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Interchain Proteolysis, in the Absence of a Dimerization Stimulus, Can Initiate Apoptosis-associated Caspase-8 Activation*

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Caspases coordinate the internal demolition of the cell that is seen during apoptosis. Proteolytic processing of caspases is observed during apoptosis, and this correlates with conversion of inactive caspase proenzymes into their active two-chain forms. However, recent studies have suggested that caspase-8 is activated through dimerization and that interchain proteolysis is not sufficient for activation of this caspase. This proposal casts doubt upon whether caspase-8 is productively activated by granzyme B during granule-dependent cytotoxic T lymphocyte or natural killer cell-mediated killing, for example. Contrary to the dimerization model, we show that direct proteolysis of caspase-8 by the cytotoxic T lymphocyte protease granzyme B, or by caspase-6, produces an active enzyme that displays robust proteolytic activity toward synthetic as well as natural caspase-8 substrates. These data suggest that enforced dimerization of caspase-8 zymogens by scaffold proteins such as Fas-associated protein with death domain (FADD), although important in certain contexts, is not a prerequisite for activation of this protease.

The caspase family of cysteine proteases plays a central role in apoptosis through controlled demolition of the cellular architecture in response to diverse stimuli (1–3). During apoptosis, restricted proteolysis of an array of caspase substrates results in the morphological and biochemical alterations characteristic of this mode of cell death (4, 5). Caspases are normally present as dormant proenzymes in viable cells and achieve catalytic competence at the onset of apoptosis. Although a common subset of caspases becomes activated in essentially all forms of apoptosis, caspase activation is achieved in stimulus-specific ways. Certain caspases, such as caspases-8 and -9, possess long N-terminal pro-domains that are used to recruit these enzymes to activation complexes. Caspase activation complexes are typically assembled at the onset of apoptosis and promote activation of caspases recruited to these complexes (6). Caspases that are activated in this way (termed initiator caspases) can then further propagate death signals by proteolytically processing downstream caspases, such as caspases-3, -6, and -7, within the cell.

Caspase-8 acts as the initiator caspase in cell death pathways that result from ligand-driven engagement of certain members of the tumor necrosis factor/nerve growth factor receptor family (7, 8). In this context, the adaptor molecule FADD[1] recruits caspase-8 to plasma membrane-associated receptor complexes and promotes activation of this caspase. Receptor-driven clustering of caspase-8 is thought to activate the enzyme through facilitating autoproteolysis of full-length caspase-8 molecules to produce a two-chain active enzyme. However, recent studies have proposed the alternative view that caspase-8 aggregation serves primarily to facilitate dimerization of the enzyme and that this is sufficient for activation (9, 10). In this model, dimerization, rather than interchain proteolysis, is the critical requirement for caspase activation, and subsequent autoprocessing of the enzyme has been proposed to act merely to stabilize caspase-8 dimers (9). A corollary of this model is that direct proteolytic processing of caspase-8, by other caspases or proteases with similar specificity such as granzyme B, in the absence of a dimerization platform may not be sufficient for activation for this caspase.

Interchain processing of caspases is observed in most, if not all, forms of apoptosis and has become widely accepted as a hallmark of caspase activation. If correct, the dimerization model suggests that the appearance of proteolytically processed forms of caspase-8 is not a reliable measure of the activation status of this protease. Interchain proteolysis of caspase-8 is observed in at least two important contexts where a stimulus for dimerization is not apparent. In the context of cytotoxic T lymphocyte/natural killer-mediated killing, caspase-8 is processed directly by the granule protease granzyme B, and this has previously been assumed to correlate with activation of this protease (11, 12). Furthermore, in the stress-initiated/apoptosome pathway to apoptosis, caspase-8 becomes proteolytically processed via direct proteolysis by caspase-6 (4, 13, 14). The dimerization model of caspase-8 activation predicts that caspase-8 processing in the aforementioned contexts will result in catalytically inactive protease. Here, we have tested this prediction, and, contrary to the dimerization model, we find that interchain processing is sufficient to produce catalytically active caspase-8.

EXPERIMENTAL PROCEDURES

Materials—The following antibodies were used for immunoblotting and/or immunoprecipitation: mouse anti-caspase-3 (BD Biosciences), rabbit anti-caspase-3 D175 (Cell Signaling Technology, UK), mouse anti-caspase-8 (clone C15; Apotech), mouse anti-caspase-8 (clone 12F5; Apotech), mouse anti-caspase-8 (clone 1C12; Cell Signaling Technology), and mouse anti-caspase-8 (clone 5F7; Upstate Biotechnology, Inc.). Anti-Fas IgM (clone CH-11) was purchased from Upstate Biotechnology and was used to initiate apoptosis by adding it to cell culture

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[1] The abbreviations used are: FADD, Fas-associated protein with death domain; AFC, amino-4[trifluoromethyl]-coumaride; CEB, cell extract buffer; cmk, chloromethyl ketone; Cyt *c*, cytochrome *c*; fmk, fluoromethyl ketone; mAb, monoclonal antibody.

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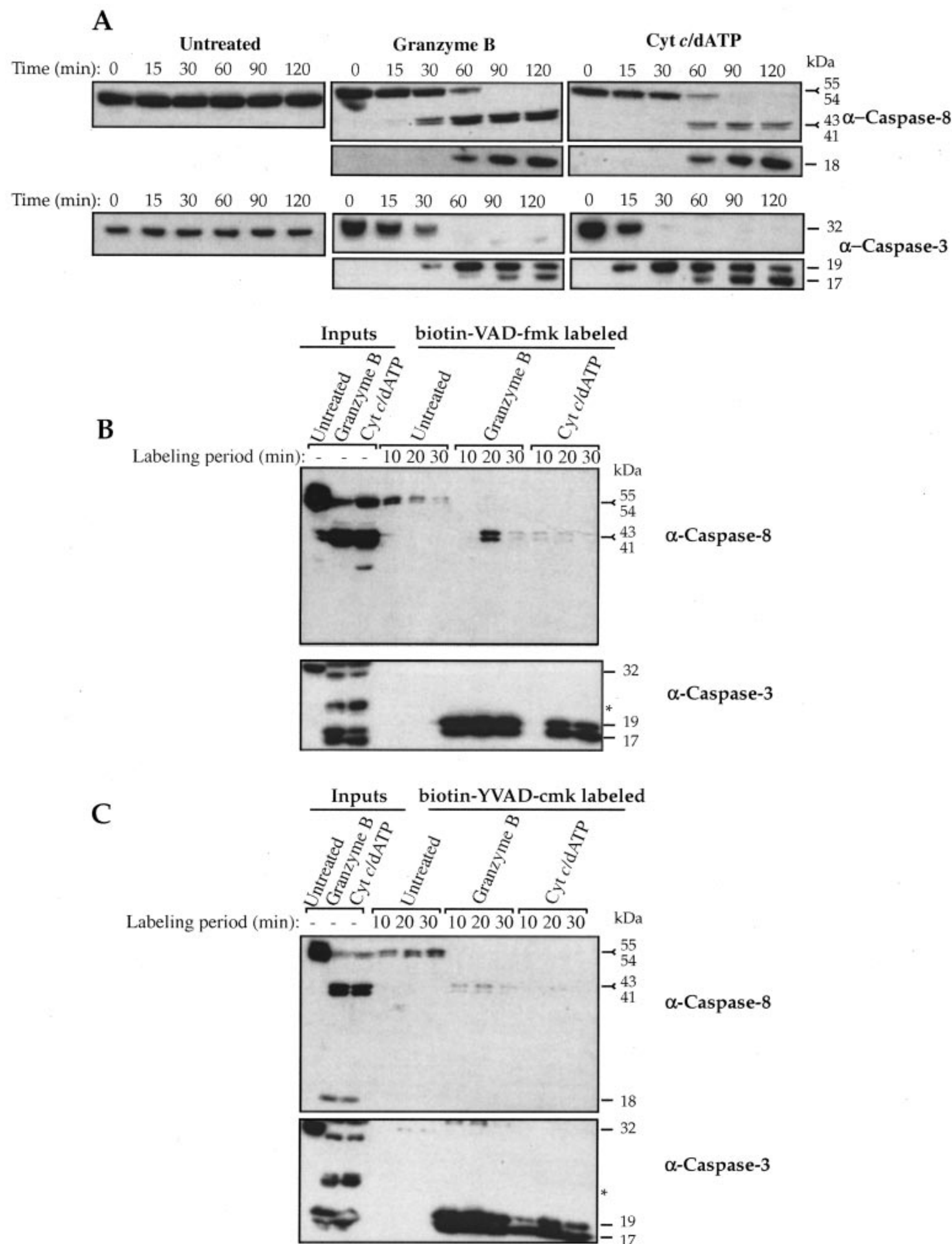

FIG. 1. **Peptide affinity labeling of caspase-8 after proteolytic processing by granzyme B or caspase-6.** *A*, Jurkat cell-free extracts were incubated at 37 °C in the presence or absence of 100 nM granzyme B, or 50 μg/ml Cyt *c* and 1 mM dATP, as indicated. Samples were removed at the indicated time points and were subsequently analyzed for caspase-8 and caspase-3 processing status by Western blotting. *B*, Jurkat cell-free extracts were incubated for 2 h at 37 °C in the presence or absence of granzyme B, or Cyt *c*/dATP, as shown. After the 2-h incubation period, biotin-VAD-fmk was then added to the extracts to a final concentration of 10 μM, and affinity labeling was allowed to proceed for 10, 20, and 30 min as indicated. Affinity-labeled caspases were captured on agarose-streptavidin beads as described under "Experimental Procedures." Bead complexes were then probed for the presence of caspase-8 (*top*) or caspase-3 (*bottom*), as indicated. Inputs represent 20% of the cell-free lysate volume that was used for affinity labeling reactions. *Asterisks* denote a nonspecific band detected with anti-caspase-3 antibody. *C*, Jurkat cell-free extracts, treated as described in *B* above, were affinity labeled with biotin-YVAD-cmk, and streptavidin-agarose-captured complexes were probed for caspase-8 or caspase-3 as shown. Inputs represent 20% of the cell-free lysate volume that was used for affinity labeling reactions. Results are representative of three separate experiments.

medium at a final concentration of 100 ng/ml. Biotin-VAD-fmk (biotin-Val-Ala-Asp-[Ome]-fluoromethyl ketone) was purchased from ICN. Biotin-YVAD-cmk (biotinyl-Tyr-Val-Ala-Asp-chloromethyl ketone) was purchased from Bachem, and the caspase-8 synthetic substrate Ac-IETD-AFC (Ac-Ile-Glu-Thr-Asp-7-amino-4[trifluoromethyl]-coumaride) was purchased from ICN.

Preparation of Cell-free Extracts—Cell-free cytosolic S-15 extracts were generated from Jurkat cells as described previously (4, 15). Briefly, 5×10^8 cells were packed into a 2 ml of Dounce homogenizer, and an equal volume of ice-cold cell extract buffer was added (CEB: 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 250 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml aprotinin). Cells were allowed to swell in CEB for 20–30 min on ice and were then lysed by homogenization with ~15–20 strokes of a B-type pestle. Crude extracts were then centrifuged for 30 min at 15,000 × *g* to remove nuclei, unbroken cells, and other debris. Extracts (25–30 mg/ml protein) were stored in aliquots at −70 °C until required.

Initiation of Caspase-8 Proteolytic Processing—Caspase-8 processing was initiated in Jurkat cell-free extracts by the addition of 50 μg/ml bovine heart cytochrome *c* (Cyt *c*) and 1 mM dATP, which triggers assembly of Apaf-1/caspase-9 apoptosomes, followed by incubation of

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cell-free reactions for 2 h at 37 °C (4, 13, 15). Alternatively, 100 nM purified human granzyme B (Calbiochem) was added to cell-free extracts followed by incubation as described above. Fas/CD95-driven caspase-8 processing was initiated in Jurkat cells by stimulation with 100 ng/ml anti-Fas IgM for 4 h at 37 °C.

Caspase-8 Immunoprecipitation and Assessment of Catalytic Activity—Caspase-8 was immunoprecipitated from cell-free reactions using various monoclonal antibodies (mAbs) as detailed above. Briefly, at appropriate time points cell-free reactions (50–100 μl/immunoprecipitation) were diluted to 250 μl with CEB followed by the addition of 1–2 μg of anti-caspase-8 mAbs and 40 μl of protein A/G-agarose (Santa Cruz). Immunoprecipitations were rotated for 4 h at 4 °C followed by pelleting of immunocomplexes and washing three times in CEB (1 ml/wash). For synthetic peptide hydrolysis assays, protein A/G-agarose-immobilized caspase-8 complexes were resuspended in 50 μl of CEB and transferred to black 96-well Fluotrac 200 plates. Ac-IETD-AFC (50 μl) was then added to each well (to a final concentration of 50 μM), and liberation of free AFC was monitored for 1 h at 37 °C at excitation and emission wavelengths of 430 and 535 nm, respectively.

^{35}S-Labeled Caspase-3 Cleavage Assays—Immunoprecipitated caspase-8 was washed as above and brought up in CEB containing *in vitro* transcribed and translated ^{35}S-caspase-3 (in a final reaction volume of 15 μl). Reactions were allowed to proceed for 3 h at 37 °C and were stopped by the addition of SDS-PAGE sample buffer followed by analysis by SDS-PAGE/autoradiography.

Affinity Labeling of Caspases—Caspases were active site labeled with biotinylated caspase substrate peptides, as follows. Cell-free extracts were incubated for 2 h at 37 °C in the presence or absence of 100 nM granzyme B, or 50 μg/ml Cyt *c* and 1 mM dATP to initiate processing of caspases. Extracts (150-μl aliquots) were then incubated at 37 °C with 10 μM biotin-VAD-fmk, or 10 μM biotin-YVAD-cmk, and 50-μl samples were removed after 10, 20, and 30 min for assessment of caspase labeling. Biotinylated proteins were captured by dilution of 50-μl reactions to 250 μl with CEB, followed by the addition of agarose-streptavidin beads (30 μl/reaction) and incubation at 4 °C under continuous rotation for 3 h. Bound proteins were washed three times with CEB (1 ml/wash) and were eluted from beads by the addition of SDS-PAGE sample buffer followed by boiling for 7 min.

RESULTS

Experiments based upon recombinant noncleavable forms of caspase-8 have suggested that dimerization of caspase-8 may be a prerequisite for activation of this protease (9, 10). The dimerization model predicts that proteolytic processing of caspase-8, in the absence of a dimerization platform, may be insufficient for activation of this enzyme (9). To explore whether this model holds for endogenous full-length caspase-8, we have used two stimuli (granzyme B and caspase-6) that promote interchain proteolytic processing of caspase-8 in a dimerization-independent manner. As illustrated in Fig. 1*A*, caspase-8 underwent rapid proteolytic maturation upon introduction of recombinant granzyme B into cell-free extracts of Jurkat cells. Similar, but less rapid, processing of caspase-8 was also observed when Cyt *c*/dATP was added to Jurkat extracts to initiate assembly of Apaf-1/caspase-9 apoptosomes (Fig. 1*A*). Previous studies have established that caspase-8 processing in the apoptosome pathway is catalyzed by caspase-6, which is activated downstream of the apoptosome (4, 13, 14). As expected, caspase-3 interchain processing also occurred in response to the addition of granzyme B or Cyt *c*/dATP to the extracts (Fig. 1*A*; Ref. 16).

To assess whether proteolytic processing of caspase-8 in these contexts produced catalytically active enzyme, we used two different biotinylated synthetic caspase substrates (biotin-VAD-fmk and biotin-YVAD-cmk) to label active caspases in the extracts after treatment with granzyme B or Cyt *c*/dATP. Using this approach, we found that although caspase-3 was labeled very efficiently by both peptides in extracts that had been treated with granzyme B or Cyt c/dATP, only trace amounts of proteolytically processed caspase-8 were labeled by comparison (Fig. 1, *B* and *C*). Similar observations have been made by Salvesen and colleagues (9) (using biotin-VAD-fmk as an affinity label) and appear to support their proposal that direct interchain proteolysis of caspase-8 is insufficient for activation of this caspase.

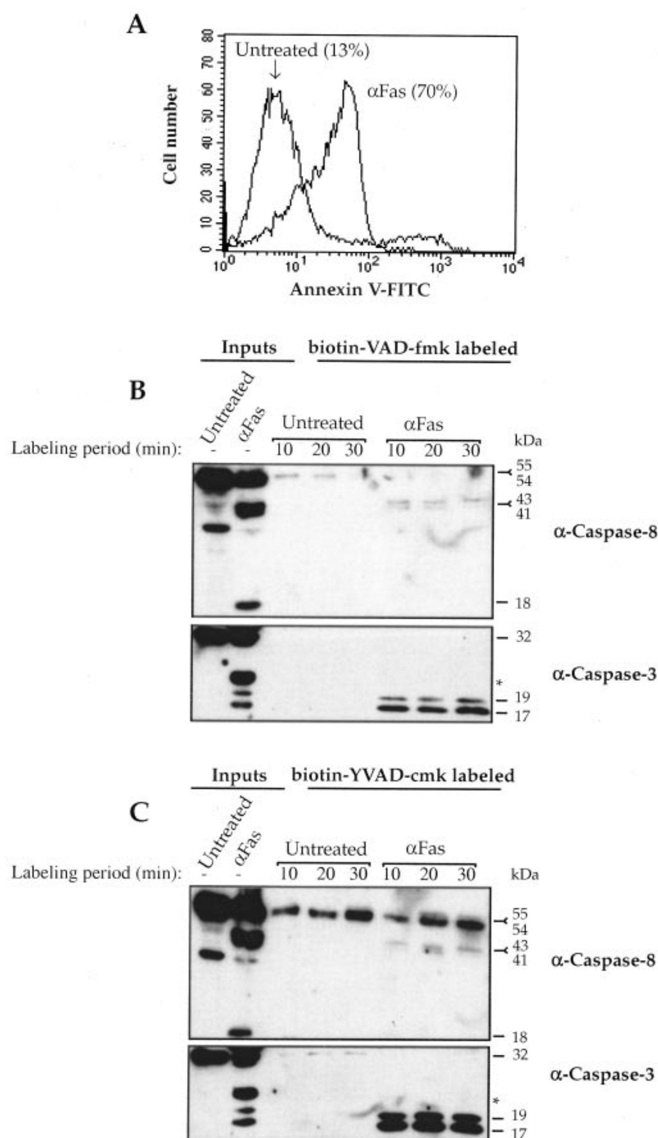

FIG. 2. **Peptide affinity labeling of caspase-8 after Fas stimulation.** *A*, Jurkat cells were cultured in the presence or absence of 100 ng/ml anti-Fas IgM for 4 h followed by assessment of apoptosis by staining with fluorescein isothiocyanate (*FITC*)-conjugated annexin V and analysis by flow cytometry. Percentages of apoptotic cells are indicated in *parentheses*. *B* and *C*, cell-free extracts, generated from control or Fas-stimulated Jurkat cells as described in *A*, were affinity labeled for the times indicated with either biotin-VAD-fmk (*B*) or biotin-YVAD-cmk (*C*). Biotinylated proteins were captured on streptavidin-agarose, and complexes were probed for caspase-8 or caspase-3 as shown. Inputs represent 33% of the cell-free lysate volume that was used for the affinity labeling reactions. *Asterisks* denote a nonspecific band detected with anti-caspase-3 antibody. Results are representative of two separate experiments.

However, we sought to validate the biotinylated peptide labeling method as a means to detect catalytically active caspase-8 using extracts from cells that had been induced to undergo apoptosis through stimulation with anti-Fas IgM. In this context, it has been well established that caspase-8 is activated through FADD-dependent recruitment of the latter to receptor complexes (7, 8). Thus, Jurkat cells were treated with 100 ng/ml anti-Fas mAb for 4 h at which time ~70% of these cells were annexin V-positive and thus undergoing apoptosis (Fig. 2*A*). Cell-free extracts prepared from the latter cells

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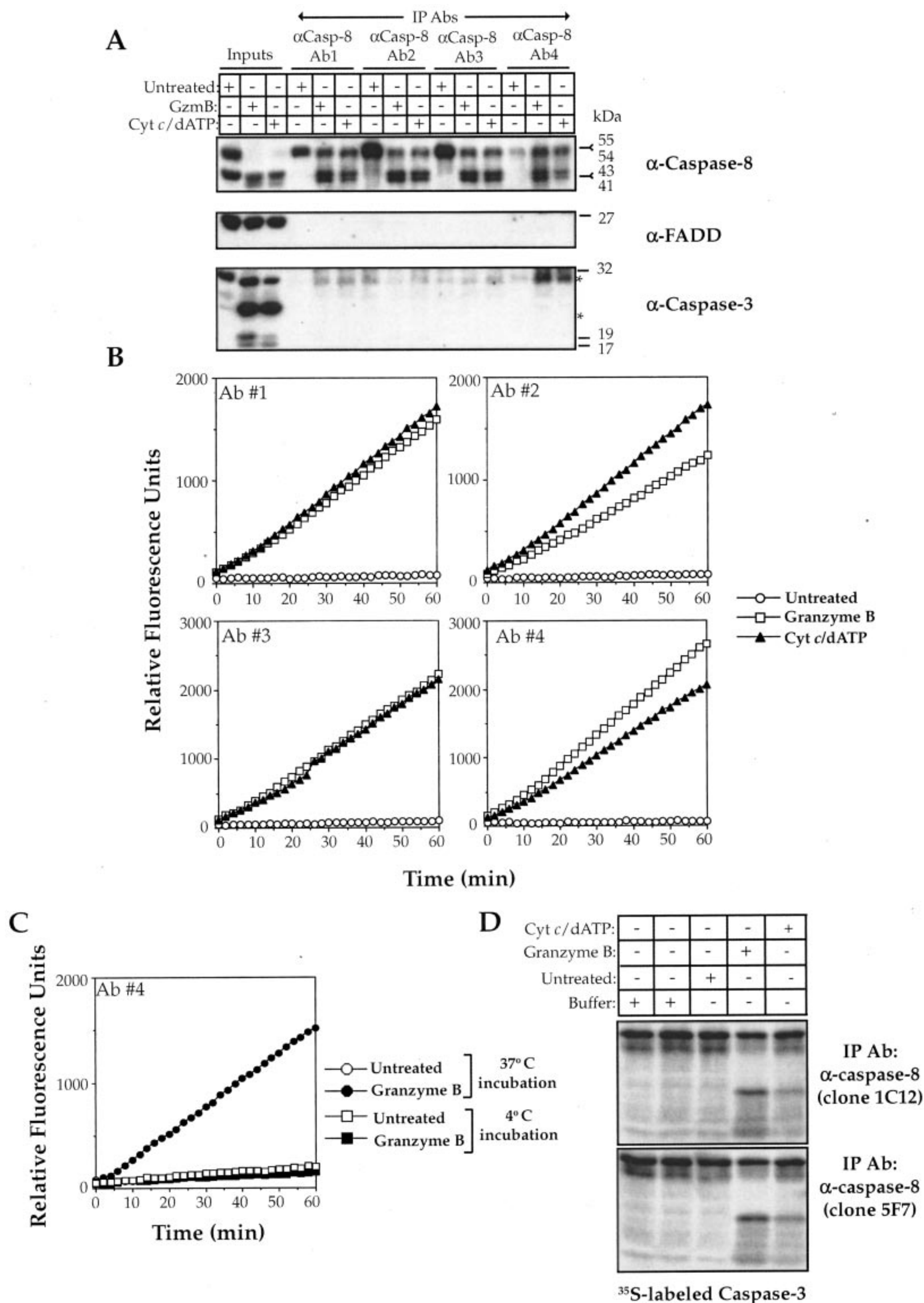

FIG. 3. **Interchain proteolysis is sufficient to activate caspase-8.** Jurkat cell-free extracts (200-μl reactions) were incubated at 37 °C in the presence or absence of 100 nM granzyme B, or Cyt *c*/dATP, as indicated. After the 2-h incubation period, each reaction was divided into four aliquots (50 μl each), and caspase-8 was immunoprecipitated using four different anti-caspase-8 mAbs, as indicated. The caspase-8 mAbs used were as follows: Ab1, clone C15; Ab2, clone 12F5; Ab3, clone 5F7; and Ab4, clone 1C12. Caspase-8 immunocomplexes were washed three times, and aliquots of each were used to assess the efficiency of caspase-8 immunoprecipitation (*A*), IETD-AFC hydrolysis activity (*B*), or proteolytic activity toward ^{35}S-caspase-3 (*D*). *A*, immunoprecipitation of proteolytically processed caspase-8 after treatment with granzyme B or Cyt *c*/dATP. Caspase-8 immunocomplexes were also probed for the presence of coprecipitated FADD (*middle panel*) or caspase-3 (*bottom panel*). *B*, Jurkat cell-free extracts were incubated for 2 h at 37 °C, in the presence or absence of granzyme B or Cyt *c*/dATP followed by immunoprecipitation of caspase-8 using different caspase-8 mAbs, as detailed above. Caspase-8 immunocomplexes were then assessed for their ability to hydrolyze Ac-IETD-AFC as described under "Experimental Procedures." Results are representative of four separate experiments. *Asterisks* are as in Figs. 1 and 2. *C*, Jurkat cell-free extracts were incubated for 2 h, either at 4 or at 37 °C as indicated, in the presence or absence of granzyme B followed by immunoprecipitation of caspase-8. Caspase-8 immunocomplexes were then assessed for their ability to hydrolyze Ac-IETD-AFC (hydrolysis assays were carried out at 37 °C) as described under "Experimental Procedures." Note that caspase-8 activity was seen only where granzyme B was incubated with cell-free extracts at 37 °C. *D*, caspase-8 was immunoprecipitated (*IP*) from Jurkat cell-free extracts after treatment with granzyme B or Cyt *c*/dATP, as indicated. Immunocomplexes were then washed and resuspended in CEB containing ^{35}S-labeled procaspase-3. Reactions were then incubated at 37 °C for 3 h followed by SDS-PAGE and fluorography for assessment of caspase-3 proteolysis. Results are representative of three separate experiments.

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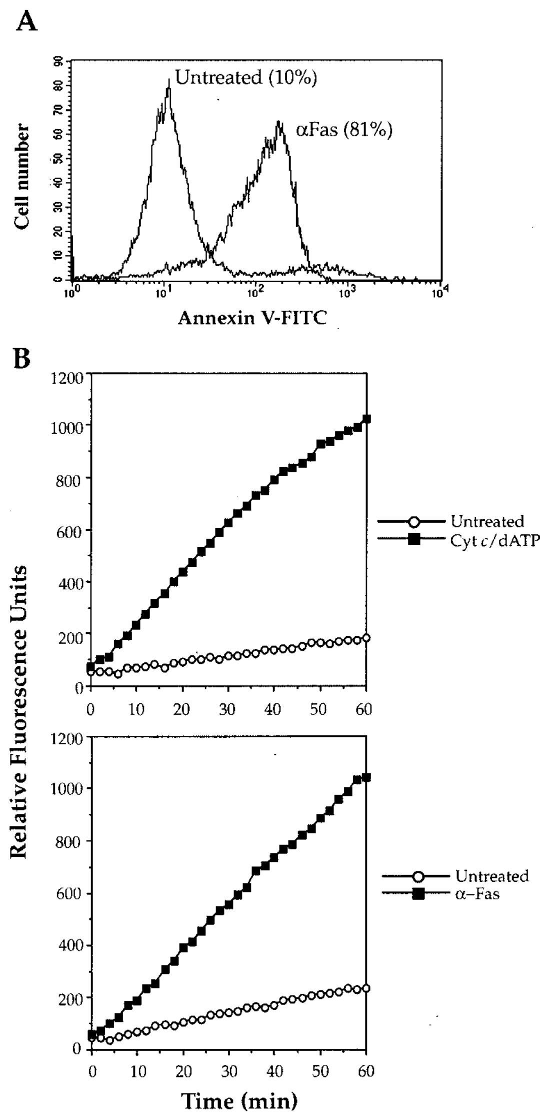

FIG. 4. **Interchain proteolysis and induced proximity-driven caspase-8 activation yield similar levels of catalytically active caspase.** *A*, Jurkat cells were cultured in the presence or absence of 100 ng/ml anti-Fas IgM for 4 h followed by assessment of apoptosis by staining with fluorescein isothiocyanate (*FITC*)-conjugated annexin V and analysis by flow cytometry. Percentages of apoptotic cells are indicated in *parentheses*. Cell-free extracts were then generated from the control and anti-Fas-stimulated cell populations. *B*, *upper panel*, Jurkat cell-free extracts were incubated for 2 h in the presence or absence of Cyt *c*/dATP followed by immunoprecipitation of caspase-8 and assessment of Ac-IETD-AFC hydrolysis activity as described under "Experimental Procedures." *Lower panel*, caspase-8 was immunoprecipitated from Jurkat cell-free extracts prepared from untreated *versus* anti-Fas IgM-treated Jurkat cell populations as shown in *A*. Caspase-8 immunocomplexes were then assessed for Ac-IETD-AFC hydrolysis activity. Note that caspase-8 immunocomplexes were prepared from the same volumes of cell-free lysates, with normalized protein concentrations, in all cases.

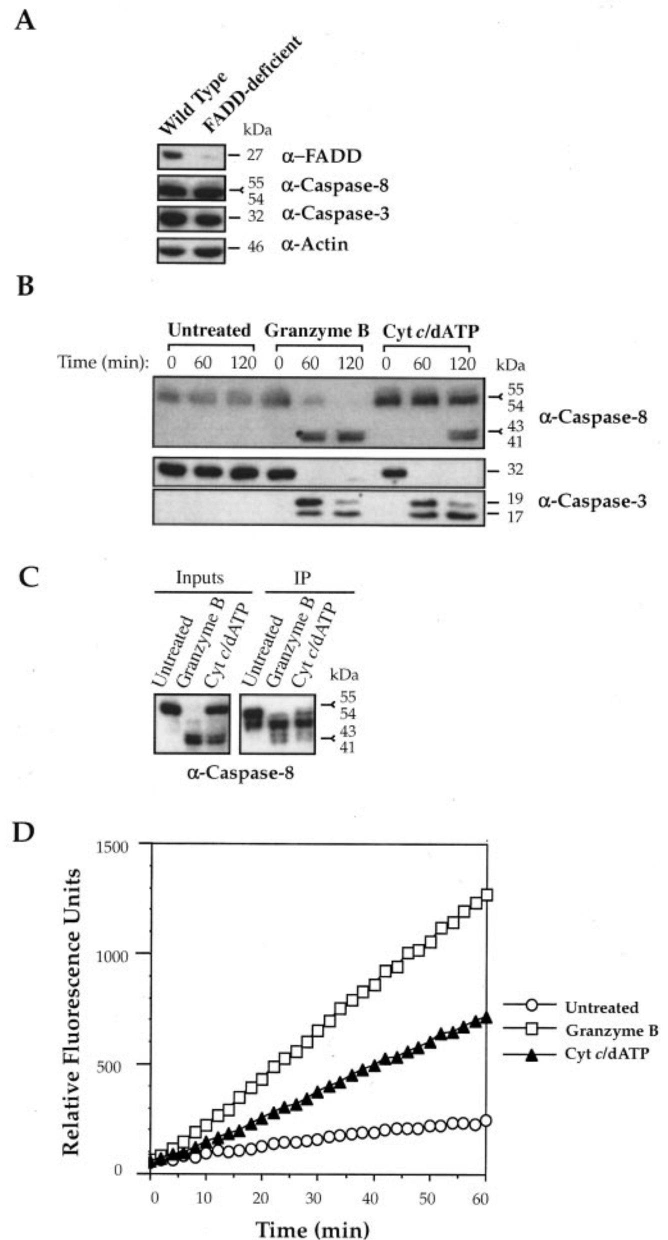

FIG. 5. **FADD is not required for granzyme B- or caspase-6-initiated caspase-8 activation.** *A*, cell-free extracts were generated from wild type and FADD-deficient Jurkat cells. Equal amounts of protein were loaded (~50 μg/*lane*), and lysates were probed for the indicated proteins. *B*, FADD-deficient Jurkat cell-free extracts were incubated at 37 °C in the presence or absence of 100 nM granzyme B, or 50 μg/ml Cyt *c* and 1 mM dATP, as indicated. Samples were removed at the indicated time points and were subsequently analyzed for caspase-8 and caspase-3 processing status by Western blotting. *C*, caspase-8 was immunoprecipitated (*IP*) from FADD-deficient Jurkat cell-free extracts after treatment for 2 h at 37 °C with 100 nM granzyme B or Cyt *c*/dATP, as indicated. *D*, caspase-8 immunoprecipitates were generated as described in *C* were then assayed for Ac-IETD-AFC hydrolysis activity.

were incubated with biotin-VAD-fmk or biotin-YVAD-cmk to label active caspases using the same procedures as before. However, once again we found that only trace amounts of caspase-8 could be labeled using this method (Fig. 2, *B* and *C*). In contrast, active caspase-3 was labeled very efficiently in the same samples (Fig. 2, *B* and *C*). These data strongly suggest that biotinylated caspase substrate peptides preferentially label certain caspases (such as caspase-3) where a mixture of different active caspases is present.

Because of the problems associated with peptide affinity labeling as discussed above, we used a more direct approach to assess the catalytic competence of caspase-8 after direct processing by granzyme B or caspase-6. Using a panel of caspase-8 mAbs, we immunoprecipitated caspase-8 from control, granzyme B-treated, or Cyt *c*/dATP-treated cell-free extracts. As illustrated in Fig. 3*A*, these antibodies immunoprecipitated both the full-length caspase-8 zymogen as well as the proteolytically processed form of this protease. Importantly, neither FADD nor caspase-3 coprecipitated with caspase-8 under the conditions used for immunoprecipitation (Fig. 3*A*). We then assessed the ability of immunocaptured caspase-8 to cleave the

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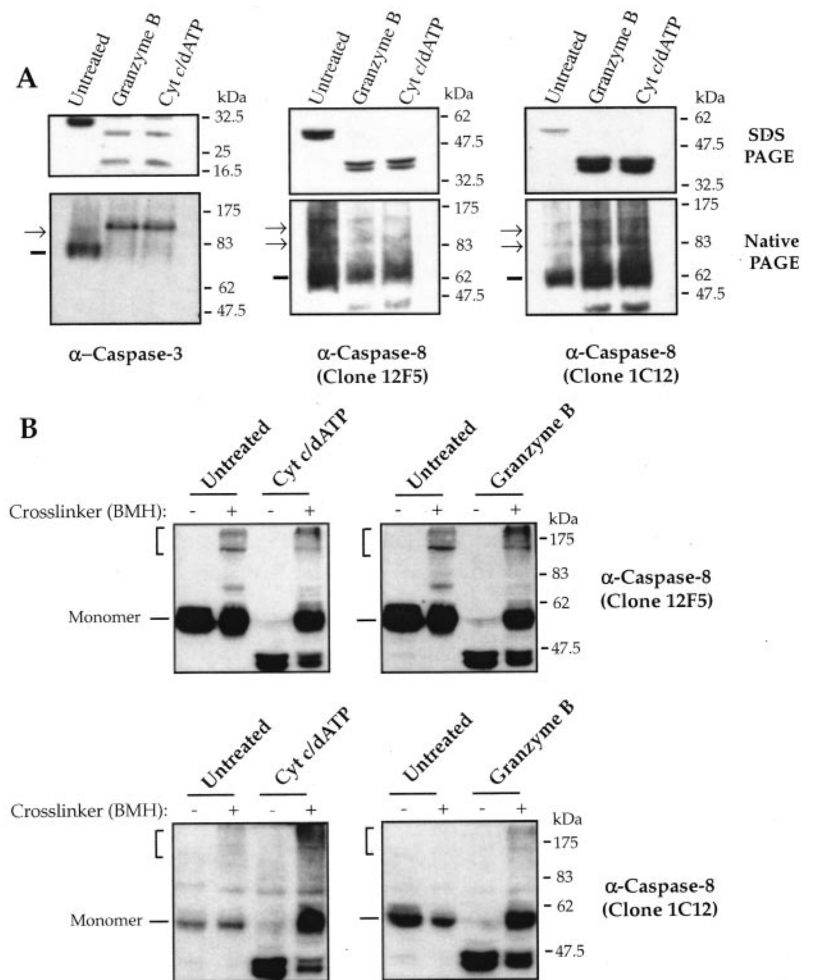

FIG. 6. **Effect of interchain processing on caspase-8 monomer *versus* dimer status.** *A*, Jurkat cell-free extracts were incubated for 2 h at 37 °C in the presence or absence of 100 nM granzyme B, or 50 μg/ml Cyt *c* and 1 mM dATP. Cell-free reactions were then analyzed by SDS-PAGE (*top panels*) or native PAGE (*bottom panels*), in 10% acrylamide gels followed by immunoblotting for caspase-3 or caspase-8, as indicated. Note that two different caspase-8 mAbs gave essentially identical results, as shown. Results are representative of three independent experiments. *Arrows* indicate putative dimeric/oligomeric species. *B*, Jurkat cell-free extracts were incubated for 1.5 h at 37 °C in the presence or absence of 100 nM granzyme B, or 50 μg/ml Cyt *c* and 1 mM dATP. Incubations were then continued for a further 30 min at room temperature in the presence or absence of 1 mM covalent cross-linking agent bismaleimidohexane (*BMH*). Cell-free reactions were then analyzed by SDS-PAGE and probed for caspase-8, as indicated. Note that two different caspase-8 mAbs gave essentially identical results as shown. Results are representative of three independent experiments.

caspase-8 preferred substrate, Ac-IETD-AFC. As Fig. 3*B* shows, whereas immunocaptured procaspase-8 failed to display any activity in this assay, proteolytically processed caspase-8 derived from cell-free extracts that had been incubated for 2 h at 37 °C with granzyme B, or Cyt *c*/dATP, was catalytically active. In contrast, caspase-8 immunoprecipitates generated from cell-free extracts treated with granzyme B at 4 °C (where granzyme B did not proteolytically process caspase-8) did not display any IETD-AFC hydrolysis activity (Fig. 3*C*). The latter result excluded the possibility that granzyme B was nonspecifically captured during the caspase-8 immunoprecipitations and contributed to the IETD-AFC hydrolysis activity seen. Essentially similar results were observed using four different caspase-8 antibodies, with granzyme B-mediated processing, or Cyt *c*/dATP-stimulation, generating catalytically active caspase-8 in all cases (Fig. 3*B*). Furthermore, caspase-8 immunocaptured from granzyme B or Cyt *c*/dATP-treated extracts also displayed proteolytic activity toward one of its natural substrates, caspase-3 (Fig. 3*D*).

We also explored whether caspase-8 immunocaptured from Fas-stimulated cells displayed enhanced catalytic activity compared with caspase-8 captured from granzyme B- or Cyt *c*/dATP-treated cell-free extracts. However, as Fig. 4 illustrates, similar rates of IETD-AFC hydrolysis were observed irrespective of whether caspase-8 was immunoprecipitated from cell-free extracts generated from anti-Fas-stimulated cells, or Cyt *c*/dATP-treated cell-free extracts.

To determine whether the caspase-8 adaptor protein FADD plays any role in the activation of caspase-8 in granzyme B- or Cyt *c*/dATP-stimulated cell-free extracts, we used extracts generated from FADD-deficient Jurkat cells (Fig. 5 *A*; Ref. 17). Similar to extracts derived from wild-type Jurkat cells, FADD-deficient extracts readily supported granzyme B- or Cyt *c*/dATP-induced caspase-8 proteolytic processing, as expected (Fig. 5*B*). Moreover, immunoprecipitation of caspase-8 from FADD-deficient extracts after treatment with granzyme B or Cyt *c*/dATP yielded catalytically active protease (Fig. 5 *C* and *D*). These data exclude the possibility that FADD plays a role as a dimerization platform for caspase-8 in the context of granzyme B- or caspase-6-initiated caspase-8 activation.

It has been reported that the inactive caspase-8 zymogen exists primarily as a monomer (9). Because we observed that caspase-8 acquired catalytic competence upon direct proteolytic processing by granzyme B or caspase-6, we wondered whether we could detect a shift of caspase-8 from a monomeric state to a dimeric state under these conditions. To address this question, we ran native PAGE of cell-free extracts that were either untreated or were exposed to granzyme B or Cyt *c*/dATP to initiate caspase-8 processing and activation. As Fig. 6*A* illustrates, no significant shift to a dimeric state was detectable after proteolytic processing of caspase-8. In sharp contrast, caspase-3 displayed a very significant increase in apparent molecular mass in the same assay (Fig. 6*A*). However, it should be noted that two bands of caspase-8-immunoreactive material, which may represent dimeric or oligomeric forms of this protease, were seen under all conditions (Fig. 6*A*). This could indicate that caspase-8 may exist in an equilibrium between a monomeric and dimeric state even in healthy cells. Proteolytic processing of the small fraction of preexisting dimers may therefore liberate the catalytic activity of this enzyme. To ex-

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plore further whether proteolytic processing of caspase-8 could promote dimerization of this protease we also used a covalent cross-linker, bismaleimidohexane (Fig. 6*B*). Using this approach, we again observed a small fraction of caspase-8-immunoreactive material that may represent dimeric or oligomeric caspase-8 complexes even in control cell lysates. A small increase in the abundance of these complexes was seen after proteolytic processing of caspase-8 by granzyme B or caspase-6 (Fig. 6*B*). However, the majority of caspase-8 clearly remained as a monomer (Fig. 6*B*).

DISCUSSION

The data presented herein argue that direct interchain proteolysis of caspase-8 is sufficient to generate catalytically active protease. Thus, the appearance of caspase-8-processing signatures is a valid means of assessing caspase activation status in the context of cytotoxic T lymphocyte/natural killer-mediated killing or apoptosome-initiated caspase-8 activation. Based upon native PAGE analysis and size exclusion chromatography, it has been argued that caspase-8 exists primarily in monomeric form within cells (9). If this is correct, our data suggest that proteolytically processed caspase-8 may be active as a monomer, or alternatively, that proteolysis of this enzyme promotes dimer formation. In support of the latter view, it has been found that substrate binding promotes formation of caspase-8 dimers (10). Moreover, it has also been shown that although recombinant caspase-8 autoprocesses and exists in an equilibrium between dimers and monomers, noncleavable caspase-8 exists predominantly (>97%) as a monomer. The latter observation argues that proteolytic processing of caspase-8 facilitates dimer formation. Thus, in the context of granzyme B or caspase-6-mediated activation, caspase-8 monomers may form dimers spontaneously upon proteolytic processing to a two-chain enzyme. However, using native PAGE analysis as well as cross-linking experiments, we did not find convincing evidence in support of the latter idea. Alternatively, the proteolytic activity we observe in our experiments may be the result of interchain proteolysis of a small proportion of preexisting caspase-8 dimers in healthy cells. Indeed, native PAGE and cross-linking experiments consistently detected caspase-8-immunoreactive material running at a molecular mass higher than expected in cell-free extracts derived from healthy cells. Because we did not detect proteolytic activity associated with procaspase-8 immunoprecipitated from extracts generated from viable cells, preexisting dimers, if present, are catalytically inactive.

Although our data do not rule out the possibility that caspase-8 may acquire catalytic competence in the absence of proteolytic maturation in certain contexts, we show that enforced dimerization is not essential for caspase-8 activation. Thus, there may be situations in which induced dimerization may indeed be sufficient to achieve catalytic competence of caspase-8, as proposed recently (9). However, notwithstanding this possibility, our data argue that interchain proteolysis of native caspase-8 is sufficient to drive activation of this caspase and that a dimerization platform is not a prerequisite for activation of this protease.

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