FLIP<sub>L</sub> induces caspase 8 activity in the absence of interdomain caspase 8 cleavage and alters substrate specificity

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Caspase 8 is an initiator caspase that is activated by death receptors to initiate the extrinsic pathway of apoptosis. Caspase 8 activation involves dimerization and subsequent interdomain autoprocessing of caspase 8zymogens, and recently published work has established that elimination of the autoprocessing site of caspase 8 abrogates its pro-apoptotic function while leaving its proliferative function intact. The observation that the developmental abnormalities of caspase 8-deficient mice are shared by mice lacking the dimerization adapter FADD (Fas-associated death domain) or the caspase parologue FLIP<sub>L</sub> [FLICE (FADD-like interleukin 1β-converting enzyme)-inhibitory protein, long form] has led to the hypothesis that FADD-dependent formation of heterodimers between caspase 8 and FLIP<sub>L</sub> could mediate the developmental role of caspase 8. In the present study, using an inducible dimerization system we demonstrate that cleavage of the catalytic domain of caspase 8 is crucial for its activity in the context of activation by homodimerization. However, we find that use of FLIP<sub>L</sub> as a partner for caspase 8 in dimerization-induced activation rescues the requirement for intersubunit linker proteolysis in both protomers. Moreover, before processing, caspase 8 in complex with FLIP<sub>L</sub> does not generate a fully active enzyme, but an attenuated species able to process only selected natural substrates. Based on these results we propose a mechanism of caspase 8 activation by dimerization in the presence of FLIP<sub>L</sub>, as well as a mechanism of caspase 8 functional divergence in apoptotic and non-apoptotic pathways.

Key words: activation mechanism, apoptosis, caspase 8, protein dimerization.

INTRODUCTION

The caspases are a family of proteases, 11 in humans, that cleave substrate proteins at aspartic-acid-containing motifs (reviewed in [1—3]). They have diverse biological functions, with ‘inflammatory’ caspases 1, 4 and 5 playing key roles in cytokine maturation and inflammation, caspase 14 being required for keratinocyte maturation [4], and the remaining caspases being involved primarily in apoptosis, but possibly also in proliferative events [5]. The role of caspases in different cell-fate decisions (death or proliferation) is best exemplified by caspase 8.

Caspase 8 (UniProt I.D. Q14790) initiates apoptosis in response to ligation of the ‘death receptors’, a subset of the TNF (tumour necrosis factor) receptor superfamily. Death receptor ligation leads to receptor clustering and recruitment of a variety of proteins to the receptors’ cytosolic tails to form the DISC (death-inducing signalling complex) [6,7]. Among the proteins recruited are FLIP<sub>L</sub> [FLICE (FADD-like interleukin 1β-converting enzyme)-inhibitory protein] (UniProt I.D. O15519) and FADD (UniProt I.D. Q13158) protein, an adapter that in turn recruits FLIP and caspase 8 via their DEDs (death effector recruitment domains) [8]. Caspase 8 is thought to be activated in the DISC by homodimerization, and subsequently undergoes a series of internal proteolytic cleavages [9,10]. The result is a mature dimeric caspase that signals by cleaving a number of protein substrates, including executioner caspases and the Bcl-2 family protein Bid, to engage the pathway of apoptosis.

In addition to its role as an inducer of apoptosis, genetic evidence has revealed a requirement for caspase 8 in normal development and innate immunity. Caspase 8-deficient mice do not survive to birth owing to a defect in cardiac development [11], and creation of tissue-specific caspase 8-knockout animals revealed that T- and B-cells lacking caspase 8 fail to undergo normal activation when stimulated via their antigen receptors [12—16]. Roles for non-catalytic caspase 8 in epithelial cell survival have been proposed [17,18], but we have recently shown that rescue of caspase 8 deficiency in the haematopoietic compartment requires the catalytically active form [19].

The homodimerization process outlined above represents a minimalist view of the mechanism of caspase 8 activation, since other proteins are also present in the DISC, most importantly FLIP<sub>L</sub> which is expressed as a short and a long isoform: FLIP<sub>S</sub> and FLIP<sub>L</sub>, respectively [20]. The short isoform mimics the pro-domain of caspase 8 and blocks caspase 8 activation in a dominant-negative fashion. The long isoform broadly homologous with the full-length caspase 8zymogen in that it contains a pro-domain and regions related to the catalytic domain, as well as interdomain linker regions containing aspartic-acid-cleavage sites. However, FLIP<sub>L</sub> lacks crucial residues responsible for proteolytic activity, and therefore cannot by itself be a protease (reviewed in [21]).

Abbreviations used: Ac-IETD-afc, N-acetyl-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethylcoumarin; B-VAD-fmk, biotinylated Val-Ala-Asp-fluoromethylketone; CHX, cycloheximide; DED, death effector recruitment domain; DISC, death-inducing signalling complex; DTT, dithiothreitol; FADD, Fas-associated death domain; FBS, fetal bovine serum; FKBP, FK506-binding protein; FLIP, FLICE (FADD-like interleukin 1β-converting enzyme)-inhibitory protein; FLIP<sub>L</sub>, long form of FLIP; FLIP<sub>S</sub>, short form of FLIP; FRB, FKB12 rapamycin binding; HDAC, histone deacetylase; HEK, human embryonic kidney; IPTG, isopropyl β-D-thiogalactopyranoside; RIPK, RIP (receptor-interacting protein) kinase; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; Z-VAD-fmk, benzyl-oxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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Thus, in addition to forming a homodimer, the caspase 8 zymogen can form a heterodimeric complex with FLIP, resulting in an enzyme with only one active site. The influence of FLIP\(_1\) on caspase 8 activation has been controversial, with various studies reporting that FLIP\(_1\) either inhibits or enhances caspase 8 activation (reviewed in [22]). Later reports have explained these apparently contradictory findings with the observation that the monomeric caspase 8 zymogen preferentially binds FLIP\(_1\), rather than a second molecule of caspase 8 [23]. Therefore high FLIP\(_1\) concentrations at the DISC lead to competition with caspase 8 zymogens for binding to FADD, whereas at concentrations comparable with caspase 8 zymogen, FLIP\(_1\) preferentially forms heterodimers leading to caspase 8 activation. Importantly, FLIP\(_1\), can also bind to and activate non-cleavable caspase 8 mutants [9,23]. The structures of the caspase 8 zymogen, as well as the FLIP\(_1\), caspase 8 heterodimer have been solved, revealing that, as predicted [23], binding to non-cleavable caspase 8 by FLIP\(_1\), allows rearrangement of the catalytic loops to form an active site in the absence of autoprocessing [24,25].

Clues to the role of caspase 8 in normal development come from the finding that the cardiac defect observed in caspase 8-deficient mice is shared by both FLIP\(_1\), and FADD-deficient animals [26,27]. This observation, coupled with the finding that non-cleavable caspase 8 can rescue the developmental, but not the apoptotic, defects of the caspase 8-deficient animal [28], has led to the hypothesis that FADD-dependent formation of a heterodimer composed of non-cleaved caspase 8 and FLIP\(_1\), could allow limited activation of caspase 8. A recent report describes that this heterodimer is only able to cleave DISC-proximal substrates [29], and additional reports have indicated that, in the context of immune cell proliferation, caspase 8 is required to inactivate the process of programmed necrosis [15], possibly via cleavage of the DISC-associated RIPs [RIP (receptor-interacting protein) kinases] RIPK-1 and/or RIPK-3 [30,31].

Despite significant evidence indicating its importance, the FLIP\(_1\), caspase 8 heterodimer has been difficult to study biochemically. In earlier reports, high concentrations of the kosmotropic salt sodium citrate was used to induce dimerization of purified FLIP\(_1\), and caspase 8 proteins lacking DED-containing pro-domains (termed ΔDED) [23]. Although informative, these studies could not rule out formation of homodimeric as well as heterodimeric species, and when interpreting these results, one must also take into account the very high non-physiological salt concentrations used. A more recent study used purified proteins to reconstitute the DISC in vitro [29]. However, the insoluble nature of the caspase 8 and FLIP\(_1\), pro-domains necessitated use of in vitro transcription and translation to produce these proteins, confounding precise quantification of the amounts of active protein present and precluding large-scale activity and substrate studies.

In the present study, we employ an inducible heterodimerization system to specifically induce the formation of FLIP\(_1\),—caspase 8 heterodimers. This system takes advantage of the naturally occurring heterodimer-inducing properties of rapamycin, which binds to FKBP (FK506-binding protein) as well as to mTOR (mammalian target of rapamycin) with high affinity, effectively joining the two together [32,33]. Optimization of both of these binding domains through mutation, as well as chemical alteration of rapamycin, has produced a technology where proteins of interest can be induced to heterodimerize with high specificity and affinity, using an engineered dimerization domain known as FRB (FKB12 rapamycin binding) [34]. Using this technology we have created fusion proteins corresponding to caspase 8 and FLIP\(_1\), in which the pro-domains are replaced by the heterodimerization domains, whereas the introduction of a series of specific point mutations at the interdomain cleavage sites of both proteins allows us to study the relevance of cleavage of either protein in caspase 8 activation. Armed with these reagents we sought to test the relationship between interdomain cleavage, homodimerization and heterodimerization of caspase 8, as well as substrate specificity on natural proteins.

**EXPERIMENTAL**

**Cloning**

Full-length caspase 8 isoform a was cloned into pcDNA3 with a C-terminal HA (haemagglutinin) tag and mutations were carried out by overlapping PCR [35]. FKBP—caspase 8 constructs were obtained by cloning the region of caspase 8 corresponding to amino acid 206 to the C-terminus into the SpeI site of the pC\(_2\)R\(_1\)E vector (Ariad Pharmaceuticals). A four-glycine linker was added between the FKBP domain and the caspase domain. Transient expression studies using FBKP—caspase 8 were carried out using this vector. FRB—FLIP\(_1\), constructs were generated by replacing the first 187 amino acids of human FLIP\(_1\), with the FRB domain obtained from the pC\(_2\)-R\(_1\)E plasmid from Ariad Pharmaceuticals. For in vitro expression FKBP—caspase 8 genes were subcloned into the pET28b vector and FRB—FLIP\(_1\), mutants were subcloned into pET29b.

**Protein expression and purification**

All proteins except mouse Bid were based on the human sequences. FKBP—caspase 8 mutants were expressed in *Escherichia coli* BL21(DE3) as N-terminal His-tagged constructs in pET28b. Upon induction with 0.4 mM IPTG (isopropyl β-D-thiogalactopyranoside), cells were grown at 25°C for 4 h and proteins were purified by Ni\(^{2+}\)-affinity chromatography, followed by further purification on the mono-Q anion-exchange Sepharose using 50 mM Tris/HCl (pH 8) buffer and a linear gradient from 0 to 1 M NaCl. FRB—FLIP\(_1\), mutants were expressed as C-terminal His-tagged proteins in pET29b. Upon induction with 1 mM IPTG, cells were grown at 25°C for 16 h and purified by Ni\(^{2+}\)-affinity chromatography. FRB—FLIP\(_1\), was stored at 4°C for up to 1 month. A small amount of 0.1 M NaCl and 10% glycerol. ADED caspase 8 (active and catalytic mutant) was purified as described previously [39]. Full-length caspase 8 with uncleavable pro-domain and solubilization mutants (D210A, D216A, D223A, F122S and I27S), were cloned into pET29b with a C-terminal His tag and expressed in *E. coli* BL21(DE3) after induction with 0.3 mM IPTG at 18°C for 16 h. DEPs of caspase 8, DED1+2 (1–189, F122Y and L123S) and DED1 (1–80, F24S and I27S), were cloned into pET29b with a C-terminal His tag and expressed in *E. coli* after induction with IPTG at 0.4 mM at 20°C for 5 h. Pro-caspase 3 (C285A), pro-caspase 6 (C285A), pro-caspase 7 (C285A), Bid and ΔDED—FLIP\(_1\), were expressed as His-tagged proteins and purified following the protocol for caspase purification [36]. Full-length HDAC7 (histone deacetylase 7) [37] and RIPK-1 in pcDNA3 (a gift from Dr Jurgen Tschopp, Department of Biochemistry, University of Lausanne, Lausanne, Switzerland) were transiently expressed for 24 h as FLAG-tagged proteins in HEK (human embryonic kidney)-293A cells and purified using M2 anti-FLAG beads (Sigma) as described previously [38]. Except for FRB—FLIP\(_1\), all proteins were stored at −80°C.

**Caspase assays and titration**

FKBP—caspase 8 mutants were diluted to 10–50 nM into caspase buffer [10 mM Pipes (pH 7.2), 0.1 M NaCl, 1 mM EDTA,
10% sucrose, 0.05% CHAPS and 5 mM DTT (dithiothreitol), followed by addition of homodimerizer compound AP20187 at stoichiometric concentrations. For activation with kosmotropes, assay buffer contained 30 mM Tris/HCl (pH 7.4), 1 M sodium citrate, 5 mM DTT and 0.05% CHAPS. Mixtures were incubated at 25°C for 30–45 min, and the activity was determined at 30°C by using 100 μM Ac-IETD-afc (N-acetyl-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethylcoumarin). Heterodimerization experiments were performed as described above, except that FRB–FLIP, and the heterodimerization compound AP21967 were added at a 4–5-fold molar excess to FKBP–caspase 8. Titration of caspase 8 with Z-VAD-fmk (benzyl-oxycarbonyl-Val-Ala-Asp-fluoromethylketone) has been described previously [39]. Briefly, upon formation of homo- or hetero-dimer, caspase 8 was incubated with serially diluted Z-VAD-fmk for 45 min at room temperature (25°C) and the remaining activity was determined with Ac-IETD-afc.

### Positional-scanning libraries

Positional-scanning substrate combinatorial libraries (P1, P2, and P3 positions) carrying the ACC fluorescent group were synthesized as described in previously published methods [40]. Activated caspase 8 as homo- or hetero-dimer was diluted into caspase buffer at 100–200 nM final concentration and the enzymatic activity was determined against 200 μM total library compounds. Activity was normalized to the highest activity displayed within the same combinatorial library.

### Cleavage of natural substrates

Purified protein substrates were diluted in caspase buffer at 2–3 μM final concentration (HDAC-7 and RIPK-1 were at ~100 nM) and mixed with serially diluted caspase 8, pre-activated as specified in the corresponding Figures. A caspase 8 concentration range was chosen such that the IETD-ase activity was similar for the following three species: caspase 8(mature) 0.1–100 nM, caspase 8(mature–FLIP) 0.2–200 nM and caspase 8(Site-1 mutant–FLIP) 0.4–400 nM. The reaction mixture was incubated for 3 h at 25°C and then terminated by the addition of 3×SDS/PAGE loading buffer. Samples were run on SDS/PAGE (8–18% gels), and the gels were either stained with Coomassie Blue or subjected to transfer on to PVDF membrane for Western blotting (for HDAC-7 and RIPK-1).

### Antibodies

Monoclonal anti-human-(caspase 8) (C15) was a gift from Dr Marcus Peter (Ben May Department for Cancer Research, University of Chicago, Chicago, IL, U.S.A.) (1:100). Anti-FLAG M2 antibodies were from Sigma (1:5000). The anti-FLIP-1 antibody (1:1000) was from Cell Signaling Technologies and the anti-Hsp90 (heat-shock protein 90) antibody (1:5000) was obtained from BD Biosciences. High-capacity neutravidin beads were from Thermoscientific.

### Tissue culture and transfection

NB7 cells [kindly provided by Dr Dwayne Stupack (Pathology Department, University of California San Diego, San Diego, CA, U.S.A.) and Dr Jill Lahti (Tumor Cell Biology Department, St Jude Children’s Research Hospital, Memphis, TN, U.S.A.)] were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (fetal bovine serum), 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Transient transfection of NB7 cells (60–70% confluent) was performed using Nanojuice (1 μg of total DNA plus 0.5 μg of Nanojuice core reagent in serum-free medium per well of a six-well dish) following the manufacturer’s instructions (Novagen). Medium was changed 3 h post-transfection. Efficiency of transfection reached 55–60% as judged by FACS of GFP (green fluorescent protein)-transfected cells. For overexpression studies, NB7 cells were transfected with 0.5 μg of caspase 8 plasmid/well in 12-well dishes for 16 h. For low-expression studies, NB7 cells were transfected with 0.05 μg of caspase 8 plasmid/well in 12-well dishes for 16 h, followed by treatment with 100 ng/ml TNFα and 2 μg/ml CHX (cycloheximide) for an additional 18 h. Cells were harvested by trypsinization, washed with PBS and stained with propidium iodide for determining the sub-G1 population by FACS. At least three independent experiments were performed. HEK-293A cells were maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% heat-inactivated FBS, 2 mM glutamate, 100 units/ml penicillin and 100 μg/ml streptomycin. TNFα was obtained from PeproTech, CHX was from Calbiochem, propidium iodide was from Sigma–Aldrich and TRAIL (TNF-related apoptosis-inducing ligand) was from Alexis Biochemicals. Streptavidin beads were from Pierce.

### Biotoxin pulldowns

NB7 cells plated in 6-cm-diameter dishes were transfected as described above for the low-expression studies for 16 h and treated with B-VAD-fmk (biotinylated-Val-Ala-Asp-fluoromethylketone) (50 μM) for 1 h prior to addition of TNFα (100 ng/ml)/CHX (1 μg/ml) for 18 h. HEK-293A cells were plated at 80% confluence in 10 cm dishes (~5 × 10^6 cells) and treated with B-VAD-fmk (100 μM) and MG132 (5 μM) for 1 h. TRAIL (70 ng/ml) was added and incubated with cells for an additional 3 h. Cells were harvested by scraping, washed in PBS and lysed in mRIPA buffer (50 mM Tris/HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate and 1% Nonidet P40, pH 7.4) for 15 min on ice. High-capacity neutravidin beads (10–20 μl, 50% slurry) were added to the lysate prepared from one plate and incubated overnight at 4°C. Beads were washed three times with mRIPA buffer and once with PBS and resuspended in 35–50 μl of 2×SDS/PAGE buffer per culture dish, boiled and run on SDS/PAGE (8–18% gels).

### RESULTS

#### Construction of regulated dimerization hybrid proteins

Upon expression and purification from E. coli, caspase 8 is produced as a mixture of monomers and homodimers, the latter of which dissociate to monomers with a half-life in the region of 30 min [36,41]. Previous biochemical studies performed by us and others suggested that dimerization of the caspase 8zymogen in the absence of intersubunit cleavage was sufficient to induce pro-apoptotic caspase 8 activity [35,42]. Catalytic domain autoproteolysis that follows dimerization-induced activation stabilizes recombinant ΔDED-caspase 8, but is dispensable for its activation in vitro [41]. Since this result was based on studies using recombinant ΔDED-caspase 8 activated by kosmotropic salts, we wanted to investigate whether this hypothesis is substantiated for caspase 8 activated in conditions mimicking the physiological settings more closely. To do this we adapted a conditional dimerization strategy that we and others have previously used by generating hybrids containing dimerization domains fused to the N-terminus of caspase 8 or FLIP, catalytic domains [9,43–45] (Figure 1). This strategy allows us to enforce homodimerization or heterodimerization of proteins in vitro and in cells by using small molecules, mimicking the expected mode of dimerization.

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Recent studies from our laboratory [45] and other groups [29] have shown that intracellular cleavage of dimeric caspase 8 is not a neutral consequence of its activation, but a critical requirement of caspase 8-mediated cell death. To further dissect the activation mechanism of full-length caspase 8 in cells with respect to its cleavage, we generated autocleavage-prohibitive aspartate/alanine mutations in two locations: between the prodomain and the catalytic domain, and between the large and small subunits of the catalytic domain, and expressed these constructs in either mammalian cells or *E. coli* (Figure 1A). Some mutants contained the natural tandem DEDs, some contained artificial dimerization domains substituting the DEDs. Constructs of caspase 8 and FLIPL carrying the dimerization domains FKBP(Fv) or FRB (Figure 1B), were generated and expressed in *E. coli* (Figure 1C). To construct these FKBP−caspase chimaeric proteins we replaced caspase 8 residues 1−206, which contains the tandem DED domains that drive recruitment to the adaptor molecules of the DISC, with the FKBP domain, leaving intact the linker between DED2 and the catalytic domain. This inducible-dimerization system uses two bifunctional small molecules that stoichiometrically bind to the FKBP or FRB domains, allowing specific and quantitative homodimerization (caspase 8−caspase 8) or heterodimerization (caspase 8−FLIPL) (Figure 1B).

In full-length wild-type caspase 8, the linker between the second DED and the catalytic domain contains aspartate residues potentially cleavable by active caspase 8 (Figure 1A, Site-2). To simulate the spatial relationships as closely as possible we maintained this linker between the artificial dimerization domains and the catalytic domain. Although our purification strategy produced no evidence for cleavage at any of these three sites (Figure 1C), we also generated aspartate/alanine mutations for these residues and performed dimerization experiments with these to control for potential autolytic removal of the dimerization domain. No substantial differences in the activation and activity of Site-2 mutants compared with wild-type were observed (Supplementary Table S1 at http://www.BiochemJ.org/bj/433/bj4330447add.htm). This indicates that these residues are not likely to be targeted for cleavage during the course of our experiments. Indeed, upon addition of the dimerizer compound, the caspase 8 dimerization domain hybrid was able to cleave its prodomain at a very low rate (Supplementary Figure S1A at http://www.BiochemJ.org/bj/433/bj4330447add.htm), showing ~25 % cleavage after 4 h, with preservation of its enzymatic activity up to 6 h (Supplementary Figure S1B). To avoid complications of pro-domain removal, most of our experiments were performed within 45−120 min of addition of the dimerizer. Almost all of our subsequent autocleavage studies focused on Site-1, which contains the combined VETD/S- and LEMD/L-cleavage sites, and the definition of the mutations used to generate the respective constructs is outlined in Figure 1(A).

To measure dimerization-driven activation, the monomeric status of all caspase 8 proteins was ensured by obtaining monomeric fractions from size-exclusion chromatography. As a control for the Site-1 mutant, caspase 8 pre-cleaved in the intersubunit linker during expression in *E. coli*, referred to as ‘mature’ caspase 8, was used.
N-terminal domain-driven homodimerization is insufficient to activate pro-caspase 8

Caspase 8-deficient mammalian cells reconstituted with a caspase 8 Site-1 mutant do not undergo apoptosis upon stimulation with anti-Fas [29,45]. We have confirmed this and demonstrated that, in contrast with wild-type and Site-2 mutant, the caspase 8 Site-1 mutant is incapable of binding B-VAD-fmk when introduced into caspase 8-null NB7 cells, and stimulated to die via the extrinsic pathway (Supplementary Figure S2 at http://www.BiochemJ.org/bj/433/bj4330447add.htm), revealing that catalytic activity is not generated in the Site-1 mutant under these transfection conditions. To explore the mechanism behind this and similar results we designed an inducible dimerization system using fully purified monomeric components that can accurately and quantitatively generate caspase 8 and FLIPζ homodimers and heterodimers. We compared activity generated by controlled dimerization with activity generated in 1.0 M sodium citrate, which maximally activates caspase 8 by a process that includes dimerization [35,41].

Homodimerization fully activated both mature caspase 8 and the Site-2 mutant, but failed to activate variants containing non-cleavable mutations at Site-1 (Figure 2A and Supplementary Table S1), despite evidence that this homodimeric compound efficiently induced dimerization of the purified caspase 8zymogen (Figure 2B). This enhancement is most likely to be due to stabilization of the active dimer against dissociation, since a control heterobifunctional dimerizer failed to activate the caspase (Figure 2D). Importantly, although the Site-1 mutant dimer was inactive in normal buffer, in the presence of equimolar homodimerization compound AP20187, the concentration of sodium citrate required to activate the non-cleavable mutant was substantially decreased (Figure 2C). This implies that the kinetic barrier to activation is lowered by dimerization via the engineered N-terminal domain.

Inactivity of the Site-1 mutant homodimer could be due to limitations of the experimental conditions. However, all scenarios to reconstitute dimerization failed to robustly activate single-chain caspase 8. For example, we tried to assemble an active Site-1 mutant by homodimerization with a catalytic caspase 8(C/A) mutant, which provides a protomer with a cleavable linker, we varied the concentrations of caspase 8 and/or dimerizer over a broad range, and we varied the duration of incubation, pH or the number of dimerization domains added at the N-terminus. Activity was checked using either peptidic substrates Ac-IETD-afc and Ac-LEHD-afc (N-acetyl-L-lys-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin), or the natural substrate pro-caspase 8 in complex with FLIPζ.

We propose that association of uncleaved caspase 8 monomers via their N-terminal dimerization domains, which simulates the natural model of dimerization mediated by the caspase 8 DEDs, is not sufficient to promote catalytic competence, but that it lowers the barrier for efficient formation of the final productive dimeric interface within the activated species.

N-terminal domain heterodimerization of pro-caspase 8 with FLIPζ is sufficient to activate pro-caspase 8

Findings of the present study, as well as our previously published data [45], indicate that Site-1-uncleavable caspase 8 cannot be activated by homodimerization, and neither can it support apoptosis. However, genetic studies have suggested that this species is able to carry out the ill-defined developmental role of caspase 8, indicating that it can still play a role in signalling in the cell [28]. In an effort to reconcile these findings, we sought to determine the effect of heterodimerization of Site-1-caspase 8 with FLIPζ.

The inactive caspase 8 parologue FLIPζ is able to activate caspase 8 by heterodimerization [23]. To generate ordered FLIPζ−caspase 8 heterodimers, we replaced the DEDs of FLIPζ, with the dimerization domain FRB. In the presence of a heterobifunctional small molecule (AP21967), FKBP hybrid proteins dimerize with FRB hybrids, and we used this principle to specifically obtain caspase 8−FLIPζ heterodimers (Figure 1B). Addition of the heterobifunctional dimerizer in the presence of FLIPζ, activated mature caspase 8 and the Site-2 mutant (Figure 2A and Supplementary Table S2 at http://www.BiochemJ.org/bj/433/bj4330447add.htm), to the same extent as their activation by homodimerization (Figure 2A and Supplementary Table S1). Importantly, caspase 8 mutants uncleavable in Site-1 that could not previously be activated by N-terminal homodimerization, were responsive to heterodimerization with FLIPζ and generated robust enzymatic activity, accounting for ∼25% of the enzymatic activity of mature caspase 8 (Figure 2D). This activity was comparable with the activity of the Site-1 homodimer mutants produced by activation in sodium citrate (Figure 2A). Judging by active-site titration experiments (Supplementary Figure S4 at http://www.BiochemJ.org/bj/433/bj4330447add.htm), both cleavable and uncleavable caspase 8 in complex with FLIPζ were maximally activated by heterodimerization, implying that heterodimerization with FLIPζ can fully activate caspase 8 without the need for a kosmotropic salt. Sodium citrate additionally increased the activity of the caspase 8−FLIPζ heterodimers only 2−3-fold (results not shown), consistent with active-site loop ordering, and not-induced dimerization [41].

FLIPζ, similar to caspase 8, carries an aspartate residue in the region of its intersubunit linker, and we determined whether cleavage at this site (LEV/D) influenced caspase 8 activation. During heterodimerization of FLIPζ with uncleavable Site-1 caspase 8, FLIPζ was indeed cleaved (Figure 2D). We mutated the FLIPζ-cleavage site to alanine (D/A), which abrogated cleavage during heterodimerization, but this mutation had no effect on the activation of caspase 8 (Figure 2D). This indicates that, although it is part of the heterodimer, cleaved FLIPζ, is not responsible for improving enzymatic activity. To rule out catalytic differences between wild-type and uncleavable FLIPζ, we co-monitored the kinetics of caspase 8 activation and FLIPζ cleavage during heterodimerization (Supplementary Figure S5 at http://www.BiochemJ.org/bj/433/bj4330447add.htm). Interestingly, the Site-1 mutant of caspase 8 achieved much of its activation before FLIPζ was cleaved, and its activity increased only ∼1.5-fold post-FLIPζ cleavage (Supplementary Figure S5). Indeed, when we quantitatively compared active-site-titrated heterodimers containing FLIPζ with heterodimers containing FLIPζ(D/A) we found no major differences between their catalytic parameters measured against four different artificial substrates (Supplementary Table S2).

Consequently, dimerization of caspase 8 with FLIPζ is sufficient to generate robust enzymatic activity in vitro in the absence of intersubunit linker proteolysis of either protomer.

In cells, uncleavable caspase 8 is activated by dimerization with FLIPζ but not with itself

So far, the results of the present study suggest that caspase 8 bearing prohibitive mutations at cleavage Site-1 is unable...
Figure 2  Caspase 8 uncleavable at Site-1 can only be activated by heterodimerization with FLIP.

(A) Homo- and hetero-dimerization activity assays of FKBP—caspase 8. For homodimerization, FKBP—caspase 8 mutants (25 nM) were dissolved in the assay buffer containing either the homodimerization compound AP20187 (25 nM) or the activator kosmotrope sodium citrate. For heterodimerization, FKBP—caspase 8 mutants (25 nM) were incubated with FRB—FLIP (125 nM) and the heterodimerization compound AP21967 (125 nM). Samples were incubated at 25°C for 30 min to permit complete activation. Relative activity was determined at 30°C with the fluorescent substrate Ac-IETD-afc. Data represent the means for three independent experiments (±S.E.M.). (B) Size-exclusion separation of FKBP—Site-1 mutant (3 μM) in the presence and absence of homodimerizer (3 μM). The caspase 8 mutant (100 μl) was pre-activated in the assay buffer as described above and applied to a Superdex 200 size-exclusion column. Calculation of the apparent molecular mass was based on column pre-calibration with protein standards. (C) FKBP—caspase 8 (Site-1 mutant) (50 nM) activation in the presence of sodium citrate and homodimerizer (500 nM). Caspase 8 was added to a mixture of homodimerizer and sodium citrate dissolved in assay buffer, to reach the indicated concentration of kosmotrope. The mixture was incubated for 30 min at 25°C and activity was monitored using Ac-IETD-afc as described above. (D) Cleavage of FRB—FLIP, at LEVD/G is not required for FKBP—caspase 8 (Site-1 mutant) activation during heterodimerization. FKBP—caspase 8 (Site-1 mutant) (0.7 μM) was activated in the presence of the heterodimerizer AP21967 and FRB—FLIP (wild-type) or FRB—FLIP(D/A) as detailed above. The mixture was split and subjected to IETD-ase activity testing or electrophoresis separation by SDS/PAGE (4–20% gels), followed by Coomassie Blue staining. (E) FRB—FLIP rescues the inactive FKBP—caspase 8 (Site-1 mutant) during co-expression in HeLa cells, inducing cell death. The caspase mutants shown were transiently transfected in HeLa cells with pcDNA3 vectors encoding the FKBP and FRB hybrids, as indicated. Transfection was followed, after 24 h, by treatment with 500 nM heterodimerization compound. Apoptotic cell death was quantified after an additional 24 h by annexin V staining (means ± S.E.M. for three independent experiments). In all panels, IETD-ase is expressed as relative fluorescence units (RFU)/min. For gels, the molecular mass in kDa is indicated on the left-hand side. WT, wild-type.
Figure 3 Substrate specificity of caspase 8 dimers

Specificity was tested for P₂−P₄ positions of a tetrapeptide on a substrate library with fixed P₁ position (aspartate) and an ACC group in P₁'. Mutants of caspase 8 dimers used in the analysis are specified on the side. FKBP−caspase 8 mutants (100–400 nM) were activated either by homodimerization (in the case of mature−mature) or heterodimerization (in the case of mature−FLIPₘ and Site-1 mutant−FLIPₘ) prior to library screening. Full-length caspase 8 containing uncleavable pro-domain and mutations for increased solubility (D210A, D216A, D223A, L122S and F123Y) (0.5 μM), as well as the ΔDED-caspase 8 mutant (50 nM) did not require artificial-induced activation, as they displayed spontaneous enzymatic activity in the assay buffer under the same conditions. The concentration of assembled dimers was chosen based on similar amounts of activity on Ac-IETD-afc prior to library testing.

to self-activate or to induce apoptosis, but heterodimerization with FLIPₘ rescues this phenotype. To assess the functional significance of the FLIPₘ activation of caspase 8 we performed binary co-transfections of HeLa cells with pcDNA3 vectors encoding constructs containing either the FKBP or FRB hybrids. This heterodimerization strategy allowed us to dissect the roles of controlled enforced dimerization in a cellular context, with cell death determined by annexin V binding. Transfection was followed, after 24 h, by treatment with 500 nM heterodimerizer for 6 h to enforce specific caspase 8 or caspase 8−FLIPₘ homo- or hetero-dimers. Enforced homodimerization of wild-type caspase 8 using this strategy resulted in an increase in cell death, as determined by annexin V staining. As expected, and in agreement with the results shown in Figure 2(D), no increase in cell death over background was observed with a Site-1 mutant (Figure 2E). Similar results were previously observed when homodimeric caspase 8 mutants were generated using a homodimerization strategy [45].

Wild-type caspase 8 produced even more pronounced cell death when FLIPₘ heterodimerization was enforced, regardless of the FLIPₘ cleavage status (Figure 2E). Most importantly, when a caspase 8 Site-1 uncleavable mutant was used instead of the wild-type for heterodimerization with FLIPₘ, robust cell death was generated, at levels similar to that induced by the wild-type−wild-type homodimer (Figure 2E). To a lesser extent, but reproducibly consistent, heterodimers of the uncleavable Site-1 mutant with FLIPₘ(D/A) were also able to kill cells. Therefore FLIPₘ was indeed able to rescue the caspase 8 uncleavable mutant from its inert state and promoted it as a productive apoptotic initiator inside cells. In contrast, a heterodimer between inactive caspase 8(C/A) and uncleavable Site-1 caspase 8 was harmless to the cells, supporting the experimental results using recombinant proteins (Supplementary Figure S3). Additionally, owing to the fact that the heterodimer between wild-type caspase 8 and uncleavable caspase 8 Site-1 mutant was almost as toxic as the wild-type−wild-type dimer (Figure 2E), we conclude that cleavage of one protomer is sufficient for activity, in agreement with recent findings [29].

The active-site cleft is restricted in caspase 8−FLIPₘ heterodimers

It has previously been suggested that cleaved and uncleavable caspase 8 have divergent substrate specificities [9,29]. To investigate whether caspase 8 maturation in Site-1 or caspase 8 heterodimerization with FLIPₘ would affect the substrate specificity of caspase 8, we employed a positional-scanning substrate library to scan the P₂, P₃ and P₄ subsite preferences, with P₁ set at aspartate. Because the caspase 8 Site-1 mutant homodimer was mostly inactive in our hands, we turned instead to the caspase 8 heterodimer with uncleavable FLIPₘ. We compared specificities of these heterodimers with homodimers composed of wild-type caspase 8 obtained by enforced dimerization (mature−mature), obtained as the dimeric fraction from freshly expressed ΔDED-wild-type caspase 8, and obtained as the dimeric fraction from freshly expressed wild-type full-length caspase 8. To produce soluble full-length caspase 8 we mutated specific residues in the DEDs, as described in the Experimental section.

Caspase 8 heterodimers with cleavable or uncleavable FLIPₘ had similar substrate profiles. Similarly, only very minor differences in substrate profile were detected for the wild-type caspase 8 homodimer species (Figure 3). However, we noticed a reproducible and substantial difference between homodimers
and heterodimers regarding the tolerance in the P_4 position, and a small preferential difference in the P_3 position (Figure 3). The relatively tolerant P_3 position was tightened up considerably in FLIPL-containing dimers, so that the ability to cleave tetrapeptides with negatively charged (aspartate and glutamate) and aromatic or β-branched (threonine, tryptophan, tyrosine and valine) amino acids at this position was almost completely abrogated in FLIPL heterodimers compared with caspase 8 homodimers. Interestingly, we could not explain this specificity difference when comparing the active-site clefts in the crystal structures of homodimeric caspase 8 (PDB code 1F9E) [46] with caspase 8—FLIPL heterodimer (PDB code 3H11) [24]. Both catalytic clefts are highly superimposable, but each structure is bound to an inhibitor reflecting a snapshot, probably the ground-state binding mode. Given the high mobility of caspase catalytic clefts during substrate binding [1], we propose that these structural snapshots do not reflect the dynamic events that occur during substrate hydrolysis, and therefore are not very useful in interpreting subtle differences in substrate specificity.

In addition to disclosing differences in the substrate specificity between heterodimers and homodimers, this positional-scanning analysis demonstrates that the artificial dimerization domain and the natural DEDs do not interfere with caspase 8 specificity. To confirm this result, we isolated soluble recombinant mutants of DEDs and added them in trans to the active ∆DED-mature-caspase 8. Caspase 8 activity did not change, even in the presence of 60-fold excess of DEDs (results not shown). Taken together these data reveal that the DEDs do not affect the activity or specificity of caspase 8, at least when measured on small peptide substrates.

A limitation of substrate library screens is the inability to predict realistic substrate specificities in the context of residues scoring 100% tolerance in the mixture. Similarly, excluded residues (0% acceptance in the library) may improve their tolerance in a good substrate owing to the influence of preferred nearby or distal residues, as demonstrated in previous structure/sequence-based analyses of protease substrate specificity [47,48].

The non-cleavable caspase 8—FLIPL heterodimer has attenuated activity on most apoptotic substrates, but high activity on HDAC-7

Table 1 Estimated k_{cat}/K_m for cleavage of natural substrates by caspase 8 mutants at 25°C

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cleavage sequence</th>
<th>k_{cat}/K_m (×10^3 M^{-1} s^{-1})</th>
<th>Mature—mature</th>
<th>Mature—FLIPL</th>
<th>Site-1—FLIPL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-caspase 3</td>
<td>IETD/S</td>
<td>3.4</td>
<td>1.7</td>
<td>0.16</td>
<td>&lt;0.08</td>
</tr>
<tr>
<td>Pro-caspase 6, pro-domain</td>
<td>TETD/A</td>
<td>1.2</td>
<td>0.2</td>
<td>&lt;0.08</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Pro-caspase 6, linker</td>
<td>TEVD/A</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Pro-caspase 7</td>
<td>IQAD/S</td>
<td>1.8</td>
<td>1.2</td>
<td>&lt;0.08</td>
<td>&lt;0.08</td>
</tr>
<tr>
<td>Pro-caspase 8, monomer</td>
<td>VETD/S</td>
<td>0.6</td>
<td>0.16</td>
<td>&lt;0.08</td>
<td>&lt;0.08</td>
</tr>
<tr>
<td>Pro-caspase 8, dimer</td>
<td>LEMD/L</td>
<td>0.6</td>
<td>0.08</td>
<td>&lt;0.08</td>
<td>&lt;0.08</td>
</tr>
<tr>
<td>FLIPL</td>
<td>LEVD/G</td>
<td>3.5</td>
<td>2.5</td>
<td>0.21</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Bid</td>
<td>IEDD/S</td>
<td>10.3</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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<tr>
<td>RIPK1</td>
<td>LQLD/C</td>
<td>0.6</td>
<td>0.64</td>
<td>0.16</td>
<td>0.42</td>
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<tr>
<td>HDAC-7</td>
<td>LETD/G</td>
<td>120</td>
<td>120</td>
<td>5.1</td>
<td></td>
</tr>
</tbody>
</table>

All caspases used as caspase 8 substrates harboured the catalytically inactive mutation C285A (caspase 1 nomenclature).

Given the limitations of small substrate libraries, we next sought to explore the activity of caspase 8 and caspase 8—FLIPL on natural substrates. We intended to determine which substrates caspase 8—FLIPL could cleave before processing its own intersubunit linker, an event that controls its transition from attenuated to high activity. In this manner, we might determine the substrates that are cleaved at ‘low’ (non-apoptotic) caspase 8 activity compared with ‘high’ (apoptotic) caspase 8 activity.

To do this, we expressed and purified 11 known caspase 8 substrates and subjected them to in vitro limited proteolysis (Table 1); sample data with two of these substrates are shown in Figure 4. Using a previously described enzymatic assay [49], we serially diluted active-site titrated caspase 8 in assay buffer, such that the activities on the artificial substrate Ac-IETD-afc were comparable among all three species (Figure 4, bottom panels). The proteolytic substrate of interest was added at a constant amount and incubated with caspase 8 over a fixed period of time. The apparent catalytic efficiency parameter, k_{cat}/K_m, was estimated based on the enzyme concentration needed to cleave half of the substrate [49], indicated by an arrow in Figure 4. To ensure that FLIPL was not cleaved during the assay, or could not compete with the natural substrates, non-cleavable FLIPL(D/A) mutant was used in all conditions.

A few salient features were apparent from this analysis. Caspase 8 in trans was not a good substrate for itself, unless it was dimerized prior to exposure to active caspase 8 (Table 1), a result that supports a previous observation [9]. Dimerization of caspase 8 as a substrate increased the k_{cat}/K_m ∼7-fold, suggesting repositioning of the linker to a more exposed environment, as previously predicted [25]. Monomeric caspase 8 or caspase 10, as well as RIPK-1, were equally poor substrates for caspase 8 (Table 1). Interestingly, caspase 8 cleaved pro-caspase 6 only in the pro-domain site at TETD/A, but not at a similar site TEVD/A situated between small and large subunit. By far the highest catalytic efficiency of caspase 8 was shown towards HDAC-7, complementing our previous finding [37].

Finally, as we reported above, the uncleavable Site-1 caspase 8 mutant in a heterocomplex with FLIPL has a decreased activity on the peptide reporter substrate Ac-IETD-afc, approx. 15% compared with mature homodimers or mature caspase 8—FLIPL heterodimers. This lower activity is recapitulated, for the most part (with some exceptions), on protein substrates (Table 1). Overall these data confirm the restricted specificity of heterodimers compared with homodimers, implying that FLIPL narrows the specificity of caspase 8. Perhaps more importantly from a
Functional viewpoint is the observation that the highest activity of Site-1 mutant caspase 8—FLIP₉ relative to mature homodimers was seen for RIPK-1, accounting for more than 25% of the maximum rate, and the high specificity for HDAC-7 was recapitulated in the Site-1 mutant caspase 8—FLIP₉ heterodimer.

**DISCUSSION**

Hughes et al. [29] elegantly demonstrated that reconstitution of a functional DISC using purified Fas, FADD and pro-caspase 8 yielded active caspase 8 with a restricted substrate repertoire, and we have demonstrated that cleavage of caspase 8 at Site-1 is required for full activation [45]. However, the nature of their reconstruction system precludes testing ordered homodimers of caspase 8 and ordered heterodimers of caspase 8 with FLIP₉, and to overcome this limitation we employed the regulatable homo-/hetero-dimerization strategy. Using this strategy we confirm that there was no activation of a non-cleavable caspase 8 species by enforced homodimerization via N-terminal dimerization domains in vitro, consistent with recent reports [29,45]. Since dimerization is a required step for activity of caspases there are three scenarios that could explain caspase 8 activation in vivo: (i) caspase 8 is somehow cleaved before dimerization, (ii) other factors stabilize the uncleaved dimer, and (iii) an early event in caspase 8 activation is heterodimerization with FLIP₉. Scenario (i) is unlikely given that the earliest forms of caspase 8 that can be detected by B-VAD-Fmk correspond to full-length, uncleaved caspase 8 (Supplementary Figure S2 and [19]), indicating that cleavage is not necessary for activity in vivo. Scenario (ii) is consistent with additional forces that promote a ‘zipper effect’ for stabilization of the uncleaved dimer, without the need for FLIP₉ (Figure 5), and indeed Cullin3-mediated ubiquitination of caspase 8 has been reported to stabilize nascent dimers [50]. Heterodimerization with FLIP₉ fulfils the third scenario because it rescues the ability to activate uncleaved caspase 8 without the need for additional factors.

The results of the present study suggest that, at least in some circumstances, FLIP₉ is needed to ignite the lag phase of caspase 8 activation in vivo. This hypothesis is supported because caspase 8 has a higher affinity for FLIP₉ than for itself. Thus it has been shown previously that the $K_d$ for dissociation of FLIP₉—caspase-8 (Site-1 mutant) dimer is smaller than the $K_d$ for dissociation of the mature—mature dimer [23,42] owing to increased hydrogen bonding at the interface visible in the caspase 8—FLIP₉ structure [24]. Consequently, assuming similar affinities of caspase 8 and FLIP₉ to the DISC, heterodimerization would tend to predominate in the early part of caspase 8 activation. Naturally this would depend on the absolute concentrations of caspase 8 and FLIP₉, such that only at FLIP₉ concentrations lower or equivalent to caspase 8 would heterodimerization be quantitative in vivo; high FLIP₉ levels would tend to obliterate caspase activation because the DISC would become saturated with FLIP₉ [51].

Although FLIP₉ activates the caspase 8 Site-1 mutant by heterodimerization, it produces only approx. 20% of the activity of mature caspase 8. This may be due to differences in the primary structures of the intersubunit linker of FLIP₉ and caspase 8, and subsequently due to a different energy of interaction of the linker with the surrounding residues, as suggested by the crystal structure of single-chain caspase 8 in complex with two-chain FLIP₉ [24]. However, it is still not known how the intersubunit linker in the uncleaved FLIP₉ is positioned so that it enables caspase 8 activation at all. It had previously been suggested that...
processing of FLIP<sub>L</sub> dramatically increased the activity of the caspase 8−FLIP<sub>L</sub> heterodimer [24], but our studies disagree with this result (Figure 2D and Supplementary Figure S5) and we believe that the authors have recorded an increase in the generation of new heterodimers after FLIP<sub>L</sub> cleavage, rather than an increase in the activity of pre-formed heterodimers. Possibly by using a large excess of ΔDED-FLIP<sub>L</sub> over ΔDED-caspase 8 to enforce formation of an unknown fraction of heterodimer, the authors missed the fact that, once cleaved, FLIP<sub>L</sub> increases its affinity for caspase 8 [23]. Therefore accumulated free cleaved FLIP<sub>L</sub> incorporated more unbound caspase 8, generating additional heterodimers and skewing the concentration of the total active sites. Our system, ensuring efficient heterodimerization and using active-site-titrated enzymes, has shown that cleavage of FLIP<sub>L</sub> increased the activity of pre-formed heterodimers only ∼ 1.5-fold (Figure 2D and Supplementary Figure S5). The fact that wild-type FLIP<sub>L</sub> was slightly more efficient in killing cells than the non-cleavable FLIP<sub>L</sub>(D/A) during co-expression experiments with caspase 8 (Figure 2D) is thus likely to be due to its more facile association with caspase 8.

Based on the results shown in the present study, we propose distinct mechanisms for caspase 8 activation that depend on FLIP<sub>L</sub> homodimers with pro-caspase 8 would provide the first active protease in the DISC. Certainly FLIP<sub>L</sub> is not required for apoptosis, since it is dispensable for apoptosis induction via the extrinsic pathway as demonstrated by ablation of the gene in mice [26]. However, FLIP<sub>L</sub>, like caspase 8, is required to overcome the developmental block that leads to death at embryonic day 10.5, leading to the suggestion that both are involved in regulating the same proliferation and differentiation events [5]. Therefore caspase 8 is required for both apoptotic and proliferative/differentiation events, whereas FLIP<sub>L</sub> is required only for the proliferative/differentiation ones. We suggest the possibility that it is the heterodimer that is primarily responsible for driving these proliferative/differentiation events.

The mildly restricted specificity of caspase 8−FLIP<sub>L</sub> heterodimer compared with the caspase 8 homodimer on tetrapeptides cannot explain the substantial alterations seen with natural protein substrates for cleavage. Intriguingly, the FLIP<sub>L</sub>−caspase 8 heterodimer, which displayed attenuated activity towards the Ac-IETD-afc substrate, cleaved HDAC-7 much more efficiently than it cleaved well-known apoptotic substrates such as Bid, caspase 3 or FLIP<sub>L</sub>, and RIPK1 was cleaved equally well by homodimers and heterodimers (Table 1). These two substrates stand out as potentially non-apoptotic targets of caspase 8, since RIPK1 has previously been suggested to be involved in the proliferative function of caspase 8 [52] and, although we cannot classify HDAC-7 as a genuine non-apoptotic substrate, previous studies have shown that HDAC-7 is processed at concentrations of active caspase 8 that were inherently non-toxic/non-apoptotic to the cell [37]. However, it is not the purpose of the present study to quantitatively define ‘non-apoptotic’ caspase 8 activity. For example, the construct that generated the weakest enzymatic activity <i>in vitro</i>, namely caspase 8(Site-1 mutant)−FLIP<sub>L</sub>(D/A) heterodimer, was actually mildly apoptotic when introduced into cells (Figure 2E) and it also cleaved Bid <i>in vitro</i> (Table 1). Therefore we cannot set a threshold of non-apoptotic events to the activity of this mutant, although under certain cellular conditions (fast FLIP<sub>L</sub> degradation, for example, or transient DISC formation), the heterodimer could be a good candidate for initiating brief spikes of enzymatic activity that would in principle support a non-apoptotic role for caspase 8.

**AUTHOR CONTRIBUTION**

Cristina Pop, Andrew Oberst and Bram van Raam designed experiments, analysed results and wrote the manuscript; Marcin Drag designed and synthesized the positional-scanning substrate library; Stefan Riedl designed full-length caspase 8 solubility mutants; Stefan Riedl, Douglas Green and Guy Salvesen designed the study, analysed results and wrote the manuscript.

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FLIP\textsubscript{L} induces caspase 8 activity in the absence of interdomain caspase 8 cleavage and alters substrate specificity

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Figure S1 Stability of FKBP–caspase 8 activity

The majority of FKBP–caspase 8(mature) activation occurs within 15–30 min post-homodimerizer addition, prior to autocleavage of the pro-domain. (A) Kinetics of pro-domain autocleavage. Dimerized FKBP–caspase 8(mature) (left-hand panel) or control sample FKBP–caspase 8(Site-2 mutant) (right-hand panel) were dissolved in assay buffer at 0.5 \mu M and incubated for the specified duration at 25°C. Reactions were stopped with loading buffer and analysed by SDS/PAGE (4–20 % gels) stained with Coomassie Blue. The molecular mass in kDa is indicated on the left-hand side. (B) Kinetics of caspase activation by homodimerization. At the end of the incubation time, samples from (A) were diluted at 25 nM in caspase buffer and their IETD-ase activity (relative fluorescence units (RFU)/min) was immediately recorded. Most experiments for the present study were performed within 45 min–1 h post-dimerization.

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2 Correspondence may be addressed to either of these authors (email douglas.green@stjude.org or gsalvesen@sanfordburnham.org).
**Figure S2** NB7 cells reconstituted with caspase 8 mutants

(A) Scheme of full-length caspase 8 and \( \Delta \)DED-caspase 8 constructs used for cellular expression. (B) Active-site labelling of caspase 8 in cells. NB7 cells devoid of caspase 8 were transiently reconstituted with non-toxic amounts of caspase 8 mutants (50 ng/well for 12-well plates) and then treated with TNF\( \alpha \)/CHX. B-VAD-fmk (50 \( \mu \)M) was added to the cells 1 h prior to the addition of TNF\( \alpha \)/CHX, followed by 18 h incubation. Cell lysates were subjected to pull-down using avidin beads followed by Western blotting against the specified proteins. The molecular mass in kDa is indicated on the left-hand side. IP, immunoprecipitation; WB, Western blot.

**Figure S3** Activation of FKBP-caspase 8(Site-1 mutant) (50 nM) by the homodimerization compound (50 nM) in the presence of catalytically inactive FKBP-caspase 8(C/A) (500 nM), sodium citrate (0.7 M) or \( \Delta \)DED–FLIP\(_L\) (50–500 nM)

For experiments where the concentration of FKBP–caspase 8(Site-1) was varied, 50–500 nM enzyme was used with proportional amounts of dimerizer. IETD-ase is expressed as relative fluorescence units (RFU)/min.
**FKBP-Casp-8 + FRB-FLIP<sub>L</sub> + AP21967 heterodimerizer**

Figure S4  Active-site titration of caspase 8 heterodimers

FKBP–caspase 8 (50 nM based on absorbance at 280 nm) was activated with FRB–FLIP<sub>L</sub> (250 nM) and heterodimerization compound (250 nM) as described in the Experimental section of the main text. Equal volumes of activated caspase 8 and Z-VAD-fmk solutions were mixed to generate 25 nM caspase 8 and the final concentrations of Z-VAD-fmk shown, followed by 30 min incubation at 25°C. The remaining enzymatic activity was quantified using the Ac-IETD-afc substrate at 30°C. The Figure shows that 80–100% of the active sites are available for catalysis.

**FKBP-Casp-8(Site-1) + FRB-FLIP<sub>L</sub> + AP21967 heterodimerizer**

Figure S5  FLIP<sub>L</sub> cleavage at LEVD/G by caspase 8 following its heterodimerization with FLIP

FKBP–caspase 8(Site-1 mutant) (500 nM), FRB–FLIP<sub>L</sub> (2 μM) and the heterodimerization compound (2 μM) were mixed in the assay buffer and incubated at 25°C for the duration shown. The reaction was stopped with 3×SDS buffer and samples were run on SDS/PAGE (4–20% gels), followed by Coomassie Blue staining (right-hand panel). IETD-ase activity (left-hand panel) was determined for the samples containing FKBP–caspase 8(Site-1 mutant). The molecular mass in kDa is indicated on the left-hand side.
Table S1  Catalytic parameters of FKBP-caspase 8 homodimers at 30°C

(a) $k_{cat}$ (s$^{-1}$)

<table>
<thead>
<tr>
<th>Caspase 8 dimer</th>
<th>Mature</th>
<th>Site-2 mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>IETD</td>
<td>0.325 ± 0.004</td>
<td>0.45 ± 0.004</td>
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<td>LEHD</td>
<td>1.08 ± 0.05</td>
<td>1.56 ± 0.1</td>
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<tr>
<td>DEVD</td>
<td>0.12 ± 0.003</td>
<td>0.17 ± 0.003</td>
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<tr>
<td>LEVD</td>
<td>0.023 ± 0.0005</td>
<td>0.033 ± 0.0005</td>
</tr>
</tbody>
</table>

(b) $K_m$ (μM)

<table>
<thead>
<tr>
<th>Caspase 8 dimer</th>
<th>Mature</th>
<th>Site-2 mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>IETD</td>
<td>21.7 ± 1.1</td>
<td>28.3 ± 1.1</td>
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<tr>
<td>LEHD</td>
<td>135 ± 13.24</td>
<td>160 ± 22</td>
</tr>
<tr>
<td>DEVD</td>
<td>8 ± 2.5</td>
<td>24.9 ± 1.7</td>
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<tr>
<td>LEVD</td>
<td>0.9 ± 0.2</td>
<td>2.5 ± 0.2</td>
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</tbody>
</table>

(c) $k_{cat}/K_m$ (M$^{-1}$.s$^{-1}$)

<table>
<thead>
<tr>
<th>Caspase 8 dimer</th>
<th>Mature</th>
<th>Site-2 mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>IETD</td>
<td>1.5 × 10$^4$</td>
<td>1.6 × 10$^7$</td>
</tr>
<tr>
<td>LEHD</td>
<td>0.8 × 10$^7$</td>
<td>0.97 × 10$^7$</td>
</tr>
<tr>
<td>DEVD</td>
<td>0.5 × 10$^3$</td>
<td>0.7 × 10$^3$</td>
</tr>
<tr>
<td>LEVD</td>
<td>1.2 × 10$^3$</td>
<td>1.3 × 10$^3$</td>
</tr>
</tbody>
</table>

Table S2  Catalytic parameters of FKBP-caspase 8–FRB–FLIP$_2$ heterodimers at 30°C

(a) $k_{cat}$ (s$^{-1}$)

<table>
<thead>
<tr>
<th>Caspase 8 dimer</th>
<th>Mature–FLIP$_2$</th>
<th>Mature–FLIP$_2$(D/A)</th>
<th>Site-2–FLIP$_2$</th>
<th>Site-2–FLIP$_2$(D/A)</th>
<th>Site-1–FLIP$_2$</th>
<th>Site-1–FLIP$_2$(D/A)</th>
<th>Site-1+2–FLIP$_2$</th>
<th>Site-1+2–FLIP$_2$(D/A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IETD</td>
<td>0.27 ± 0.006</td>
<td>0.36 ± 0.003</td>
<td>0.53 ± 0.01</td>
<td>0.52 ± 0.01</td>
<td>0.073 ± 0.002</td>
<td>0.121 ± 0.016</td>
<td>0.09 ± 0.004</td>
<td>0.124 ± 0.003</td>
</tr>
<tr>
<td>LEHD</td>
<td>0.89 ± 0.02</td>
<td>1.15 ± 0.02</td>
<td>1.41 ± 0.02</td>
<td>1.48 ± 0.01</td>
<td>0.18 ± 0.002</td>
<td>0.196 ± 0.02</td>
<td>0.2 ± 0.001</td>
<td>0.215 ± 0.004</td>
</tr>
<tr>
<td>DEVD</td>
<td>0.10 ± 0.005</td>
<td>0.129 ± 0.003</td>
<td>0.18 ± 0.008</td>
<td>0.18 ± 0.005</td>
<td>0.05 ± 0.003</td>
<td>0.066 ± 0.002</td>
<td>0.06 ± 0.003</td>
<td>0.071 ± 0.002</td>
</tr>
<tr>
<td>LEVD</td>
<td>0.02 ± 0.0006</td>
<td>0.03 ± 0.0006</td>
<td>0.049 ± 0.001</td>
<td>0.03 ± 0.0006</td>
<td>0.02 ± 0.001</td>
<td>0.028 ± 0.0007</td>
<td>0.029 ± 0.001</td>
<td>0.032 ± 0.006</td>
</tr>
</tbody>
</table>

(b) $K_m$ (μM)

<table>
<thead>
<tr>
<th>Caspase 8 dimer</th>
<th>Mature–FLIP$_2$</th>
<th>Mature–FLIP$_2$(D/A)</th>
<th>Site-2–FLIP$_2$</th>
<th>Site-2–FLIP$_2$(D/A)</th>
<th>Site-1–FLIP$_2$</th>
<th>Site-1–FLIP$_2$(D/A)</th>
<th>Site-1+2–FLIP$_2$</th>
<th>Site-1+2–FLIP$_2$(D/A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IETD</td>
<td>16.5 ± 1.5</td>
<td>20.4 ± 0.7</td>
<td>26.5 ± 1.9</td>
<td>25.7 ± 2</td>
<td>31.5 ± 3.4</td>
<td>59.4 ± 2.1</td>
<td>27.6 ± 4.6</td>
<td>59.3 ± 3</td>
</tr>
<tr>
<td>LEHD</td>
<td>60.1 ± 4.1</td>
<td>73.5 ± 3.7</td>
<td>64.5 ± 3.2</td>
<td>71.9 ± 2.1</td>
<td>123.4 ± 30.3</td>
<td>122.7 ± 3.6</td>
<td>57.8 ± 9.4</td>
<td>112.5 ± 5</td>
</tr>
<tr>
<td>DEVD</td>
<td>21.8 ± 3.8</td>
<td>20.3 ± 2.1</td>
<td>25.6 ± 3.9</td>
<td>21.9 ± 2.4</td>
<td>101.9 ± 17</td>
<td>172.2 ± 11</td>
<td>75.7 ± 10</td>
<td>175.7 ± 11</td>
</tr>
<tr>
<td>LEVD</td>
<td>4.0 ± 0.46</td>
<td>3.7 ± 0.4</td>
<td>5.2 ± 0.9</td>
<td>4.1 ± 0.2</td>
<td>29.2 ± 5</td>
<td>40.9 ± 3</td>
<td>21.6 ± 3</td>
<td>42.6 ± 2.7</td>
</tr>
</tbody>
</table>

(c) $k_{cat}/K_m$ (M$^{-1}$.s$^{-1}$)

<table>
<thead>
<tr>
<th>Caspase 8 dimer</th>
<th>Mature–FLIP$_2$</th>
<th>Mature–FLIP$_2$(D/A)</th>
<th>Site-2–FLIP$_2$</th>
<th>Site-2–FLIP$_2$(D/A)</th>
<th>Site-1–FLIP$_2$</th>
<th>Site-1–FLIP$_2$(D/A)</th>
<th>Site-1+2–FLIP$_2$</th>
<th>Site-1+2–FLIP$_2$(D/A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IETD</td>
<td>1.66 × 10$^4$</td>
<td>1.76 × 10$^4$</td>
<td>2.01 × 10$^4$</td>
<td>2.05 × 10$^4$</td>
<td>0.23 × 10$^4$</td>
<td>0.204 × 10$^4$</td>
<td>0.35 × 10$^4$</td>
<td>0.209 × 10$^4$</td>
</tr>
<tr>
<td>LEHD</td>
<td>1.49 × 10$^4$</td>
<td>1.56 × 10$^4$</td>
<td>2.18 × 10$^4$</td>
<td>2.06 × 10$^4$</td>
<td>0.14 × 10$^4$</td>
<td>0.159 × 10$^4$</td>
<td>0.35 × 10$^4$</td>
<td>0.191 × 10$^4$</td>
</tr>
<tr>
<td>DEVD</td>
<td>0.48 × 10$^4$</td>
<td>0.63 × 10$^4$</td>
<td>0.71 × 10$^4$</td>
<td>0.82 × 10$^4$</td>
<td>0.04 × 10$^4$</td>
<td>0.038 × 10$^4$</td>
<td>0.08 × 10$^4$</td>
<td>0.040 × 10$^4$</td>
</tr>
<tr>
<td>LEVD</td>
<td>0.72 × 10$^4$</td>
<td>0.93 × 10$^4$</td>
<td>0.72 × 10$^4$</td>
<td>1.11 × 10$^4$</td>
<td>0.07 × 10$^4$</td>
<td>0.070 × 10$^4$</td>
<td>0.13 × 10$^4$</td>
<td>0.073 × 10$^4$</td>
</tr>
</tbody>
</table>