Bcl-2 and Bcl-xL Inhibit CD95-mediated Apoptosis by Preventing Mitochondrial Release of Smac/DIABLO and Subsequent Inactivation of X-linked Inhibitor-of-Apoptosis Protein*

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Xiao-Ming Sun‡, Shawn B. Bratton‡, Michael Butterworth, Marion MacFarlane, and Gerald M. Cohen§

From the Medical Research Council Toxicology Unit, Hodgkin Building, University of Leicester, P. O. Box 138, Lancaster Road, Leicester LE1 9HN, United Kingdom

Bcl-2 and Bcl-xL are reported to inhibit CD95-mediated apoptosis in “type II” but not in “type I” cells. In the present studies, we found that stimulation of CD95 receptors, with either agonistic antibody or CD95 ligand, resulted in the activation of caspase-8, which in turn processed caspase-3 between its large and small subunits. However, in contrast to control cells, those over-expressing either Bcl-2 or Bcl-xL displayed a distinctive pattern of caspase-3 processing. Indeed, the resulting p20/p12 caspase-3 was not active and did not undergo normal autocatalytic processing to form p17/p12 caspase-3, because it was bound to and inhibited by endogenous X-linked inhibitor-of-apoptosis protein (XIAP). Importantly, Bcl-2 and Bcl-xL inhibited the release of both cytochrome c and Smac from mitochondria. However, since Smac alone was sufficient to promote caspase-3 activity in vitro by inactivating XIAP, we proposed the existence of a death receptor-induced, Smac-dependent and apoptosome-independent pathway. This type II pathway was subsequently reconstituted in vitro using purified recombinant proteins at endogenous concentrations. Thus, mitochondria and associated Bcl-2 and Bcl-xL proteins may play a functional role in death receptor-induced apoptosis by modulating the release of Smac. Our data strongly suggest that the relative ratios of XIAP (and other inhibitor-of-apoptosis proteins) to active caspase-3 and Smac may dictate, in part, whether a cell exhibits a type I or type II phenotype.

Apoptosis, or programmed cell death, can be induced through two basic and distinct cell death signaling pathways, both of which culminate in the activation of cysteineyl aspartate-specific proteases or caspases. Stimulation of death receptors, such as CD95, TNFR1, and DR5, leads to formation of the death-inducing signaling complex (DISC),1 which minimally contains the receptor, the adapter protein Fas-associated death domain, and caspase-8 (1–5). Similarly, various stressors, including toxicants and radiation, can induce mitochondrial release of cytochrome c and formation of the apoptosome, a complex that contains the adapter protein apoptotic protease-activating factor-1 (Apaf-1) and caspase-9 (6–9). All caspases are synthesized as single chain zymogens, possessing a prodomain, a large (~20 kDa) subunit, and a small (~10 kDa) subunit. Caspases-8 and -9 are referred to as apical caspases, because they contain long prodomains that allow them to interact with their respective adapter proteins and undergo proximity-induced, autocatalytic activation. In this model, one caspase-8 or caspase-9 molecule within the DISC or apoptosome, respectively, activates another by cleaving between its large and small subunits. The activated apical caspases then propagate the death signal by activating short prodomain effector caspases-3 and/or -7, which proteolytically dismantle the cell (10, 11).

Inhibitor-of-apoptosis proteins (IAPs) are a family of proteins that contain baculoviral repeat (BIR) domains and, in some cases, a zinc RING-finger domain (12). X-linked IAP (XIAP), Livin/ML-IAP, cIAP-1, and cIAP-2 are believed to inhibit apoptosis through their direct inhibition of caspases, although some of these proteins are also involved in additional signaling pathways (13–18).2 XIAP, the most potent of these caspase inhibitors, selectively inhibits one of the active forms of caspase-9 (p35/p12 heterotetramer) through an interaction involving its BIR3 domain and the small subunit (p12) of caspase-9 (19–21). In contrast, the BIR2 domain of XIAP, along with a few critical adjacent residues, is required to inhibit active caspases-3 and -7 (22–25). Consequently, XIAP is thought to inhibit death receptor-induced apoptosis by inhibiting effector caspases and mitochondrial induced apoptosis by inhibiting both apical and effector caspases (26). Recently, a structural homologue of the Drosophila proteins, Reaper, Hid, and Grim, has been identified and termed Smac/DIABLO (27, 28). This protein is normally localized to mitochondria but, like cytochrome c, is released into the cytosol during the early stages of apoptosis, where it appears to promote caspase activity by inhibiting IAPs, particularly XIAP (27–32).

In some cells, stimulation of death receptors can lead to caspase-8-mediated cleavage and activation of the proapoptotic Bcl-2 protein, BID (33–35). Truncated Bid stimulates formation of Bax (and/or Bak) pores in the outer mitochondrial membrane, which mediate the release of cytochrome c and, consequently, formation of the Apaf-1/caspase-9 apoptosome (36). In

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‡ Both authors contributed equally to this work.

§ To whom correspondence should be addressed. Tel.: 44-116-252-5601; Fax: 44-116-252-5616; E-mail: gmc2@le.ac.uk.

1 The abbreviations used are: DISC, death-inducing signaling complex; XIAP, X-linked inhibitor-of-apoptosis protein; IAP, inhibitor-of-apoptosis protein; BIR, baculoviral repeat; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PARP, poly(ADP-ribose) polymerase; TRAIL, TNF-related apoptosis-inducing ligand (where TNF is tumor necrosis factor); DEVDase, effector caspase activity to cleave Z-DEVD.AFC (benzoxylcarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin).
contrast, antiapoptotic Bcl-2 and Bcl-xL proteins, in addition to inhibitors of Bax, such as the viral protein E1B 19K, appear to inhibit cell death by blocking formation of these cytochrome c-releasing pores (36, 37). Thus, in death receptor-induced apoptosis, protection by Bcl-2 or Bcl-xL is an indicator of the requirement for mitochondrial involvement, and for the sake of convenience, those cells that are protected by Bcl-2 and Bcl-xL have been termed type II cells, whereas those that are not protected are termed type I cells (38, 39). However, the role of mitochondria in death receptor-induced apoptosis has been strongly challenged, in part, because many cells isolated from cytotoxicity c-/-, apaf-1-/-, and caspase-9-/- mice appear to respond normally to death ligands, such as CD95L (40). In the present study, we provide an explanation for some of the conflicting results previously observed and, in doing so, help to redefine or update the type I/type II cell model. We show that Bcl-2 and Bcl-xL, in addition to inhibiting the release of cytochrome c, also block release of Smac from mitochondria and thus can modulate a second, unique type II signaling pathway. Indeed, XIAP can prevent death receptor-induced apoptosis solely through its inhibition of active caspase-3. Therefore, by inhibiting the release of Smac, Bcl-2 and Bcl-xL can prevent the inactivation of XIAP and inhibit cell death.

MATERIALS AND METHODS

Cell Culture Experiments/Apoptosis Measurements—Jurkat T-cells (36) and T-lymphoblastic leukemia CEM cells, stably transfected with either empty vector, Bcl-xL, or Bcl-xL (41, 42), were cultured in RPMI 1640 containing 10% fetal calf serum, 1% Glutamax, and 0.4 mg/ml G418 in 5% CO2 at 37 °C. Cells were treated for 4 h with the agonistic anti-CD95 antibody, CH-11 (10–50 ng/ml) (Upstate Biotechnology, Inc., Lake Placid, NY), or recombinant CD95 ligand (50 ng/ml), cross-linked with M2 anti-FLAG antibody, according to the manufacturer’s suggested protocol (Alexis Biochemicals, Nottingham, UK). After treatment, the percentage of annexin V-positive cells (% apoptosis) was determined by fluorescence-activated cell sorter analysis, as described previously (43). Annexin V/fluorescein isothiocyanate was purchased from Bender Medsystems (Vienna, Austria).

Caspase Processing and Cytochrome c/Smac Release—After treatment, cells were pelleted and washed with ice-cold phosphate-buffered saline. Some of the cells were then mixed with Laemmli loading buffer and briefly sonicated. The whole cell protein samples were subsequently resolved by 12–15% SDS-PAGE and immunoblotted for caspase-8, caspase-3, cleaved caspase-6 (recognizes only the processed caspase-8, p43 and p41 caspase-8 splice forms), procaspase-3, procaspase-6, and poly(ADP-ribose) polymerase (PARP). The remaining cells were incubated with buffer containing 0.25% digitonin on ice for 20 min. The samples were then centrifuged at 20,000 × g for 10 min, and the cytosolic fractions (supernatants) were collected. These fractions were separated by SDS-PAGE and immunoblotted for cytochrome c (BD Pharmingen) and Smac. The rabbit polyclonal antibodies to caspase-8 (large subunit) (43) and Smaβ (residues 1–186) (44) were generated in our laboratory.

Immunoprecipitation Experiments—Lysates prepared from control and CD95-stimulated Jurkat cells were incubated with protein G-Sepharose beads (Amersham Biosciences), blocked with 3% bovine serum albumin, and precoated with anti-caspase-3 antibodies (BD Transduction Laboratories, Franklin Lakes, NJ). The resulting protein complexes were obtained by centrifugation, washed 4 times in buffer (100 mM HEPES, 0.1% CHAPS, 10 mM dithiothreitol, 10% sucrose, pH 7.0), and immunoblotted for XIAP (Transduction Laboratories, Franklin Lakes, NJ), cIAP-1, or cIAP-2 (R & D Systems, Minneapolis, MN). For the XIAP blots, a secondary antibody specific for mouse IgG was used.

Reconstitution of Death Receptor, XIAP/Smac-dependent Pathway—Purified recombinant caspase-8 (pg19/p12 form; 5–120 nmol), procaspase-3 (200 nmol), XIAP (1–40 nmol), and Smac (5–100 nmol) were incubated in various combinations at 37 °C for 1 h in caspase assay buffer (100 mM HEPES, 0.1% CHAPS, 10 mM dithiothreitol, 10% sucrose, pH 7.0), and caspase-3 was subsequently immunoblotted, as described above. DEVDase activities were determined using the substrate, benzoylcarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin, as described previously (8).

RESULTS

Bcl-2 and Bcl-xL Inhibit Caspase-3 Processing in CD95-stimulated Cells—As already noted, during death receptor-induced apoptosis, caspase-8 processes caspase-3 between its large and small subunits. However, recent studies (37, 45) indicate that the prodomain of caspase-3 is not subsequently removed in cells overexpressing Bcl-2, Bcl-xL, or the viral protein E1B 19K. Because these cells were also resistant to cell death, we found this observation intriguing and thus began to search for a causal relationship. Perez and White (37) suggested that maturation of caspase-3 from its p20/p12 to p17/p12 form was required for its catalytic activity and that this second cleavage step was carried out by the Apaf-1/caspase-9 apoptosome. However, this explanation seemed unlikely to us, given that removal of the prodomain from caspase-3 is normally associated with its autocatalytic activity (46, 47).

Therefore, we initiated studies in which Jurkat T-cells, stably expressing either Bcl-2 or Bcl-xL, were treated with an agonistic CD95 antibody (CH-11). As expected, in vector control-treated cells, CD95 stimulation led to increased apoptosis, caspase-8 processes caspase-3 between its large and small subunits. However, recent studies (37, 45) indicate that the prodomain of caspase-3 is not subsequently removed in cells overexpressing Bcl-2, Bcl-xL, or the viral protein E1B 19K. Because these cells were also resistant to cell death, we found this observation intriguing and thus began to search for a causal relationship. Perez and White (37) suggested that maturation of caspase-3 from its p20/p12 to p17/p12 form was required for its catalytic activity and that this second cleavage step was carried out by the Apaf-1/caspase-9 apoptosome. However, this explanation seemed unlikely to us, given that removal of the prodomain from caspase-3 is normally associated with its autocatalytic activity (46, 47).

Therefore, we initiated studies in which Jurkat T-cells, stably expressing either Bcl-2 or Bcl-xL, were treated with an agonistic CD95 antibody (CH-11). As expected, in vector control-treated cells, CD95 stimulation led to increased apoptosis in a concentration-dependent manner (Fig. 1A). Both pro-caspase-8 splice forms were processed to the p43 and p41 forms (prodomain + large subunit), respectively (Fig. 1A, lanes 2 and 3), and at the higher concentration of CH-11, some p18 fragment (large subunit only) was also observed (Fig. 1A, lane 3). Caspase-3 was processed primarily to its p19 form or its fully mature p17 form (Fig. 1A, lanes 2 and 3), and the caspase-3 substrates, caspase-6 and poly(ADP-ribose) polymerase-
ase (PARP), were significantly processed (Fig. 1A, lanes 2 and 3; Fig. 2A, lane 2). In addition, there was a dramatic increase in caspase-3-like DEVDase activity (data not shown).

In cells overexpressing either Bel-2 or Bel-x<sub>L</sub>, cell death was markedly reduced (Fig. 1A). Caspase-8 was processed normally to its p43 and p41 forms, although to a lesser extent than in control cells, and no p18 fragment was observed (Fig. 1A, lanes 5, 6, 8, and 9). Caspase-8 was active, as it processed caspase-3 between its large and small subunits, but interestingly, almost all of the processed caspase-3 was present in its p20 form (Fig. 1A, lanes 5, 6, 8, and 9). Cleavage of caspase-6 and PARP, as well as DEVDase activity, was significantly reduced in cells overexpressing Bel-2 or Bel-x<sub>L</sub>, indicating that caspase-3 activity was reduced (Fig. 1A, lanes 5, 6, 8, and 9; Fig. 2A, lanes 3 and 4; data not shown). Furthermore, this lack of caspase-3 activity and, consequently, activation of caspase-6 probably explained the observed decrease in caspase-8 processing compared with control cells (Fig. 1A), because caspase-6 is known to process additional procaspase-8 (48). Thus, similar to previous reports (38, 39), Bel-2 and Bel-x<sub>L</sub> inhibited DEVDase activity by preventing release of the IAP antagonist, Smac/DIABLO, from mitochondria. Vector control (VC), Bel-2, and Bel-x<sub>L</sub>-overexpressing cells were treated with CH-11 antibody (50 ng/ml) for 4 h. Cytosolic lysates from control and treated cells were then prepared and assayed for processing of PARP (A), basal levels of XIAP, cIAP-1, and cIAP-2 (B), and release of cytochrome c (Cyt. c) and Smac/DIABLO from mitochondria (C, D). Total caspase-3 (Casp-3) was immunoprecipitated (IP) from each lysate, and the pellets were subsequently Western blotted (WB), for XIAP and caspase-3. For clarity, XIAP was subsequently Western blotted (WB) for XIAP and caspase-3. For clarity, XIAP blots were also incubated with a mouse anti-IgG secondary (2<sup>nd</sup>) antibody, because it was difficult to separate the heavy chain (HC) of the mouse polyclonal antibody from XIAP. LC, light chain. E, lysates from untreated vector control (Con) and CD95-stimulated Bel-2/Bel-x<sub>L</sub>-overexpressing cells were also incubated (0–60 min) with exogenous Smac (500 nm) at 37 °C and subsequently assayed for DEVDase activity, as described under “Materials and Methods.”

Although the Jurkat T-cells in the present study clearly underwent death receptor-mediated apoptosis following CD95 ligation with CH-11 antibodies, and did so in a Bel-2/Bel-x<sub>L</sub>-inhibitable manner, there has been considerable debate in the literature as to the existence of type II cells, and particularly the use of anti-CD95 antibodies to induce apoptosis (40, 49, 50).

Therefore, to verify that Bel-2 could influence CD95-mediated processing of caspase-3 and cell death in another cell line, we treated T-cell acute lymphoblastic leukemia CEM cells with either CH-11 antibodies or cross-linked CD95L (50 ng/ml). Remarkably, both treatments induced almost identical levels of apoptosis, and the processing of caspases-8, -3, and -6, in the CH-11 and CD95L-treated cells was virtually indistinguishable (Fig. 1B, lanes 11 and 12). More importantly, however, in Bel-2 stably transfected CEM cells, cell death was inhibited by ~50–60% in both treatments, and processing of caspase-8 was significantly reduced (Fig. 1B, lanes 13 and 14). As before, caspase-3 was processed primarily to its p20 form, and there was no autoprocessing of caspase-3 to its fully mature p17 form (Fig. 1B, lanes 13 and 14). Furthermore, the p20 form of caspase-3 was inhibited, as no processing of caspase-6 could be observed (Fig. 1B, lanes 13 and 14). Thus, the inhibition of caspase-3-processing in Bel-2-transfected cells was not limited to Jurkat T-cells, and the phenomenon could be observed with either anti-CD95 antibody or CD95L.

Bel-2 and Bel-x<sub>L</sub> Prevent Release of Smac from Mitochondria and Allow XIAP to Bind and Inhibit p20/p12 Caspase-3—The inability of p20/p12 caspase-3 to remove its prodomain suggested to us that the enzyme might be inhibited. Therefore, because IAPs, including XIAP, cIAP-1, and cIAP-2, are known to inhibit caspase-3 activity with inhibitory constants (K<sub>i</sub>) of ~0.7, ~108, and ~35 nm, respectively (13, 15), we initially investigated whether Bel-2 or Bel-x<sub>L</sub> might influence their expression. The levels of XIAP, cIAP-1, and cIAP-2, however, were not elevated in Bel-2 or Bel-x<sub>L</sub>-transfected cells compared with vector control cells (Fig. 2B, lanes 1–3). Therefore, we next examined if these IAPs might be directly associated with caspase-3 in cells. No interaction between caspase-3 and cIAP-1 or cIAP-2 could be detected (data not shown). However, immunoprecipitation of total caspase-3 from unstimulated and stimulated cells indicated that XIAP was almost exclusively associated with processed caspase-3 (Fig. 2D, compare lane 2 with lanes 3–5). Strikingly, far more XIAP was associated with the partially processed p20 subunit of caspase-3, in cells overexpressing Bel-2 or Bel-x<sub>L</sub>, than with the p17 subunit in vector control cells, even though significantly more of the p17 subunit was immunoprecipitated (Fig. 2D, compare lane 3 with lanes 4 and 5). Because XIAP can bind and inhibit both p17/p21 caspase-3 and p20/p12 caspase-3 in vitro (22), these data suggested that Bel-2 and Bel-x<sub>L</sub> specifically inhibited the release of some factor from mitochondria that could bind to XIAP and prevent its interaction with processed caspase-3.

Smac/DIABLO contains an N-terminal “AVPI” motif, which allows it to bind the BIR3 domain in XIAP and displace caspase-9, as well as a second region that is sufficient to displace processed caspases-3 and -7 from the BIR2 domain in XIAP (29–31, 51). Therefore, we examined the cytosols from
FIG. 3. Reconstitution of Smac-dependent type II pathway. A, processing of purified procaspase-3 (200 nM) and its resulting DEVDase activity was determined following incubation with active caspase-8 (0–40 nM). autoprocessing of p20/p12 caspase-3 was then determined in the presence of recombinant XIAP (1–40 nM) (B) and in the presence of XIAP (30 nM) and its antagonist, Smac (0–100 nM) (C). D, inhibition of caspase-3 autoprocessing by XIAP (30 nM) was examined in the presence of increasing concentrations of active caspase-8 (30–120 nM).

stimulated and unstimulated cells for the presence of Smac. Indeed, Smac was released in CD95-stimulated vector control cells but not in cells transfected with Bcl-2 or Bcl-xL (Fig. 2C), confirming that antiapoptotic Bcl-2 proteins are capable of inhibiting the release of Smac following an apoptotic stimulus (32, 51). These data were important because they supported the potential existence of a novel type II pathway. In this pathway, Bcl-2 and Bcl-xL could inhibit death receptor-induced apoptosis by preventing the release of Smac and thus inactivation of XIAP. Consequently, XIAP would be allowed to maintain its association with and inhibit the activity of p20/p12 caspase-3, as well as cell death. Importantly, however, Bcl-2 and Bcl-xL also inhibited the release of cytochrome c, compared with vector control cells (Fig. 2C). Therefore, the possibility remained that Bcl-2 and Bcl-xL might inhibit cell death, primarily by preventing formation of the Apaf-1/caspase-9 apoptosome. In theory, active caspase-9 might still be required to process additional procaspase-3 or to further process p20/p12 caspase-3 to its p17/p12 form.

To prove that the lack of caspase-3 activity was due to inhibition by endogenous XIAP, we incubated lysates prepared from CD95-stimulated Jurkat T cells with recombinant Smac. Addition of Smac led to a time-dependent increase in caspase-3 DEVDase activity in lysates from Bcl-2 and Bcl-xL-expressing cells but not from control cells (Fig. 2E). As expected, Smac had no significant effect on lysates from CD95-stimulated vector control cells (data not shown), because caspase-3 was already active and only weakly inhibited by XIAP (Fig. 1A, lanes 2 and 3; Fig. 2D, lane 3). The effect of Smac was far more profound in lysates prepared from Bcl-2-overexpressing cells than from cells transfected with Bcl-xL (Fig. 2E). This result, however, can be easily explained. In the case of the Bcl-xL-transfected cells, CD95 stimulation did induce partial release of cytochrome c, whereas none was observed in the Bcl-2-overexpressing cells (Fig. 2C, lanes 3 and 4). Therefore, it is likely that in vitro incubation of the lysates, from the Bcl-xL but not the Bcl-2-transfected cells, initiated cytochrome c-dependent formation of the Apaf-1/caspase-9 apoptosome and, consequently, increased activation of caspase-3. Indeed, in the absence of Smac, incubation of lysates from vector control and Bcl-2-transfected cells did not lead to a substantial increase in DEVDase activity, whereas lysates from Bcl-xL-transfected cells exhibited a marked increase in caspase-3 activity (Fig. 2E).

Interestingly, overexpression of cytosolic Smac has previously been achieved using an artificial green fluorescent protein-(IETD AVPI)-Smac, which could be cleaved by caspase-8 following death receptor stimulation, to release mature Smac that contains its requisite N-terminal AVPI motif (51). In these studies, mature Smac potentiated TRAIL-induced apoptosis in caspase-3-deficient, MCF-7 type II cells stably transfected with Bcl-xL (51). Thus, consistent with the results obtained in our studies, Smac overexpression could bypass the protection afforded by Bcl-xL and potentiate death receptor-induced apoptosis, probably by antagonizing XIAP-mediated inhibition of active caspase-7.

In Vitro Reconstitution of a Novel Death Receptor-mediated Type II Pathway Involving XIAP and Smac—To verify the minimal proteins required for the proposed type II model, we next reconstituted the pathway in vitro, using only purified recombinant proteins. All known death receptor-induced apoptotic pathways, including CD95, utilize caspase-8 as their apical caspase. Therefore, we utilized fully mature caspase-8 (p18/p12) and confirmed its ability to process recombinant procaspase-3 (Fig. 3A). Low concentrations of caspase-8 (5–20 nM) processed procaspase-3 between its large and small subunits (IETD 5 S), and importantly, this resulted in immediate detection of substantial DEVDase activity, even though caspase-3 retained its promdomain (Fig. 3A, lanes 2–4). This effector caspase activity was due to the activation of caspase-3, because caspase-8 (40 nM) alone had very little DEVDase activity (Fig. 3A, lane 7).

Removal of the promdomain from caspase-3 occurred only at higher concentrations of caspase-8 (>20 nM) (Fig. 3A, lanes 5 and 6), which suggested (i) that a certain threshold concentration of active p20/p12 caspase-3 had to be attained before autocatalytic removal of its promdomain could proceed, or (ii) that at higher concentrations, caspase-8 might also remove the promdomain from caspase-3. The latter interpretation was ruled out, however, because caspase-8 (100 nM) processes catalytically inactive caspase-3 (C287A) only between its large and small subunit (26). Therefore, these data confirmed that p20/p12 caspase-3 must be active (or uninhibited) and must be
The addition of XIAP did not inhibit the initial processing of procaspase-3 by caspase-8 to its p20 form (Fig. 3B, lanes 1–7), in agreement with the previous observation that XIAP does not inhibit caspase-8 (14). However, XIAP did inhibit p20/p12 caspase-3 activity, as determined by a decrease in DEVDase activity and inhibition of autocatalytic processing (i.e. conversion of the p20 subunit to the p17 subunit) (Fig. 3B, lanes 2–7). Thus, at physiological concentrations of caspase-8 (30 nM), procaspase-3 (200 nM), and XIAP (30 nM) (47), we observed the identical pattern, or “footprint,” of caspase-3 processing as was observed in CD95-stimulated cells overexpressing Bcl-2 and Bcl-xL. Next, because Smac was released from mitochondria in the CD95-stimulated control cells, we titrated this IAP inhibitor into our incubations and observed a concentration-dependent increase in both caspase-3 activity and autoprocessing (Fig. 3C). Thus, we were able to reproduce in vitro the effects of Bcl-2 and Bcl-xL on caspase-3 activity and processing in vivo by altering the levels of XIAP and Smac.

It has been suggested that type I cells do not require mitochondrial involvement, because they recruit and activate more caspase-8 at the DISC than do type II cells (38). Therefore, we questioned whether the effects of XIAP on caspase-3 processing might be overcome by increasing the amount of active caspase-8 in the absence of Smac. As the concentration of caspase-8 was increased, we observed a concomitant increase in the total processing of procaspase-3 as well as a shift from the predominantly p20 to p17 form (Fig. 3D). Thus, at high concentrations, caspase-8 can cleave sufficient procaspase-3 to exceed the capacity of endogenous XIAP to inhibit all of the processed, active caspase-3. However, the concentrations of caspase-8 required were significantly higher than physiological levels, which suggests that a death receptor-mediated, Smac-and cytochrome c-independent, apoptotic pathway (i.e. type I pathway) may only exist in cells expressing low to moderate levels of IAPs. In type II cells, there are likely two distinct pathways that may act in concert or independently of one another. In the proposed pathway, the release of Smac appears to be sufficient to antagonize XIAP and promote activation of caspase-3, independent of any contribution from the apoptosome (Fig. 4). However, in some cases, the Apaf-1/caspase-9 apoptosome might also participate with caspase-8 in generating additional p20/p12 caspase-3 and aid in overcoming the inhibitory effect of XIAP.

**DISCUSSION**

The simple observations that CD95-stimulated cells overexpressing Bcl-2 or Bcl-xL exhibit only partial processing of caspase-3 and that they are also resistant to apoptosis made us question if the two effects were causally linked. Because both the partially processed (p20/p12) and fully processed (p17/p12) forms of caspase-3 were equally active in vitro, removal of the prodomain from caspase-3 was not required for its activity or ability to execute the apoptotic program. In fact, this autocatalytic step did not occur in CD95-stimulated cells overexpressing Bcl-2 or Bcl-xL, because p20/p12 caspase-3 was strongly inhibited by XIAP. Indeed, the pattern or footprint of caspase-3 processing merely served as a signature for caspase-3 inhibition. Additional IAPs, such as cIAP-1 and cIAP-2, also inhibit autocatalytic processing of caspase-3 and produce a similar footprint (14, 15). However, we detected no association of cIAP-1 or cIAP-2 with caspase-3 in our studies (data not shown). Bcl-2 and Bcl-xL indirectly preserved the inhibition of p20/p12 caspase-3 by XIAP, and thus blocked cell death, by preventing release of the IAP antagonist, Smac. We demonstrated in vitro that Smac is sufficient to promote caspase activation in a death receptor-mediated pathway, without an absolute requirement for the Apaf-1/caspase-9 apoptosome. In support of this hypothesis, Cidlowski and co-workers (52) recently demonstrated that Jurkat T-cells, expressing a dominant negative form of caspase-9, were resistant to UV-induced apoptosis but responded normally to agonistic CD95 antibodies. Indeed, in the caspase-9 dominant-negative cells, DEV-Dase activity was only marginally lower than in CD95-stimulated control cells, and caspase-3 was processed predominantly to its p17/p12 form (52).

In most cells, Bcl-2 and Bcl-xL also prevent or attenuate the release of additional proapoptotic mitochondrial proteins, including cytochrome c, apoptosis-inducing factor, and endonuclease G (53–55). Despite the difficulties in determining their relative importance to death receptor-induced apoptosis, there are some important considerations that can be addressed. In the debate over type I versus type II cells, one of the strongest arguments made against the existence of type II cells, is that many cytochrome c<sup>−/−</sup>, apaf-1<sup>−/−</sup>, and caspase-9<sup>−/−</sup> cells appear to respond normally to anti-CD95 antibodies, implying that the apoptosome is not required for death receptor-induced apoptosis (56–58). However, others argue that mice deficient in
Bid are resistant to anti-CD95 antibodies, particularly in the liver, implying that mitochondrial involvement is required for death receptor-induced apoptosis (59). We propose a model in which Bcl-2 and Bcl-\(x_l\) protect against apoptosis in type II cells via at least two distinct pathways: one involving the release of cytchrome c and formation of the apoptosome, and the other involving the release of Smac and inactivation of XIAP (Fig. 4). Thus, even in cells that are incapable of forming an apoptosome, permeabilization of mitochondria may still be necessary for efficient death receptor-mediated apoptosis in cells expressing moderate to high levels of IAPs.

In most situations, both the apoptosis- and Smac-dependent type II pathways will function synergistically, as Smac can also enhance the function of the apoptosome by preventing IAP inhibition of caspase-9 (20, 27, 29, 31). However, it is interesting to consider situations in which Smac release may be vitally important for death receptor-induced apoptosis. Indeed, Apaf-1 inactivation or deletion has been detected in a number of tumors, rendering them resistant to common chemotherapy (60). TRAIL is currently in clinical trials for the treatment of cancer, as this death ligand appears to selectively target tumor cells (61). In particular, TRAIL could prove invaluable for the treatment of chemoresistant tumors, such as those deficient in Apaf-1. In this regard, we have also observed that Jurkat T-cells overexpressing Bcl-2 or Bcl-\(x_l\) are similarly resistant to TRAIL-induced apoptosis, exhibiting only partial caspase-3 processing (data not shown). Thus, mitochondrial release of Smac will prove particularly important for TRAIL-induced apoptosis in tumor cells lacking Apaf-1, but expressing high levels of XIAP (62).

Finally, these studies underscore the complexity of defining type I versus type II cells. Even in the case of Smac, modulation of its release by Bcl-2 or Bcl-\(x_l\) would be relatively unimportant in cells expressing very low levels of IAPs, as there would be no benefit from its release. Thus, the focal point for the proposed Smac-dependent type II pathway rests on IAP-dependent inhibition of caspases. Interestingly, it has been suggested that in contrast to type I cells, type II cells do not activate sufficient caspase-8 at the DISC, necessitating mitochondrial-dependent caspase amplification. However, given that active caspase-3 initiates a feed-forward amplification loop, which results in additional processing of caspase-8, it is plausible that XIAP can rapidly inhibit caspase-3 and prevent processing of caspase-8 (Fig. 4). Thus, the levels of IAPs may indirectly affect the apparent degree to which caspase-8 is processed in death receptor-stimulated cells. Indeed, the relative levels of active caspase-3, IAPs, and Smac likely dictate, in part, the degree to which death ligands induce apoptosis.

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