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Improved Artificial Death Switches Based on Caspases and FADD

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ABSTRACT

A number of "suicide genes" have been developed as safety switches for gene therapy vectors or as potential inducible cytotoxic agents for hyperproliferative disorders, such as cancer or restenosis. However, most of these approaches have relied on foreign proteins, such as HSV thymidine kinase, that primarily target rapidly dividing cells. In contrast, novel artificial death switches based on chemical inducers of dimerization (CIDs) and endogenous proapoptotic molecules function efficiently in both dividing and nondividing cells. In this approach, lipid-permeable, nontoxic CIDs are used to conditionally cross-link target proteins that are fused to CID-binding domains (CBDs), thus activating signaling cascades leading to apoptosis. In previous reports, CID-regulated Fas and caspases 1, 3, 8, and 9 were described. Since the maximum efficacy of these artificial death switches requires low basal and high specific activity, we have optimized these death switches for three parameters: (1) extent of oligomerization, (2) spacing between CBDs and target proteins, and (3) intracellular localization. We describe improved conditional Fas and caspase 1, 3, 8, and 9 alleles that function at subnanomolar levels of the CID AP1903 to trigger apoptosis. Further, we demonstrate for the first time that oligomerization of the death effector domain of the Fas-associated protein, FADD, is sufficient to trigger apoptosis, suggesting that the primary function of FADD, like that of Apaf-1, is oligomerization of associated caspases. Finally, we demonstrate that nuclear-targeted caspases 1, 3, and 8 can trigger apoptosis efficiently, implying that the cleavage of nuclear targets is sufficient for apoptosis.

OVERVIEW SUMMARY

Ligand-based suicide gene therapies have the potential to confer regulated viability on a target cell population. We have developed an improved panel of suicide genes based on the Fas signaling pathway that includes inducible alleles of Fas, FADD, and caspases 1, 3, 8, and 9. In this approach, lipid-permeable dimeric ligands, called chemical inducers of dimerization (CIDs), are used to conditionally cross-link, and therefore activate, proteins that are fused to CID-binding domains. We demonstrate here improved artificial death switches that function at lower CID concentrations than previously reported and include the first description of inducible FADD. These CID-based alleles should satisfy many, if not all, of the criteria for a widely applicable suicide gene. They function in dividing and nondividing cells, are based on endogenous genes bestowing reduced immunogenicity, act with fast kinetics, and have low basal activity. These conditional cytotoxic signaling molecules should have broad biomedical applications.

INTRODUCTION

THE SAFE DEPLOYMENT of gene therapy will require a mechanism to terminate treatment if the therapy becomes deleterious to the host. For example, insertional mutagenesis by gene therapy vectors could lead to the activation of oncogenes. Moreover, cell-based therapies, such as allogeneic bone marrow transplants to treat leukemia, will require a mechanism to reduce or eliminate the therapeutic cells if donor T lymphocytes cross-react too vigorously with healthy host tissue and initiate graft-versus-host disease (Bordignon *et al*., 1995). Hence, there has been an escalation in the number of characterized drug-activated "suicide genes."

The most highly characterized suicide gene is the herpes simplex virus thymidine kinase gene, which activates the dideoxynucleoside prodrug ganciclovir, leading to DNA chain termination and cell death (Field *et al.*, 1983). Other well-characterized suicide genes that convert relatively nontoxic agents to highly cytotoxic agents include the *Escherichia coli* proteins cytosine deaminase, which converts 5-fluorocytosine into 5-flu-

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orouracil (Mullen *et al.,* 1992); purine nucleoside phosphorylase, which converts 6-mythylpurine-deoxyriboside into 6-methylpurine (Sorscher *et al.,* 1994); and nitroreductase, which converts monofunctional alkylating agents into difunctional alkylating agents (Bridgewater *et al.,* 1995). Since each of these gene–prodrug combinations targets DNA or RNA synthesis, their efficacy in killing nonreplicating cells is reduced. Furthermore, for long-term gene therapy applications, suicide genes would need to be based on endogenous, and therefore less immunogenic, proteins.

Conditional alleles based on endogenous Fas signaling intermediates have been used to regulate cellular apoptosis (Belshaw *et al.,* 1996; Spencer *et al.,* 1996; MacCorkle *et al.,* 1998; Muzio *et al.,* 1998; Yang *et al.,* 1998). These novel alleles have been designed to respond to nontoxic, lipid-permeable ligands, called chemical inducers of dimerization (CIDs) (Spencer *et al.,* 1993). In this approach, target proteins are fused to one or more CID-binding domains (CBDs) so that they are noncovalently cross-linked after interaction with CIDs (Spencer *et al.,* 1993). In those studies the CID FK1012, and its cognate binding protein FKBP12 (where FKBP is FK506-binding protein), or more specific variants of the two, were used (Spencer *et al.,* 1993; Clackson *et al.,* 1998).

The details of Fas signaling are unfolding rapidly and have been frequently reviewed (Adams and Cory, 1998; Ashkenazi and Dixit, 1998; Green and Reed, 1998; Thornberry and Lazebnik, 1998). Fas signaling commences with Fas ligand (FasL)-mediated cross-linking of the Fas receptor, aggregation of Fas cytoplasmic domains, termed "death domains" (DDs), and the creation of high-affinity binding sites for the DD-containing adapter protein FADD (Chinnaiyan *et al.,* 1995). FADD, in turn, interacts via its amino-terminal "death effector domain" (DED) with a homologous DED within the prodomain of the aspartic acid-directed cysteine protease (caspase), caspase 8/FLICE (Muzio *et al.,* 1996). Thus, Fas cross-linking is linked to caspase 8 cross-linking and processing to its fully active form, likely via cross-proteolysis. Since caspases cleave substrates after aspartic acid and are themselves activated by aspartic acid-directed cleavages, family members, such as caspase 8, are able to undergo autoproteolysis and trigger the induction of a caspase cascade that includes downstream "effector" caspases, such as caspase 3/YAMA (Stennicke *et al.,* 1998). In addition, caspase 8 can activate proapoptotic Bcl-2 family members, such as Bid (Li *et al.,* 1998) or Bax (Gross *et al.,* 1998), leading to mitochondrial targeting, the release of cytochrome *c* and apoptosis-inducing factor (AIF), and the loss of inner membrane potential (Ψ_m). In turn, cytochrome *c* helps displace the inhibitory WD40 domains of the adapter molecule Apaf-1, permitting Apaf-1 multimerization, and subsequent oligomerization and autoprocessing of associated procaspases 2, 8, and 9 (Srinivasula *et al.,* 1998a; Yoshida *et al.,* 1998). Finally, caspase 9 can also process effector caspases 3 and 7 that proteolyze multiple cytoplasmic and nuclear proteins, leading to apoptosis. To balance proapoptotic signals, multiple antiapoptosis, "gatekeeper" proteins, such as c-FLIP (Irmler *et al.,* 1997), Bcl-2 family members, and inhibitors of apoptosis, called IAPs (Deveraux *et al.*, 1998), serve to mitigate the effects of Fas signaling by preventing the activation of caspases. On the basis of this detailed knowledge of Fas signaling, multiple Fas signaling molecules have been converted to CID-inducible artificial death switches (ADSs), including the Fas receptor (Belshaw *et al.,* 1996; Spencer *et al.,* 1996), caspase 8 (Muzio *et al.,* 1998; Yang *et al.,* 1998). caspase 1/ICE (MacCorkle *et al.,* 1998; Yang *et al.,* 1998), caspase 3 (MacCorkle *et al.,* 1998), caspase 9, Apaf-1 (Hu *et al.,* 1998), and Bax (Gross *et al.,* 1998).

Improving the specific activity of these molecules should allow a lower level of expression to maintain CID-dependent efficacy while minimizing CID-independent basal toxicity. We have now investigated a number of critical parameters that could effect CID-dependent activation, such as protein interdomain spacing, degree of aggregation, and intracellular localization. We report here that dimerization of caspase 3 is sufficient for maximum activation, and plasma membrane targeting can increase CID sensitivity while concomitantly raising basal activity. We also report significantly improved caspase 1, 8, and 9-based ADSs that are responsive to the highly selective FK1012 analog AP1903 (Clackson *et al.,* 1998) at subnanomolar levels. Further, we show that dimerization of the DED of FADD is sufficient to trigger apoptosis with reduced basal toxicity relative to Fas and caspases 1, 8, and 9 and with greater AP1903 sensitivity than caspase 3. Finally, we demonstrate that the activation of nuclear-localized caspases 1, 3, or 8 is sufficient to trigger apoptosis. Thus, combined with other reports, virtually all Fas signaling intermediates can be regulated by CIDs. The exquisitely sensitive AP1903-responsive Fas signaling intermediates described here should be able to function as suicide switches not only for gene therapy vectors but also for a variety of animal models based on temporally regulated and tissue-specific cell ablation.

MATERIALS AND METHODS

Plasmid construction

All constructs were assembled from *Pfu*I-amplified fragments typically flanked by a 5′ *Xho*I site and a 3′ *Sal*I site. Polymerase chain reaction (PCR) products were initially subcloned into pCR-Blunt (InVitrogen, Carlsbad, CA) and sequenced. All expression plasmids were prepared by two-spin CsCl centrifugation and checked for expression by Western blot. Parent expression plasmid pSH1/S-F_{pk}3-E, containing three tandem copies of ~330-bp hFKBP12`$_{P89,\ K90}$ and a 3′ hemagglutinin epitope (E) cloned into expression vector pSH1 (a high copy version of pBJ5), was described previously (MacCorkle *et al*., 1998). Inserts S-F_v1-E, S-F_v2-E, and S-F_v3-E were made by substituting F_{pk}3 with one to three tandem F_vs hFKBP12$_{V36}$ previously described (Clackson *et al*., 1998; MacCorkle *et al*., 1998). In S-F_{vls}1-E and S-F_{vls}2-E, F_{pk}3 is replaced with one or two copies of "short" linkered F_v ("F_{vls}"). F_{vls} was amplified from F_v using primers hFK5X (5′-gcgacactcgag ggagtgcaggtggaaa cc-3′) and hFKL3S1 (5′-acagtcgac tccggatc-caccgccagattccagt tttagaagctccac-3′). In S-F_v1-F_{vls}-E, F_{vls} is subcloned into the 3′ *Sal*I site of S-F_v1-E. To make N2-F_v2-E (and other variations), oligonucleotides 5′-tcgac cctaagaagaa-gagaaaggta *c*-3′ and 5′-tcgag tacctttctcttcttcttagg g-3′, containing the nuclear localization sequence PKKKRKV from Simian virus 40 (SV40) large T antigen (Boulikas, 1993), were annealed and subcloned in tandem into the 5′ *Xho*I site of S-F_v2-E. To make Mas70$_{34}$-F_v2-E, the mitochondria-targeting se-

quence from pMas70 (residues 1–34) (Hase *et al*., 1984) was PCR amplified from M-Raf (Wang *et al*., 1996) using primers 5SCMAS70 (5′cgacaccgcggccac c atgaagagcttcattaca aggaac-3′) and 3XMAS70P (5′-acactcgagttgttgc aattggttgtaataatag tag-gcaccgatggc-3 ′). The resulting ~120-bp *SacI*I–*Xho*I fragment (Mas70$_{34}$ was subcloned into *SacI*I–*Xho*I-digested S-F$_v$2-E. M-F$_v$2-E and M-F$_v$2-FAS-E were described previously (MacCorkle et al., 1998).

Human caspase 1 (Casp1), 3 (Casp3), 8 (Casp8), and 9 (Casp9) inserts were PCR amplified from plasmids pcDNA3/hICE-AUI, pcDNA3/YAMA, pcDNA3/FLICE, and pcDNA3/Casp9, respectively, using primers hICE5X (5′-ccga-cactcgag gccgacaaggtcctgaa ggag-3′) and hICE3ST (5′-agagtc-gac ttaatgtcctgggaaga ggtagaaac-3′), primers YAMA5X (5′-ccgacactcgag gagaacactgaaaact cagtg-3′) and YAMA3S (5′-cg-tagagtcgac gtgataaaaatagagtt cttttgt-3′, primers FLICE5S (5′-agagtcgac atggacttcagcagaaa tctttatg-3′) and FLICE3S (5′cgta-gagtcgac atcagaagggaagaca agttttttc-3′), and primers 5Casp9S (5′-gatagtcgac atggacgaagcggatc ggcggc-3′) and 3Casp9S (5′ctatgtcgac tgatgttttaaagaaaag ttttttccgg-3′). Resulting *Xho*I–*Sal*I (caspases 1 and 3) or *Sal*I (caspases 8 and 9) fragments were subcloned into the *Sal*I sites of the appropriate vectors. Human FADD$_{125}$ and FADD$_{100}$ were PCR amplified from plasmid pcDNA3/AU1-FADD, using 5′ primer FADD5X (5′-ccgacactcgag gacccgttcctggtgc tgc-3′) and 3′ primer FAD-ΔDD3X (5′-ccgacactcgagcttg gtgtctgagactttgag c-3′ or FAD-ΔC3X (5′acactcgag tgctgcacacaggtctt cttccc-3′, respectively. FADD$_{80}$ is from the ,240-bp *Xho*I–*Sal*I fragment of FADΔ125. Human $_{25}$FADD$_{125}$ was amplified using primers Δ25Fad5x (5′-acactcgag ctatgcctcgggcgcg tgggc-3′) and FADD5X. To make S-F$_{pk}$3-FADD$_{125}$V82, residues 81 to 125 of FADD$_{125}$ were reamplified using primers 5SFADV82 (5′-cgcgtcgac gacgtc-gaggcgggggcggcg g-3′) and FADΔDD3X. The resulting ~140-bp *Sal*I–*Xho*I fragment was then subcloned into the *Sal*I site of pSH1/S-F$_{pk}$3-FADD$_{80}$-E. Reporter plasmid SRα-SEAP was described previously (MacCorkle et al., 1998). Cloning sites are underlined and codons are separated.

Tissue culture

Jurkat-TAg cells were grown in RPMI 1640 medium, 10% fetal bovine serum (FBS), 10 m*M* HEPES (pH 7.4), penicillin (100 units/ml), and streptomycin (100 μg/ml). HeLa, A375, HT1080, B16, 293, and PC3 cells were grown in Dulbecco's modified Eagle's medium, 10% FBS, and penicillin–streptomycin.

Secreted alkaline phosphatase assays

Jurkat-TAg cells (10^7) in log-phase growth were electroporated (950 μF, 250 V) with expression plasmid and 2 μg of SRα-SEAP (SEAP, secreted alkaline phosphatase). Other cell lines (~2×10^5 cells per 35-mm dish) in log phase were transfected with 6 μl of FuGENE-6 (Beoehringer Mannheim, Indianapolis, IN) in Opti-MEM I (GIBCO-BRL, Gaithersburg, MD). After 24 hr, transformed cells were stimulated with CID. After an additional 20 hours, supernatants were assayed for SEAP activity as described previously (Spencer *et al.,* 1993). Units of SEAP activity are reported directly and as a percentage of activity relative to no stimulation ("percent relative SEAP activity"). All experiments were repeated at least three times and representative experiments performed with duplicate samples are shown.

Western blot analysis

Approximately 10^6 Jurkat-TAg cells were lysed in 20 μl of radioimmuno precipitation assay (RIPA) buffer (0.01 M Tris-HCl [pH 8.0], 140 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1:100 protease inhibitor cocktail [Sigma, St. Louis, MO] on ice for 20 min. Cell debris was pelleted and supernatants were boiled in 1:1 sample buffer (5% 2-mercaptoethanol in Bio-Rad [Hercules, CA] Laemmli buffer) for 3–5 min. Alternatively, cells were lysed directly in 2× Laemmli buffer to detect nuclear proteins. Equal volumes of extracts were separated on a 15% SDS-polyacrylamide gel. Membranes were blotted with anti-HA antibody HA.11 (BAbCO, Richmond, CA) and then with polyclonal horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody. Bands were detected with SuperSignal chemiluminescent substrate (Pierce, Rockford, IL).

Immunofluorescent staining protocol

HeLa cells were plated at 2×10^5 cells per 10-cm dish the night before transfection. Plated cells were incubated with 2 μg of indicated expression plasmids, containing various HA-tagged fusion proteins, resuspended in 3 μl of FuGENE-6 (Boehringer Mannheim) in OPTI-MEM I. On day 2, transfected cells were transferred to staining slides (10^4 cells per spot) and incubated overnight at 37°C. Adhered cells were fixed in 4% paraformaldehyde (10 min), permeabilized in −20°C methanol (2 min), rinsed three times in phosphate-buffered saline (PBS), and incubated for 1 hr at room temperature with HA.11 diluted 100× in PBS–3% serum. After three PBS rinses, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse polyclonal Ig (PharMingen, San Diego, CA) in PBS–3% serum for 45 min in the dark at room temperature. After three 10-min PBS rinses, cells were treated with Vectashield anti-bleach mounting medium (Vector Laboratories, Burlingame, CA) and stored in darkness at 4°C until analysis with a Multiprobe 2001 confocal system using Image Space software (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Dimerization is sufficient for caspase 3 activation

After administration of CIDs, caspases containing amino-terminal CBDs should be cross-linked, leading to intermolecular processing in some cases (Fig. 1A). Since prodomains with attached CBDs are removed, CID-activated proteins should be indistinguishable from physiologically activated caspases.

To determine the number of FKBPs that are needed for optimal CID-mediated caspase activation, we attached zero, one, two, or three copies of FKBP12$_{V36}$ (abbreviated "F$_v$") to the amino terminus of caspase 3 (Fig. 1B). Since AP1903 binds with high affinity to F$_v$ (K_d ~0.1 n*M*) (Clackson *et al.,* 1998) but with low affinity to wild-type FKBP12 (K_d = 67 n*M*), high specificity for F$_V$ is achieved. Individual constructs were transiently transfected into Jurkat-TAg cells along with the reporter plasmid SRα-SEAP, containing secreted alkaline phosphatase

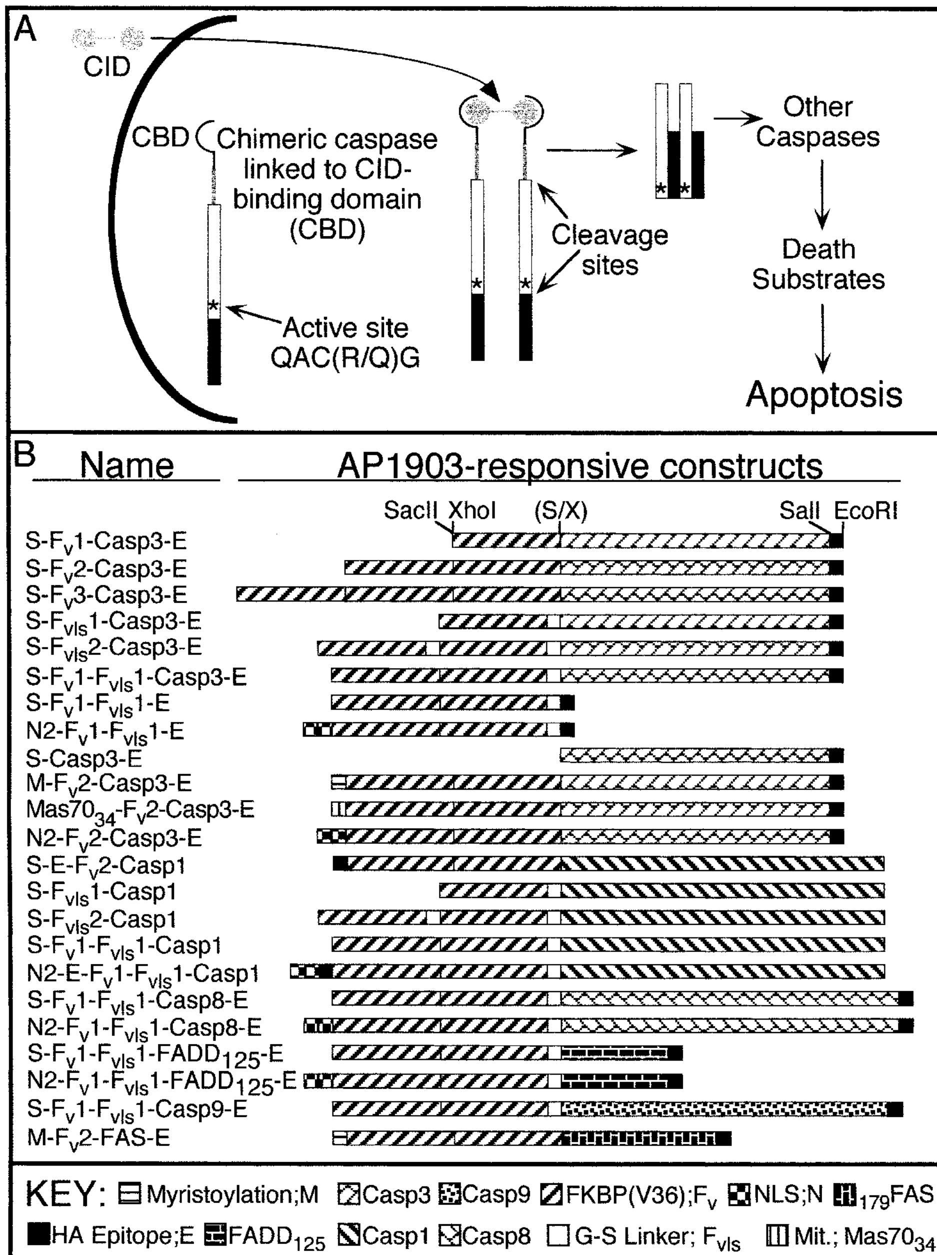

FIG. 1. Design of conditional Fas signaling intermediates. **(A)** Model of CID-regulated caspases. Transmembrane diffusion of CIDs (e.g., FK1012, AP1903) leads to the cross-linking of intracellular procaspases that are genetically fused to one or more CID-binding domains (e.g., FKBP12), leading to transproteolysis and processing to their fully active forms. The caspase active site consensus sequence, QAC(R/Q)G, is shown. **(B)** Schematic of CID-regulated proapoptotic molecules showing the CID-binding domain (i.e., F_v = FKBP12$_{V36}$), intracellular targeting sequences (i.e., M [myristoylation-targeting sequence], N [nuclear localization sequence], and Mas70$_{34}$ [mitochondria-targeting sequence]), proapoptotic molecules (i.e., caspase 1, 3, 8, and 9; Fas cytoplasmic domain [residues 179–319]; FADD$_{125}$ [death effector domain], and hemagglutinin epitope tag (E).

(SEAP) under the transcriptional control of the constitutively active promoter SRα. Twenty-four hours later, cell aliquots were treated with increasing amounts of AP1903. After an additional 20 hr, cell supernatants were assayed for reporter activity. Although all constructs were expressed at comparable levels (Fig. 2A), constructs containing either one or two F_vs were equally sensitive to AP1903 as reflected by the dramatic decrease in SEAP activity (IC$_{50}$ ~3 n*M*), whereas constructs containing three F_vs were much less sensitive (IC$_{50}$ ~150 n*M*) to AP1903. S-F_v3-Casp3 is, however, still sensitive to the larger (i.e., MW ~1800 Da) CID FK1012 (IC$_{50}$ ~20 n*M*; data not shown). Reductions in reporter activity by this assay faithfully reflect apoptosis as determined by flow cytometry (MacCorkle *et al.,* 1998; and data not shown). These results demonstrate that dimerization of caspase 3 is sufficient for its activation while excess cross-linking by the relatively small CID AP1903

(MW ~1200 Da), may "lock" caspases into inactivatable conformations.

Caspase 3 activation is not sterically hindered by amino-terminal FKBP12

Two possible, nonexclusive models can account for CID-mediated caspase activation: (1) CIDs increase the proximity of procaspases, increasing the likelihood that transproteolysis will occur and (2) CIDs actively maintain the correct orientation for caspase processing. If model 1 is correct, then molecules should be relatively insensitive to orientation and spacing between FKBPs and caspases. If model 2 is correct, then the converse is true. To test these two possibilities, we engineered a small, six-amino acid (G-S-G-G-G-S) linker/spacer between F_v and caspase 3, permitting increased flexibility (Fig. 1B). Again, constructs were transiently transfected into Jurkat-TAg cells and treated after 24 hr with AP1903. We observed no significant difference in the dose response to AP1903 between constructs with or without the small linker, regardless of whether one or two F_vs were fused to caspase 3 (compare S-F_v1-Casp3 with S-F_{vls}1-Casp3 and S-F_v2-Casp3 with S-F_v1-F_{vls}1-Casp3; Fig. 2B). In contrast, construct S-F_{vls}2-Casp3, containing two linkered FKBPs ("F_{vls}"), was less sensitive to AP1903, presumably

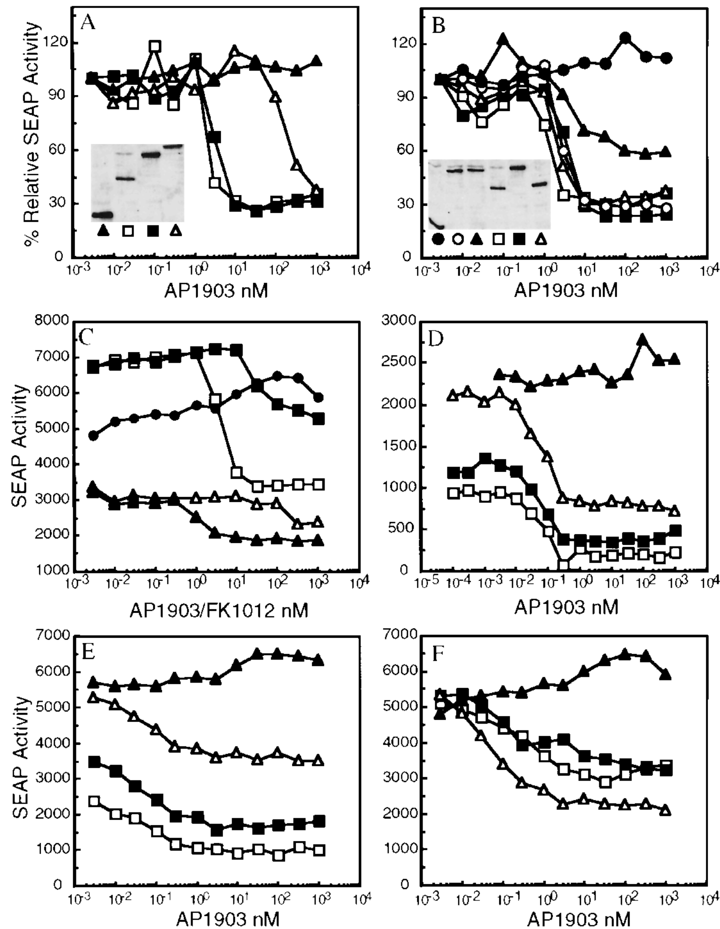

FIG. 2. Activation of caspase 1 and 8, but not 3, by high-specificity CID AP1903 requires a flexible linker between FKBP12 and caspase domains. **(A–F)** Jurkat-TAg cells were transiently transfected with 2 μg of reporter plasmid SRα-SEAP, along with the indicated amount of expression plasmid pSH1, containing F_v/caspase fusion proteins or control F_vs. After 24 hr cells were treated with half-log dilutions of AP1903 (or FK1012) and incubated for an additional 20 hr before extracts were assayed for SEAP activity. **(A)** Dimerization is sufficient for caspase 3 activation. Cells received 4 μg of S-Casp3 (▲), S-Fv1-Casp3 (□), S-F_v2-Casp3 (■), or S-F_v3-Casp3 (△). **(B)** Caspase 3 activation is not sterically hindered by amino-terminal FKBP12. Cells received 4 μg of S-F_v1-F_{vls}1 (●), S-F_v1-F_{vls}1-Casp3 (○), S-F_{vls}2-Casp3 (▲), S-F_v1-Casp3 (□), S-F_v2-Casp3 (■), or S-F_{vls}1-Casp3 (△). **(C)** Caspase 1 activation is sterically hindered by amino-terminal FKBP12. Cells received 4 μg of S-F_v2-Casp3 (+AP1903, □), (+FK1012, ■), S-F_v2-Casp1 (+AP1903, △), +FK1012, (▲), or S-F_v1-F_{vls}1 (+AP1903, ●), **(D)** A flexible linker confers AP1903 sensitivity to caspase 1. Cells received 2 μg (□), 1 μg (■), or 0.5 μg (△) of S-F_v1-F_{vls}1-Casp1, or 4 μg of control plasmid S-F_v1-F_{vls} (▲), **(E)** A flexible linker confers AP1903 sensitivity to caspase 8. Cells received 4 μg (□), 2 μg (■), or 1 μg (△) of S-F_v1-F_{vls}1-Casp8, or 4 μg of control plasmid S-F_v1-F_{vls}1 (▲). **(F)** A single short G-S linker augments the AP1903 sensitivity of caspase 1. Cells received 1 μg of S-F_v1-F_{vls}1-Casp-1 (△), S-F_{vls}1-Casp1 (□), or S-F_{vls}2-Casp1 (■), or 4 μg of S-F_v1-F_{vls}1 (▲). *Insets* (**A** and **B**): Equal aliquots of cell extracts were analyzed by Western blot with MAb to the HA epitope ("E" in Fig. 1B).

because the increased rotational freedom of this construct reduces the probability that the correct conformation for cleavage occurs. These results support the model that the orientation by which caspases are brought together is important for their activation, and that AP1903 fortuitously cross-links S-F_v1-Casp3 in an appropriate orientation that is not improved, and may be decreased, by increasing the flexibility and rotational freedom of the cross-linked molecules.

The activation of caspases 1 and 8 by AP1903 is sterically hindered by amino-terminal FKBP12

While F_v2-Casp3 does not require a flexible linker for efficient activation by AP1903, caspases 1 and 8 fused to two F_vs, S-F_v2-Casp1 and S-F_v2-Casp8, cannot be activated efficiently by AP1903 (IC_{50} caspase 1 ~200 n*M*; Fig. 2C and data not shown). However, the larger CID, FK1012, can activate S-F_v2-Casp1 (IC_{50} ~1 n*M*), despite the lower affinity (by ~10-fold) of FK1012 for F_v versus AP1903 and the fact that FK1012 does not discriminate against endogenous FKBPs. In contrast, the advantages of AP1903 versus FK1012 are readily apparent on the activation of S-F_v2-Casp3. These results imply that either AP1903 brings S-F_v2-Casp1 and F_v2-Casp8 into unfavorable orientations for processing or that steric hindrance prevents the efficient cross-linking of these FKBP–caspase chimeras.

Therefore, to increase AP1903 sensitivity, we fused caspases 1 and 8 to the linkered FKBP, F_{vls}, as described above. As hypothesized, the use of a flexible linker in S-F_v1-F_{vls}1-Casp1 (Fig. 2D) and in S-F_v1-F_{vls}1-Casp8 (Fig. 2E) led to alleles of caspases 1 and 8 that were exquisitely sensitive to AP1903 (IC_{50} caspase 1 ≤100 p*M*; IC_{50} caspase 8 ~100 p*M*). Again, providing too much flexibility, as in S-F_{vls}2-Casp1, reduced the responsiveness to AP1903 (Fig. 2F), and adding the longer linker, (G-G-S-G-G-G-S-G-G-G) almost completely abrogated

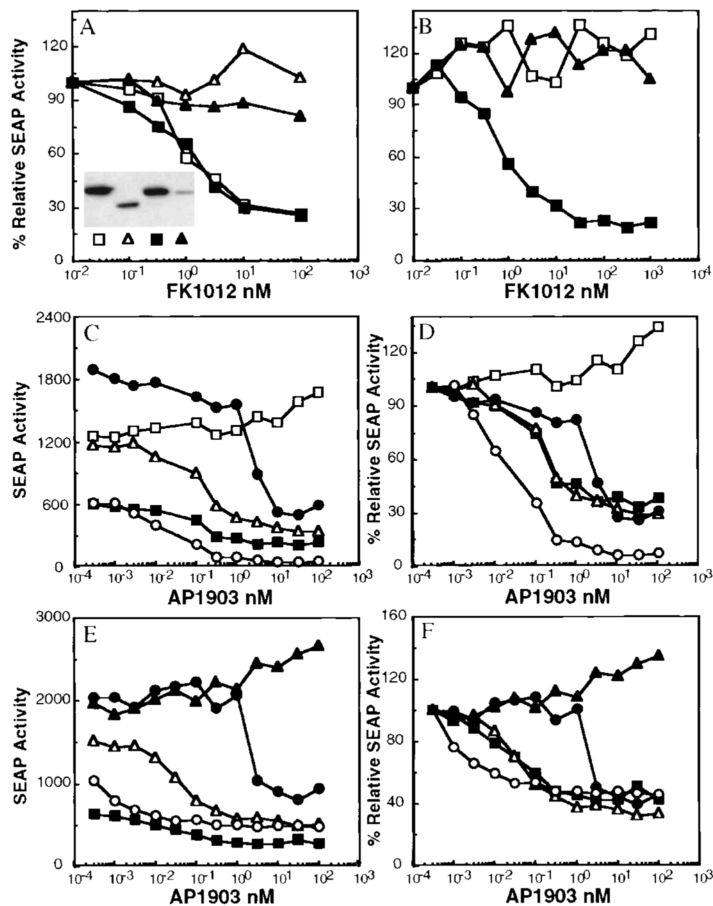

FIG. 3. Cross-linking the death effector domain of FADD is sufficient for triggering apoptosis with reduced basal toxicity relative to Fas and caspases 1, 8, and 9. **(A–F)** As described in Fig. 2, Jurkat-TAg cells were transfected with 2 μg of SRα-SEAP plus the indicated expression plasmids. **(A)** $FADD_{100}$ is sufficient for FK1012-mediated cytotoxicity. Cells received 4 μg of S-F_{pk}3-$FADD_{125}$ (□), S-F_{pk}3-$FADD_{80}$ (△), S-F_{pk}3-$FADD_{100}$ (■), or S-F_{pk}3-Δ25$FADD_{125}$ (▲). *Inset:* Equal aliquots of cell extracts were analyzed by Western blot as described in Fig. 2. **(B)** Cells received 4 μg of S-F_{pk}3 (□), S-F_{pk}3-$FADD_{125}$V82 (▲), or S-F_{pk}3-$FADD_{125}$ (■). **(C)** Fas and caspase 1 have high basal activity relative to $FADD_{125}$ and caspase 3. **(D)** Caspase 1 is more AP1903 sensitive than Fas, FADD, or caspase 3. **(C and D)** Cells received 2 μg of S-F_v1-F_{vls}1 (□), S-F_v2-Fas (■), S-F_v1-F_{vls}1-$FADD_{125}$ (△), S-F_v1-F_{vls}1-Casp1 (○), or S-F_v1-F_{vls}1-Casp3 (●). **(E)** Caspases 1, 8, and 9 have higher basal activity relative to caspase 3. **(F)** Caspases 1, 8, and 9 are more AP1903 sensitive than caspase 3. **(E and F)** Cells received 2 μg of S-F_v1-F_{vls}1-Casp1(CTS) (▲), S-F_v1-F_{vls}1-Casp1 (△), S-F_v1-F_{vls}1-Casp3 (●), S-F_v1-F_{vls}1-Casp8 (■), or S-F_v1-F_{vls}1-Casp9 (○).

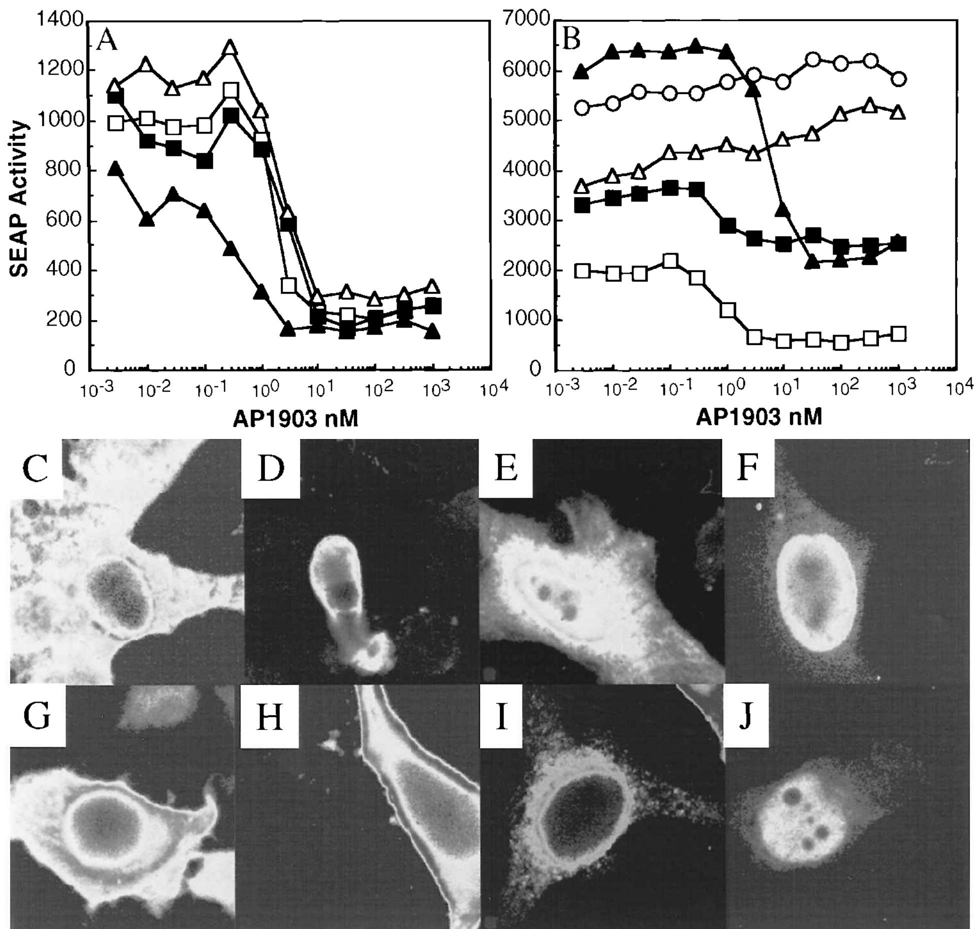

FIG. 4. Plasma membrane targeting of caspase 3 increases its AP1903 sensitivity and basal activity. **(A and B)** Transient transfection assay was performed as described in Fig. 2. **(A)** Cells received 2 μg of SRα-SEAP plus 4 μg of N2-F_v2-Casp3 (□), Mas70_{34}-F_v2-Casp3 (■), or S-F_v2-Casp 3 (△), or 1 μg of M-F_v2-Casp3 (▲). **(B)** Cells received 2 μg of SRα-SEAP plus 4 μg (□), 1μg (■), or 0.25 μg (△) of M-F_v2-Casp3, or 4 μg of S-F_v2-Casp3 (▲), or 4 μg of control plasmid S-F_v1-F_{vls}1 (○). **(C–J)** Localization of caspase 3 to different intracellular membranes. HeLa cells transiently transfected with cytoplasmic S-F_v2-Casp3-E **(C),** plasma membrane-localized M-F_v2-Casp3-E **(D),** mitochondria-localized Mas70_{34}-F_v2-Casp3-E **(E),** or nuclear N2-F_v2-Casp3-E **(F)** were fixed, stained with anti-HA antibodies, and examined by confocal microscopy. Alternatively, control proteins were localized, including S-F_v2-E **(G),** M-F_v2-E **(H),** Bcl-x_L-E **(I),** or Gal4-VP16-E **(J).** In each case, cells shown are representative of several transfected cells.

AP1903 responsiveness (data not shown). In each case, however, the basal drug-independent cytotoxicites of linkered caspase 1 and 8 constructs were the same as those of unlinkered constructs, implying that amino-terminal F_vs do not sterically hinder stochastic caspase interactions. Nevertheless, since S-F_v1-F_{vls}1-Casp1 and -Casp8 are highly sensitive to AP1903, reducing protein expression to levels that are still sensitive to AP1903 (Fig. 2D and E) should largely eliminate these basal toxicities.

Cross-linking the death effector domain of FADD is sufficient for triggering apoptosis with reduced basal toxicity

Since caspases such as caspases 1 and 8 can have high basal activity when overexpressed, and because Fas is autotoxic owing to the tendency of death domains to self-associate (Boldin *et al.,* 1995), we investigated whether CID-mediated cross-linking of the adapter molecule FADD could trigger apoptosis with lower basal activity. Therefore, we fused the amino terminus of FADD ($FADD_{125}$; residues 1–125), containing the DED, to a trimer of $FKBP12_{P89,\ K90}$ to obtain S-F_{pk}3-$FADD_{125}$. As described above, we cotransfected reporter plasmid into Jurkat-TAg cells along with S-F_{pk}3-$FADD_{125}$ or variants, including S-F_{pk}3-Δ25$FADD_{125}$ (residues 26–125), S-F_{pk}3-$FADD_{100}$ (residues 1–100), or S-F_{pk}3-$FADD_{80}$ (residues 1–80). While cross-linking $FADD_{125}$ and $FADD_{100}$ led to FK1012-dependent diminution of reporter activity, further truncation of FADD, as in Δ25$FADD_{125}$ and $FADD_{80}$, eliminated FK1012-dependent toxicity (Fig. 3A). The lower stability of these truncated proteins may contribute only partially to this lack of activity (Fig. 3A, inset) since S-F_{pk}3-$FADD_{125}$ and -$FADD_{100}$ still function better than S-F_{pk}3-$FADD_{80}$ even after normalizing transfections for steady state protein levels (data not shown). Control point mutant S-F_{pk}3-$FADD_{125}$V82 was also unable to trigger apop-

tosis after dimerization (Fig. 3B). Thus, cross-linking the DED of FADD is sufficient to trigger the Fas pathway.

To quantitate the differences in specific and basal activities between AP1903-inducible versions of caspases 1, 3, 8, and 9 (see Fig. 1), FADD, and Fas (M-F_v2-Fas; not shown), equivalent amounts of each expression vector were transfected into Jurkat cells. Twenty hours after AP1903 administration, SEAP activity was determined as described above (Fig. 3C) and results were further normalized to drug-free control wells (Fig. 3D). Averaging slight experimental variation between transfections, CID-independent basal toxicities had the following rankings: Fas, caspase 8, and caspase 9 > caspase 1 > $FADD_{125}$ > caspase 3. Sensitivity to AP1903 follows a somewhat different order than basal toxicity: caspase 9 (IC_{50} ~10 p*M* > caspase 1 (IC_{50} ~50 p*M*) > Fas (not shown), FADD, caspase 8 (IC_{50} ~200 p*M*) > caspase 3 (IC_{50} ~2 n*M*). Thus, caspases 1 and 9 are likely to be the most effective ADS for most applications owing to their exquisite sensitivity, while caspase 3 may be more appropriate when long-term expression is required, owing to its low basal activity. In contrast to caspases 1, 8, and 9, however, caspase 3 requires higher expression levels for CID-inducible toxicity.

Plasma membrane targeting of caspase 3 increases its CID sensitivity and basal activity

Since FKBP–caspase 3 chimeras display low basal activity, we considered the possibility that intracellular localization of caspase 3 might increase CID sensitivity without a commensurate increase in basal toxicity. Therefore, F_v2-Casp3 was fused to a myristoylation-targeting sequence (M) as in M-F_v2-Casp3, a mitochondrial-targeting sequence as in $Mas70_{34}$-F_v2-Casp3, or a nuclear-localization sequence as in N2-F_v2-Casp3. Once again, the various constructs were transfected into Jurkat cells and assayed for inducible apoptosis. Surprisingly, only the plasma membrane-localized chimeric caspase 3 was significantly more sensitive to AP1903 than the nonlocalized construct, S-F_v2-Casp3 (IC_{50} ~300 p*M* versus ~3 n*M*; Fig. 4A). Moreover, M-F_v2-Casp3 was significantly more autotoxic than the other caspase 3 constructs even when fourfold less plasmid was transfected (Fig. 4A). While reducing the expression levels of M-F_v2-Casp3 reduces basal activity, AP1903 sensitivity disappears before basal activity does, rendering low, completely nontoxic levels insufficient for triggering apoptosis (Fig. 4B). Further, we observed that plasma membrane-localized FADD and caspase 8 were even more autotoxic than membrane-localized caspase 3, consistent with our observations that nontargeted versions of FADD and caspase 8 have higher basal activity than caspase 3 chimeras.

To ensure that the (M, $Mas70_{34}$, and N2) targeting sequences used in this study conferred predicted localization to heterologous proteins, immunofluorescence experiments were performed with cells transiently transfected with the differentially targeted, HA-tagged caspase 3 alleles or with epitope (E)-tagged control proteins. Since intracytoplasmic staining in Jurkat cells is difficult to visualize owing to a low cytoplasm-to-nucleus ratio, caspase 3-sensitive HeLa cells were used (MacCorkle *et al.,* 1998). As expected, nontargeted S-F_v2-Casp3-E is distributed throughout the cytoplasm (Fig. 4C); however, staining is somewhat punctate, suggesting possible intracellular membrane interactions. Plasma membrane-targeted M-F_v2-Casp3-E stains at the plasma membrane; however, trans-

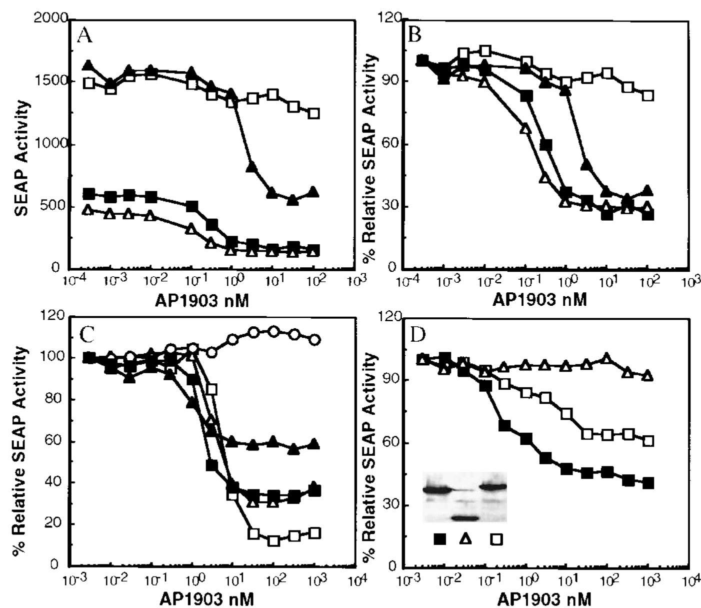

FIG. 5. Nuclear-targeted caspases 1, 3, and 8 trigger apoptosis. **(A and B)** Transient transfection assay was performed as described in Fig. 2. Cells received 2 μg of SRα-SEAP plus 2 μg of nuclear-targeted caspases, including N2-F_v1-F_{vls}1-Casp1 (△), N2-F_v1-F_{vls}1-Casp3 (▲), N2-F_v1-F_{vls}1-Casp8 (■), or control construct N2-F_v1-F_{vls}1 (□). **(C)** Nuclear-targeted caspase 3 functions efficiently. Cells received SRα-SEAP plus 4 μg (□) or 1 μg (△) of S-F_v2-Casp3, 4 μg (■) or 1 μg (▲) N2-$F_2$2-Casp, or 1 μg of S-F_v1-F_{vls}1 (○). **(D)** Nuclear-targeted $FADD_{125}$ has reduced activity. Cells received reporter plasmid plus 2 μg of S-F_v1-F_{vls}1-$FADD_{125}$ (■), N2-F_v1-F_{vls}1-$FADD_{125}$ (□), or N2-F_v1-F_{vls}1 (△). Inset: Equal aliquots of cell extracts were analyzed by Western blot as described in Fig. 2.

fected cells are primarily shrunken and apoptotic, reflecting the high basal toxicity of this construct (Fig. 4D). Mitochrondria-targeted Mas70$_{34}$-F$_v$2-Casp3-E stains in a perinuclear punctate pattern consistent with mitochondria staining (Fig. 4E), and nuclear-targeted N2-F$_v$2-Casp3-E stains in the nucleus (Fig. 4F). Control constructs S-F$_v$2-E (Fig. 4G), M-F$_v$2-E (Fig. 4H), Bcl-x$_L$-E (Fig. 4I), and Gal4-VP16-E (Fig. 4J) all localized to their predicted intracellular locations.

Nuclear-targeted caspases trigger apoptosis

Since multiple caspase targets, such as poly(ADP-ribose) polymerase (PARP), lamin A and B, DNA-dependent protein kinase catalytic subunit (DNA-PK$_{cs}$), histone H1, MDM2, and topoisomerases, are localized in the nucleus, it is not surprising that nuclear activation of caspase 3 can trigger apoptosis. To test whether caspase 3 is unique in this ability, we also targeted caspases 1 and 8 to the nucleus and triggered their activation with AP1903. Interestingly, all three caspases were fully functional in the nucleus and had basal activities and AP1903 sensitivities similar to their cytoplasmic activities (Fig. 5A and B). Although immunofluorescence studies suggested that nuclear targeting is efficient, we cannot rule out that a small amount of cytoplasmic protein is responsible for this cytotoxicity. To minimize this possibility we titrated both cytoplasmic and nuclear F$_v$2-Casp3 and compared their AP1903 sensitivities. Consistent with localization studies and Fig. 4A, N2-F$_v$2-Casp3 can trigger apoptosis with a similar AP1903 dose response relative to S-F$_v$2-Casp3, even at low levels (Fig. 5C). Nuclear N2-F$_v$-F$_{vls}$-FADD$_{125}$, however, was unable to activate apoptosis as efficiently as cytoplasmic S-F$_v$-F$_{vls}$-FADD$_{125}$, likely reflecting the fact that the interaction with cytoplasmic caspase 8 does not normally occur in the nucleus (Fig. 5D). Thus, cleavage of nuclear substrates by caspases in intact cells is sufficient to trigger apoptosis.

CID-responsive caspase 1 triggers apoptosis in a broad panel of cells

Since inhibitor of apoptosis (IAP) family proteins can directly inhibit caspase activation and other antiapoptosis proteins may prevent apoptosis after caspase activation, we investigated whether CID-sensitive caspase 1 could trigger apoptosis in non-T cell lineages. Initially, this panel included human and murine melanomas A375 and B16, respectively, and human fibrosarcoma HT1080, cervical carcinoma HeLa, kidney embryonic carcinoma 293, and prostate adenocarcinoma PC3. In each case, cells were transiently transfected with HA-tagged S-E-F$_v$-F$_{vls}$-Casp1 or control plasmid S-E-F$_v$-F$_{vls}$-Casp1(CTS), containing a cysteine-to-serine point mutation in the active site (C285). After administration of 100 n*M* AP1903, there was a dramatic diminution of reporter activity in cells expressing wild-type caspase 1 but not in the control cells expressing mutant caspase 1$_{S285}$ (Fig. 6). Interestingly, even in the absence of AP1903, there is at least partial processing of caspase 1, probably reflecting cleavage between the large and small subunits. This breakdown product is not detectable in cells expressing mutant caspase 1 (Fig. 6, inset). Similar results were obtained with conditional caspase 3, but typically 2- to 10-fold higher levels of caspase 3 needed to be expressed to achieve equivalent effects on reporter activity (data not shown). Finally, using adenovirus vectors expressing CID-sensitive caspase 1, we can achieve up to 100% killing of transduced cells after CID administration (data not shown).

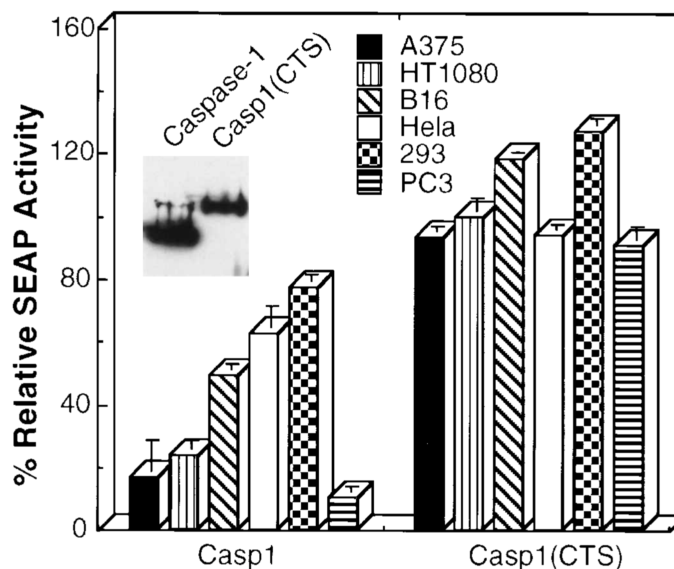

FIG. 6 Sensitivity of multiple cell lineages to conditional caspase 1. The cells indicated were transiently transfected along with SRα-SEAP. After 24 hr, transfected cells were split into duplicate cultures and 100 n*M* AP1903 was added to one culture for an additional 24 hr. The percent relative reporter activity is determined as described in text. Error bars represent the SD of the mean activity of three transfections assayed in duplicate.

DISCUSSION

By using the high-specificity CID, AP1903, and its cognate binding domain F_v, we have developed a panel of highly sensitive artificial death switches (ADSs) based on Fas and the Fas signaling intermediates FADD and caspases 1, 3, 8, and 9. In independent studies, FK1012/AP1510-activated Apaf-1 and Bax have also been described (Gross *et al.,* 1998, Hu *et al.,* 1998). Since different tissues or tumor lines are likely to be inhibited by one or more anti-apoptosis "gatekeeper" proteins, such as c-FLIP, Bcl-x_L, or IAPs, a broad repertoire of conditional proapoptotic proteins is likely to be useful to find the ADS(s) that work best for every cell type. In fact, we find that many tumor types and cell lines are insensitive to the upstream signaling molecules Fas, FADD, and caspase 8 (our unpublished data). Nevertheless, every cell tested from a broad panel of cells has proven to be sensitive to conditional alleles of caspase 1 and caspase 3 after 20-hr stimulation with the CID AP1903. These cells include the prostate cancer cells LNCaP, PC3, TSU-Pr1, and DU145, the cervical carcinoma HeLa, the kidney carcinoma 293, the fibrosarcoma HT1080, the melanomas A375 and B16, the T cell lymphoma Jurkat, the IL-3-dependent pro-B cell BaF/3, and primary smooth muscle cells from rat arteries and prostates from benign prostate hypertrophy (BPH) patients (Fig. 6; MacCorkle *et al.,* 1998, and our unpublished data). In contrast to the rapid killing that we observed by caspases, when we expressed a drug-specific HSV thymidine kinase mutant, based on pCMV:75, into Jurkat cells, there was little (~20%) 20-hr effect on reporter expression even after incubation with 30 μM ganciclovir, which is at least 10-fold more GCV than is necessary to kill mammalian cells after several days using this *tk* mutant (data not shown; Black *et al.,* 1996).

Furthermore, to optimize fully the efficacy of these novel reagents, we have investigated several parameters relevant to caspase activation: (1) extent of cross-linking (2) interdomain flexibility, and (3) intracellular localization. These improved ADSs now meet many, if not all, of the desiderata for a broadly applicable suicide switch: (1) they trigger apoptosis within hours of activation, (2) function independently of the cell cycle, (3) have low basal activity when expressed at minimal functional levels, (4) are based on endogenous proteins to minimize immunogenicity in humans, and (5) can trigger apoptosis in multiple tissues independently when different CID–CBD combinations are used (Spencer *et al.,* 1993; Belshaw *et al.,* 1996).

We find that dimerization of caspase 3 is sufficient for maximum CID sensitivity, while higher order multimerization is somewhat more efficient for caspase 1 activation using these reagents. We also find that optimized activation of caspases 1 and 8, and $FADD_{125}$, by AP1903 (but not by the larger CID, FK1012) requires a short G-S-G-G-G-S linker between the cognate CBD (i.e., F_vs) and the procaspase. The crystal structure of AP1903 bound to two F_vs reveals that the $FKBP12_{V36}$ moieties are brought into closer proximity than the two FKBP12 moieties of FK1012A/FKBP12 (M. Gilman, personal communication, 1998). Thus, the more "intimate" AP1903-mediated F_v interactions may "lock" cross-linked chimeric proteins into conformations that are incompatible with their activation. Interestlingly, constructs with two interdomainal linkers (e.g., S-F_{vis}2-Casp3) or with a longer G-G-S-G-G-G-S-G-G-G linker (data not shown) are less sensitive to activation, perhaps owing to too much flexibility. This may imply that CIDs do more than increase the proximity of proteins; they could also hold proteins in the correct (or incorrect) orientation for activation. Further, we find that plasma membrane localization of conditional caspases 3 and 8, or $FADD_{125}$, increases their sensitivity for AP1903 by about 10-fold, while simultaneously increasing their basal activities. Interestingly, mitochondrial localization of $Mas70_{34}$-F_v2-Casp3 did not increase its basal activity relative to cytoplasmic S-F_v2-Casp3 even though some antiapoptosis caspase 3 targets, such as Bcl-2 and Bcl-x_L, are mitochondria localized (Cryns and Yuan, 1998). Perhaps the topography of the mitochondrial outer membrane, distinct membrane fluidity, or larger collective surface area may reduce the local concentration of $Mas70_{34}$-F_v2-Casp3 relative to M-F_v2-Casp3.

Nuclear-targeted caspases trigger apoptosis

In addition to multiple cytoplasmic changes that occur during apoptosis, such as membrane blebbing, redistribution of phosphatidylserine to the outer plasma membrane, and mitochondrial dysfunction, cellular apoptosis includes a number of well-characterized nuclear events. These include the disassembly of the nuclear matrix, condensation of chromatin, and internucleosomal DNA degradation (Cryns and Yuan, 1998). Further, among the caspase substrates that have been identified, several are primarily found in the nucleus, including PARP, DNA-PK_c, MDM2, Rb, $p21^{Cip1}$, $p27^{Kip1}$, topoisomerase I, lamin A and B, nuclear mitotic apparatus protein (NuMA), and histone H1. There is also evidence that some procaspases, such as caspase 2, are targeted to the nucleus (Colussi *et al.,* 1998a). Consistent with these findings, we find that nuclear-targeted caspases 1, 3, and 8 trigger apoptosis in the nucleus with similar efficacy relative to their cytoplasmic counterparts. In contrast, nuclear-targeted $FADD_{125}$ N2-F_v1-F_{vis}1-$FADD_{125}$ functions less efficiently than its cytoplasmic counterpart. Since caspase 8 is thought to be cytoplasmic, we were somewhat surprised that nuclear FADD functioned at all. Possible explanations are that a small, but functionally significant, fraction of N2-F_v1-F_{vis}1-$FADD_{125}$ was present in the cytoplasm or that $FADD_{125}$ can interact weakly with the prodomains of other caspases that are found in the nucleus, such as caspase 2.

Dimerization of caspases represents a physiological control mechanism

The initial discovery that procaspase 8 is associated with the Fas receptor complex via FADD led to the hypothesis that caspase 8 may be activated by autoproteolysis. This study and others support this hypothesis (Boldin *et al.,* 1996; Muzio *et al.,* 1996, 1998; Martin *et al.,* 1998; Yang *et al.,* 1998). While the primary role for FADD may be to convert Fas cross-linking into procaspase 8 cross-linking, the fact that the DED of FADD has moderate basal toxicity implies that this domain may have additional roles in caspase 8 activation. One possibility is that $FADD_{125}$ is able to self-aggregate if overexpressed without abrogating FLICE-binding capacity, consistent without one report of FADD "filaments" in overexpressing cell lines (Siegel *et al.,* 1998). Alternatively, $FADD_{DED}$ may be able to increase the basal aggregation of caspase 8. Similarly, Apaf-1, the human homolog of *Caenorhabditis elegans* CED4, appears to be an

adapter that can activate several procaspases, including caspases 2, 8, and 9 (Yoshida *et al.,* 1998). The ATP/cytochrome *c*-regulated multimerization of Apaf-1 appears to trigger the multimerization, and therefore activation, of associated caspases via interactions between the amino-terminal CARD domain of Apaf-1 and the homologous prodomains of some caspases (Srinivasula *et al.,* 1998a). Consistent with this observation is the direct demonstration that forced dimerization of either the Apaf-1 amino terminus or procaspase 9 can activate procaspase 9 and trigger apoptosis (Hu *et al.,* 1998). In addition, procaspase 2 can bind to the adapter RAIDD that associates with the TNFRI complex to trigger apoptosis after TNF-α or LTα binding (Chou *et al.,* 1998). Finally, the Apaf-1-like protein FLASH has been reported to interact with caspase 8 in the death-inducing signaling complex (DISC) and contribute to its activation (Imai *et al.,* 1999). Therefore, the multimerization and activation of caspases constitute a common mechanism for the activation of at least some family members, which can be reproduced with CIDs.

The high basal toxicity of several caspases, such as caspases 1, 2, 8, and 9, when overexpressed is consistent with the idea that the long prodomains of several caspases can contribute to their unstimulated basal activation. We and others have observed that the prodomain of caspase 8 increases its basal activity (Yang *et al.,* 1998; and our unpublished results). However, truncation of the prodomain of caspase 1 does not appear to affect its basal activity significantly (our unpublished results). In contrast, the short prodomains of caspases 3 and 7 may inhibit their activity, as the removal of these two prodomains leads to higher basal activity (Duan *et al.,* 1996; MacCorkle *et al.,* 1998). Interestingly, a chimeric caspase containing the prodomain of caspase 2 with the catalytic domains of caspase 3 can increase the basal activity of caspase 3 (Colussi *et al.,* 1998b). An alternative explanation for the low basal activity of caspase 3 is that it makes a poor substrate for itself because one or more cleavage sites are inaccessible to the catalytic site or of low affinity. While the 5′ prodomain cleavage site for caspase 3, ESMD^{28}S, is close to the preferred caspase 3 consensus DxxD the 3′ cleavage site IETD^{175}S is closer to the consensus site (I/L/V)ExD for caspases 6, 8, and 9, which have been shown to cleave caspase-3 (Orth *et al.,* 1996; Li *et al.,* 1997; Muzio *et al.,* 1997). However, the idea that the cleavage site is inaccessible is also supported by a report that shuffling the domains of caspase 3 (and caspase 6) increases basal activity (Srinivasula *et al.,* 1998b). Finally, postranslational modifications of caspase 3 can inhibit its activation *in vivo.* Regardless of the recalcitrance of caspase 3 to undergo spontaneous activation, forced dimerization of caspase 3 can clearly overcome these obstacles.

Although others reported that dimerization of caspase 3 or FADD could not trigger apoptosis (Grimm *et al.,* 1996; Yang *et al.,* 1998), those contradictory results are actually reconcilable with our empirical observations that the spacing between target proteins and dimerization domains is often important. For example, we found that S-F_v2-$FADD_{125}$, S-F_v2-Casp1, and S-F_v2-Casp8 were not cytotoxic after AP1903 administration, while the corresponding constructs containing the six-amino acid G-S linker between the F_v and the target genes were sensitive to AP1903. Further, we and others observed that S-F_{pk}3-Δ180Casp8, lacking the first 180 residues of caspase 8, is sensitive to FK1012 (Yang *et al.,* 1998), while S-F_{pk}3-Δ170Casp8, lacking the first 170 residues, is unresponsive to FK1012 (data not shown). Therefore, although dimerization or oligomerization may be a common control mechanism for caspases, the correct orientation of dimerization appears to be important as well.

Potential therapeutic applications

Gene therapy applications using suicide switches have already reached phase II clinical trials for the treatment of several cancers, including prostate, ovarian, and brain tumors (Singhal and Kaiser, 1998). The most widely characterized approach has been the HSV-*tk*/GCV suicide switch, which relies on both direct and indirect "bystander' mechanisms to achieve efficacy. Unlike HSV-*tk* and other suicide genes that rely on cell cycling, CID-regulated ADSs may be more useful for treating slow-growing cancers, such as prostate cancer or benign hyperplastic disorders, such as benign prostate hypertrophy or atherosclerosis. Further, triggering apoptosis in tumors, especially in the context of the appropriate cytokine milieu (e.g., IL-12, GM-CSF), may increase the transfer of putative tumor-associated antigens to professional APCs, such as dendritic cells (Albert et al., 1998). Nevertheless, further characterization of any direct or indirect bystander effect of ADSs will be necessary before the utility of ADSs for the treatment of hyperproliferative disorders can be fully appreciated.

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