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Caspase-9, Bcl-X_L, and Apaf-1 Form a Ternary Complex*

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Genetic analysis of apoptosis in the nematode *Caenorhabditis elegans* has revealed the cell death machine to be composed of three core interacting components. CED-4 (equivalent to mammalian Apaf-1) is a nucleotide binding molecule that complexes with the zymogen form of the death protease CED-3, leading to its autoactivation and cell death. CED-9 blocks death by complexing with CED-4 and attenuating its ability to promote CED-3 activation. An equivalent ternary complex was found to be present in mammalian cells involving Apaf-1, the mammalian death protease caspase-9, and Bcl-X_L, an anti-apoptotic member of the Bcl-2 family. Consistent with a central role for caspase-9, a dominant negative form effectively inhibited cell death initiated by a wide variety of inducers.

Programmed cell death, or apoptosis, is an evolutionarily conserved and genetically regulated biological process that plays an important role in the development and homeostasis of multicellular organisms (1–4). The nematode *Caenorhabditis elegans* has served as a model system for defining core components of the death machine (5, 6). CED-3 represents the effector arm of the cell death machine and belongs to a family of related mammalian proteases termed caspases for cysteine proteases that cleave following an Asp residue (7, 8). Caspases exist as zymogens composed of a prodomain plus large and small catalytic subunits. Generation of the active enzyme requires accurate processing at internal Asp residues to liberate the prodomain and produce the two chain active enzyme (7–9). Caspases can be classified according to whether they possess a large or a small prodomain. Large prodomains function as signal integrators as they bind adapter molecules involved in signal transduction. For example, the death effector domain within the prodomain of caspase-8 binds to the corresponding motif in the adapter molecule FADD[1] allowing for its recruitment to the CD-95 death receptor signaling complex (10, 11).

The death effector domain is a specific example of a more global homophilic interaction domain termed CARD (for caspase recruitment domain) that is present in other large prodomains including those of caspase-2 (ICH-1) and caspase-9 (ICE-LAP6, Mch6; Ref. 12). Caspase-2 is recruited to the TNFR-1 signaling complex through an interaction involving the respective CARD domains within the adapter molecule RAIDD and the prodomain of caspase-2 (13). To date, the other large prodomain-containing caspase, caspase-9, has not been implicated in any specific signaling pathway (14). We find that caspase-9 is part of a ternary signaling complex analogous to the one present in *C. elegans* involving CED-3, CED-4, and CED-9 (6, 15–17). CED-9 is an inhibitor of apoptosis in the nematode and corresponds to mammalian cell death inhibitors including Bcl-2 and Bcl-X_L (18). It can be found complexed with the nematode caspase equivalent CED-3 in the presence of the bridging molecule CED-4 (15–17). This suggests that a molecular mechanism based on the physical interaction of these components could potentially account for the inhibitory function of CED-9 and, by extension, Bcl-X_L and Bcl-2 (5). The ability of the worm genes to function in mammalian cells underscores their conservation and the interchangeability of key death components (15, 16). For example, in transfected human embryonic kidney cells, CED-4 bound CED-9 or its mammalian counterpart Bcl-X_L. Similarly, CED-4 bound CED-3 or corresponding large prodomain mammalian caspases (including caspase-1 and caspase-8) but not small prodomain caspases like caspase-3. The inability of dominant negative versions of caspase-1 or caspase-8 to block CED-4-induced cell death suggested that either another distinct large prodomain caspase was the primary target or that CED-4 activated multiple caspases (15). The exact mechanism deployed by CED-4 to activate CED-3 and/or caspases remains unclear. It has been shown, however, that CED-4 is a P-loop-containing nucleotide binding protein that is capable of promoting the activation of CED-3 and that this is blocked by CED-9 (19–21).

Recently, a human CED-4 homologue (Apaf-1) has been identified that possesses an NH_2-terminal CED-3 prodomain-like region that includes a CARD domain, a CED-4-like segment including conserved P-loop and a COOH-terminal extension composed of multiple WD-40 repeats that are lacking in nematode CED-4 (Fig. 1*A*; Ref. 22). Apaf-1, in the presence of cytochrome *c*, nucleotide (dATP), and a previously unidentified factor (Apaf-3) that has recently been shown to be caspase 9 (31), is able to promote the activation of caspase-3 (a small prodomain downstream caspase) by a mechanism that awaits definition (23–25). We found that caspase-9, but not other large prodomain caspases, and Bcl-X_L bound distinct regions in Apaf-1 and that dominant negative caspase-9 effectively blocked cell death induced by a variety of effectors. Thus, caspase-9 likely represents a direct downstream target of Apaf-1 and its activation appears critical for the propagation of death signals.

EXPERIMENTAL PROCEDURES

Expression Constructs—cDNAs encoding Apaf-1 or its truncated forms were obtained by polymerase chain reaction based on the published Apaf-1 DNA sequence (22). The full-length Apaf-1 was cloned into pcDNA3 (Invitrogen) with a NH_2-terminal Flag tag. Apaf-1(3+4)-Myc (amino acids 1–412), Apaf-1(3)-Myc (amino acids 1–102), and Apaf-1(4)-Myc (amino acids 86–412) were cloned into pcDNA3.1(−)/Myc-His

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[1] The abbreviations used are: FADD, Fas-associated death domain; RAIDD, RIP-associated ICH-1/CED-3-homologous protein with a death domain; TRADD, tumor necrosis factor-associated death domain. TNF, tumor necrosis factor; mAb, monoclonal antibody.

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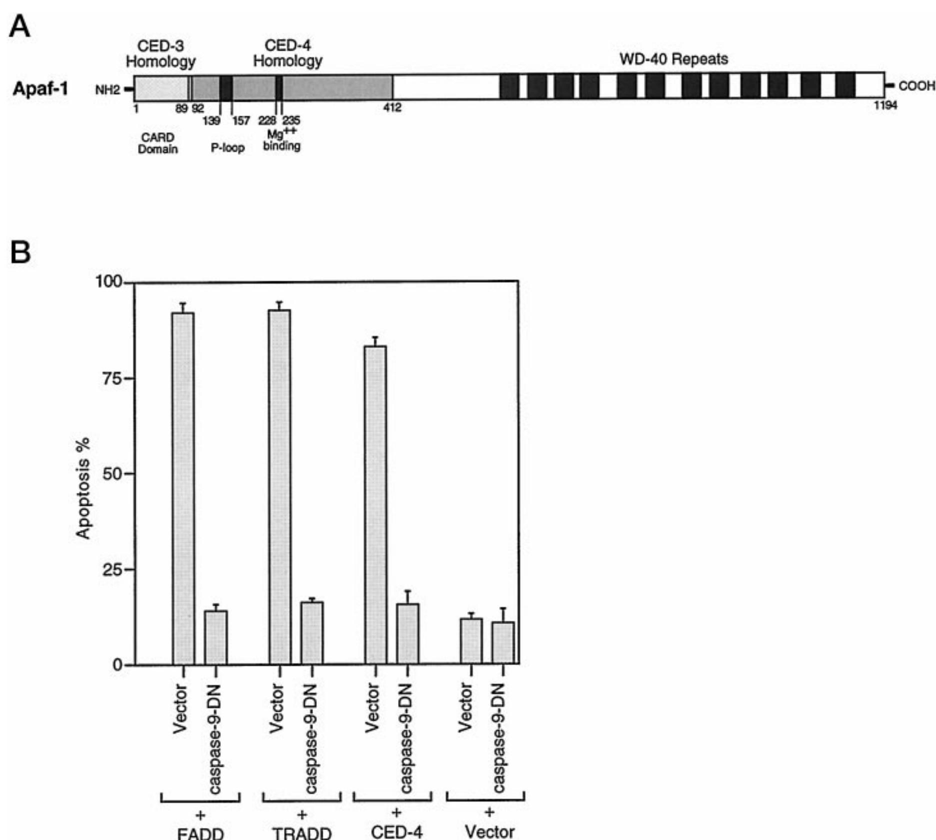

FIG. 1. **Dominant negative caspase-9 (*caspase-9-DN*) blocked apoptosis induced by FADD, TRADD, and CED-4.** *A*, a schematic presentation of Apaf-1. Apaf-1 contains an NH_2-terminal CED-3-homologous region (amino acids 1–89) that includes a CARD domain as indicated, a CED-4-homologous segment (amino acids 92–412) and a COOH-terminal sequence that includes 12 WD-40 repeats as shown. The P-loop sequence for nucleotide binding and a putative Mg^{2+} binding site are also indicated. *B*, FADD-, TRADD-, and CED-4-induced cell death is inhibited by caspase-9-DN. MCF7 cells were co-transfected with FADD, TRADD, or CED-4 together with a β-galactosidase-expressing reporter construct in the presence of a 3-fold excess of either vector or caspase-9-DN. Fifteen hours after transfection, cells were stained and examined as described (28). The data (mean ± S.D.) represent the percentage of round, apoptotic cells as a function of total β-galactosidase-positive cells (n = 3). *DN*, dominant negative.

B (Invitrogen) with a COOH-terminal Myc tag provided by the vector. The construct expressing caspase-9-prodomain (amino acids 1–168) was cloned by polymerase chain reaction into pcDNA3 with a COOH-terminal Flag tag. The constructs encoding HA-BAX, HA-BAK, HA-BIK, Bcl-XL-Myc, Bcl-X_L-Flag, Bcl-$X_L$mt1-Flag, Bcl-XLmt7-Flag, caspase-1-Flag, caspase-2-prodomain-Flag, caspase-3-Flag, caspase-8-DN-Flag, caspase-9-DN-Flag, caspase-9 p30-Flag (amino acids 130–416), CED-4-Myc, FADD, RAIDD, TRADD-Myc, cIAP1, and CrmA have been described elsewhere (11, 13–15, 26).

Apoptosis Assays—Cell death assays were performed as described (26, 27). MCF7 cells were transfected using the lipofectAMINE procedure (Life Technologies, Inc.) according to the manufacturer's instructions.

Co-immunoprecipitation—*In vivo* interaction assays have been described elsewhere (26, 28). 293 cells were transfected by means of calcium phosphate precipitation.

RESULTS AND DISCUSSION

Dominant Negative Caspase-9 (Caspase-9-DN) Blocks Apoptosis Induced by FADD, TRADD, and CED-4—As shown in Fig. 1*B*, dominant negative caspase-9 (caspase-9-DN) inhibited cell death induced by the receptor associated death adapter molecules FADD and TRADD in human breast carcinoma MCF7 cells, consistent with caspase-9 functioning downstream of these two adapter molecules. Importantly, CED-4-induced apoptosis, which had previously been shown not to be blocked by dominant negative forms of the other large prodomain caspases (including caspase-2 and caspase-8) (9), was blocked by dominant negative caspase-9. It is therefore probable that nematode CED-4 induces apoptosis in mammalian cells by activating caspase-9. In keeping with this observation, we have previously noted that caspase-9 physically interacts with CED-4 (14).

Caspase-9 but Not Other Caspases Interacts with Apaf-1—To determine if caspase-9 similarly bound the mammalian CED-4 equivalent Apaf-1, co-immunoprecipitation was undertaken in human embryonic kidney 293 cells. Flag-Apaf-1 was found to co-precipitate with caspase-9 (Fig. 2*A*). Since caspase-9-DN-Flag also bound truncated Apaf-1 (Apaf-1(3+4); residues 1–412) that contained only the CED-3 and CED-4 homologous regions (Fig. 2*B, left*), further analysis used only this truncated form. Caspase-9-DN-Flag, but not other large prodomain-containing caspases, specifically immunoprecipitated with Apaf-1 (Fig. 2*B, left*). Therefore, unlike CED-4, which appears to promiscuously bind large prodomain caspases, Apaf-1 was specific for caspase-9. Additionally, as expected, the small prodomain-containing caspase-3 did not bind Apaf-1. The specificity of this interaction was confirmed by the finding that caspase-2, a CARD-containing large prodomain caspase, bound its cognate adapter molecule RAIDD through a CARD-mediated interaction, yet did not interact with the Apaf-1 CARD domain (Fig. 2*B, right*). To delineate the Apaf-1 interacting domain in caspase-9, both a prodomainless form (caspase-9 p30-Flag) and a prodomain only expressing form were assessed in binding studies. Consistent with the expected involvement of the prodomain in recruitment to signaling complexes, only the prodomain of caspase-9 was required to interact with Apaf-1(3+4)-Myc (Fig. 2*C*).

Bcl-X_L Interacts with Apaf-1—Given that CED-9 binds CED-4 (15–17), we asked whether an equivalent interaction existed between the corresponding mammalian counterparts Bcl-X_L and Apaf-1. Upon co-transfection, Flag-Apaf-1 co-precipitated with Bcl-X_L-Myc (Fig. 2*A*). We consistently observed that Apaf-1 expression was enhanced by co-expressing Bcl-X_L (15), suggesting that Bcl-X_L may stabilize Apaf-1. Bcl-X_L-Flag also co-immunoprecipitated with Apaf-1(3+4)-Myc as well as CED-4-Myc (Fig. 2*D, left* and *middle*). Previous studies had shown that epitope-tagging Bcl-X_L at the NH_2 terminus disrupts its ability to interact with CED-4 and this similarly inhibited binding to Apaf-1 (Ref. 9; Fig. 2*D, left*). A previously characterized dicodon mutant form of Bcl-X_L (mt7) that does not inhibit cell death did not bind Apaf-1, while an alternate dicodon mutant form Bcl-X_L (mt1), which blocks apoptosis but

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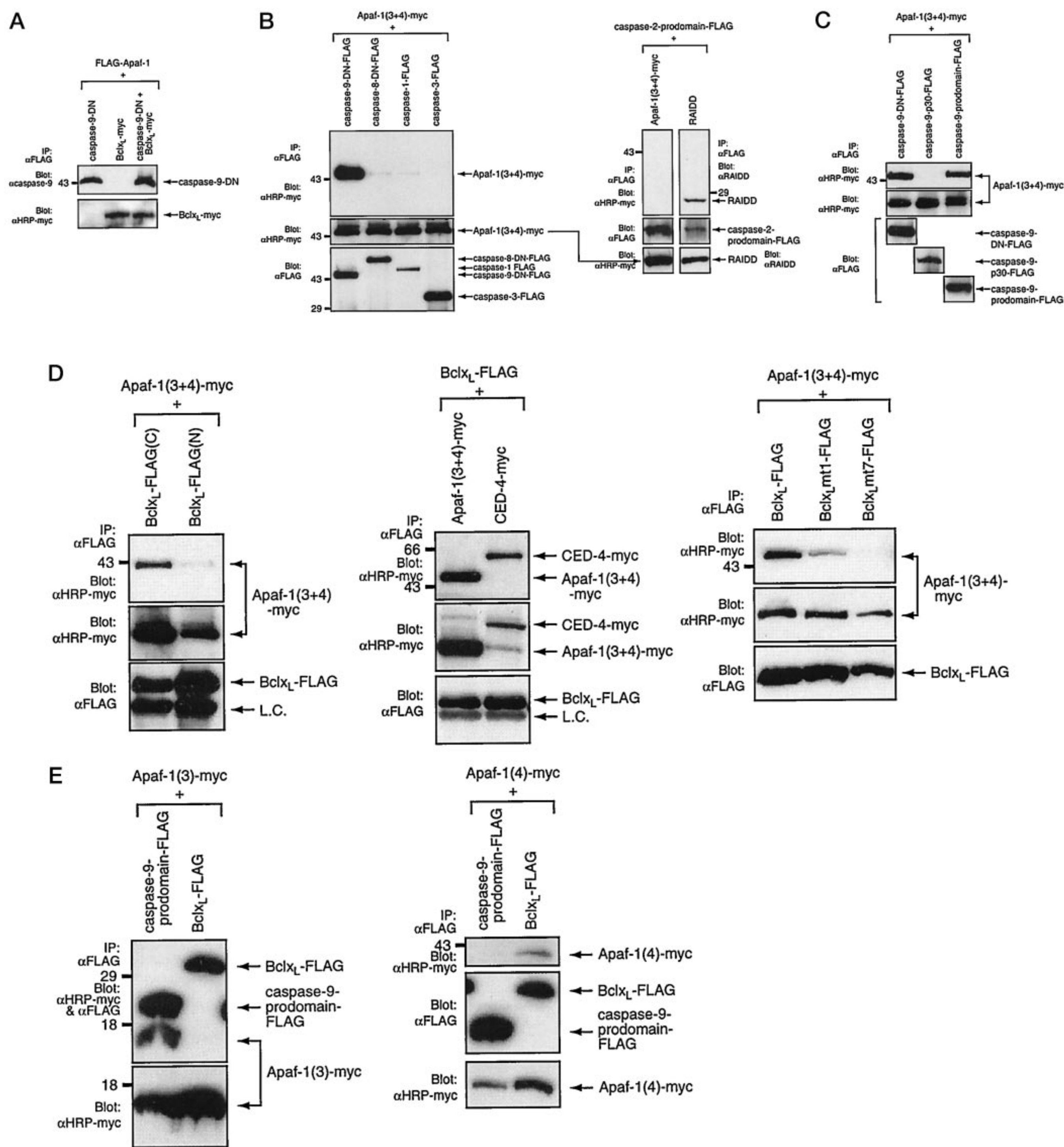

FIG. 2. **Both caspase-9 and Bcl-X$_L$ co-immunoprecipitate with Apaf-1.** *A*, Apaf-1 binds caspase-9 and Bcl-X$_L$. 293 cells were transfected with indicated expression constructs for Flag-Apaf-1, caspase-9-DN, and Bcl-X$_L$-Myc (18, 19). After 36–40 h, extracts were prepared and immunoprecipitated (*IP*) with anti-Flag M$_2$ affinity gel (Kodak Scientific Imaging Systems). The presence of caspase-9, Bcl-X$_L$-Myc, and Flag-Apaf-1 was detected by immunoblotting with polyclonal antibody against caspase-9, horseradish peroxidase (*HRP*)-conjugated anti-Myc (*BMB*), and anti-Flag (Babco), respectively, as indicated. *B*, caspase-9, but not other caspases, interacts with Apaf-1. 293 cells were co-transfected with Apaf-1(3+4)-Myc and various caspase constructs as described under "Experimental Procedures." Cell lysates were co-immunoprecipitated with anti-Flag affinity gel, and immunoblotting was performed with various monoclonal antibodies (mAb) or polyclonal anti-RAIDD as indicated. The *middle* and *bottom panels* show the expression of the individual proteins. *C*, the prodomain of caspase-9 binds Apaf-1. Apaf-1(3+4)-Myc was co-expressed in 293 cells with various caspase-9 constructs as described under "Experimental Procedures." Cell lysates were co-immunoprecipitated with anti-Flag affinity gel, and immunoblotting was done with mAbs as indicated. *D*, Bcl-X$_L$ binds Apaf-1. Apaf-1(3+4)-Myc was co-expressed with either COOH-terminal-tagged Bcl-X$_L$ (*Bcl-X$_L$-FLAG(C)*) or NH$_2$-terminal tagged Bcl-X$_L$ (*Bcl-X$_L$-FLAG(N)*). Co-immunoprecipitation was done with anti-Flag affinity gel, and immunoblotting was performed with various mAbs as shown. *L.C.*, light chain. *E*, caspase-9 and Bcl-X$_L$ bind distinct regions in Apaf-1. Either caspase-9-prodomain-Flag or Bcl-X$_L$-Flag was co-transfected with Apaf-1(3)-Myc that contains only the CED-3-homologous region and Apaf-1(4)-Myc that contains only the CED-4-homologous region as described under "Experimental Procedures." Cell extracts were prepared and co-immunoprecipitated with anti-Flag affinity gel. The presence of each protein was detected by immunoblotting with various antibodies as indicated.

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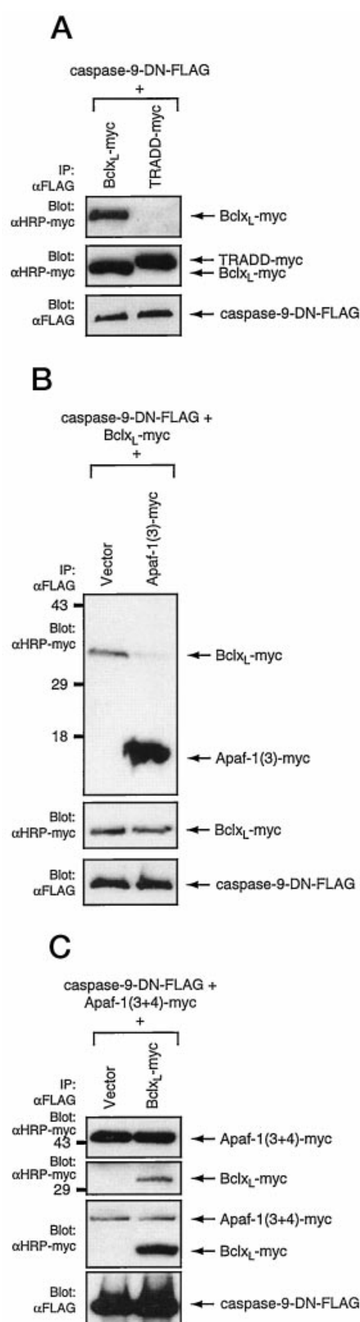

FIG. 3. **Caspase-9 and Bcl-X_L form a ternary complex with Apaf-1.** *A*, caspase-9 associates with Bcl-X_L through an endogenous Apaf-1-like activity. 293 cells were co-transfected with caspase-9-DN together with Bcl-X_L-Myc or a control molecule TRADD-Myc as indicated. Cell lysates were prepared and co-immunoprecipitated with anti-Flag affinity gel, and individual protein was detected by immunoblotting with the indicated mAbs. *B*, co-expression of the CED-3-homologous region of Apaf-1 disrupts the association of caspase-9 with Bcl-X_L. 293 cells were co-transfected with caspase-9-DN-Flag and Bcl-X_L-Myc in the presence of a vector or a construct expressing Apaf-1(3)-Myc. Co-immunoprecipitation was performed with anti-Flag affinity gel, and immunoblotting was done as indicated. *C*, Bcl-X_L does not affect the interaction between caspase-9 and Apaf-1. Caspase-9-DN-Flag was co-expressed with Apaf-1(3+4)-Myc in the presence of either a vector or a Bcl-X_L-Myc construct. Co-immunoprecipitation was done with anti-Flag affinity gel and proteins were detected by immunoblotting as indicated.

does not heterodimerize with other Bcl-2 family members (29, 30), retained binding to Apaf-1, albeit to a lesser extent (Fig. 2*D, right*). Regardless, the data are consistent with the notion that Bcl-X_L may function by interacting with Apaf-1.

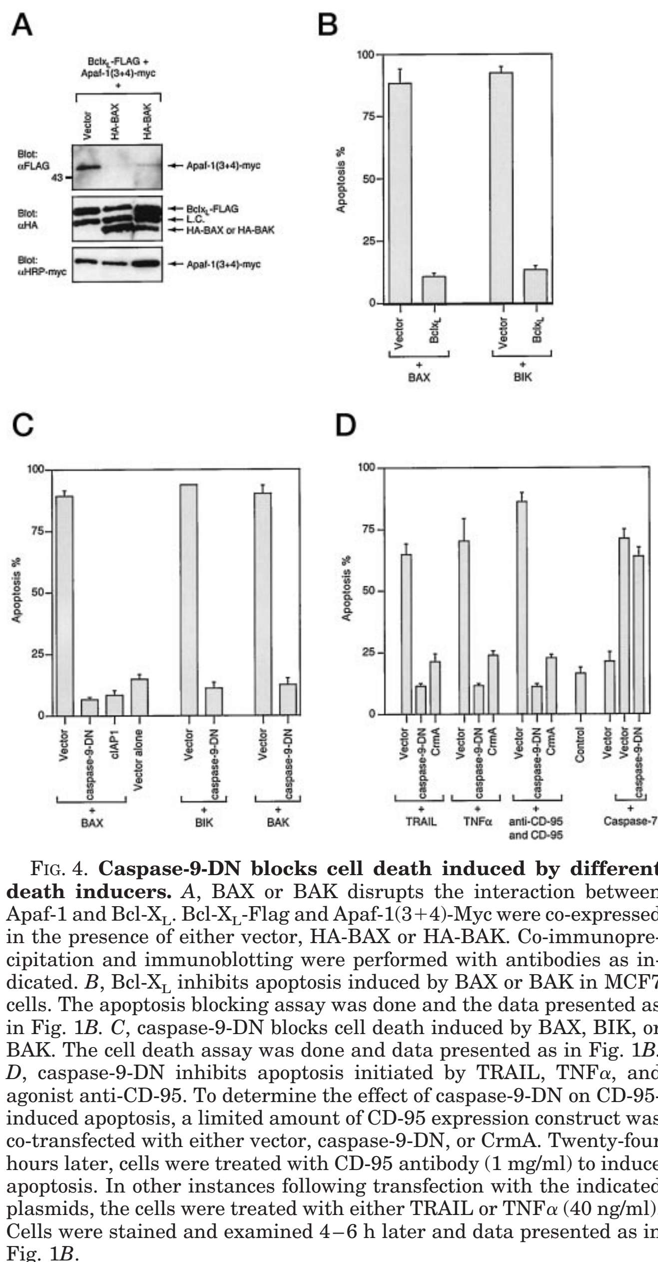

FIG. 4. **Caspase-9-DN blocks cell death induced by different death inducers.** *A*, BAX or BAK disrupts the interaction between Apaf-1 and Bcl-X_L. Bcl-X_L-Flag and Apaf-1(3+4)-Myc were co-expressed in the presence of either vector, HA-BAX or HA-BAK. Co-immunoprecipitation and immunoblotting were performed with antibodies as indicated. *B*, Bcl-X_L inhibits apoptosis induced by BAX or BAK in MCF7 cells. The apoptosis blocking assay was done and the data presented as in Fig. 1*B*. *C*, caspase-9-DN blocks cell death induced by BAX, BIK, or BAK. The cell death assay was done and data presented as in Fig. 1*B*. *D*, caspase-9-DN inhibits apoptosis initiated by TRAIL, TNFα, and agonist anti-CD-95. To determine the effect of caspase-9-DN on CD-95-induced apoptosis, a limited amount of CD-95 expression construct was co-transfected with either vector, caspase-9-DN, or CrmA. Twenty-four hours later, cells were treated with CD-95 antibody (1 mg/ml) to induce apoptosis. In other instances following transfection with the indicated plasmids, the cells were treated with either TRAIL or TNFα (40 ng/ml). Cells were stained and examined 4–6 h later and data presented as in Fig. 1*B*.

Caspase-9 and Bcl-X_L Bind to Distinct Regions in Apaf-1—To determine if caspase-9 and Bcl-X_L bind to distinct domains in Apaf-1 (3+4), the CED-3-homologous region alone (Apaf-1(3)) and a truncated form that contained only the CED-4-homologous region (Apaf-1(4)) were assessed separately for binding in a co-transfection assay. Caspase-9 bound the CED-3-homologous region (Apaf-1(3); Fig. 2*E, left*), while Bcl-X_L interacted with the CED-4-homologous region (Apaf-1(4); Fig. 2*E, right*). Therefore, caspase-9 and Bcl-X_L bind to distinct domains in Apaf-1, raising the possibility that they can form a ternary complex with Apaf-1.

Caspase-9 and Bcl-XL Form a Ternary Complex with Apaf-1—To assess this, we asked whether caspase-9 and Bcl-X_L might co-precipitate through an endogenous Apaf-1-like activity. 293 cells were co-transfected with caspase-9-Flag and Bcl-X_L-Myc. Caspase-9 co-precipitated with Bcl-X_L but not a control protein, TRADD-Myc (Fig. 3*A*). To confirm that the observed association was indeed mediated by an endogenous Apaf-1-like molecule, the CED-3-homologous domain of Apaf-1 (Apaf-1(3)-Myc) was co-expressed in the same cells. We reasoned that this domain, when present in excess, should com-

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petitively inhibit the binding of caspase-9 to endogenous Apaf-1, thereby disrupting the association between caspase-9 and Bcl-X_L if the bridging molecule was indeed Apaf-1. As anticipated, Apaf-1(3)-Myc on co-expression attenuated the association of caspase-9-Flag and Bcl-X_L-Myc (Fig. 3*B*). Furthermore, Apaf-1(3)-Myc was observed in complex with caspase-9-Flag (Fig. 3*B*), confirming the competitive nature of the inhibition. Additional validation for ternary complex formation was provided by the observation that overexpressing Bcl-X_L-Myc in the same cells did not compete for the association of caspase-9 with Apaf-1(3+4)-Myc (Fig. 3*C*). This result is consistent with the existence of independent binding sites on Apaf-1 for caspase-9 and Bcl-X_L.

BAX and BAK Disrupt Interaction between Bcl-X_L and Apaf-1—The anti-apoptotic ability of Bcl-X_L is antagonized by pro-apoptotic members of the Bcl-2 family, including BAX, BAK, and BIK that are capable of forming heterodimers with Bcl-X_L (29, 30). Given this, we asked if the pro-apoptotic family members may function by interfering with the ability of Bcl-X_L to bind Apaf-1. In keeping with this hypothesis, co-expression of HA-BAX or HA-BAK attenuated the interaction between Bcl-X_L-Flag and Apaf-1(3+4)-Myc (Fig. 4*A*), with HA-BAX or HA-BAK being found in complex with Bcl-X_L-Flag (Fig. 4*A*). Consistent with the suggested mechanism, Bcl-X_L effectively inhibited BAX-, BIK-, or BAK-induced cell death (Fig. 4*B*). Since CED-9 functions upstream of CED-4 and CED-3, Bcl-2 family members likely also function upstream of Apaf-1 and caspase-9 (5). Supporting this viewpoint, we found that cell death induced by BAX, BIK, or BAK was effectively inhibited by dominant negative caspase-9 (Fig. 4*C*).

Dominant Negative Caspase-9 Inhibits Cell Death Initiated by Death Ligands and Agonist CD-95 Antibody—In agreement with the notion that activation of caspase-9 serves as a common conduit for the flow of death signals, dominant negative caspase-9 also blocked apoptosis induced by members of the TNF receptor family activated with either cognate ligand or agonist antibody (Fig. 4*D*).

In conclusion, we have shown that both caspase-9 and Bcl-X_L specifically and simultaneously interact with Apaf-1. Therefore, the formation of a complex involving caspase-9, Apaf-1, and Bcl-X_L may play a regulatory role in modulating the mammalian cell death machine.

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