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FLIP_L induces caspase-8 activity in the absence of interdomain caspase-8 cleavage and alters substrate specificity

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Abstract

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-8 zymogens, 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 or the caspase paralog FLIP_L has led to the hypothesis that FADD-dependent formation of heterodimers between caspase-8 and FLIP_L could mediate the developmental role of caspase-8. 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_L 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_L does not generate a fully active enzyme, but an attenuated species able to process only select natural substrates. Based on these results we propose a mechanism of caspase-8 activation by dimerization in the presence of FLIP_L, as well as a mechanism of caspase-8 functional divergence in apoptotic and non-apoptotic pathways.

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Author Contributions Cristina Pop, Andrew Oberst and Bram Van Raam designed and experiments, analyzed 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, Doug Green and Guy Salvesen designed the study, analyzed results, and wrote the manuscript.

Keywords

apoptosis; activation mechanism; 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 required for keratinocyte maturation [4], and the remaining caspases primarily involved in apoptosis, but possibly also involved 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 tumor necrosis factor (TNF) receptor superfamily. Death receptor ligation leads to receptor clustering and recruitment of a variety of proteins to the receptors’ cytosolic tails to form the death-inducing signaling complex, or DISC [6, 7]. Among proteins recruited are the FLICE-like inhibitory protein (FLIP – UniProt I.D. O15519) and the Fas-associated death domain (FADD - UniProt I.D. Q13158) protein, an adapter that in turn recruits FLIP and caspase-8 via their death effector recruitment domains (DEDs) [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 due 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 hematopoietic 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 which is expressed as a short and a long isoform - FLIP_S and FLIP_L respectively [20]. The short isoform mimics the prodomain of caspase-8 and blocks caspase-8 activation in a dominant-negative fashion. The long isoform is broadly homologous to the full-length caspase-8 zymogen in that it contains a prodomain and regions related to the catalytic domain, as well as interdomain linker regions containing aspartic acid cleavage sites. However, FLIP_L lacks crucial residues responsible for proteolytic activity, and therefore cannot by itself be a protease – reviewed in [21].

Thus, in addition to forming a homodimer, the caspase-8 zymogen can form a heterodimeric complex with FLIP_L, resulting in an enzyme with only one active site. The influence of FLIP_L on caspase-8 activation has been controversial, with various studies reporting that FLIP_L 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_L, rather than a second molecule of caspase-8 [23]. Therefore, high FLIP_L concentrations at the DISC lead to competition with caspase-8 zymogens for binding to FADD, while at concentrations comparable to caspase-8 zymogen, FLIP_L preferentially forms heterodimers leading to caspase-8 activation. Importantly, FLIP_L can also bind to and activate non-cleavable caspase-8 mutants [9, 23]. Recently, the structures of the caspase-8 zymogen, as well as the FLIP_L-caspase-8 heterodimer have been solved, revealing that, as predicted [23], binding to non-cleavable caspase-8 by FLIP_L allows rearrangement of the catalytic loops to form an active site in the absence of autoprocessing [24, 25].

Clues on 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_L 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_L 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 recently-described process of programmed necrosis [15] possibly via cleavage of the DISC-associated receptor interacting protein (RIP) kinases, RIPK-1 and/or RIPK-3 [30, 31].

Despite significant evidence indicating its importance, the FLIP_L-caspase-8 heterodimer has been difficult to study biochemically. In earlier reports, a high concentrations of the kosmotropic salt sodium citrate was used to induce dimerization of purified FLIP_L and caspase-8 proteins lacking DED-containing prodomains (termed DED) [23]. While informative, these studies could not rule out formation of homodimeric besides 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_L prodomains 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 current study, we employ an inducible heterodimerization system to specifically induce the formation of FLIP_L-caspase-8 heterodimers. This system takes advantage of the naturally occurring heterodimer-inducing properties of rapamycin, which binds to the FK506-binding protein (*FKBP*) as well as to mTOR with high affinity, effectively joining the two together [32, 33]. Optimization of both 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* [34]. Using this technology we have created fusion proteins corresponding to caspase-8 and FLIP_L in which the prodomains are replaced by the heterodimerization domains, while 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 inter-domain 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 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 *Spe*I site of the pC₄-F_V1E vector (Ariad Pharmaceuticals). A 4-glycine linker was added between the *FKBP* domain and the caspase domain. Transient expression studies using *FKBP*-caspase-8 were carried out using this vector. *FRB*-FLIP_L constructs were generated by replacing the first 187 amino acids of human FLIP_L with the *FRB* domain obtained from the pC₄-R_HE plasmid from Ariad Pharmaceuticals. For *in vitro* expression *FKBP*-Caspase-8 genes were sub-cloned into the pET28b vector and *FRB*-FLIP_L mutants were sub-cloned into pET29b.

Protein expression and purification

All proteins except mouse BID were based on the human sequences, and BID. *FKBP*-caspase-8 mutants were expressed in *E. coli* B121DE3 as N-terminal His-constructs in pET28b. Upon induction with 0.4 mM IPTG, cells were grown at 25 °C for 4 h and proteins purified by Ni-affinity chromatography, followed by further purification on the mono-Q anion exchange Sepharose using 50 mM Tris pH 8/NaCl buffer. *FRB*-FLIP_L 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 on Ni-affinity chromatography. *FRB*-FLIP_L was stored at 4 °C for up to one month in 50 mM Tris pH 7.6, 0.1 M NaCl, 10% glycerol.

DED caspase-8 (active and catalytic mutant) was purified as described [39]. Full-length caspase-8 with uncleavable prodomain and solubilization mutants (D210A, D216A, D223A, L122S, F123Y) was cloned in pET15b with a 8xHis-tag at N-terminus and expressed in *E. coli* B121DE3 after induction with 0.3 mM IPTG at 18 °C for 16 h. Death effector domains of caspase-8 DED1+2 (1-189, F122Y, L123S) and DED1 (1-80, F24S, I27S) were cloned in pET29b with C-terminal His tag and expressed in *E. coli* after induction with IPTG at 0.4 mM at 20 °C for 5 h. Procaspace-3 (C285A), procaspase-6 (C285A), procaspase-7 (C285A), BID, and DED-FLIP_L were expressed as His-tagged proteins and purified following the protocol for caspase purification [36]. Full length HDAC-7 [37] and RIPK-1 in pcDNA3 (a kind gift from Dr. Jurg Tschoopp) were transiently expressed for 24 h as FLAG tagged proteins in HEK293A cells and purified using M2 anti FLAG beads (Sigma) as described previously [38]. Except for *FRB*-FLIP_L, 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, 5 mM DTT), followed by addition of homodimerizer compound AP20187 in stoichiometric concentrations. For activation with kosmotropes, assay buffer contained 30 mM Tris/HCl, pH 7.4, 1 M Na-citrate, 5 mM DTT, 0.05% CHAPS. Mixtures were incubated at 25 °C for 30-45 min, and the activity was determined at 30 °C by using 100 μ M Ac-IETD-afc. Heterodimerization experiments were performed as above, except that *FRB*-FLIP_L and the heterodimerization compound AP21967 were added in 4-5 fold molar excess to *FKBP*-caspase-8. Titration of caspase-8 with Benzyl-oxy carbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) was previously described [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 and the remaining activity was determined with Ac-IETD-afc.

Positional scanning libraries

Positional scanning substrate combinatorial libraries (P4, P3 and P2 positions) carrying the ACC fluorescent group were synthesized by previously published methods [40]. Activated caspase-8 as homo- or heterodimer was diluted into caspase buffer at 100-200 nM final concentration and the enzymatic activity was determined against 200 μ M 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 μ M 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_L) 0.2-200 nM, caspase-8 (Site-1 mutant/FLIP_L) 0.4-400 nM. The reaction mixture was incubated for 3 h at 25 °C and then terminated by the addition of 3x SDS-PAGE loading buffer. Samples were run in 8-18 % SDS-PAGE and the gels were either stained with Coomassie blue or subjected to transfer on PVDF membrane for Western blotting (for HDAC-7 and RIPK-1).

Antibodies

Monoclonal anti-human-Caspase-8 (C15) was a kind gift from Dr. Marcus Peter (1:100). Anti FLAG M2 antibodies were from Sigma (1:5000). The anti RIPK-1 antibody (1:1000) was from Cell Signaling Technologies and the anti Hsp90 antibody (1:5000) was obtained from BD Biosciences. High capacity neutravidin beads were from Thermoscientific.

Tissue culture and transfection

NB7 cells (kindly provided by Dwayne Stupack and Jill Lahti) were maintained in RPMI1640 medium supplemented with 10% heat inactivated FBS, 2 mM glutamine, and penicillin/streptomycin (Invitrogen). Transient transfection of NB7 cells (60-70% confluent) was done using Nanojuice (1 μ g total DNA plus 0.5 μ g Nanojuice core reagent in serum-free

medium per well of 6-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 transfected cells. For overexpression studies, NB7 cells were transfected with 0.5 µg caspase-8 plasmid/well in 12-well dishes for 16 h. For low expression studies, NB7 cells were transfected with 0.05 µg caspase-8 plasmid/well in 12-well dishes for 16 h, followed by treatment with 100 ng/mL of TNFα and 2 µg/mL of cycloheximide for an additional 18 h. Cells were harvested by trypsinization, washed with PBS and stained with propidium iodide for determining the subG1 population by FACS. At least three independent experiments were performed. HEK293A cells were maintained in DMEM medium supplemented with 10% heat inactivated FBS, 2 mM glutamine, and penicillin/streptomycin. TNFα was obtained from PeproTech, cycloheximide was from Calbiochem, propidium iodide was from Sigma Aldrich and TRAIL was from Alexis Biochemicals. Streptavidin beads were from Pierce.

Biotin pull-downs

NB7 cells plated in 6 cm dishes were transfected as described above for the low expression studies for 16 h and treated with B-VAD-fmk (50 µM) for 1 h prior to addition of the TNFα (100 ng/mL)/cycloheximide (CHX) (1 µg/mL) for 18 h. HEK293A cells were plated at 80% confluence in 10 cm dishes (~5×10⁶ 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 additional three hours. Cells were harvested by scrapping, washed in PBS and lysed in mRIPA buffer 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 one time with PBS and resuspended in 35-50 µL 2xSDS-PAGE buffer per culture dish, boiled and run in 8-18% SDS-PAGE 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-8 zymogen in the absence of intersubunit cleavage was sufficient to induce pro-apoptotic caspase-8 activity [35, 42]. Catalytic domain auto-proteolysis that follows dimerization-induced activation stabilizes recombinant DED-caspase-8, but is dispensable for its activation *in vitro* [41]. Since this result was based on studies using recombinant DED-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_L catalytic domains [9, 43-45] – see Fig 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 induced by the natural N-terminal DED domains of caspase-8 and FLIP_L.

Recent studies from our laboratory [45] and other groups [29] showed 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 Asp/Ala mutations in two locations: between the pro-domain 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* (Fig 1A). Some mutants contained the natural tandem DEDs, some contained artificial dimerization domains substituting the DEDs. Constructs of caspase-8 and FLIP_L carrying the dimerization domains *FKBP*(Fv) or *FRB* (Fig1B), were generated and expressed in *E. coli* (Fig1C). To construct these *FKBP*/caspase chimeric 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/FLIP_L) (Fig1B).

In full-length wild type caspase-8, the linker between the second DED and the catalytic domain contains Asp residues potentially cleavable by active caspase-8 (Fig 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 (Fig 1C), we also generated Asp/Ala 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 versus wild type were observed (Supplementary Table 1). 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 pro-domain at a very slow rate (Fig S1A), showing ~25% cleavage after 4 h, with preservation of its enzymatic activity up to 6 h (Fig S1B). To avoid complications of pro-domain removal, most of our experiments were performed within 45-120 min of dimerizer addition. 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 Fig 1A. 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 procaspase-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 confirm this and demonstrate that, in contrast to WT and Site-2 mutant, the caspase-8 Site-1 mutant is incapable of binding Biotinyl-Val-Ala-Asp-Fluoromethylketone (B-VAD-fmk) when introduced into caspase-8 null NB7 cells, and stimulated to die via the extrinsic pathway (Fig S2), 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 FLIP_L homodimers and heterodimers. We compared activity generated by controlled dimerization with activity generated in 1.0 M Na-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 (Fig 2A, Supplemental Table 1), despite evidence that this homodimeric compound efficiently induced dimerization of the purified caspase-8 zymogen (Fig 2B). This enhancement is most likely due to stabilization of the active dimer against dissociation, since a control hetero-bifunctional dimerizer failed to activate the caspase (Fig 2D). Importantly, although the Site-1 mutant dimer was inactive in normal buffer, in the presence of equimolar homodimerization compound AP20187, the concentration of Na-Citrate required to activate the non-cleavable mutant was substantially decreased (Fig 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; we varied time 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, or the natural substrate procaspase-3. Except for Na-Citrate addition, none of the above conditions successfully resulted in assembling active Site-1 uncleavable species (Figure S3 and not shown).

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 *per se* 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 procaspase-8 with FLIP_L is sufficient to activate procaspase-8

Findings presented here, as well as our previously published data [45] indicate that Site-1 uncleavable caspase-8 cannot be activated by homodimerization, nor 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 signaling 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 FLIP_L.

The inactive caspase-8 paralogue FLIP_L is able to activate caspase-8 by heterodimerization [23]. To generate ordered FLIP_L/caspase-8 heterodimers, we replaced the DEDs of FLIP_L with the dimerization domain *FRB*. In the presence of a hetero-bifunctional small molecule (AP21967), *FKBP* hybrid proteins dimerize with *FRB* hybrids, and we used this principle to specifically obtain caspase-8/FLIP_L heterodimers (Fig 1B). Addition of the hetero-bifunctional dimerizer in the presence of FLIP_L activated Mature caspase-8 and the Site-2 mutant (Fig 2A and Supplemental Table 2) to the same extent as their activation by homodimerization (Fig 2A and Supplemental Table 1). Importantly, caspase-8 mutants uncleavable in Site-1 that could not previously be activated by N-terminal homodimerization, were responsive to heterodimerization with FLIP_L and generated robust enzymatic activity, accounting for ~25% of the enzymatic activity of mature caspase-8 (Fig 2D). This activity was comparable to the activity of the Site-1 homo-dimer mutants produced by activation in Na-Citrate (Fig 2A). Judging by active site titration experiments (Fig S4), both cleavable and uncleavable caspase-8 in complex with FLIP_L were maximally activated by heterodimerization, implying that heterodimerization with FLIP_L can fully activate caspase-8 without the need for a kosmotropic salt. Na-Citrate additionally increased the activity of the caspase-8/FLIP_L heterodimers by only 2-3 fold (data not shown), consistent with active site loop ordering, and not-induced dimerization [41].

FLIP_L, similar to caspase-8, carries an Asp residue in the region of its inter subunit linker, and we determined whether cleavage at this site (LEVD/G) influenced caspase-8 activation. During heterodimerization of FLIP_L with uncleavable Site-1 caspase-8, FLIP_L was indeed cleaved (Fig 2D). We mutated the FLIP_L cleavage site to Ala (D/A), which abrogated cleavage during heterodimerization, but this mutation had no effect on the activation of caspase-8 (Fig 2D). This indicates that, although it is part of the heterodimer, cleaved FLIP_L is not responsible for improving enzymatic activity. To rule out catalytic differences between wild-type and uncleavable FLIP_L, we co-monitored the kinetics of caspase-8 activation and FLIP_L cleavage during heterodimer reconstitution (Fig S5). Interestingly, the Site-1 mutant of caspase-8 achieved much of its activation before FLIP_L was cleaved, and its activity increased by only ~1.5 fold post FLIP_L cleavage (Fig S5). Indeed, when we quantitatively compared active site-titrated heterodimers containing FLIP_L with heterodimers containing FLIP_L(D/A) we found no major differences between their catalytic parameters measured against four different artificial substrates (Supplementary Table 2).

Consequently, dimerization of caspase-8 with FLIP_L 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 FLIP_L but not with itself

So far, our results suggest that caspase-8 bearing prohibitive mutations at cleavage Site-1 is unable to self activate or to induce apoptosis, but heterodimerization with FLIP_L rescues this phenotype. To assess the functional significance of the FLIP_L 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 hours, by treatment with 500 nM heterodimerizer for 6 h to enforce specific caspase-8 or caspase-8/FLIP_L homo- or heterodimers. Enforced homodimerization of caspase-8 WT using this strategy resulted in an increase of cell death, as determined by annexin V staining. As expected, and in agreement with the earlier results shown in Fig 2D, no increase in cell death over background was observed with a Site-1 mutant (Fig 2E). Similar results were previously observed when homodimeric caspase-8 mutants were generated using a homodimerization strategy [45].

Caspase-8 WT produced even more pronounced cell death when FLIP_L heterodimerization was enforced, regardless of the FLIP_L cleavage status (Fig 2E). Most importantly, when a caspase-8 Site-1 uncleavable mutant was used instead of the WT for heterodimerization with FLIP_L, robust cell death was generated, at levels similar to that induced by the WT/WT homodimer (Fig 2E). To a lesser extent but reproducibly consistent, heterodimers of the uncleavable Site-1 mutant with FLIP_L (D/A) were also able to kill cells. Therefore, FLIP_L was indeed able to rescue 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 caspase-8 Site-1 was harmless to the cells, supporting the experimental results using recombinant proteins (Fig S3). Additionally, due to the fact that the heterodimer between caspase-8 WT and uncleavable caspase-8 Site-1 mutant was almost as toxic as the WT/WT dimer (Fig 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/FLIP_L 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 FLIP_L 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 Asp. Because the caspase-8 Site-1 mutant homodimer was mostly inactive in our hands, we turned instead to the caspase-8 heterodimer with uncleavable FLIP_L. We compared specificities of these heterodimers with homodimers composed of WT caspase-8 obtained by enforced dimerization (Mature/Mature), obtained as the dimeric fraction from freshly expressed DED-WT caspase-8, and obtained as the dimeric fraction from freshly expressed WT full length caspase-8. To produce soluble full length caspase-8 we mutated specific residues in the DEDs, as described under Methods.

Caspase-8 heterodimers with cleavable or uncleavable FLIP_L had similar substrate profiles. Similarly, only very minor differences in substrate profile were detected for the caspase-8 WT homodimer species (Fig 3). However, we noticed a reproducible and substantial difference between homodimers and heterodimers regarding the tolerance in the P₄ position, and a small preferential difference in the P₂ position (Fig 3). The relatively tolerant P₄ position was tightened up considerably in FLIP_L containing dimers, so that the ability to cleave tetrapeptides with negatively charged (Asp, Glu) and aromatic or β -branched amino acids (Thr, Trp, Tyr, Val) at this position was almost completely abrogated in FLIP_L heterodimers compared to 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 1F9E [46] with caspase-8/FLIP_L 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 (data not shown). Together these data reveal that the DEDs do not affect the activity or specificity of caspase-8, at least when measured on small peptidyl substrates.

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

The non-cleavable caspase-8/FLIP_L heterodimer has attenuated activity on most apoptotic substrates but high activity on HDAC-7

Given the limitations of small substrate libraries, we next sought to explore the activity of caspase-8 and caspase-8/FLIP_L on natural substrates. We intended to determine which substrates caspase-8/FLIP_L could cleave before processing its own inter-subunit linker, an event that controls its transition from attenuated to high activity. In this manner, we might determine the substrates that get cleaved at “low” (non-apoptotic) caspase-8 activity versus “high” (apoptotic) caspase-8 activity.

To do this, we expressed and purified eleven known caspase-8 substrates and subjected them to *in vitro* limited-proteolysis (Table 1) – sample data with two of these substrates are shown in (Fig 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 (Fig 4, bottom). The proteolytic substrate of interest was added in a constant amount and incubated with caspase-8 over a fixed period of time. The apparent catalytic efficiency parameter, $k_{cat}/K_{M(app)}$, was estimated based on the enzyme concentration needed to cleave half the substrate [49], indicated by an arrow in Fig 4. To ensure that FLIP_L was not cleaved during the assay, nor could compete with the natural substrates, non-cleavable FLIP_L (D/A) mutant was used in all conditions.

A few salient features are 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 by ~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 procaspase-6 only in the prodomain 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 toward HDAC-7, complementing our previous finding [37].

Finally, as we reported above, the uncleavable Site-1 caspase-8 mutant in a hetero-complex with FLIP_L has a decreased activity on the peptidyl reporter substrate Ac-IETD-afc, about 15% compared to Mature homodimers or Mature caspase-8/FLIP_L 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 versus homodimers, implying that FLIP_L 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/FLIP_L 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/FLIP_L heterodimer.

DISCUSSION

Hughes *et al.* elegantly demonstrated that reconstitution of a functional DISC using purified Fas, FADD, and procaspase-8 yielded active caspase-8 with a restricted substrate repertoire, and that cleavage of caspase-8 at Site-1 is required for full activation [29, 45]. However, the nature of their reconstitution system precludes testing ordered homodimers of caspase-8 and ordered heterodimers of caspase-8 with FLIP_L, and to overcome this limitation we employed the regulatable homo/heterodimerization 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*: 1) caspase-8 is somehow cleaved before dimerization, 2) other factors stabilize the uncleaved dimer, 3) an early event in caspase-8 activation is heterodimerization with FLIP_L. Scenario 1 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 (Fig S2) and reference [19], indicating that cleavage is not necessary for activity *in vivo*. Scenario 2 is consistent with additional forces that promote a ‘zipper effect’ for stabilization of the uncleaved dimer – without the need for FLIP_L - (Fig 5), and indeed Cullin3-mediated ubiquitination of caspase-8 has been reported to stabilize nascent dimers [50]. Heterodimerization with FLIP_L fulfills the third scenario because it rescues the ability to activate uncleaved caspase-8 without the need for additional factors.

Our results suggest that, at least in some circumstances, FLIP_L 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 FLIP_L than for itself. Thus, it has previously been shown that the K_d for dissociation of FLIP_L/caspase-8(Site-1 mutant) dimer is smaller than the K_d for dissociation of the Mature/Mature dimer [23, 42] due to increased hydrogen bonding at the interface visible in the caspase-8/FLIP_L structure [24]. Consequently, assuming similar affinities of

caspase-8 and FLIP_L 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 FLIP_L, such that only at FLIP_L concentrations lower or equivalent to caspase-8 would heterodimerization be quantitative *in vivo*; high FLIP_L levels would tend to obliterate caspase activation because the DISC would become saturated with FLIP_L [51].

Although FLIP_L activates caspase-8(Site-1) mutant by heterodimerization, it produces only about 20% of the activity of Mature caspase-8. This may be due to differences in the primary structures of the inter subunit linker of FLIP_L 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 FLIP_L [24]. However, it is still not known how the inter subunit linker in the uncleaved FLIP_L is positioned so that it enables caspase-8 activation at all. It had previously been suggested that processing of FLIP_L dramatically increased the activity of the caspase-8/FLIP_L heterodimer [24], but our studies disagree with this result (Fig 2D, Fig S5) and we believe that the authors have recorded an increase in the generation of new heterodimers after FLIP_L cleavage, rather than an increase in the activity of pre-formed heterodimers. Possibly by using a large excess of DED-FLIP_L over DED-caspase-8 to enforce formation of an unknown fraction of heterodimer, the authors missed the fact that, once cleaved, FLIP_L increases its affinity for caspase-8 [23]. Therefore, accumulated free, cleaved FLIP_L incorporated more unbound caspase-8, generating additional heterodimers and skewing the concentration of the total active sites. Our system, ensuring efficient hetero-dimerization and using active-site titrated enzymes, has shown that cleavage of FLIP_L increased the activity of pre-formed heterodimers by only ~1.5 fold (Fig 2D, Fig S5). The fact that wild-type FLIP_L was slightly more efficient in killing cells than the non-cleavable FLIP_L(D/A) during co-expression experiments with caspase-8 (Fig 2D) is thus likely due to its more facile association with caspase-8.

Based on the results shown in this study, we propose distinct mechanisms for caspase-8 activation that depend on FLIP_L (Fig 5). Our data predict that FLIP_L heterodimers with procaspase-8 would provide the first active protease in the DISC. Certainly FLIP_L 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_L, 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_L 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 caspase-8/ FLIP_L heterodimer compared to the caspase-8 homodimer on tetrapeptides cannot explain the substantial alterations seen with natural protein substrates for cleavage. Intriguingly, the FLIP_L/caspase-8 heterodimer, which displayed attenuated activity toward 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_L, 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 *in vitro*, namely caspase-8(Site-1 mutant)/FLIP_L(D/A) heterodimer, was actually mildly apoptotic when introduced into cells (Fig 2E) and it also cleaved Bid *in vitro* (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_L 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Ac-IETD-afc	N-Acetyl-Ile-Glu-Thr-Asp-7-Amino-4-trifluoromethylcoumarin
BVAD-fmk	Biotinylated-Val-Ala-Asp-fluorofethylketone
CHX	Cycloheximide
DED	Death Effector Domain
DISC	Death Inducing Signaling Complex
FADD	Fas-Associated Death Domain
FKBP	FK506 Binding Protein
FLIP	FLICE-Like Inhibitory Protein
FRB	FKB12 Rapamycin Binding
HDAC-7	Histone DeAcetylase-7
RIPK	Receptor Interacting Protein Kinase
TNFα	Tumor Necrosis Factor α

WT

Wild Type

Z-VAD-fmk

Benzyl-oxycarbonyl-Val-Ala-Asp-fluoromethylketone

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Figure 1. Caspase-8 mutants and activation scheme

(A) Cleavage mutants designed in the context of full-length caspase-8 used for cellular expression, or in the context of chimeric caspase-8 used for *in vitro* activation studies. Mutations are shown in bold. Chimeric caspase-8 consists of the catalytic domain fused with the artificial dimerization domain *FKBP* (~14 kDa) [34]. Note that upon expression in *E. coli*, chimeric caspase-8 underwent proteolysis at Site-1 producing a two-chain species that we refer to as “Mature” throughout the manuscript. We refer to caspase-8 as wild-type (WT) only in the case of caspase-8 expressed in mammalian cells. (B) Diagram of homo- and hetero-dimerization between caspase-8 and FLIP_L in the presence of the specified dimerization compounds (AP20187 and AP21967, respectively). *FRB* is fused to FLIP_L to provide a specific heterodimerization with *FKBP* of caspase-8 fusions [34]. (C) Coomassie stained SDS-PAGE gel showing purified *FKBP*-caspase-8 and *FRB*-FLIP_L mutants expressed *in vitro*. Two-chain species underwent autoproteolysis during expression, whereas one-chain species displayed no autoproteolytic activity.

Figure 2. Caspase-8 uncleavable at Site-1 can only be activated by hetero-dimerization with FLIP_L

(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 homodimerizer compound AP20187 (25 nM) or the activator kosmotrope Na-citrate. For heterodimerization, *FKBP*-caspase-8 mutants (25 nM) were incubated with FRB-FLIP (125 nM) and 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-IETF-afc. Data represent the mean of three independent experiments ($\pm$ SEM); (B) Size exclusion separation of *FKBP*-Site-1 mutant (3 μ M) in the presence and absence of homo-dimerizer (3 μ M). The caspase-8 mutant (100 μ L) was pre-activated in the assay buffer as described above and applied to a Superdex200 size exclusion column. Calculation of apparent molecular weight was based on column pre-calibration with protein standards; (C) *FKBP*-Site-1 mutant caspase-8 (50 nM) activation in the presence of Na-citrate and homodimerizer (500 nM). Caspase-8 was added to a mixture of homodimerizer and Na-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-IETF-afc as described above; (D) Cleavage of FRB-FLIP_L at LEVD/G is not required for *FKBP*-Site-1 mutant caspase-8 activation during heterodimerization. *FKBP*-casp8(Site-1 mutant) (0.7 μ M) was activated in the presence of the heterodimerizer AP21967 and FRB-FLIP_L(wt) or FRB-FLIP_L(D/A) as detailed above. The mixture was split and subjected to IETF-ase activity testing or electrophoresis separation in 4-20% SDS-PAGE gels, followed by Coomassie staining; (E) FRB-FLIP_L rescues the inactive *FKBP*-Site-1 mutant caspase-8 during co-expression in HeLa cells,

inducing cell death. The depicted caspase mutants were transiently transfected in HeLa cells with pcDNA3 vectors encoding the *FKBP* and *FRB* hybrids, as indicated. Transfection was followed, after 24 hours, by treatment with 500 nM heterodimerization compound. Apoptotic cell death was quantified after an additional 24 hours by Annexin V staining (average $\pm$ SEM of three independent experiments). In all panels, IETD-ase is expressed as RFU/min.

Figure 3. Substrate specificity of caspase-8 dimers

Specificity was tested for P₂-P₄ positions of a tetra-peptide on a substrate library with fixed P₁ position (Asp) 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/FLIP_L and Site-1 mutant/FLIP_L) prior to library screening. Full-length caspase-8 containing uncleavable pro-domain and mutations for increased solubility (D210A,D216A,D223A,L122S,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.

Figure 4. Cleavage of natural substrates by caspase-8 homo/heterodimers

Caspase-8 dimers were pre-activated for 45 min following addition of the appropriate dimerization compound and/or *FRB-FLIP_L*. Protein substrates (~0.1-3 μ M final concentration) were added to serially diluted caspase-8. Final caspase concentrations were 100 nM (Mature/Mature), 100-200 nM (Mature/*FLIP_L*) or 400 nM (Site-1 mutant/*FLIP_L*), as indicated. The protein mix was incubated at 25°C for 3 h and reactions were stopped by the addition of 3 $\times$ SDS buffer. Cleavage was analyzed by either staining the gels with Coomassie or by Western blotting (HDAC-7). Caspase-3 used as a caspase-8 substrate harbored the catalytically inactive mutation C285A (caspase-1 nomenclature). The lower panels compare IETD-ase activity (RFU/min) generated by the same caspase-8 dilutions as in the protein cleavage assays. Arrows point to the enzyme concentration producing 50% substrate cleavage. * *FKBP-Caspase-8*(Mature) runs at the same size as caspase-3 zymogen.

Figure 5. Hypothetical model for caspase-8 dimerization

Homodimerization primes the active state of caspase-8, but additional stabilization or interaction, such as cleavage or perhaps C-terminal ubiquitination, is required. In contrast, heterodimerization with FLIP_L primes the active state via interface recruitment, and activates without a requirement of further stabilization. On the other hand, kosmotropes promote the activation by uniformly priming the entire caspase surface for association.

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

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

Substrate	Cleavage Sequence	k_{cat}/K_{Mapp} ($\times 10^3 M^{-1} s^{-1}$)		
		Mature-Mature	Mature-FLIP _L	Site-1-FLIP _L
Procasp-3	IED/S	3.4	1.7	0.16
Procasp-6, prodomain	TETD/A	1.2	0.2	<0.08
Procasp-6, linker	TEVD/A	<<0.01	<<0.01	<<0.01
Procasp-7	IQAD/S	1.8	1.2	<0.08
Procasp-8, monomer	VETD/S	0.6	0.16	<0.08
	LEMD/L	0.6	0.08	<0.08
Procasp-8, dimer	VETD/S	4.2	1.8	<0.08
	LEMD/L	4.2	0.08	<0.08
Procasp-10	IEAD/A	0.2	0.1	<0.08
FLIP _L	LEV/D/G	5.3	2.5	0.21
BID	IEAD/S	10.3	<<0.01	<<0.01
RIPK1	LQTD/G	10.3	5.1	0.42
	LQLD/C	0.6	0.64	0.16
HDAC-7	LETD/G	128	128	5.1

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