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Synthetic activation of caspases: Artificial death switches

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ABSTRACT **The development of safe vectors for gene therapy requires fail-safe mechanisms to terminate therapy or remove genetically altered cells. The ideal "suicide switch" would be nonimmunogenic and nontoxic when uninduced and able to trigger cell death independent of tissue type or cell cycle stage. By using chemically induced dimerization, we have developed powerful death switches based on the cysteine proteases, caspase-1 ICE (interleukin-1β converting enzyme) and caspase-3 YAMA. In both cases, aggregation of the target protein is achieved by a nontoxic lipid-permeable dimeric FK506 analog that binds to the attached FK506-binding proteins, FKBPs. We find that intracellular cross-linking of caspase-1 or caspase-3 is sufficient to trigger rapid apoptosis in a Bcl-x_L-independent manner, suggesting that these conditional proapoptotic molecules can bypass intracellular checkpoint genes, such as Bcl-x_L, that limit apoptosis. Because these chimeric molecules are derived from autologous proteins, they should be nonimmunogenic and thus ideal for long-lived gene therapy vectors. These properties should also make chemically induced apoptosis useful for developmental studies, for treating hyperproliferative disorders, and for developing animal models to a wide variety of diseases.**

The routine acceptance of gene therapy as a therapeutic option will require that it is proven to be safe. To guarantee this safety, a mechanism must be used to eliminate transferred gene products or genetically modified cells in the event that they become deleterious to the host. For this reason, "suicide genes," such as herpes simplex virus thymidine kinase (*tk*), are often incorporated into gene therapy vectors (1, 2). The *tk* gene kills proliferating cells by converting the dideoxynucleoside analog ganciclovir into a form that can be incorporated into elongating DNA, leading to chain termination (3). In contrast, regulating apoptosis by controlling the multimerization of Fas with anti-Fas antibodies, Fas ligand, or synthetically with the dimerizing drug FK1012 has been shown to be possible in nonproliferating cells, including $CD4^+$ $CD8^+$ thymocytes (4, 5), differentiated neutrophils and monocytes (6), and hepatocytes (7). Further, unlike the viral *tk* gene, conditional Fas alleles can be made from human proteins, minimizing potential immunogenicity (5, 8).

Fas is a member of the tumor necrosis factor receptor superfamily whose members can induce pleiotropic responses, including proliferation, activation, differentiation, and apoptosis, depending primarily on their cytoplasmic signaling domains (for review, see ref. 9). The molecular details of Fas-mediated apoptosis are rapidly emerging and frequently reviewed (10). The interaction of Fas with the Fas ligand FasL leads to the aggregation of Fas cytoplasmic death domains (DD) and increases the affinity of the Fas DD for the DD of the adapter molecule FADD (Fas-associated with death domain) (MORT1). FADD, in turn, interacts with the cysteine protease caspase-8 [FADD-like interleukin 1β converting enzyme (ICE), FLICE/MACH], via a conserved death effector domains (DED) found in both proteins. Thus, Fas cross-linking leads to caspase-8 cross-linking. Like all caspases, caspase-8 is an aspartic acid-directed protease that is activated by the proteolytic removal of its amino-terminal prodomain and by an additional internal cleavage, producing a fully active molecule composed of two p17 and two p12 subunits. Probably by transproteolysis, the aggregation of caspase-8 contributes to this activation and the initiation of a protease cascade that includes caspase-1 (ICE)-related and caspase-3 (YAMA/CPP32)-related enzymes, ultimately leading to the irreversible cleavage of multiple proapoptotic targets. Mitochondrial-derived factors and proteins, including cytochrome *c* and the protease AIF (apoptosis inducing factor) seem to be essential for the activation of the most downstream members of this protease cascade, including caspases 3, 6, and 7 (for review, see ref. 11). Members of the Bcl-2 family, such as Bax or Bcl-x_L, are primarily localized in the mitochondria and help to modulate the release of these factors that occurs concomitant with an apoptosis-associated increase in the permeability of the mitochondrial membrane (12). Because many of the events in Fas signaling are regulated by protein–protein interactions, Fas signaling intermediates, such as caspases, are ideal candidates for designing conditional alleles based on chemically induced dimerization (CID) (13–15).

In this report we describe conditional alleles of the zymogens caspase-1 and caspase-3. The CID-binding domain (CBD), FKBP12 (where FKBP is FK506-binding protein), has been placed at the amino terminus of these proteins adjacent to the prodomains of the inactive proteases. Upon administration of a lipid-permeable dimerizing drug, aggregation of caspases occurs, leading to autoproteolysis and activation. We show that this chemical activation of either caspase-1 or caspase-3 is sufficient to trigger apoptosis in target cells, and we call this technique chemically induced apoptosis (CIA) and term these molecules artificial death switches (ADS). Although conditional caspase-1 alleles are somewhat "autotoxic," like previously described conditional Fas alleles (5), the conditional caspase-3 alleles are completely nontoxic in the absence of CID, even at high levels of expression. Interestingly, a truncated caspase-3 lacking its prodomain is somewhat autotoxic, consistent with other reports that the prodomains of caspases contribute to maintaining their quiescence in unstimulated cells (16). Further, the conditional caspase-1 allele appears to be completely insensitive to excess Bcl-x_L, whereas the conditional caspase-3 allele can be blocked by an excess of Bcl-x_L levels. Finally, both conditional caspase-1 and caspase-3

Abbreviations: CID, chemically induced dimerization or chemical inducers of dimerization, depending on context; CBD, CID-binding domain; FKBP, FK506-binding protein; CIA, chemically induced apoptosis; ADS, artificial death switch; DD, death domain; DED, death effector domain; E, epitope; SEAP, secreted alkaline phosphatase; FBS, fetal bovine serum; GFP, green fluorescent protein.

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alleles can trigger apoptosis in a broad range of tissues. These results confirm that cross-linking caspases can lead to their activation in intact cells and demonstrate an expanded repertoire of proteins that can be activated by CID.

MATERIALS AND METHODS

Plasmid Construction. To make M-$F_{pk}2$, $F_{pk}3$, and F_v2, F_{pk} [hFKBP12 (P89,K90)] and F_v [hFKBP12(V36)] were amplified by *Pfu*I PCR using primers 5′-GCGACACTCGAGGGAGTGCAGGTGGAAACC-3′ and 5′-CGACAGTCGACTTCCAGTTTTAGAAGC-3′ and the F_{pk} template hFKBP(P89,K90) (17) or the F_v template M46 (where underlined bases are restriction sites). The resulting products (and all other PCR fragments) were blunt-end ligated into *Eco*RV-digested pBluescript (Stratagene) to create pKS/F_{pk} and pKS/F_v and sequenced. The 330-bp *Xho*I–*Sal*I fragments from pKS/F_{pk} and pKS/F_v were ligated in tandem into *Xho*I/*Sal*I-digested MF3E and SF1E (described in ref. 18) to make $F_{pk}3$-E (three copies of F_{pk}), M-$F_{pk}2$-E (two copies F_{pk}), and F_v2-E (two copies F_v). An additional 5′-epitope (E) was added to $F_{pk}3$-E to produce E-$F_{pk}3$-E by cloning hybridized oligonucleotides 5′-TCGACTATCCGTACGACGTCCCAGACTACGCAC--3′ and 5′-TCGAGTGCGTAGTCTGGGACGTCGTACGGATAG-3′ into the 5′ *Xho*I site. M-$F_{pk}2$-Fas was constructed by subcloning the *Xho*I–*Sal*I Fas fragment from pKS/Fas (described in ref. 5) into *Sa*lI-digested M-$F_{pk}2$-E vector. Caspase-1, caspase-3, and Δ20caspase-3 inserts were PCR-amplified from plasmids pCDNA3/hICE/AU1 and pCDNA3/YAMA by using the following primers containing *Xho*I sites (5′) and *Sa*lI sites (3′): HICE5X, 5′-CCGACACTCGAGGCCGACAAGGTCCTGAAGGAG-3′; HICE3S, 5′-CGTAGAGTCGACGTCCTGGGAAGAGGTAGAAAC-3′; YAMA5X, 5′-CCGACACTCGAGGAGAACACTGAAAACTCAGTG-3′; YAMA3S, 5′-CGTAGAGTCGACGTGATAAAAATAGAGTTCTTTTGT-3′; 20Yam5x, 5′-ACACTCGAGATACATGGAAGCGAATCAATGG-3′. PCR products were subcloned into pBluescript to create pKS/ICE, pKS/YAMA, and pKS/Δ20YAMA. *Xho*I–*Sal*I fragments from these plasmids were then ligated into *Sal*I-digested E-$F_{pk}3$-E (abbreviated $F_{pk}3$) and F_v2-E vectors, to produce $F_{pk}3$-casp-1, F_v2-casp-3, $F_{pk}3$-casp-3, and $F_{pk}3$-Δ20casp-3. To make casp-3/S163, the 340-bp *Stu*I–*Sal*I fragment of pKS/YAMA was reamplified by using primers 5′-ATTCAGGCCTCCCGTGGTACCGAACTGGACTGTGGCATTGAG-3′ and YAMA3S, subcloned into pBluescript to make pKS/YAMAS, and sequenced. The mutant *Stu*I–*Sal*I fragment was substituted with the wild-type fragment in pKS/YAMA to make pKS/YAMA/S136 and ultimately $F_{pk}3$-casp-3/S136 and F_v2-casp-3/S136. The SRα-SEAP reporter plasmid was created by cloning the secreted alkaline phosphatase (SEAP) cDNA from NFAT-SX into the polylinker of pBJ5 (13). Bcl-x_L was amplified from a Bcl-x_L cDNA by using primers 5′-CCGACACTCGAGTCTCAGAGCAACCGGGAGCTGG-3′ and 5′-CGTAGAGTCGACTTTCCGACTGAAGAGTGAGCCCA-3′ and subcloned into *Xho*I/*Sal*I-digested F1-E. All plasmids were prepared by two rounds of CsCl centrifugation.

Tissue Culture. Jurkat-TAg cells (19) were grown in RPMI 1640 medium, 10% fetal bovine serum (FBS), 10 mM Hepes (pH 7.4), penicillin (100 units/ml), and streptomycin (100 μg/ml). HeLa and 293 cells were grown in DMEM containing 10% FBS and antibiotics.

SEAP Assays. Jurkat-TAg cells (10^7) in logarithmic-phase growth were electroporated (950 μF, 250 V; Gene Pulser II) with the expression plasmid and 1–2 μg of SRα-SEAP. After 24 h, transformed cells were stimulated with CID or anti-Fas antibody (CH.11, Kamiya Biomedical, Thousand Oaks, CA). After an additional 20 h, supernatants were assayed for SEAP activity as described (15). Units of SEAP activity are reported directly and as a percentage of activity relative to no stimulation within the same transfections (percent relative activity).

Western Blot Analysis. Jurkat-TAg cells were electroporated with 2 μg of plasmid, cultured 36 h, and stimulated with drug for the indicated time period. Approximately 5×10^5 cells were lysed in 100 μl of RIPA buffer (0.01 M Tris·HCl, pH 8.0/140 mM NaCl/1% Triton X-100/1 mM phenylmethylsulfonyl fluoride/1% sodium deoxycholate/0.1% SDS) on ice for 30 min. Cell debris were pelleted and supernatants were boiled in 1:1 Laemmli sample buffer (with 5% 2-mercaptoethanol) for 5 min. Equal volumes of extracts were separated by SDS/PAGE on a 15% gel. Membranes were incubated with anti-HA epitope monoclonal antibody HA.11 (Babco, Richmond, CA) followed by polyclonal horseradish peroxidase-conjugated goat anti-mouse antibody (Bio-Rad). Bands were detected with SuperSignal chemiluminescent substrate (Pierce).

Lipofection and Cell Selection. Jurkat-TAg cells (4×10^6 cells) were transfected with 12 μl of DMRIE-C reagent (GIBCO/BRL), 2 μg of green fluorescent protein (GFP) expression vector pEGFP (CLONTECH), 2 μg of the pMACS-H2-K^k vector (Miltenyi Biotec, Sunnyvale, CA), and 2–4 μg of CID-responsive plasmids in Opti-MEM-I reduced serum medium. Transfected cells were purified to approximately 60% by MACSelect magnetic bead selection on MiniMACS separation columns (Miltenyi) as assessed by flow cytometry analysis (described below). Selected cells were resuspended in 2 ml of Jurkat-TAg medium and split into two aliquots, and one aliquot was treated with drug.

Flow Cytometry. Cells were washed with PBS/1% FBS, resuspended in staining buffer [PBS/1% FBS/propidium iodide (0.5 μg/ml)] and analyzed within 1 h. Two-color flow cytometry of propidium iodide (band pass = 620 nm) and GFP (band pass = 525 nm), was performed with a Coulter Epics XL MCL cytometer. Greater than 20,000 events were counted per sample. Gates were set by using mock-transfected cells, unstained cells transfected with GFP, and stained nontransfected cells. Data are reported as the percent of propidium iodide-negative/GFP-positive cells after drug addition relative to untreated cells in triplicate cultures.

FuGENE6 Transfection and Luciferase Assays. HeLa and 293 cells were plated at 2×10^5 cells per 35-mm dish 18 h before transfection. Plated cells were transfected with 2 μg of constitutive luciferase reporter pGL2-Control (Promega), 2 μg of test plasmid, and 6 μl of FuGENE6 (Boehringer–Mannheim) in Opti-MEM-I medium. Jurkat-TAg cells (4×10^6 cells) were transfected as described above with 2 μg of pGL2-Control, 2 μg of test plasmid, and 12 μl of DMRIE-C in Opti-MEM-I medium. After 24 h, cells were split into two groups, and one group was treated with 500 nM AP1903 (F_v constructs) or FK1012 (F_{pk}). After an additional 24 h, cells were lysed in 100 μl of reporter lysis buffer (Promega) with three freeze/thaw cycles, and 10 μl of the supernatants (or constant dilutions) was assayed with 90 μl of Luciferase assay substrate (Promega) and a Turner TD-20e luminometer. Data are reported as the percent of luciferase activity after drug addition relative to luciferase activity without drug in duplicate cultures. All transfections were performed at least three times and averaged.

RESULTS

Strategy. The strategy used in this study to develop sensitive conditional cytotoxic molecules relies on CID (15–17) and the discovery that full-length caspase zymogens can undergo intermolecular processing to become fully active (for review, see ref. 20). A chemical inducer of dimerization (CID) is defined as a dimer of the ligand for a CBD. Responsiveness to a CID is achieved by fusing a CBD to target proteins. In this manner, CID administration leads to protein cross-linking. The CIDs

used in this study include the previously described nonimmunosuppressive FK506 dimer FK1012 (13) and a novel fully synthetic FK1012 analog, AP1903, that is analogous to FK1012 acetylated at the C_9 position of the FK506 moieties. This modification of FK506 prevents binding of AP1903 to the highly expressed endogenous FKBPs (K_m for FKBP12 ~ 250 nM) but leads to subnanamolar affinity to a mutant FKBP12 (F36V, abbreviated "F_v") (M. Gilman, personal communication). The valine substitution of F_v creates a deeper drug-binding pocket that accommodates the acetyl groups of AP1903.

The proapoptotic conditional alleles were designed based on currently accepted models of Fas signaling (Fig. 1*A*). In the design of conditional Fas (Fig. 1*B*), FKBP12 (or mutants) replaces the extracellular domain of Fas, and the myristoylation-targeting domain of c-Src (residues 1–14) directs membrane localization (5). For conditional caspases (Fig. 1*C*), CBDs are attached to their prodomains. After CID, prodomains should be cleaved and the resulting fully active proteases should be indistinguishable from wild-type proteins. All constructs described follow the "cassette" cloning strategy outlined in Fig. 1*D*.

Cross-Linking Caspase-1 Triggers Apoptosis in Mammalian Cells. Two critical factors that are required for a broadly applicable ADS are high sensitivity to drug and low basal toxicity. In previously reported experiments, it was demonstrated that a Fas-based ADS could trigger apoptosis in mammalian cells after FK1012 administration (5). However, overexpression of this Fas allele was somewhat autotoxic, consistent with reports that the cytoplasmic domain (DD) of Fas can spontaneously multimerize when expressed at high levels (21). Nevertheless, relatively high expression of chimeric M-$FKBP_2$-Fas was needed to kill cells efficiently probably because FK1012 binds equally well to endogenous and ectopic FKBPs. In addition, Fas signaling does not always lead to apoptosis due to intracellular checkpoint genes such as Bcl-2 and Bcl-x_L (22, 23), regulation of caspase-8 activation (24), or other mechanisms (25). Therefore, we reasoned that downstream effectors of apoptosis, such as caspase-1 or caspase-3, may be superior to Fas by bypassing most checkpoints.

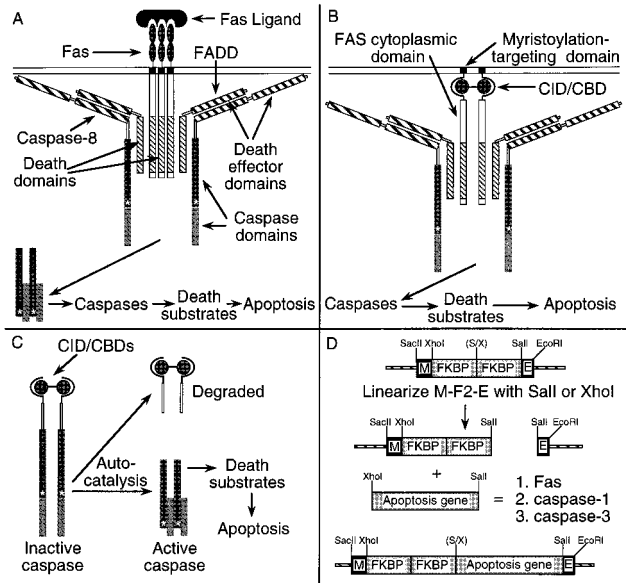

FIG. 1. Design of conditional alleles of Fas and caspases. (*A*) Model of Fas ligand-mediated Fas signaling. Fas cross-linking causes association with FADD, triggering caspase-8 activation, and signaling cascades that lead to apoptosis. (*B* and *C*) In each example, administration of a CID leads to the cross-linking of chimeric proteins by their CBDs. Asterisk, active site QACRG. (*B*) Conditional Fas receptor showing Fas-associated proteins, FADD, and caspase-8. (*C*) Conditional caspases illustrating CID-mediated removal of the prodomain and conversion to fully processed form. (*D*) Schematic of CID-regulated proapoptotic molecules. Signaling molecules can be cloned 5′ or 3′ of FKBPs. (S/X), *Sal*I/*Xho*I composite site, destroying both; M, myristoylation targeting peptide; E, hemagglutinin epitope.

To test this idea, Jurkat-TAg cells were cotransfected with reporter plasmid SRα-SEAP, constitutively expressing secreted alkaline phosphatase, along with caspase-1 fused to three tandem FKBP12s, F_{pk}3-casp-1, or control tandem FKBPs, F_{pk}3 (Fig. 2). Twenty-four hours after transfection, cells received half-logarithmic dilutions of CID for an additional 20 h. Apoptosis of cells is indicated by a net reduction in reporter activity. The FKBP12 variant F_{pk} (P89,K90) binds FK1012 as well as wild-type FKBP12 but the two amino acid changes prevent CID-independent interactions with cellular proteins, such as calcineurin (17). This eliminates a mild toxicity associated with overexpressing wild-type FKBP12. Similar to M-$FKBP_3$-Fas, in the presence of FK1012, there is a dramatic reduction of reporter activity in cells expressing F_{pk}3-casp-1 that is not present in cells expressing control F_{pk}3 (Fig. 2*B*). Also like conditional Fas, caspase-1 is autotoxic, as indicated by the CID-independent reduction in reporter activity (Fig. 2*A*). However, this autotoxicity can be greatly reduced with only a marginal decrease in efficacy by transfecting less caspase-1 plasmid (Fig. 2).

Conditional Caspase-3 Displays Extremely Low Basal Activity yet Triggers Apoptosis Efficiently in the Presence of CIDs. In contrast to caspase-1 and Fas, conditional caspase-3 alleles are not apparently autotoxic (Fig. 3 *A–C*). In the absence of the CID AP1903, reporter activity is the same in cells transfected with F_v2-casp-3, control F_v2 (data not shown), or control F_v2-casp-3/S163, which is inactive due to the substitution of serine for cysteine within the conserved active-site QACRG motif (Fig. 3 *A* and *B*) (20). However, in the presence of AP1903 (EC_{50} ~ 1 nM), cross-linking caspase-3 was sufficient to trigger a dramatic reduction in reporter activity of ~65%, comparable to Fas or caspase-1 signaling (Fig. 3*B*). Enzymatically inactive caspase-3, F_v2-casp-3/S163, could not reduce reporter activity even in the presence of 100 nM AP1903. The absence of toxicity of F_v2-casp-3 (and F_{pk}3-casp-3, Fig. 3*C*) suggests that caspase-3 is less likely than caspase-1 to be spontaneously activated in Jurkat cells. The increased specificity of AP1903 for F_v is reflected by an increased drug efficacy (Fig. 3, compare *A* and *C*).

To test whether the prodomain of caspase-3 contributes to low basal activity, we removed 20 residues from the amino terminus of caspase-3, containing most of the prodomain. Interestingly, removal of the prodomain makes caspase-3 somewhat autotoxic (Fig. 3*C*), although CID induces a more

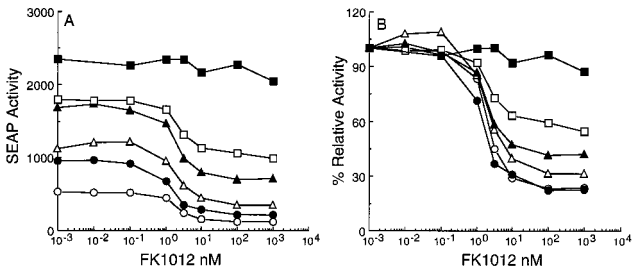

FIG. 2. Cross-linking caspase-1 triggers apoptosis in mammalian cells. Jurkat-TAg cells were transiently transfected with 4 μg of F_{pk}3-casp-1 (○), 2 μg of F_{pk}3-casp-1 (●), 1 μg of F_{pk}3-casp-1 (△), 0.5 μg of F_{pk}3-casp-1 (▲), 0.25 μg of F_{pk}3-casp-1 (□), or 4 μg of F_{pk}3 (■). After 20 h, transfected cells were treated with FK1012. After an additional 24 h, SEAP activity was assayed and reported directly (*A*) or as a percentage of activity from untreated cells in identical aliquots from the same transfections (*B*). Data are representative of three experiments performed in duplicate.

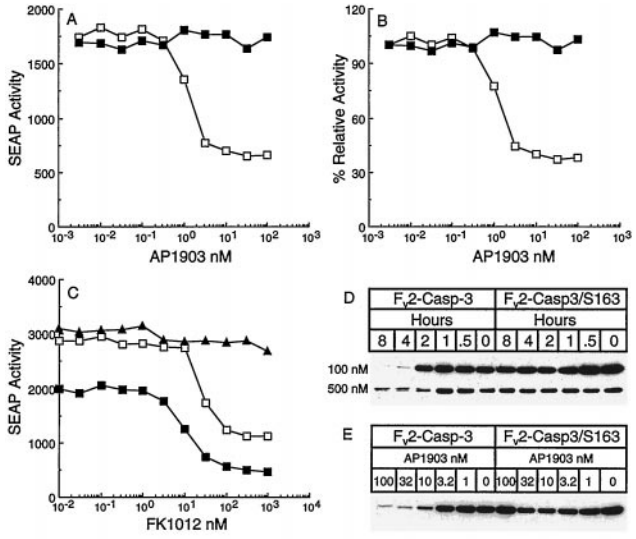

FIG. 3. Conditional caspase-3 triggers apoptosis in mammalian cells. Jurkat-TAg cells were transiently transfected as follows: (*A* and *B*) F_v2-casp-3 (2 μg; □) or F_v2-casp-3/S163 (2 μg; ■). (*C*) F_{pk}3-casp-3/S163 (2 μg; ▲), F_{pk}3-casp-3 (2 μg; □), or F_{pk}3-Δ20casp-3 (2 μg; ■). After 20 h, transfected cells were treated and analyzed as before. Data are representative of at least three experiments performed in duplicate. (*D*) Anti-hemagglutinin epitope immunoblot of extracts from Jurkat TAg cells transiently transfected with 2 μg of F_v2-casp-3 or F_v2-casp-3/S163 and treated with 100 or 500 nM AP1903 as indicated. (*E*) Anti-hemagglutinin epitope immunoblot of extracts from similarly transfected Jurkat-TAg cells treated for 8 h with half-logarithmic dilutions of AP1903 as indicated.

complete diminution of reporter. This is consistent with previous reports that caspases are regulated by proteins interacting with their inhibitory prodomains (for review, see ref. 11).

Administration of CID Causes Rapid Processing of F_v2-Caspase-3. Processing of caspase-3, and other caspases, ordinarily involves two or more cleavages after aspartic acid residues. To confirm that the CID-mediated diminution of reporter activity was caused by activation of the conditional caspases, we directly examined the processing and degradation of conditional caspase-3. In Fig. 3*D*, Jurkat cells were transfected with F_v2-casp-3 plasmid or control F_v2-casp-3/S163. After 36 h, aliquots of cells were treated with 100 nM or 500 nM AP1903 for various times. After these incubations, cell extracts were analyzed by Western blotting using monoclonal antibodies to an epitope tag (hemagglutinin) placed at both ends of the construct. When 500 nM AP1903 was administered, degradation of full-length caspase-3 was complete within 2 h, but no decrease in control caspase-3/S163 was detected. When cells were treated with 100 nM AP1903, full degradation of F_v2-casp-3 took 4 h. In Fig. 3*E*, aliquots of these cells were treated for 8 h with half-logarithmic dilutions of AP1903. As little as 10 nM AP1903 was sufficient to cause the processing and degradation of the majority of F_v2-casp-3. Curiously, we did not see the breakdown products of caspase-3 activation even though epitope tags were present at both ends of the protein. This was probably due to the inherent instability of the caspase-3 fragments and the fact that cells expressing fully processed caspase-3 are apoptotic. Similar results were seen with F_{pk}3-casp-1 (data not shown) although a reduced amount of chimeric protein was seen, presumably due to autoprocessing of caspase-1. Therefore, homomultimerization of the caspases, caspase-1 and caspase-3, is sufficient for their activation, which may reflect physiological activation mechanisms.

Conditional Caspase-1 and Caspase-3 Trigger Apoptosis in the Presence of Excess Bcl-x_L. To determine whether conditional caspases can bypass inhibition by the checkpoint gene Bcl-x_L, we coexpressed conditional caspase-1 and caspase-3 alleles along with an excess of Bcl-x_L. Although ectopically expressed Bcl-x_L inhibits Fas-mediated apoptosis by ~50% in Jurkat cells (Fig. 4*C,* lanes 2–5), it consistently had no inhibitory effect on F_{pk}3-casp-1-mediated apoptosis (Fig. 4 *A* and *E*), even when a large molar excess of Bcl-x_L was expressed (Fig. 4*A*). However, Bcl-x_L is able to block caspase-3 mediated signaling at a similar 4-fold molar excess (Fig. 4*B*). Likewise, the presence of F_v2-casp 3 is able to reduce the inhibition of Fas signaling by Bcl-x_L by about 50% (Fig. 4*D,* lanes 2–4). Control F_v2-casp-3/S163 does not attenuate the protein function of Bcl-x_L, suggesting that a functional protease domain is necessary for the partial neutralization of Bcl-x_L (compare Fig. 4*D*, lanes 2–5). These results are also consistent with a reported direct or indirect interaction between caspase-3 and Bcl-x_L,

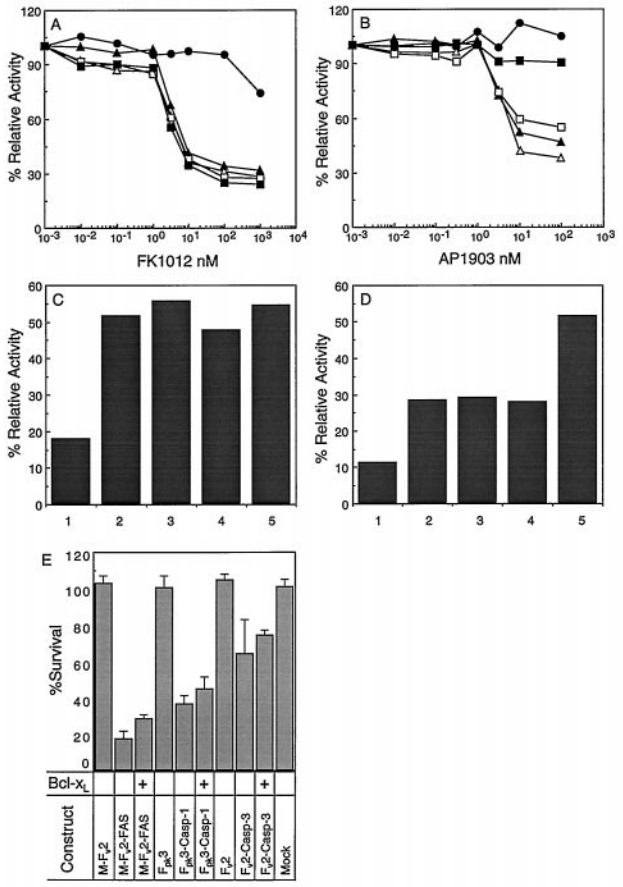

FIG. 4. Conditional caspase-1 and caspase-3 can bypass Bcl-x_L. Jurkat-TAg cells were transiently transfected with the following plasmids: (*A*) F_{pk}3-casp-1 (△; *C*, bar 1), F_{pk}3-casp-1 + 1 μg of Bcl-x_L (▲; *C*, bar 2), F_{pk}3-casp-1 + 2 μg of Bcl-x_L (□; *C*, bar 3), F_{pk}3-casp-1 + 4 μg Bcl-x_L (■; *C*, bar 4), or F_{pk}3 + 4 μg Bcl-x_L (●; *C*, bar 5). (*B*) F_v2-casp-3 (△; *D*, bar 1), F_v2-casp-3 + 1 μg of Bcl-x_L (▲; *D*, bar 2), F_v2-casp-3 + 2 μg of Bcl-x_L (□; *D*, bar 3), F_v2-casp-3 + 4 μg of Bcl-x_L (■; *D*, bar 4), or F_v2-casp-3/S163 + 4 μg of Bcl-x_L (●; *D*, bar 5). Cells were transfected and assayed as above. Data are representative of at least three experiments. (*C* and *D*) Aliquots of cells from *A* and *B* were treated with anti-Fas antibody CH-11 and assayed as above. Bars 1–5 are described in *A* and *B*. Data are given relative to untreated cells from the same transfection. (*E*) Jurkat-TAg cells were transiently transfected with a constitutive GFP reporter and the indicated plasmids. Transfected cells were enriched to greater than 60% and split into two equal cultures, one of which was treated with 500 nM AP1903 (F_v chimeras) or FK1012 (F_{pk} chimeras). After 24 h, cells were stained with propidium iodide and analyzed by flow cytometry to determine the percentage of viable GFP-positive/propidium iodide-negative cells. The percent survival indicated is the percentage of viable cells after treatment with drug relative to the untreated aliquots.

constituting an antiapoptotic control mechanism (for reviews, see refs. 13 and 26).

Direct Demonstration of Apoptosis in Jurkat Cells. Because the above experiments are based on an indirect reporter assays for apoptosis and cannot definitively rule out that conditional molecules are reducing transcription, translation or protein stability of reporter, we reexamined the efficacy of CIA more directly. To enrich for ADS-expressing Jurkat cells, cells receiving conditional alleles were cotransfected with a selectable surface marker, consisting of the extracellular domain of the murine major histocompatibility complex molecule K^k and GFP to mark transfected cells. Twenty-four hours later, cells were sorted by using magnetic bead-conjugated K^k antibodies, leading to an enrichment of 60% GFP^+ cells from ~15% unsorted. Sorted cells were split into two groups, one was treated with 500 nM FK1012 (or AP1903), and the other was untreated. Also, in separate transfections, a 1:1 ratio of Bcl-x_L-containing plasmid to ADS-containing plasmid was added. Cells were analyzed by flow cytometry to determine the percentage of GFP^+ cells that survived after treatment with drug. Although Bcl-x_L could inhibit endogenous Fas signaling by ~50% (Fig. 4*C*), there was very little Bcl-x_L effect on CID-mediated apoptosis by conditional caspase-1 or caspase-3 (Fig. 4*E*). This demonstrates that conditional caspases can bypass at least some intracellular checkpoints, such as Bcl-x_L, and should, therefore, have broader usefulness than a Fas-based ADS.

Conditional Fas, Caspase-1, and Caspase-3 Trigger Apoptosis in a Panel of Cell Lines. To determine whether the conditional caspase-1 and caspase-3 alleles functioned in a range of cells, we reexamined CID-mediated apoptosis in the adenocarcinoma line HeLa and in the kidney epithelial line 293 (Fig. 5). Although conditional caspase-1 and caspase-3 functioned in every cell tested, conditional Fas did not trigger apoptosis in 293 cells. This mirrors previous studies that demonstrate that endogenous Fas cannot trigger apoptosis in every cell (4, 27, 28). In contrast the caspase-based ADS may lead to a more universally applicable death switch.

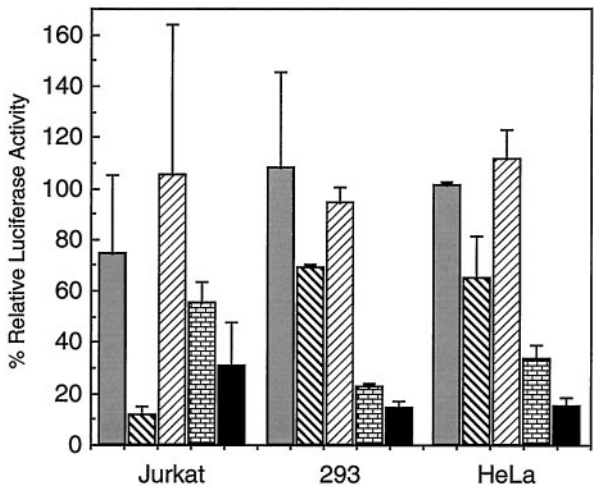

FIG. 5. Sensitivity of multiple cell lines to conditional Fas, caspase-1, and caspase-3. Jurkat-Tag, 293, and HeLa cells were transiently transfected with a constitutively expressing luciferase reporter plasmid and control vector MF_v2 (shaded bars), MF_v2-Fas plasmid (wide cross-hatched bars), control vector $F_{pk}3$ (narrow cross-hatched bars), $F_{pk}3$-casp-1 (bricked bars), or F_v2-casp-3 (solid bars). After 24 h, transfected cells were split into duplicate cultures and 500 nM drug (AP1903 for F_v or FK1012 for F_{pk}) was added to one culture for an additional 24 h. The percent relative reporter activity is the percent of luciferase activity after drug addition relative to untreated cells. Error bars represent the SD of the mean activity of three transfections.

DISCUSSION

By using CID technology, we have developed novel proapoptotic molecules based on caspase-1 and caspase-3 and have demonstrated their wide applicability as conditional cytotoxic reagents. As opposed to other cytotoxic molecules (e.g., ricin A chain or diphtheria toxin), these proteins have minimal, or no, toxicity when expressed at functional levels in target cells. Their toxicity can be "switched on" or regulated by the administration of a lipid-permeable drug; we, therefore, term these new inducible proteins ADSs and call the approach CIA.

The development of an FK1012-sensitive conditional Fas receptor, M-$FKBP_2$-Fas, was previously reported that was effective in ablating immature thymocytes (i.e., $CD4^+$ $CD8^+$) *in vivo* and a number of other tissues *ex vivo* (5, 8, 27, 29). The limitation of using the Fas receptor as a death switch is, however, that the sensitivity of target tissue to Fas signaling may vary depending on the developmental stage of the cell. The underlying causes of this resistance are not entirely clear but may include the regulation of caspase-8 activation through dominant negative caspase-8 homologs (24), inhibitors of caspase activation (25), the overexpression of checkpoint genes such as Bcl-2 and Bcl-x_L (30), and the underexpression of Fas signaling intermediates. Further, M-$FKBP_2$-Fas is only useful when expressed within a narrow range due to autotoxicity.

Caspase activation is a common integration point for diverse apoptotic stimuli and is, therefore, a more logical control point for CIA. Cytotoxic T lymphocytes contain two mechanisms to activate caspases (20). (*i*) Fas ligand expression on cytotoxic T lymphocytes initiates Fas signaling and (*ii*) proteases within cytolytic granules, such as granzyme B, can directly activate caspase-3 and related caspases (for review, see ref. 20). Other DD-containing receptors, such as tumor necrosis factor receptor I and DR4 can also signal caspase activation (31). Finally, DNA damage can lead to caspase activation by triggering the release of cytochrome *c* from the mitochondrial membrane. In each case of caspase activation, however, it is not always clear whether activation is via a homo- or heterotypic caspase or an intra- or intermolecular interaction. Overexpression of caspases in bacteria or other nonmammalian cells has led to the demonstration that caspases can activate themselves if overexpressed, presumably by interchain homotypic proteolysis (for review, see ref. 20). Also, it is assumed that Fas-mediated cross-linking leads to caspase-8 autoproteolysis (32, 33). However, it is less clear that caspase-3 can normally undergo autoactivation and may be more likely to be activated by "upstream" proteases (34). Although we now demonstrate that caspase-3 autoactivation is possible, this provocative result is not necessarily physiologically relevant because CID generates extremely high local concentrations of protein that can overcome low specificity for a target substrate. Nonetheless, these reagents should be useful to study subsequent signaling events.

Recent data suggesting that caspase prodomains are important for regulating caspase activity is also supported by our data (for reviews, see refs. 11 and 20). For example, truncation of the prodomain of caspase-7 leads to its autoactivation (16). Similarly, we find that truncation of the prodomain of caspase-3 leads to its autoactivation (Fig. 3*C*). The autotoxicity of the DED of FADD may provide additional insight into how these prodomains work (35). Because the DED of FADD interacts with the prodomain of caspase-8, it is possible that FADD displaces an otherwise autoinhibitory prodomain to induce caspase-8 activation. Alternatively, FADD helps to align FLICE for proteolysis. Interestingly, the DED shares weak homology with the *Caenorhabditis elegans* protein CED-4 that binds to the prodomain of CED-3 (a caspase-3 homolog in nematodes) (36), activating CED-3 processing in an ATP-dependent fashion (22). A good candidate for the human homolog of CED-4, Apaf-1, has recently been identified that

binds to the CARD domain within the prodomain of caspase 9 contributing to its activation (23, 26). Active caspase 9, in turn, presumably acts on caspase-3. Thus, it appears likely that caspases are regulated by proteins interacting with their inhibitory prodomains. Curiously, wild-type caspase-1 and F_{pk}3-casp-1, containing intact prodomains, are autotoxic. In these cases, overexpression of caspase-1 may overwhelm physiological control mechanisms by bringing these proteins together at supraphysiological levels, similar to the mechanism of CID.

Because Bcl-x_L has been shown to coimmunoprecipitate with caspase-1, and caspase-1 has been placed upstream of cytochrome *c* release in most models (23, 37, 38), we were surprised that Bcl-x_L had no effect on F_{pk}3-casp-1 activation. Again, potential interactions between F_{pk}3-casp-1 and Bcl-x_L may be sterically hindered by the presence of amino-terminal FKBPs. Alternatively, CID-mediated activation of caspase-1 may bypass fine-tuned intracellular checkpoints, like Bcl-x_L. We were similarly surprised that caspase-3 ADSs were somewhat sensitive to Bcl-x_L, because caspase-3 does not presumably directly interact with Bcl-x_L but acts presumably downstream (37). In a reciprocal fashion, F_v2-casp-3 attenuated the protective effect of Bcl-x_L on Fas-mediated apoptosis by ~50%. Because control F_v2-casp-3/S163 did not reduce Bcl-x_L function, the caspase-3 prodomain is not sufficient for this activity (Fig. 4). Thus, these results may reflect the fact that caspases, particularly when overexpressed, can act at multiple places in apoptotic cascades.

As gene therapy comes of age and vectors move from the laboratory to the clinic, the need for safety is becoming a serious consideration. We propose that caspase-based ADSs may lead to clinically suitable suicide switches for these vectors for the following reasons: (*i*) They can be made exclusively from syngeneic proteins, reducing the likelihood of triggering an immune response. (*ii*) They are effective in a wide variety of cells, are not restricted to dividing cells, and are not significantly blocked by intracellular checkpoint genes such as Bcl-x_L. (*iii*) CIA works with a panel of distinct dimerizing agents that are not currently used for any other purpose and will, therefore, be useful for regulating viability in multiple independent target tissues (5, 8). Finally, CIA may be useful for developmental studies or for treating hyperproliferative disorders such as cancer.

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