The p75 neurotrophin receptor (p75NTR) has been shown to mediate neuronal death through an unknown pathway. We microinjected p75NTR expression plasmids into sensory neurons in the presence of growth factors and assessed the effect of the expressed proteins on cell survival. We show that, unlike other members of the TNFR family, p75NTR signals death through a unique caspase-dependent death pathway that does not involve the “death domain” and is differentially regulated by Bcl-2 family members: the anti-apoptotic molecule Bcl-2 both promoted, and was required for, p75NTR killing, whereas killing was inhibited by its homologue Bcl-xL. These results demonstrate that Bcl-2, through distinct molecular mechanisms, either promotes or inhibits neuronal death depending on the nature of the death stimulus.

...cared neurons. We show that the p75NTR death signaling pathway is unlike the TNFR death signaling pathway as an intracellular juxta-membrane domain, and not the death domain, of p75NTR is sufficient and required to mediated death signaling. Secondly, the anti-apoptotic Bcl-2 family members that have little influence on TNFR death signaling have profound effects on the p75NTR death pathway.

MATERIALS AND METHODS

Cell Culture—Dorsal root ganglia were dissected from postnatal day zero C57Bl/6 or Bcl-2-deficient mice and plated in 3-cm tissue dishes precoated with poly-L-ornithine (500 µg/ml, Sigma) and laminin (20 µg/ml, Life Technologies, Inc.) at a density of 5000 neurons/dish (5). Cells were grown in Monomed medium (CSL, Melbourne, Australia) containing 1% fetal bovine serum and leukemia inhibitory factor (LIF, AMRAD, Australia) or nerve growth factor (2.5 S NGF, 50 ng/ml, Alomone Laboratories). Survival of sensory neurons was assessed by morphological criteria (5) and propidium iodide exclusion. Heterozygous mice containing a disrupted Bcl-2 gene, (D. Loh, Roche, Japan) were mated to produce litters containing homozygous Bcl-2 knock-out mice. The genotypes of newborn mice were determined by polymerase chain reaction, and results were confirmed by staining of thymocytes with an anti-mouse Bcl-2 antibody (PharMingen) before dissection and microinjection of neurons isolated from individual mice of appropriate genotype.

Microinjection—Sensory neurons were injected into the nucleus with a solution containing plasmid (100 µg/ml, with the exception of Bcl-2, which was at 50 µg/ml), tetramethylrhodamine dextran (“fluoro-ruby,” 0.15%, Molecular Probes), and phosphate-buffered saline. Where more than one plasmid was expressed in a single condition, only one injection of solution containing all plasmids was made, with the individual plasmid concentrations as specified above. Approximately 70 neurons/well were injected, with two or three wells comprising each condition. At least 2 h after completion of the injections, the number of fluorescent cells that had survived the injection procedure was counted, and this provided the time zero 100% value for each well.

Immunostaining—Cells were fixed with 4% paraformaldehyde for 15 min and then stained with an anti-rat-p75NTR antibody (MC192, Roche Molecular Biochemicals) (3) or anti-human-Bcl-2 antibody (Bcl-2/100, PharMingen).

DNA Constructs—The plasmid containing the full-length rat p75NTR cDNA, p75NTR, is described (4), and all p75NTR plasmids are modified versions of this original expression vector. A control plasmid, p75NTRnc (no cytoplasmic domain) is missing the entire cytoplasmic domain except the membrane anchoring Lys-274 and Arg-275. p75NTRtr is truncated with an I308A substitution followed by a stop codon, deleting the entire death domain. p75NTRΔd retains Lys-274 and Arg-275 and the last 108 amino acids creating a cytoplasmic domain including the death domain but deleted for the 33-amino acid juxta-membrane domain retained by p75NTRtr. All plasmids were constructed using polymerase chain reaction to amplify desired coding regions followed by subcloning the polymerase chain reaction products into plasmid vectors. Details of primers used in construction of these plasmids are available on request. Bcl-2 and Bcl-xL, plasmids are previously described (16, 17). The modified CrmA has the caspase recognition sequence modified from wild-type Leu-Glu-Ala-Asp to Asp-Gln-Met-Asp.

Yeast Two-hybrid Methods—The p75NTR death domain from Leu-342 on was cloned into pGBT9 (CLONTECH) and used to screen a
C57Bl/6 P2 dorsal root ganglia neuron library cloned into the HybriZap vector (Stratagene). No death domain proteins were isolated. Interactions between the p75NTR death domain and other known death signaling proteins (FADD death domain, full-length FADD, IAP1, Trafs 1–6) cloned into pGAD10 (CLONTECH) were tested by sequential yeast transformation for growth on media selective for an interaction.

RESULTS

To investigate whether p75NTR signals death through interaction with death domain containing proteins or via another pathway, we have activated the p75NTR pathway in a similar fashion to experiments that use ligand-free overexpression of TNFR to study apoptosis (18). We overexpressed p75NTR in neurons by microinjecting a rat p75NTR expression plasmid into the nucleus of mouse dorsal root ganglia sensory neurons (Fig. 1) and cultured them in the presence of the neural cytokine, LIF, to prevent p75NTR-independent neuronal death. These neurons were chosen because we had previously shown that their death was, at least in part, mediated by p75NTR (5, 6). It was found after 16 h that approximately 20–25% of neurons injected with full-length p75NTR plasmid died (Fig. 2A), compared with neurons injected with a control β-galactosidase plasmid or a truncated p75NTR protein lacking the entire cytoplasmic domain (p75NTRnc) expressed to a similar extent. The majority of the p75NTR-mediated death was observed in the first 16 h (see Fig. 3C for example), comparable...
Bcl-2 has previously been observed to increase cell death when highly expressed both in vitro and in vivo (23–25). Thus, it is possible that the high level of Bcl-2 is able to “prime” the domain adapter proteins, such as TRADD and FADD, may be responsible for p75NTR-mediated killing.

To further explore whether p75NTR killing was different from TNFR killing pathways, we examined whether overexpression of the anti-apoptotic Bcl-2 family proteins could inhibit killing by p75NTR. Bcl-2 and Bcl-xL, are well characterized inhibitors of growth factor withdrawal and stress-induced apoptosis (15). However, both proteins are poor inhibitors of CD95/Apo1/Fas and TNFR-mediated apoptosis (20, 21).

We found that overexpression of Bcl-xL protected neurons against p75NTR-induced death (Fig. 3A), supporting the hypothesis that p75NTR signals through an alternative pathway to TNFR-induced apoptosis. Whereas Bcl-2 overexpression alone had no effect on cell survival in the presence of LIF (Fig. 3B), Bcl-2 in combination with p75NTR overexpression, surprisingly, enhanced the neuronal death seen with p75NTR overexpression alone, yet in combination with p75NTRnc it had no effect (Fig. 3B). The results are surprising because Bcl-2 is functionally indistinguishable from Bcl-xL in most cell-death systems (16, 22), yet in this assay we find that they have opposite effects (Fig. 3C). The cell death observed with p75NTR and Bcl-2 overexpression was totally ablated if the cells were cultured in NGF, confirming that signaling though trk may inhibit p75NTR activity (Fig. 3D). Bcl-2 was able to protect against neuronal death induced by NGF withdrawal (Fig. 3E).

Thus, at the same expression levels in the same neuronal population, Bcl-2 was able to inhibit or enhance neuronal cell death depending on the nature of the death signal.

To determine whether the paradoxical effect of Bcl-2 on p75NTR-induced killing was related to its known anti-apoptotic activity, inactive Bcl-2 mutants were utilized (17). Like wild-type Bcl-2, expression of the Bcl-2 mutants did not affect neuronal survival. In combination with p75NTR expression, the enhanced killing effect seen with Bcl-2 co-expression was abrogated by expression of the mutant G145E Bcl-2, suggesting the protein was non-functional (Fig. 4A). Thus, an intact BH1 homology region is required for both the survival and death promoting activities of Bcl-2. Using a W188A mutated Bcl-2, we found that co-expression with p75NTR not only abolished the increased p75NTR killing but, more importantly, protected neurons from any p75NTR-induced death (Fig. 4D), reminiscent of that seen with Bcl-xL, despite being unable to protect against growth factor withdrawal-induced death (17). Thus, the mechanism by which Bcl-2 participates in the death pathway is separable from its function in the survival pathway.

Bcl-2 has previously been observed to increase cell death when highly expressed both in vitro and in vivo (23–25). Thus, it is possible that the high level of Bcl-2 is able to “prime” the...
Bcl-2-deficient neurons are significantly less susceptible to killing by control plasmid p75NTRnc. and have no increased mortality compared with neurons expressing cell death. A inhibited death induced by p75NTR and Bcl-2 expression. **CrmA, or p75NTR and Bcl-2, or all three together. Expression of mod-

vector expressing green fluorescent protein had no affect on regulation with p75NTRnc (data not shown). In addition, a control affect neuronal survival when expressed alone or in conjunc-

tion with p75NTRnc (Fig. 5). The specificity of the Bcl-2 antisense was demonstrated by its inability to affect neuronal survival when expressed alone or in conjunc-

tion with p75NTRnc (data not shown). In addition, a control vector expressing green fluorescent protein had no affect on p75NTR killing (data not shown).

The requirement for endogenous Bcl-2 for p75NTR killing was also demonstrated in neurons from newborn mice lacking Bcl-2, which showed a significant reduction in cell death induced by p75NTR compared with wild-type litter mates (Fig. 5B), supportive of the Bcl-2 antisense experiments.

To investigate whether the p75NTR-Bcl-2 death-signaling cascade was dependent on caspase activation, a modified CrmA, designed to inhibit downstream group II caspases, such as caspase 3 (28, 29), was overexpressed together with p75NTR. The modified CrmA able to block the killing induced either by p75NTR alone or by co-expression of p75NTR and Bcl-2 in wild-type neurons (Fig. 5C), suggesting that p75NTR-Bcl-2-induced apoptosis utilizes a caspase-dependent pathway.

**DISCUSSION**

Bcl-2 has been shown to be cleaved by caspases, with the cleavage product being capable of promoting apoptosis in vitro (30). Cleavage of Bcl-2 may occur in our system but the mutated Bcl-2 proteins would be similarly cleaved, arguing that cleavage of Bcl-2 is unlikely to be the dominant mechanism by which Bcl-2 promotes killing in our system. In addition, if caspase cleavage was the dominant mechanism, Bcl-xL might also be expected to promote death in our system as Bcl-xL has been shown to be cleaved in a similar way (31).

Bcl-2 and Bcl-xL are likely to have a very similar tertiary structure based on sequence similarity. The NMR structure of Bcl-xL has been determined, showing a hydrophobic cleft formed by seven α helices where other Bcl-2 family proteins interact (32). Bcl-xL, and Bcl-2, may function differently by specific interaction with proteins that mediate p75NTR-induced death. For example, pro-apoptotic Bcl-2 family members, Bak and Bad, interact strongly with Bcl-xL, but weakly with Bcl-2 (33, 34). Also, Bcl-xL, but not Bcl-2 as yet, has been shown to interact with Apaf-1, the mammalian CED-4 homologue, to regulate apoptosis via caspase-9 activation (35). Mutation of Bcl-2 at Gly-145 or Trp-188 may affect its interaction with pro-

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caspases are required for p75NTR-induced cell death. A, neurons expressing both p75NTR and antisense Bcl-2 show a significant increase in survival compared with p75NTR alone and have no increased mortality compared with neurons expressing control plasmid p75NTRnc. B, neurons from Bcl-2-deficient mice and their in wild-type littermates were injected with either p75NTR or p75NTRnc, and the degree of killing because of p75NTR was calculated. Bcl-2-deficient neurons are significantly less susceptible to killing by p75NTR overexpression. C, neurons were injected with either modified CrmA, or p75NTR and Bcl-2, or all three together. Expression of mod-

ified CrmA alone had no effect on neuronal survival, but significantly inhibited death induced by p75NTR and Bcl-2 expression. *p < 0.05; **p < 0.01 error bars indicate S.E., n = 3.

death pathway such that an apoptotic stimulus via p75NTR results in rapid cell death. We examined whether Bcl-2 may not only enhance p75NTR killing but may be essential for p75NTR killing to proceed by testing whether lowering endogenous Bcl-2 would inhibit p75NTR-mediated killing. We used a Bcl-2 antisense plasmid, which has previously been shown to effect-

tively lower Bcl-2 expression after microinjection into neurons (26, 27). When the Bcl-2 antisense plasmid was injected at the same time as the p75NTR plasmid, no diminution of the death signal was seen. If, however, the neurons were microinjected in the presence of NGF, to give time for the antisense to deplete the deple-
ete Bcl-2, and then switched into LIF the next day to permit p75NTR killing, there was a significant decrease in the ability of p75NTR to kill neurons with lowered Bcl-2 (Fig. 5A); in fact, the level of killing was not significantly different from that of neurons expressing p75NTRnc (Fig. 5A). The specificity of the Bcl-2 antisense was demonstrated by its inability to affect neuronal survival when expressed alone or in conjunc-

tion with p75NTRnc (data not shown). In addition, a control vector expressing green fluorescent protein had no affect on p75NTR killing (data not shown).
through NGF or BDNF signaling as has been recently shown (10, 41).

The apparent paradoxical actions of Bcl-2 may be a program by which rapid selection of cell survival or death occurs during neuronal development and after nerve injury. There are high levels of Bcl-2 in neurons of both the central and peripheral nervous systems during periods of developmental cell death (42), and activation of p75NTR, also expressed widely in the nervous system during periods of developmental cell death.(10, 41).

Acknowledgments—We thank Dr. D. C. Huang, Walter and Eliza Hall Institute (WEHI), for helpful discussions, critical reading of the manuscript, anti-human Bcl-2 antibody, and the Bcl-2 and Bcl-xL plasmids; Dr. Cris Print, WEHI, for assistance with Bcl-2 genotyping; and Dr. P. Ekert, WEHI, for modified CrmA plasmid.

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