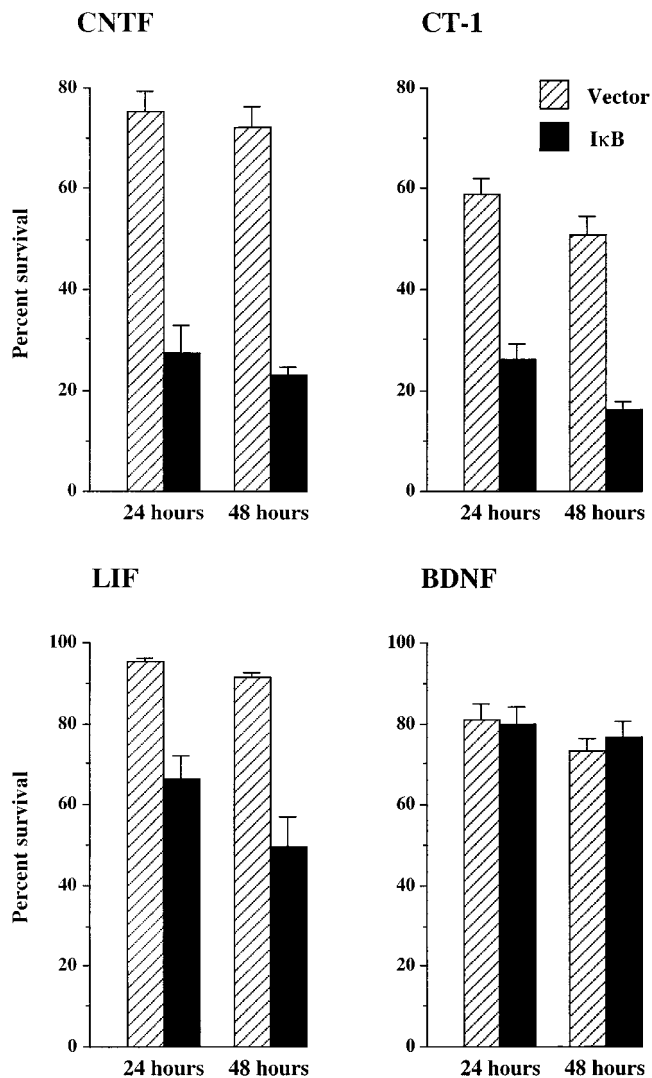

Figure 3. Bar chart of the number of E18 nodose ganglion neurons surviving 24 and 48 h after injection with either an empty pcDNAIII plasmid (vector) or the super-repressor IκB-α plasmid (IκB) expressed as a percentage of the number of injected neurons. The data from neurons grown separately with 50 ng/ml CNTF, 50 ng/ml CT-1, 50 ng/ml LIF, and 10 ng/ml BDNF are shown. The means and SEM of the results of five to ten separate experiments for each factor are shown.

shortly after this stage. Although nodose neurons are maximally responsive to neurotrophic cytokines at late fetal stages, a substantial proportion of these neurons survive in response to cytokines at stages up to E14 (Horton et al., 1998). For these experiments, adult p65$^{+/-}$ mice were crossed and pregnant females were killed after 12- or 14-d gestation and the nodose ganglia dissected out from the embryos. Because of the small size of the nodose ganglia, a rapid genotyping procedure was used so that the ganglia from either wild-type or p65$^{-/-}$ embryos could be pooled to obtain sufficient neurons for at least one dose response in each experiment. Fig. 5 shows that p65-deficient nodose neurons displayed a substantially reduced survival response to LIF, CNTF, CT-1, and IL-6 compared with wild-type embryos from the same litters. Although only either
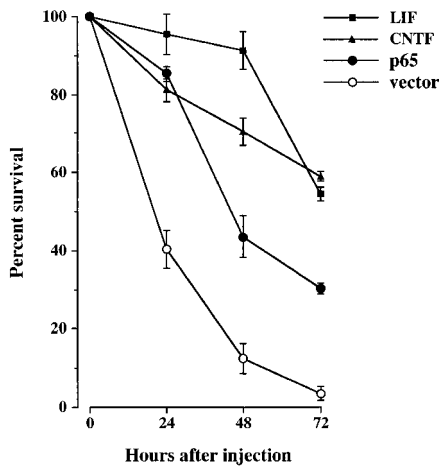
Figure 4. Graph of the number of E18 nodose ganglion neurons surviving at 24 hourly intervals after LIF deprivation and injection with either an empty pcDNAIII plasmid (vector) or the p65 plasmid (p65) expressed as a percentage of the number of injected neurons or resupplemented with LIF or CNTF. The means and SEM are shown for three separate experiments.

E12 or E14 data are illustrated for each cytokine, in several experiments carried out at both ages, the survival of p65-deficient neurons to each cytokine was markedly lower than wild-type neurons throughout the broad concentration range studied. In the case of CNTF, where the difference in survival between wild-type and p65-deficient neurons was most pronounced, the number of wild-type neurons surviving with CNTF was more than fivefold greater than the number of p65-deficient neurons surviving with this factor over virtually the entire concentration range. These results indicate that the absence of p65 substantially reduces the ability of neurotrophic cytokines to promote the survival of developing nodose neurons. In contrast to the markedly impaired survival response of p65-deficient nodose neurons to cytokines, our previous work on the response of p65-deficient neurons to neurotrophins has demonstrated that the dose response of p65-deficient nodose neurons to BDNF is completely normal (Hamanoue et al., 1999). This demonstrates that the impaired survival response of p65-deficient nodose neurons to cytokines is not some nonspecific detrimental effect of the lack of p65 on cell viability, but shows that p65 plays a highly selective role in mediating responses to certain neurotrophic factors.

Increased Apoptosis in the Nodose Ganglia of p65-deficient Embryos

To determine if the reduced survival response of p65-deficient nodose neurons to cytokines observed in vitro results in increased death of these neurons in vivo, we counted the total number of neurons in the nodose ganglia of wild-type and $p65^{-/-}$ embryos and estimated the proportion of neurons undergoing apoptosis at E14. Embryos were fixed, embedded, and serially sectioned through the nodose ganglia and stained for neurofilament protein to positively identify all neurons. The number of neurons with pyknotic nuclei and the total number of neurons were
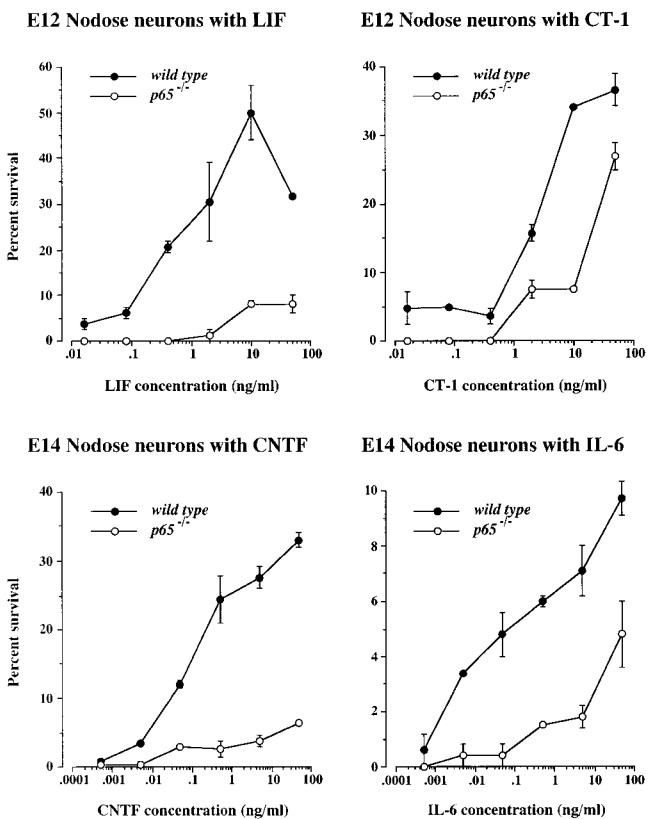

Figure 5. Graphs of the dose responses of E12 or E14 nodose neurons from wild-type and $p65^{-/-}$ embryos to LIF, CT-1, CNTF, and IL-6. The means and standard errors of typical dose responses set up in triplicate are shown. The results of two to four separate dose response experiments for each factor at each age were very similar to the results illustrated.

counted in these sections. All histology slides were coded so that these estimates were made without knowledge of the genotype.

The number of cells undergoing apoptosis in the trigeminal ganglion was estimated by counting the number of pyknotic nuclei that were recognized as one or more darkly stained spherical structures contained within a clearly visible membrane. The great majority of pyknotic nuclei were observed in large degenerating cells, suggesting that these were neurons (Oppenheim, 1991). Fig. 6 shows that the number of pyknotic nuclei in the nodose ganglia of E14 $p65^{-/-}$ embryos was almost threefold greater than in wild-type embryos of the same age. There was also a 30% reduction in the total number of neurons in the nodose ganglia of $p65^{-/-}$ embryos at this age. The total number of neurons in the nodose ganglia of $p65^{+/-}$ embryos was intermediate between the numbers in wild-type and $p65^{-/-}$ embryos, indicating a gene dosage effect. These results indicate that the survival of p65-deficient nodose neurons is impaired in vivo.

Cytokine Receptor Expression Is Unaffected in p65-deficient Nodose Neurons

As NF-κB is a transcription factor that regulates the expression of many genes, it is possible that the reduced sur-
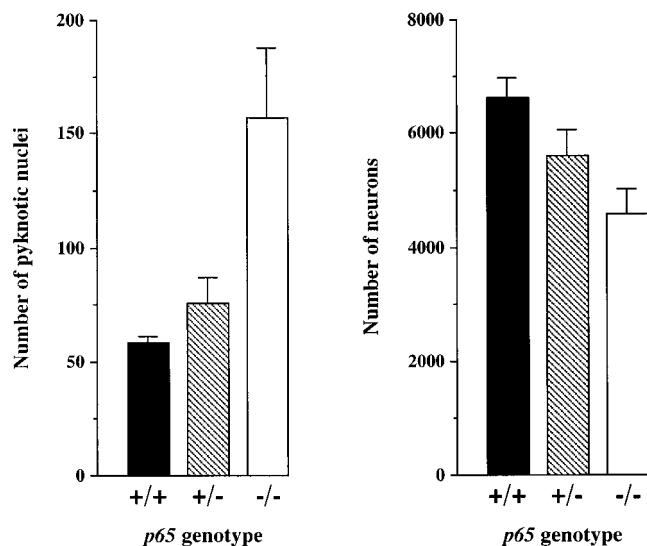
E14 Nodose ganglia

Figure 6. Bar charts of the number of pyknotic nuclei and total numbers of neurons in the nodose ganglia of E14 wild-type, $p65^{+/-}$, and $p65^{-/-}$ embryos. The means and standard errors of the data obtained from five wild-type, seven $p65^{+/-}$, and seven $p65^{-/-}$ embryos are shown.
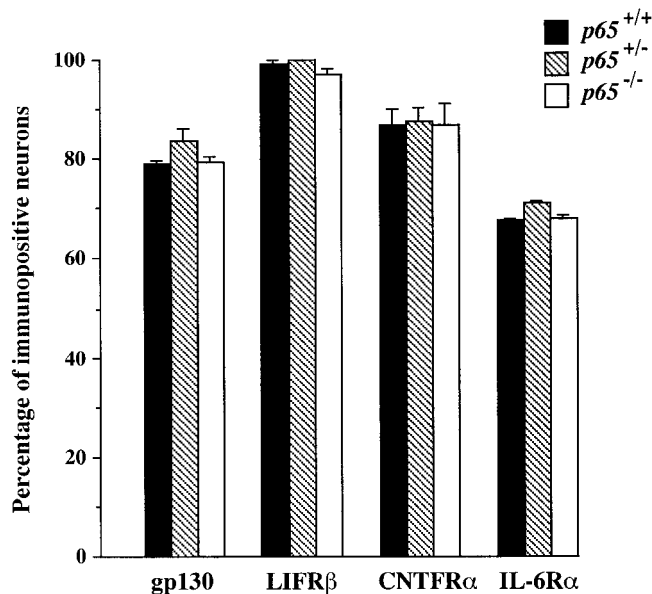

Figure 7. Bar chart of the percentage of E14 nodose neurons in short-term cultures established from wild-type, $p65^{+/-}$, and $p65^{-/-}$ embryos that are immunoreactive for gp130, LIFRβ, CNTFRα, and IL-6Rα. The means and standard errors of the percentage of labeled neurons in quadruplicate wells for each data point in a representative experiment are shown. Similar results were observed in two additional experiments carried out at E12 and E14.

vival response of p65-deficient sensory neurons to cytokines is not a direct consequence of p65 being an integral component of a cytokine signaling pathway, but may be secondary to a reduction in cytokine receptor expression. There is evidence, for example, that NF-κB regulates the expression of the IL-2 receptor (Cross et al., 1989). To explore the possibility that the selective reduction of the cytokine survival responses in p65-deficient neurons might be due to reduced expression of cytokine receptors, we used immunocytochemistry to assess the proportion of neurons that express cytokine receptor components in cultures established from the nodose ganglia of E12 and E14 wild-type, $p65^{+/-}$, and $p65^{-/-}$ embryos. In these experiments, dissociated cultures of nodose neurons were cultured for six hours with a cocktail of cytokines and BDNF to sustain the survival of the maximum number of neurons. The cultures were then fixed and stained with antibodies to gp130, LIFRβ, CNTFRα, and IL-6Rα. Fig. 7 shows that the great majority of neurons exhibited positive staining for each of these receptors and that the percentage stained for each receptor was almost identical in wild-type and p65-deficient neurons. No neurons were stained in cultures that were not incubated with primary antiserum, but otherwise, were processed normally. These findings suggest that similar levels of cytokine receptors are expressed in p65-deficient and wild-type nodose neurons and that reduced receptor expression is unlikely to account for the decreased sensitivity of p65-deficient neurons to cytokines.

Discussion

Using a variety of complementary experimental ap-

proaches, we have demonstrated that NF-κB plays a major role in mediating the survival response of embryonic sensory neurons to the neurotrophic cytokines, CNTF, LIF, CT-1, and IL-6. First, CNTF causes a marked activation of NF-κB in cultured trigeminal neurons. Second, preventing CNTF-induced NF-κB activation in these neurons with super-repressor IκBα causes a substantial reduction in the survival response of these neurons to CNTF. Likewise, super-repressor IκB causes a marked reduction in the survival response of nodose neurons to CNTF, LIF, and CT-1. Third, nodose neurons from $p65^{-/-}$ embryos have a poor survival response to these cytokines compared with neurons from wild-type embryos. Fourth, increased numbers of cytokine-dependent nodose neurons die by apoptosis in $p65^{-/-}$ embryos in vivo. Expression of the super-repressor IκB does not exert a nonspecific detrimental effect on neuronal survival because it has no effect whatsoever on the survival of nodose neurons grown with BDNF. Likewise, the poor survival of p65-deficient nodose neurons with neurotrophic cytokines does not represent a nonspecific detrimental effect of the absence of this protein on cell survival because the dose response of these neurons to BDNF is entirely normal. Furthermore, the selective reduction in the response of nodose neurons to cytokines is not due to reduced expression of cytokine receptors because the proportion of nodose neurons that express cytokine receptor subunits is completely unaffected by the *p65* null mutation.

Previous work has shown that NGF activates NF-κB in developing sensory neurons by binding to the p75 neurotrophin receptor and that inhibiting NF-κB activation

with super-repressor IκBα causes a small reduction in the number of neurons surviving with NGF (Hamanoue et al., 1999). In contrast, the level of NF-κB activation induced by CNTF in the same sensory neurons is greater and the decrease in the survival response to this and other neurotrophic cytokines brought about by super-repressor IκB is substantially greater. These findings, together with our demonstration that the response of p65-deficient sensory neurons to neurotrophic cytokines is markedly reduced, indicate that NF-κB activation plays a much more prominent role in mediating the survival response of developing sensory neurons to cytokines than to NGF.

Although it has been established that binding of CNTF to its receptor complex activates directly or indirectly a number of signaling pathways, including the JAK-Stat, PI-3 kinase, Ras/MAP kinase, and PLC-γ pathways (Boulton et al., 1994; Stahl et al., 1994; Frank et al., 1996), the mechanism by which CNTF promotes neuronal survival is not known. However, most signals that induce NF-κB do so by activation of the IκB kinases α and β. The activated kinases phosphorylate IκB-α on serines 32 and 36 leading to recognition of phosphorylated IκB-α by an SCF ubiquitin ligase complex containing βTrCP (Maniatis, 1999). Studies with knockout mice have indicated that, whereas IKKα appears to be involved in the development of skin and skeleton, IKKβ appears to participate in signaling mediated by the proinflammatory cytokines, TNFα and IL-1β (May and Ghosh, 1999). As CNTF signaling is blocked by the S32A/S36A mutant of IκBα that is unable to be phosphorylated by IKKα or IKKβ, it seems likely that CNTF signaling leads to activation of either IKKα or -β. In addition, it has recently been demonstrated that the PKB/Akt protein kinase, activated by PI-3 kinase, directly phosphorylates and activates IKKα, leading to NF-κB activation (Ozes et al., 1999; Romashkova and Makarov, 1999). Thus, a possible sequence of events after engagement of CNTF by its receptor complex is activation of PI-3 kinase, leading to activation of PKB/Akt, which in turn phosphorylates and activates IKKα, resulting in signal-induced degradation of IκBα and release of active NF-κB.

In addition to promoting neuronal survival, CNTF has many functions in different cell types, including regulation of neuroblast and oligodendrocyte proliferation (Ernsberger et al., 1989; Barres et al., 1996), neuropeptide and neurotransmitter synthesis (Ernsberger et al., 1989; Rao et al., 1992; Louis et al., 1993), rod cell differentiation (Fuhrmann et al., 1998), and induction of acute phase proteins in hepatocytes (Schooltink et al., 1992). It has yet to be ascertained which of the signaling pathways activated by CNTF mediate each of these cellular responses. Although LIF has been shown to activate NF-κB in phagocytes (Gruss et al., 1992), we have shown for the first time that CNTF activates NF-κB in neurons and that this plays a major role in mediating the trophic actions of this and other cytokines.

Although the mechanism by which NF-κB activation promotes the survival of cytokine-dependent developing sensory neurons is unknown, there is growing evidence that NF-κB induces the expression of several genes encoding proteins that oppose the cell death program. It appears that prevention of TNFα-mediated apoptosis by NF-κB is a direct consequence of increased expression of genes en-

coding TRAF1, TRAF2, c-IAP1, and c-IAP2, which block caspase 8 activation (Chu et al., 1997; Wang et al., 1998). Two other genes that appear to be important in protecting cells from TNF-induced apoptosis, A20 and IEX-1L, are also direct transcriptional targets of NF-κB (Krikos et al., 1992; Wu et al., 1998). NF-κB induces expression of the Bcl-2 homolog A1 in B and T cells, which is critical for survival during lymphocyte activation (Grumont et al., 1999; Zong et al., 1999), and NF-κB–mediated induction of Bcl-2 and Bcl-x also plays a role in the neuroprotective action of TNF against hypoxia- or nitric oxide-induced injury (Tamatani et al., 1999).

In summary, we have provided the first evidence that NF-κB plays a major role in mediating the neurotrophic actions of cytokines. These findings not only have implications for understanding survival signaling in neurons during the critical stages of development when connections are being established, but raise questions about the importance of NF-κB for sustaining the survival of mature neurons and in neurodegenerative diseases.

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