Conversion of Bcl-2 to a Bax-like Death Effector by Caspases

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Caspases are a family of cysteine proteases implicated in the biochemical and morphological changes that occur during apoptosis (programmed cell death). The loop domain of Bcl-2 is cleaved at Asp^{34} by caspase-3 (CPP32) in vitro, in cells overexpressing caspase-3, and after induction of apoptosis by Fas ligation and interleukin-3 withdrawal. The carboxyl-terminal Bcl-2 cleavage product triggered cell death and accelerated Sindbis virus–induced apoptosis, which was dependent on the BH3 homology and transmembrane domains of Bcl-2. Inhibitor studies indicated that cleavage of Bcl-2 may further activate downstream caspases and contribute to amplification of the caspase cascade. Cleavage-resistant mutants of Bcl-2 had increased protection from interleukin-3 withdrawal and Sindbis virus–induced apoptosis. Thus, cleavage of Bcl-2 by caspases may ensure the inevitability of cell death.

Bcl-2 is an integral intracellular membrane protein that inhibits programmed cell death induced by multiple insults in a wide variety of cell types (*1*). Both biochemical and genetic evidence indicates that Bcl-2 family members can regulate cell death induced by caspases (*2*). A number of substrates for the caspase proteases have now been identified, including protein kinases (*3*), the retinoblastoma protein (*4*), cytoskeletal proteins (*5*), and several autoantigens (*6*). Cleavage of these proteins by caspases may either activate or inactivate essential functions or produce cleavage products with altered activities. Cleavage of a novel protein designated DFF triggers DNA fragmentation during apoptosis (*7*). In addition, caspases cleave the proenzyme precursors to produce the active subunits of caspases themselves (*8*). Here, we investigated the possibility that Bcl-2 could also serve as a caspase substrate.

In vitro translated human Bcl-2 was digested with purified recombinant caspase-3 (CPP32). Although inefficient, treatment with active caspase-3 produced a 23-kD proteolytic fragment of Bcl-2 (Fig. 1A). However, a mutant of Bcl-2 (*9*) that lacks the loop domain (amino acids 32 to 80) was not susceptible to proteolysis, which suggested that the caspase cleavage site is localized within the loop. Two potential caspase cleavage sites occur within the loop at positions 34 and 64. Mutation of Asp^{34} to Ala (D34A) abolished cleavage by caspase-3 in vitro, whereas mutation of Asp^{64} to Ala (D64A) had no effect (Fig. 1A). Consistent with a cleavage site following Asp^{34} in the sequence Asp^{31}-Ala^{32}-Gly^{33}-Asp^{34}, the proteolytic fragment of Bcl-2 comigrated with an NH_2-terminal deletion mutant of Bcl-2 lacking amino acids 2 to 34 (ΔN34).

The P4 position of caspase cleavage sites confers protease specificity (*10*). Consistent with the preference for Asp at the P4 position of caspase-3 cleavage sites, mutation of Asp^{31} (D31A) also abolished cleavage of Bcl-2 by caspase-3 in vitro (*11*). However, Asp^{31} itself apparently does not serve as a cleavage site because this site is preserved in the loop deletion mutant Δloop, which is not cleaved. (Deletion of the loop region reconstitutes Ala^{32}.) The Asp residues at P1 and P4 in human Bcl-2 are also conserved in the rat and murine Bcl-2 proteins.

To determine whether Bcl-2 is cleaved inside cells, we cotransfected COS cells with plasmids expressing Bcl-2 and caspase-3. Approximately 50% of the Bcl-2 protein was cleaved in the presence of caspase-3 (Fig. 1B). Similar to results obtained in vitro, mutation of Asp^{31} or Asp^{34} abolished proteolysis in transfected cells, whereas mutation of Asp^{64} had no effect. Cotransfection of the baculovirus caspase inhibitor P35 abolished proteolysis of Bcl-2 (Fig. 1B), which indicates that the transfected caspase-3, or potentially other cellular proteases activated by

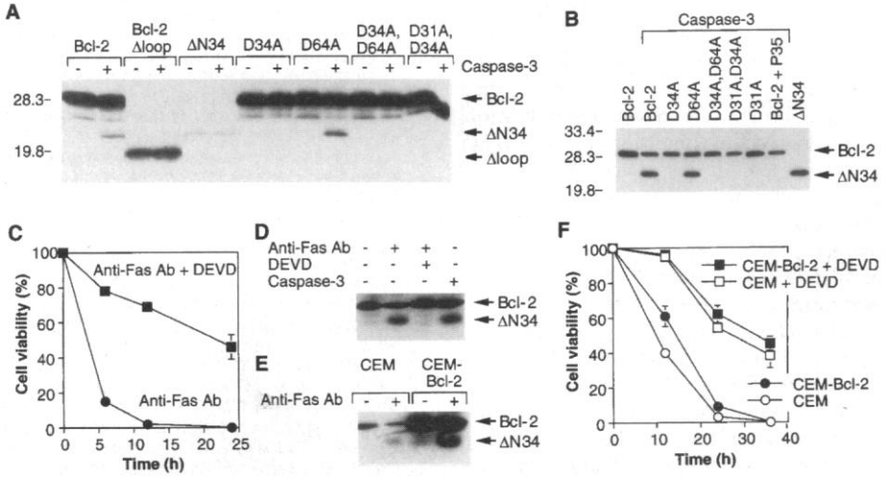

Fig. 1. Bcl-2 is cleaved by caspases both in vitro and in intact cells. (**A**) ^{35}S-labeled in vitro translated Bcl-2 and the indicated Bcl-2 mutants were digested with purified recombinant caspase-3 and analyzed on 12% SDS-polyacrylamide gels (*24*). Molecular size markers are indicated (in kilodaltons). (**B**) COS-1 cells (5×10^5) were cotransfected using lipofectamine (Life Technologies) with equal amounts of DNA [2 μg of each of the indicated expression plasmids + control vector pSG5 control vector (Stratagene) as required] and cell lysates were immunoblotted 24 hours after transfection with antibody to Bcl-2 (from D. Mason). (**C**) Jurkat cells were treated with anti-Fas (1 μg/ml) for 2 hours with or without a 30-min pretreatment with 300 mM Ac-DEVD-CHO, then assessed for viability by trypan blue exclusion in three independent experiments, and the percentage of viable cells was calculated relative to untreated cells. (**D**) Jurkat cells treated as in (C) were immunoblotted with Bcl-2 antibody 100 (Santa Cruz). Alternatively, Jurkat cell lysates were digested for 4 hours with recombinant caspase-3. (**E**) Stably transfected CEM cells or neo controls (*25*) were untreated or treated with anti-Fas and immunoblotted as in (D). (**F**) Viabilities were determined as described in (C) for the indicated CEM cell lines treated as described in (C).

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caspase-3, are responsible for cleaving Bcl-2 in intact cells. A larger fraction of Bcl-2 is cleaved by caspases in cells relative to in vitro assays, raising the possibility that protein modification such as phosphorylation may modulate the cleavage event (*12*). Alternatively, other cell factors may facilitate cleavage of Bcl-2. Similar to Bcl-2, Bcl-x_L is also cleaved in the loop domain by caspases in apoptotic cells (*11*).

To determine whether endogenous Bcl-2 was cleaved by endogenous caspases after a death stimulus, we examined proteolysis of Bcl-2 during Fas-induced apoptosis, a scenario where Bcl-2 is an inefficient death inhibitor (*13*). Treatment of Jurkat cells with antibody to Fas induced a cleavage product of endogenous Bcl-2 that comigrated with cleaved Bcl-2 produced by digestion of Jurkat cell lysates with recombinant caspase-3 (Fig. 1D). Jurkat cells treated with a caspase inhibitor blocked Fas-induced cleavage of Bcl-2 and significantly delayed cell death, indicating that caspases are responsible for cleaving Bcl-2 after Fas ligation (Fig. 1, C and D). Likewise, both endogenous and overexpressed Bcl-2 were cleaved after activation of the Fas pathway in CEM cells (Fig. 1E). Compared with the potency of a caspase inhibitor, stably expressed Bcl-2 only transiently delayed CEM cell death (Fig. 1F).

Cleavage of Bcl-2 by caspases could serve to inactivate the antiapoptotic activity of Bcl-2 during cell death. Alternatively, the cleaved product of Bcl-2 may acquire a new function. To test the function of cleaved Bcl-2, we expressed the NH_2-terminal deletion mutant of Bcl-2, ΔN34, by means of the Sindbis virus vector system (*14*). In contrast to wild-type Bcl-2, which (relative to controls) protected cells from cell death, deletion of the NH_2-terminal 34 amino acids of Bcl-2 accelerated cell death more than did reverse orientation of ΔN34 or a chloramphenicol acetyltransferase (CAT) control (Fig. 2). Similar acceleration of death was observed with Bax and Bak (*14*). Thus, cleavage of Bcl-2 by caspases can unleash a latent proapoptotic activity of Bcl-2.

To further test the proapoptotic activity of the cleavage product, we transfected a plasmid expressing the NH_2-terminal deletion mutant of Bcl-2 into BHK (baby hamster kidney) cells. Although a detectable reduction in cell viability by wild-type Bcl-2 was routinely observed, Bcl-2 ΔN34 reduced cell viability more than did wild-type Bcl-2 over the full range of DNA concentrations (Fig. 3A). In a luciferase assay, the ΔN34 mutant exhibited proapoptotic activity similar to that observed after transfection with a Bax expression plasmid (12.6% and 8.3% viability, respectively) (Fig. 3B, solid bars). The baculovirus caspase inhibitor P35 inhibited cell death induced by ΔN34 and Bax, whereas cotransfection with the poxvirus caspase inhibitor CrmA did not protect cells from ΔN34-induced cell death and modestly protected them from Bax (Fig. 3B). These results suggest that the proapoptotic activity of ΔN34 lies upstream of at least some caspases, implying that ΔN34 facilitates activation of additional proteases leading to cell death similar to Bax.

To explore the mechanism by which ΔN34 kills cells, we investigated the importance of the transmembrane domain and the BH3 homology domain. Deletion of the transmembrane domain after residue 219 abolished the proapoptotic activity of ΔN34, indicating that proper subcellular localization is important for cell killing (Fig. 3C). Although Bax may be able to induce death by a mechanism independent of BH3, the BH3 domain appears to contribute to the proapoptotic activity of Bak, Bax, and other BH3-containing proteins (*15*). This domain is also conserved in Bcl-2 and Bcl-x_L, where there is conflicting evidence for its role in blocking cell death (*16*). Mutation of the core sequence (Gly^{101}-Asp^{102}-Asp^{103} → Ala^{101}-Ala^{102}-Ala^{103}) or two flanking hydrophobic residues (Leu^{97} → Ala and Phe^{104} → Ala) of BH3 abolished the proapoptotic activity of Bcl-2 ΔN34 (Fig. 3C). The expression of ΔN34 in transfected cells was consistently reduced relative to that of wild-type Bcl-2 or other Bcl-2 mutants, presumably because of its proapoptotic activity (Fig. 3D) (*11*).

The loop region of Bcl-2 and Bcl-x_L contains a regulatory domain that impairs antiapoptotic function (9). Deletion of the loop domain of Bcl-2 converts Bcl-2 into a more potent inhibitor of growth factor withdrawal and anti-immunoglobulin M–induced apoptosis (9). The enhanced activity of the loop deletion mutant may be explained by the inability of this mutant to be cleaved by caspases (see Fig. 1A), thus preventing activation of the executioner function of Bcl-2. To explore the possibility that cleavage of the loop region is important for regulating cell death after a death stimulus, we stably transfected cleavage-resistant mutants of

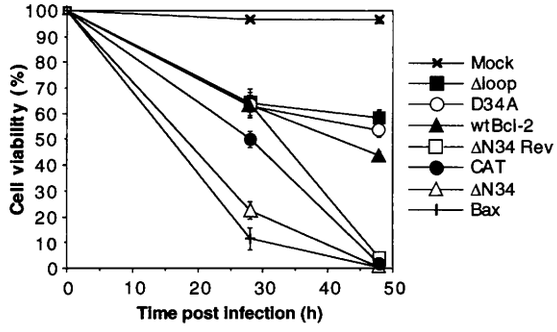

Fig. 2. Deletion of the NH_2-terminal 34 amino acids of Bcl-2 converts Bcl-2 into a proapoptotic protein. Cell viability after infection of BHK cells with recombinant Sindbis virus vectors expressing the indicated proteins was determined by trypan blue exclusion, as described (*14*) (wt, wild type). Viabilities are presented as the mean ± SD for all time points in three independent experiments. Immunoblot analysis confirmed expression of Bcl-2 family members (*11*).

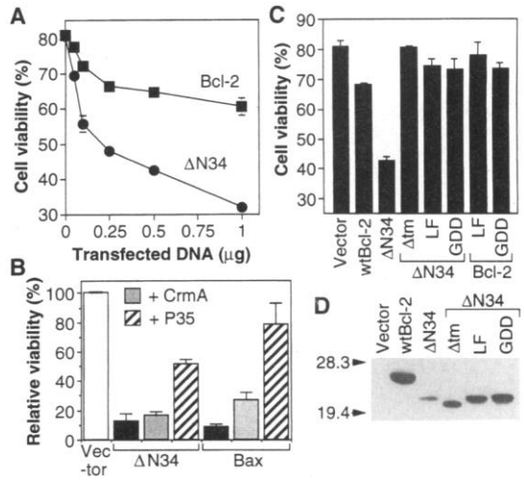

Fig. 3. Bcl-2 ΔN34–induced death of transfected cells requires the BH3 and transmembrane domains of Bcl-2 and is blocked by P35. (**A**) BHK cells were transfected as in Fig. 1B with increasing concentrations of wild-type Bcl-2 or the ΔN34 mutant, and cell viabilities were determined by trypan blue exclusion for three independent experiments (mean ± SD). (**B**) BHK cells were cotransfected with 2 μg of each expression plasmid and 0.5 μg of luciferase reporter plasmid (GL2 control, Promega) plus the required amount of pSG5 for a total of 4.5 μg (using lipofectamine). Luciferase activity was assayed 24 hours later and compared with the luciferase reporter (+ 4 μg of pSG5). (**C**) Cells were transfected with 0.5 to 1.0 μg of the indicated constructs (see text) and viabilities were determined as for (A). (**D**) Transfected cells described in (C) were immunoblotted with a monoclonal antibody to Bcl-2 (D. Mason).

Bcl-2 into Ba/F3 cells. Individual cell clones expressing similar amounts of Bcl-2 protein (Fig. 4A) were compared for their ability to block cell death after interleukin-3 (IL-3) withdrawal. Cell lines expressing either the D31A or D34A mutant exhibited 20 to 30% increased viabilities relative to wild-type Bcl-2 at 60 to 115 hours after the death stimulus (Fig. 4B). Although less pronounced, the D34A and Δloop mutants also consistently provided increased protection relative to wild-type Bcl-2 (10 to 15%) in the Sindbis virus vector assay (see Fig. 2). Cleavage of wild-type Bcl-2 in Ba/F3 cells was detected with different Bcl-2 antibodies simultaneously with a decrease in viability of Ba/F3 cells after IL-3 withdrawal (Fig. 4, C and D). In contrast, cleavage was reduced or absent after IL-3 withdrawal in cells expressing the D31A or D34A mutants, consistent with their increased viabilities (*17*).

Cleavage of Bcl-2 by caspases results in loss of the NH_2-terminal BH4 homology domain that is required for antiapoptotic activity and for interaction with Raf-1 (*18*). We have now shown that deletion of this domain in ΔN34 not only inactivated the antiapoptotic activity of Bcl-2, but also released a potent proapoptotic fragment. Although other domains of cleaved Bcl-2 may be required for proapoptotic activity, our results suggest that caspase cleavage activates the proapoptotic activity of the BH3 domain. Several viral homologs of Bcl-2 lack the loop region and have a poorly conserved BH3 domain (*16*, *19*), suggesting that these viral proteins may evade regulation by caspases. Although Bax and Bak are potent inducers of cell death, both have been reported to inhibit cell death under specific situations (*20*). Perhaps it is also possible to mask the proapoptotic domains of Bax and Bak, converting these proteins into inhibitors of apoptosis. ΔN34 may kill cells by a mechanism that resembles Bax except that the transmembrane domain is not critical for Bax-induced death (*21*). Although Bax is known to form heterodimers with Bcl-2 through use of the BH3 domain, potentially functioning as a competitive inhibitor of Bcl-2, Bax also regulates cell death independent of Bcl-2, perhaps by forming ion channels, disrupting membrane potential, or inducing the release of proapoptotic factors from mitochondria (*22*). Likewise, Bcl-2 ΔN34 may modulate cell death by multiple mechanisms.

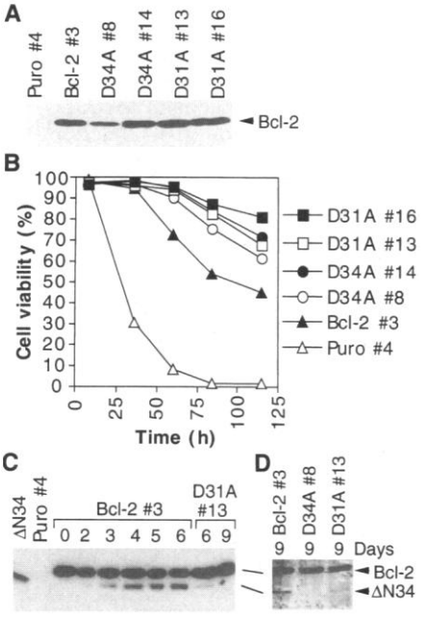

Fig. 4. Mutation of the caspase cleavage site in Bcl-2 enhances antiapoptotic activity. (**A**) Ba/F3 cell lines were established by electroporating with pSG5–Bcl-2 or Bcl-2 mutants and pBabePuro and selected in puromycin (2 μg/ml) (*26*). Cell lysates were analyzed by immunoblotting equal amounts of cellular protein for Bcl-2 with 14371E antibody (Pharmingen). (**B**) Viabilities of cells in (A) after IL-3 withdrawal were determined by propidium iodide staining and FACScan flow cytometry. The data are presented as means ± SD (concealed by the symbols) and are representative of two independent experiments performed in triplicate. (**C** and **D**) Ba/F3 cell lines were immunoblotted for Bcl-2 on the indicated days after IL-3 withdrawal with one of two antibodies to Bcl-2, from D. Mason (C) or from Pharmingen (D). Cell viabilities were determined simultaneously by propidium iodide staining and FACScan flow cytometry (*17*).

Although Bcl-2 is presumed to inhibit caspase activation by acting upstream of caspases (*23*), Bcl-2 also may be a downstream death substrate of caspases, suggesting the existence of a feedback loop between Bcl-2 and caspases. The observation that Bcl-2 cannot inhibit apoptosis in some situations implies that specific caspases may bypass the pathway inhibited by Bcl-2. In this scenario, activation of a subset of caspases that are insensitive to Bcl-2 may also promote cleavage of Bcl-2, not only inactivating its antiapoptotic function but also enhancing cell death.

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17. Percent cell viabilities for the experiment shown in Fig. 4, C and D, were determined on days 0, 2, 3, 4, 5, 6, and 9 for Bcl-2 #3: 98%, 81%, 41%, 29%, 22%, 18%, and 8%, respectively; for D34A #8: 99%, 98%, 90%, 83%, 74%, 54%, and 26%, respectively; for D31A #13: 98%, 90%, 83%, 76%, 68%, 60%, and 29%, respectively; and for Puro #4: 98%, 27%, and 3% (days 0, 2, and 3 only).
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24. Mutations in human Bcl-2 (D31A, D34A, D64A, and the double mutants) were generated by recombinant polymerase chain reaction and completely sequenced. An initiation codon was added to codons 35 to 239 to generate Bcl-2 ΔN34. In vitro translated proteins (3 μl) were mixed with 2 μl of purified caspase-3 (1 μl produces 2.5×10^3 relative fluorescence units/min using DEVD-AMC) and 5 μl of caspase reaction buffer [100 mM Hepes (pH 7.5), 10% sucrose, 0.1% CHAPS, and 20 mM dithiothreitol] at 30°C for 6 hours.
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27. We thank S. Zou and B. Plunkett for technical assistance, and D. Mason, B. Chang, C. Thompson, and K. Kikly for antibodies and reagents. R.J.C. is an American Cancer Society Fellow, and D.G.K. is supported by the NIH Medical Scientist Training Program. Supported by grants from the National Institutes of Health and the Muscular Dystrophy Association (J.M.H.).

2 September 1997; accepted 4 November 1997