with a CCD camera (n = 6). The photodiode data are shown because they provide greater time resolution.

16. The electrode tip was filled with 28 mM fura-2 in 150 mM potassium acetate that was iontophoresed for 55 min with a −1-nA current.
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Prevention of Vertebrate Neuronal Death by the *crmA* Gene

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Interleukin-1β converting enzyme (ICE) is a mammalian homolog of CED-3, a protein required for programmed cell death in the nematode *Caenorhabditis elegans*. The activity of ICE can be specifically inhibited by the product of *crmA*, a cytokine response modifier gene encoded by cowpox virus. Microinjection of the *crmA* gene into chicken dorsal root ganglion neurons was found to prevent cell death induced by deprivation of nerve growth factor. Thus, ICE is likely to participate in neuronal death in vertebrates.

The survival of sensory neurons during development of the vertebrate nervous system depends on neurotrophic factors produced by the neuronal targets (*1*). In the absence of these factors, the neurons are thought to undergo programmed cell death, an active process requiring RNA and protein synthesis (*2*). Little is known about the genes regulating cell death in vertebrates. A gene essential for cell death in the nematode C. *elegans*, *ced-3*, was recently shown to share sequence similarity with a mammalian gene, *ICE*, which encodes a cysteine protease involved in the processing of interleukin-1β (*3*). Overexpression of *ICE* induces programmed cell death in Rat1 fibroblasts and this death can be suppressed by the cowpox virus *crmA* gene, a specific inhibitor of *ICE*, and by the *BCL-2* protooncogene (*4*, *5*). To determine whether *ICE* is involved in neuronal death, we investigated the effect of *crmA* on the survival of chicken dorsal root ganglion (DRG) neurons cultured in the absence of nerve growth factor (NGF), a condition that would normally lead to cell death.

We microinjected an expression vector (pHD1.2) containing a *crmA* complementary DNA (cDNA) under the control of the chicken β-actin gene promoter, or in vitro transcribed *crmA* RNA (*6*), into cultured DRG neurons along with rhodamine-isothiocyanate–labeled dextran (*7*). About 90% of the injected neurons retained a normal morphology, and, in the presence of NGF, about 85% survived through day 6. Within 3 days of NGF deprivation, however, more than 80% of the control neurons (injected with dye alone, vector alone, or antisense *crmA* cDNA) died, with fewer than 10% surviving to day 6 (Fig. 1). In contrast, more than 60% of the *crmA*-injected neurons survived through day 6 in the absence of NGF (Fig. 1).

Expression of crmA protein was detected in almost all of the injected neurons by immunofluorescence staining with an affinity-purified rabbit polyclonal antibody to crmA (Fig. 2) (*8*). The crmA protein could be detected in neurons 9 days after injection, suggesting that it is very stable. The *crmA*-injected DRG neurons cultured in the absence of NGF were round and often devoid of extensive neurites, a morphology similar

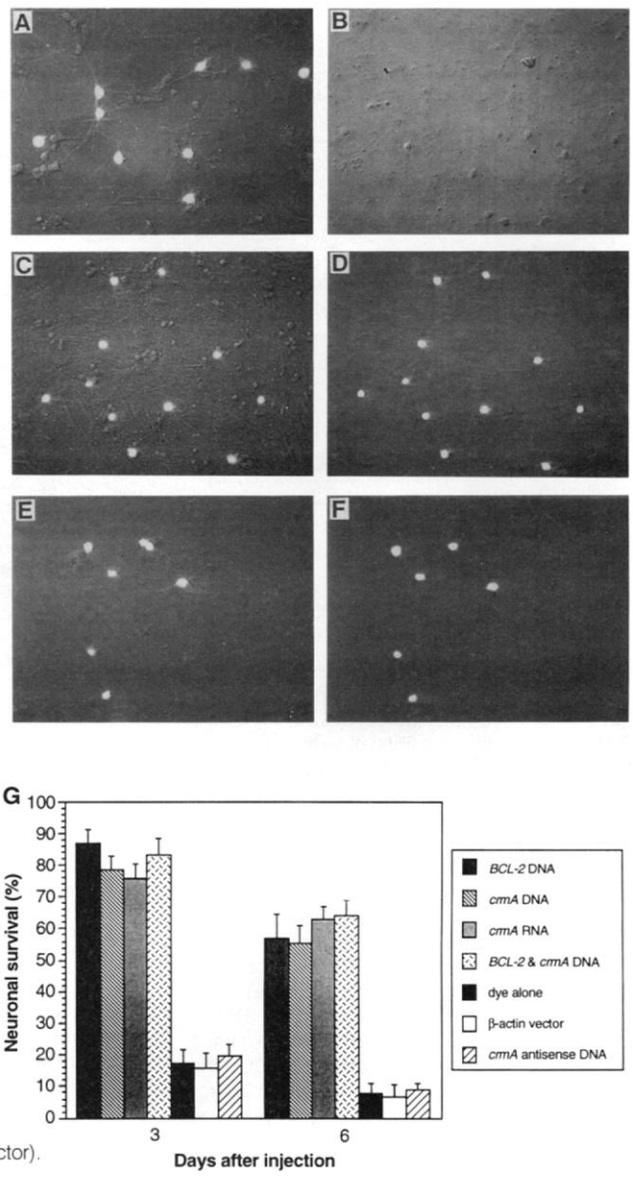

Fig. 1. Effects of *crmA* and *BCL-2* on the survival of NGF-deprived DRG neurons in culture. The neurons were microinjected with a control β- actin vector (**A** and **B**), an expression vector containing *crmA* cDNA (**C** and **D**), or an expression vector containing *BCL-2* cDNA (**E** and **F**). The neurons were photographed after being cultured in the absence of NGF for 0 days (A, C, and E) or 3 days (B, D, and F). Photographs were taken with a computerized video imaging system (Axovideo, Axon Instruments) with a rhodamine filter. Images of the fluorescent cells were amplified with a Silicon-Intensified-Target (SIT) video camera (Dage). (**G**) shows the survival of DRG neurons in the absence of NGF after injection of *BCL-2* expression vector RR/1 (*BCL2* DNA), *crmA* expression vector pHD1.2 (*crmA* DNA), antisense-*crmA* expression vector pHD2.1 (*crmA* antisense DNA), *crmA* RNA, *BCL-2* expression vectors RR/1 and *crmA* pHD1.2 (*BCL-2* and *crmA* DNA), control β-actin vector (pβactSTneoB), or dye alone. Live DRG neurons were scored as rhodamine-containing cells with neuronal morphology. Trypan blue staining was used to confirm the viability of neurons in some of the experiments (data not shown). Results are presented as the mean percentage of survival ± SEM, observed in four to five experiments (~300 microinjected neurons for each vector).

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to that of *BCL-2*–injected sympathetic neurons cultured in the absence of NGF (*9*). Addition of NGF to *crmA*-injected neurons that had been deprived of NGF for 6 days induced neurite outgrowth. The neurons could then be maintained in culture for at least 11 more days (*10*). This result indicates that although the expression of *crmA* prevents the death of NGF-deprived DRG neurons, it cannot replace the neurite-promoting activity of NGF. Thus, NGF may have two separate activities on DRG neurons—one promoting survival and the other promoting neuritogenesis.

In *C. elegans*, expression of *BCL-2* can partially prevent programmed cell death (*11*). If the death of NGF-deprived DRG neurons depends upon the ICE/CED-3 protein, then it should also be suppressed by the BCL-2 protein. To test this, we microinjected a *BCL-2* expression vector containing human *BCL-2* cDNA under the control of the chicken β-actin gene promoter (RR/1) (*12*) into cultured DRG neurons. About 57% of the *BCL-2*–injected neurons survived through day 6 in the absence of NGF (Fig. 1). This result confirms the previously observed inhibitory effect of *BCL-2* on the death of other sensory neurons (*13*), and shows that *BCL-2* and *crmA* are about equivalent in their ability to prevent the death of DRG neurons.

If *BCL-2* and *crmA* act through separate pathways, their ability to prevent the death of DRG neurons should be additive. To investigate this possibility, we injected both the *BCL-2* and *crmA* expression vectors (RR/1 and pHD1.2) into the neurons. The survival rate of these doubly injected neurons in the absence of NGF was no greater than that of the neurons singly injected with either *crmA* or *BCL-2* (64%) (Fig. 1). Thus, *crmA* and *BCL-2* are likely to act through the same pathway.

Our results suggest that genes in the *ICE/ced-3* family participate in the death of NGF-deprived DRG neurons in culture, and possibly in vivo. To test whether genes in the *ICE/ced-3* family induce programmed cell death of DRG neurons in the presence of NGF, we prepared an expression vector in which a murine *ICE* cDNA was fused with the *Escherichia coli lacZ* gene and placed under the control of the chicken β-actin gene promoter (pβactM10Z). We microinjected this vector into DRG neurons in culture in the presence of NGF, and, as controls, we injected either a murine *ICE-lacZ* fusion construct with a point mutation that changed the active site Cys to a Gly (pβactM17Z) (*5*), or the vector alone (*14*). About 80% of the neurons injected with control vector and 65% of those injected with pβactM17Z survived through day 3 in the presence of NGF (Fig. 3). In contrast, more than 65% of the *ICE*-injected neurons died 1 day after the injection and fewer than 30% survived to day 3 (Fig. 3). We stained the neurons that had survived to day 3 with an antibody to β-galactosidase. No staining was detected in the *ICE*-injected neurons. In contrast, all the neurons injected with vector alone or with the mutant *ICE* construct were stained (*15*). These results suggest that all neurons overexpressing *ICE* die and that *ICE*-mediated killing requires the same active site needed for the conversion of pro-interleukin-1β into active interleukin-1β.

During neuronal development, the presence of trophic factors may promote the activity of *BCL-2* and thereby prevent cell death, whereas the absence of trophic factors may lead to inactivation of *BCL-2* and activation of genes in the *ICE/ced-3* family, which in turn cause programmed cell death. Consistent with this hypothesis, expression of *BCL-2* has been detected in postmitotic neurons, whose survival depends on the presence of neural trophic factors (*16*), although the relationship between *BCL-2* and these factors remains to be examined. Our results also raise the possibility that the *ICE/ced-3* family of genes are involved in

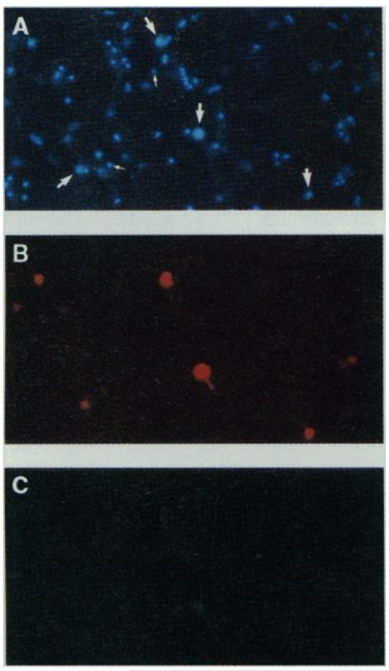

Fig. 2. Expression of crmA protein in microinjected DRG neurons. An expression vector containing a *crmA* cDNA was microinjected, together with tetramethyl rhodamine-isothiocyanate dextran, into the cultured neurons. The injected neurons were cultured for 9 days in the absence of NGF and then stained with affinity-purified rabbit antibody to crmA. (**A**) Hoechst dye staining of neuronal nuclei. (**B** and **C**) Same area as in (A), with the *crmA*-injected neurons detected by rhodamine fluorescence (B) or by the antibody to crmA (C). The injected neurons have healthy nuclei (big arrows). Some of the uninjected neurons show condensed nuclei, a typical feature of apoptosis (small arrows).

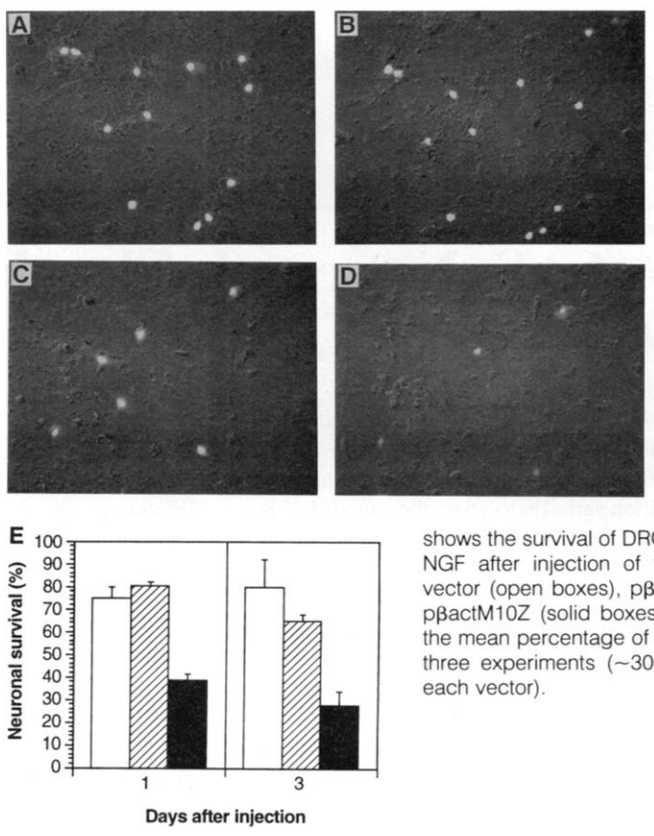

Fig. 3. Effect of ICE on the survival of DRG neurons in the presence of NGF. Neurons were microinjected with an expression vector containing a murine *ICE* gene with a point mutation that changes the Cys in the active site to a Gly (pβactM17Z) (**A** and **B**), or an expression vector containing the wild-type murine *ICE* (pβactM10Z) (**C** and **D**). The neurons were photographed after being cultured in the presence of NGF for 0 days (A and C) or 1 day (B and D). Photographs were taken with a computerized video imaging system as in Fig. 1. (**E**) shows the survival of DRG neurons in the presence of NGF after injection of the β-galactosidase control vector (open boxes), pβactM17Z (striped boxes), or pβactM10Z (solid boxes). Results are presented as the mean percentage of survival ± SEM, observed in three experiments (~300 microinjected neurons for each vector).

human diseases characterized by neuronal degeneration. Interestingly, interleukin-1β, which is generated from an inactive precursor form by ICE, is overexpressed in the brains of patients with Alzheimer's disease (*17*) and may promote the expression of the amyloid precursor protein (*18*).

REFERENCES AND NOTES

1. A. M. Davies, *Development* **100**, 185 (1987).
2. D. P. Martin *et al.*, *J. Cell Biol.* **106**, 829 (1988); R. W. Oppenheim, D. Prevette, M. Tytell, S. Homma, *Dev. Biol.* **138**, 104 (1990).
3. J. Yuan, S. Shaham, S. Ledoux, H. M. Ellis, H. R. Horvitz, *Cell* **75**, 641 (1993).
4. D. L. Vaux, S. Cory, J. M. Adams, *Nature* **335**, 440 (1988); G. Nunez *et al.*, *J. Immunol.* **144**, 3602 (1990); A. Strasser, A. W. Harris, S. Cory, *Cell* **67**, 889 (1991); C. L. Sentman, J. R. Shutter, D. Hockenbery, O. Kanagawa, S. J. Korsmeyer, *ibid.*, p. 879; C. A. Ray *et al.*, *ibid.* **69**, 597 (1992).
5. M. Miura, H. Zhu, R. Rotello, E. A. Hartwieg, J. Yuan, *Cell* **75**, 653 (1993).
6. A 1.46-kb *crmA* cDNA fragment, previously subcloned into the Eco RI site of the pBluescript plasmid, was isolated by Eco RI digestion, treated with Klenow enzyme, purified by agarose gel electrophoresis, and blunt end–ligated into the Sal I site of the expression vector pβactSTneoB. The orientation of the inserted *crmA* cDNA fragments in the pHD1.2 (sense) and pHD2.1 (antisense) expression plasmids was confirmed by restriction mapping. Plasmids were purified by passage over Qiagen columns. The *crmA* RNA was obtained by in vitro transcription with T7 polymerase. The template was the pBluescript plasmid containing the *crmA* cDNA fragment.
7. Primary cultures of embryonic sensory neurons were prepared under sterile conditions from DRG of 9- to 11-day-old chick embryos (Spafas, Preston, CT). Dissociated neurons were plated on slides treated with poly-L-lysine (Sigma, 30 μg/ml for 1 hour) and coated with laminin (Sigma, 20 μg/ml for 2 hours). Neurons were cultured for 2 to 4 days in F12 medium (Gibco) plus 10% fetal bovine serum (Hyclone), penicillin (100 U/ml; Gibco), streptomycin (100 μg/ml; Gibco), 5 μM cytosine β-D-arabinofuranoside (Sigma) as an antimitotic agent, and NGF 7S (20 ng/ml; Sigma). Cell injections were done with an Eppendorf microinjector (model 5242) equipped with glass micropipettes made from borosilicate capillaries. Micropipettes were loaded with 1 μl of the appropriate DNA or RNA, diluted to 0.8 μg/ml with 2.5% tetramethyl rhodamine-isothiocyanate dextran. Each neuron was given a brief (1- to 3-s) pulse. Three hours after injection, the NGF-containing medium was replaced with NGF-free and serum-free medium in the presence of sufficient mouse monoclonal antibody to NGF (Boehringer Mannheim) to accelerate neuronal degeneration. The medium was changed daily. To confirm that the cultured cells were neurons, we stained them with a polyclonal antibody to neurofilaments (Sigma).
8. A 1.2-kb Eco RI–Sal I fragment of *crmA* cDNA was inserted into the Eco RI and Sal I sites of the *Escherichia coli* expression vector pET21a (Novagen), and the construct (pJ434) was transformed into *E. coli* strain BL21/DE3. The crmA fusion protein was made according to a protocol provided by Novagen and purified by polyacrylamide gel electrophoresis. The excised gel slice containing the 40-kD crmA fusion protein was injected into rabbits to generate a polyclonal antibody [E. Harlow and D. Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988), pp. 92–135]. The crmA antiserum was purified by affinity chromatography over a CNBr-activated Sepharose column (Pharmacia) coupled to purified crmA fusion protein. We verified the purity of the antiserum by using it as a probe in immunoblots of crmA-expressing fibroblasts. Neurons were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature and then dehydrated for 5 min in absolute ethanol. For immunofluorescence staining, neurons were treated with blocking buffer consisting of 2% bovine serum albumin (Sigma) and 10% heat-inactivated normal goat serum (Gibco) in PBS for 30 min at room temperature. Affinity-purified rabbit antibody to crmA was diluted to 10 μg/ml in blocking buffer and incubated with the neurons overnight at 4°C. Secondary antibody (fluorescein-conjugated goat antibody to rabbit immunoglobulin, Gibco) was diluted 1:200 (final concentration 5 μg/ml) and applied with Hoechst dye 33258 (Sigma) to the slides for 45 min at room temperature. The slides were rinsed several times in PBS, mounted in 10% PBS, 90% glycerol containing *p*-phenylenediamine (1 mg/ml) (Sigma) to prevent photobleaching, and then viewed with a fluorescence microscope.
9. I. Garcia, I. Martinou, Y. Tsujimoto, J.-C. Martinou, *Science* **258**, 302 (1992).
10. V. Gagliardini and P. A. Fernandez, unpublished observations.
11. D. L. Vaux, I. L. Weissman, S. K. Kim, *Science* **258**, 1955 (1992).
12. A 0.9-kb blunt-ended Sal I–Eco RI *BCL-2* cDNA fragment was inserted into the blunt-ended Sal I site of the pβactSTneoB vector.
13. T. E. Allsopp, S. Wyatt, H. F. Paterson, A. M. Davies, *Cell* **73**, 295 (1992).
14. A. Mayawaki, T. Furuichi, N. Mueda, K. Mikoshiba, *Neuron* **5**, 1114 (1990).
15. V. Gagliardini, unpublished observations.
16. D. Hockenbery, M. Zutter, W. Hickey, M. Nahm, S. J. Korsmeyer, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6961 (1991).
17. W. S. T. Griffin *et al.*, *ibid.* **86**, 7611 (1989).
18. D. Goldgaber *et al.*, *ibid.*, p. 7606; G. Forloni, F. Demicheli, C. Bendotti, N. Angeretti, *Mol. Brain Res.* **16**, 128 (1992); J. D. Buxbaum *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10075 (1992).

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