Redesigning the procaspase-8 dimer interface for improved dimerization

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Abstract: Caspase-8 is a cysteine directed aspartate-specific protease that is activated at the cytosolic face of the cell membrane upon receptor ligation. A key step in the activation of caspase-8 depends on adaptor-induced dimerization of procaspase-8 monomers. Dimerization is followed by limited autoproteolysis within the intersubunit linker (IL), which separates the large and small subunits of the catalytic domain. Although cleavage of the IL stabilizes the dimer, the uncleaved procaspase-8 dimer is sufficiently active to initiate apoptosis, so dimerization of the zymogen is an important mechanism to control apoptosis. In contrast, the effector caspase-3 is a stable dimer under physiological conditions but exhibits little enzymatic activity. The catalytic domains of caspases are structurally similar, but it is not known why procaspase-8 is a monomer while procaspase-3 is a dimer. To define the role of the dimer interface in assembly and activation of procaspase-8, we generated mutants that mimic the dimer interface of effector caspases. We show that procaspase-8 with a mutated dimer interface more readily forms dimers. Time course studies of refolding also show that the mutations accelerate dimerization. Transfection of HEK293A cells with the procaspase-8 variants, however, did not result in a significant increase in apoptosis, indicating that other factors are required *in vivo*. Overall, we show that redesigning the interface of procaspase-8 to remove negative design elements results in increased dimerization and activity *in vitro*, but increased dimerization, by itself, is not sufficient for robust activation of apoptosis.

Keywords: caspase; apoptosis; dimerization; enzyme activation

Introduction

Apoptosis is an essential mechanism in animal development and tissue homeostasis.¹ It is a highly regulated process that is used to remove unwanted or dysfunctional cells without eliciting an inflammatory response. Apoptosis occurs in almost all stages of development. For example, during embryonic development, apoptosis is involved in shaping organs, the nervous system, and extremities.^{2,3} Apoptotic

pathways can be triggered by various intra- or extracellular signals, such as cytotoxic stress or genome damage,^{4,5} and defects in apoptosis are related to various developmental diseases, such as cancer. Because of the importance of apoptosis in maintaining cellular homeostasis in higher organisms, mechanisms that regulate apoptosis are vitally important in cell development.

Caspases, or cysteinyl aspartate-specific proteases, are primarily responsible for carrying out apoptosis. Caspases cleave substrates after aspartate residues, and the proteases specifically recognize xxxD motifs, where the requirement of the first three amino acids depends on the particular caspase.⁶ In general, there are two families of caspases, the apoptotic caspases and the inflammatory caspases. The apoptotic caspases are composed of initiator caspases, such as caspase-8, -9, and -10, and effector caspases, such as

Abbreviations: DED, death effector domain; IL, intersubunit linker, quad mutants, P466I,F468S/V,T441H,K473E.

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caspase-3, -6, and -7.^{7,8} Effector caspases are responsible for cleaving various protein substrates, resulting in characteristic morphological changes and dismantling of the cell.⁹ Caspases are synthesized as inactive zymogens, and effector caspases are activated following proteolysis by initiator caspases to separate the large and small subunits. Cleavage of the linker connecting the subunits (intersubunit linker, or IL), results in active site formation due to rearrangement of the active site loops and formation of the substrate-binding pocket.^{6,10}

In contrast to effector procaspases, the initiator procaspase-8 exists as an inactive monomer under physiological conditions.¹¹ The activation of initiator caspases depends on the formation of recruitment platforms, such as the DISC (death-inducing signaling complex), apoptosome, or PIDDosome.^{12–14} The recruitment of procaspase-8 into the DISC increases the local concentration of the monomer and induces dimerization.¹⁵ Subsequent cleavage of the IL results in full activation, while cleavage of the pro-domain releases the activated caspase-8 into the cytosol.¹⁶ In contrast to procaspase-3, monomeric procaspase-8 gains enzymatic activity upon dimerization and prior to IL cleavage.¹⁵ The structure of uncleaved procaspase-8 shows that activation occurs through the rearrangement of surface loops that form the substrate-binding cleft, which properly orients the catalytic residues.¹⁷ In addition to homodimerization, procaspase-8 also forms heterodimers with the structurally similar protein, FLIP.^{18–20} In the cell, the heterodimer inactivates RIPK1, preventing necroptosis and promoting cell survival, and it is thought that heterodimerization represents the default cellular pathway.²¹ Under strong apoptotic stimuli, procaspase-8 forms homodimers and promotes apoptosis. Thus, the oligomeric state of procaspase-8 controls the fate of the cell,⁷ although the threshold stimulus that causes the switch from hetero- to homodimers is unknown.

The difference between the activation mechanism and the oligomeric state of procaspase-8 *versus* procaspase-3 leads to the question of what structural features play a role in promoting dimerization. Comparing the structures of activated caspase-8 and caspase-3, one observes that, although the overall structure is very similar [Fig. 1(A)], the dimer interfaces are significantly different, particularly in the central strand, β -strand 8.^{22,23} The central β -strand of caspase-8 has limited, longer range, interactions between backbone atoms of F468 and P466' (3.6 Å) and P466 and T467' (3.3 Å, backbone to side-chain or 3.9 Å, backbone to backbone) [see Fig. 1(B)]. In contrast, the same region of caspase-3 contains four backbone hydrogen bonds, at distances of 2.5–2.8 Å, as well as significant hydrophobic contacts across the interface contributed by I265 and V266 from each monomer [see Fig. 1(D)]. In addition to the

interface hydrogen bonds, residues H234 and E272 from α -helices 5 and 5' of caspase-3 create a network of charge-charge interactions on the surface of the protein [Fig. 1(F)]. For caspase-3, the residues in β 8 present a flat, yet hydrophobic, face for each monomer, and the four inter-strand hydrogen bonds and α -helix 5 charge-charge interactions provide stabilizing interactions. Indeed, the K_D for dimer dissociation of procaspase-3 was estimated to be <50 nM²⁴ whereas that for procaspase-8 was estimated to be $\sim$ 5 μ M.^{16,25} In the caspase-8 interface, one observes two inter-strand interactions between P466 and F468 in the center of the interface, where the side-chain of F468 stacks on the flat surface provided by P466' from the second monomer [Fig. 1(B)]. The presence of P466 results in misalignment of the backbone atoms in β 8 and β 8' (from the second monomer) and prevents hydrogen bonding between the strands. In the heterodimer of caspase-8 and FLIP, the central strands contain three backbone-backbone H-bonds with distances of 2.8–3.3 Å and three additional side-chain and/or side-chain-backbone hydrogen bonds, all under 3.1 Å, because the central strand of FLIP does not contain the proline or phenylalanine residues observed in caspase-8 [see Fig. 1(E)]. As a result, the central β -strands of the heterodimer form a more regular anti-parallel arrangement.

Negative design elements have been described previously in β -strands and are important in preventing indiscriminate association of exposed β -sheets.²⁶ In the case of procaspase-8, the inter-strand interactions between P466 and F468 require a complementary surface for association. This negative design element in β 8 presumably lowers the dimerization potential of procaspase-8. In addition, at the corresponding position on helices α 5 and α 5', the small residue T441 and positively charged K473 do not form an efficient network of charge interactions across the interface, as observed for procaspase-3.

To examine the features of the caspase-8 interface that prevents efficient dimerization, we designed several mutants within the caspase-8 dimer interface that mimic the interface of caspase-3. It has been reported that procaspase-8 is activated *in vitro* by high concentrations of sodium citrate, which promotes dimerization.^{16,27} Utilizing this *in vitro* assay, we show that removal of the negative design elements of procaspase-8 promotes dimerization. In addition, kinetic studies show that the mutations increase the rate of association. Transfection assays of HEK293A and NB7 cells, however, show that the increased dimerization did not result in an increase in apoptosis. Overall, the data show an improved dimerization of the interface mutants, but dimerization of procaspase-8 by itself is not sufficient to initiate apoptosis.

Figure 1. Dimer interface of procaspase-8. A: Comparison of caspase-8 (colored, pdb code 1QTN) and of caspase-3 (gray, pdb code 2J30). Red box corresponds to the dimer interface, and black box corresponds to α -helices 5 and 5' interactions (the prime (') refers to the second monomer in the dimer). B: Interactions on β -strands 8 and 8' in the interface of the caspase-8 homodimer. C: Model of the redesigned caspase-8 interface with the P466I and F468V mutations. D: Interactions in the interface of caspase-3. E: The interface of the caspase-8:FLIP heterodimer shows several backbone hydrogen bonds at distances below 3 Å (pdb code 3H11). F: Comparison of charge-charge interactions on the surface of caspase-3 (gray, transparent) and of caspase-8 (T441 and K473).

Results

The procaspase-8 dimer interface

The procaspase-8 dimer interface contains long-range interactions between β -strands 8 and 8' across the interface. The backbone hydrogen bonds are weak since the distances are between 3.3 Å and 3.9 Å [Fig. 1(B)]. The interface is strengthened by ring-stacking interactions between F468 from one monomer and P466' from the second monomer, as well as the symmetry-related interactions between P466 and F468'. The presence of P466 in the interface as well as reliance upon P466-F468 ring-stacking interactions introduces negative design elements into the interface.²⁶ In order to examine the role of these elements in the interface of caspase-8, we replaced P466 and F468 with amino acids found in caspase-3, isoleucine, and serine, respectively. The rationale for the mutations was that the procaspase-3 homodimer has a more regular antiparallel β -sheet in the interface, with several hydrogen bonds below 3 Å distance [Fig. 1(D)]. In addition, the heterodimer of caspase-8 with FLIP shows that removal

of the proline from one strand improves the inter-strand hydrogen bonds [Fig. 1(E)]. The structural model of caspase-8 lacking the negative design elements demonstrates an apparent improvement of H-bond formation in the interface [Fig. 1(C)].

As shown in Figure 2(A), the mutations were made in the context of full-length as well as Δ DED-caspase-8. In this case, the DEDs (death effector domains) are known to cause insolubility when the protein is expressed in *E. coli*, so the *in vitro* assays utilized the Δ DED constructs. The phenylalanine at 468 also was replaced with valine, rather than serine, in order to increase the hydrophobicity of the interface. In addition, two surface residues on helix 5, T441, and K473, were replaced with histidine and glutamate, respectively, in order to improve charge-charge interactions as observed in procaspase-3 [Fig. 1(F)]. We note however, that the Δ DED-casp8(T441H,K473E) variant was insoluble when expressed in *E. coli*, so it was not examined in the studies presented here. Finally, in order to determine whether chain cleavage of the IL is required to further improve dimer stability in the

Figure 2. A: Procaspase-8 variants used in these studies. The interface mutants were placed in the contexts of fully uncleavable procaspase-8 (called D₅A: D210A, D216A, D223A, D374A, D384A), cleavable intersubunit linker (called D₃A - D210A, D216A, D223A), pro-less procaspase-8 (called ΔDED caspase-8), and with uncleavable linker (called D₂A - D374A, D384A). The C360S mutation removes the catalytic cysteine to produce an enzymatically inactive variant. B: Activation profiles for procaspase-8 variants. Proteins were incubated in sodium citrate-containing buffer for 4 h, as described in Methods, and activity was measured at several substrate concentrations in order to determine k_{cat} and K_M values at each concentration of citrate. C: Maximum activity for each variant. For panels B and C, the numbers correspond to the mutations shown in panel A. Here, “wild-type casp-8” refers to the cleaved two-chain protein (that is, the mature heterotetramer of caspase-8), which shows the maximum activity expected.

mutants, the mutations were made in the context of cleavable or uncleavable ILs (D374A, D384A) [Fig. 2(A)].

Procaspase-8 variants with mutated dimer interface demonstrate increased activation

The direct relationship between dimerization and activation of caspase-8 has been well-established,^{11,16,28} so the enzyme activity of procaspase-8 is proportional to the population of dimer in the sample. Salvesen and coworkers also have shown that procaspase-8 dimerization is facilitated by sodium citrate, a kosmotrope, resulting in activation.^{11,16} To examine the effects of the interface mutations on dimerization, we measured the enzyme activity of each mutant after incubation in assay buffer that contained sodium citrate between 0M and 1M [Fig. 2(B)]. The data show that the uncleavable ΔDEDcaspase-8(D₂A) [open diamonds, mutant “2” in Fig. 2(A–C)] achieves maximum activity in 0.8–0.9M citrate of $\sim 1.7 \times 10^5 M^{-1} s^{-1}$ [Fig. 2(C)].

For the ΔDEDcaspase-8(D₂A) with wild-type interface, no activity was observed below 0.6M citrate, demonstrating that the protein is monomeric below 0.6M citrate. The activity profile was similar for the P466I, F468S double mutant [open triangles, mutant “3” in Fig. 2(B,C)], the P466I, F468V double mutant [closed diamonds, mutant “4” in Fig. 2(B,C)], the single mutant T441 [open circles, Fig. 2(B), mutant “5” in Fig. 2(C)], and the triple mutant P466I, F468S, T441H [closed squares, Fig. 2(B), mutant “6” in Fig. 2(C)]. These four mutants demonstrated maximum activities in ~ 0.8 – $0.9M$ citrate, although we note that the activity of the P466I, F468S was somewhat higher than that of uncleavable ΔDEDcaspase-8(D₂A) ($\sim 3.7 \times 10^5 M^{-1} s^{-1}$ versus $\sim 1.7 \times 10^5 M^{-1} s^{-1}$) [Fig. 2(C)].

Combining the interface mutations, P466I and F468V, with the single surface mutation, T441H, resulted in a significant increase in activity as well as a shift in the activity profile to lower citrate

concentrations. As shown in Figure 2(B), the P466I, F468V, T441H triple mutant (closed triangles, mutant “8”) demonstrated a maximum activity at $\sim 0.7M$ citrate, with about 5-fold increase in activity compared with the uncleavable ΔDED caspase-8 (D₂A) [Fig. 2(C)].

We observed robust increases in activity upon combining the mutations for improved interface (P466I, F468S, or F468V) with the mutations for improved helix 5 surface interactions (T441H and K473E) to generate the quadruple mutants. Both quad mutants demonstrated maximum activities in $\sim 0.8M$ citrate [Fig. 2(B), open squares and closed circles, mutants “7” and “9”] at $\sim 1.2 \times 10^6 M^{-1} s^{-1}$ [Fig. 2(C)]. Both quad mutants and the P466I, F468V, T441H triple mutant retained approximately half their maximum activity in $0.6M$ citrate and had significant activity in $0.4M$ citrate. Indeed, the maximum activities for the two quad mutants were similar to the wild-type caspase-8 with a cleavable IL. The data further support the notion that dimerization is sufficient for activity in procaspase-8.^{15,28} Stabilizing the dimer, as observed for the two quad mutants, appears to be as efficient in forming the

active procaspase as cleavage of the IL in the wild-type caspase-8 [Fig. 2(C)], because the IL is not cleaved in these mutants.

The redesigned dimer interface accelerates the dimerization of procaspase-8

The data shown in Figure 2 represent the steady-state formation of the caspase-8 dimer resulting from incubation in citrate-containing buffer and the changes in the steady-state profile as a result of the interface mutations. To gain an understanding of how the mutations affect the dimerization of procaspase-8 monomers, we also examined the kinetics of dimerization for three of the interface mutants by monitoring gain of enzyme activity upon incubation in citrate (Fig. 3). It was shown previously that the dimer of caspase-8 is stabilized upon cleavage of the IL.^{15,16,28} In order to eliminate the influence of IL cleavage on the kinetics assay, we performed the studies on the D374A, D384A uncleavable mutants [called ΔDED , D₂A, Fig. 2(A)], which removes the cleavage sites in the linker. As the activity of procaspase-8 can only be detected in the dimer, the gain of activity over time represents dimerization of the monomers.

Figure 3. Activation kinetics for procaspase-8 variants. Proteins were added to $1M$ sodium citrate-containing buffer to start the dimerization reaction, and aliquots were measured over time to determine activity, as described in Methods. The protein concentration assayed for enzyme activity was $20 nM$, and the initial protein concentration for each experiment is shown to the right of each curve (26 – $333 nM$). Wild-type procaspase-8 (Panel A), the double mutants, P466I, F468S (Panel B) and P466I, F468V (Panel C), and the quad mutant (P466I, F468V, T441H, K473E) were monitored for ~ 250 min. In this experiment, all mutations were in the context of the pro-less (ΔDED) and IL-uncleavable (D₂A) procaspase-8. Solid lines represent simulations of the data using Kinsim and the model in Scheme 1, as described in the text. Apparent rate constants determined from the simulations are provided in Table I.

The activation profile of wild-type procaspase-8 (that is, Δ ED,D₂A) displays a protein-concentration dependence with at least two phases, the first of which is dependent on the protein concentration [Fig. 3(A)]. At higher protein concentrations, there is a rapid increase in activity in the first ~ 10 min followed by a slower increase in activity over the course of the experiment (~ 4 h).

The activation profile for Δ EDcaspase-8(D₂A,P466I,F468S) is similar to that for wild-type procaspase-8. The data show a rapid increase in activity within the first ~ 5 min [Fig. 3(B)]. In contrast, for Δ EDcaspase-8(D₂A,P466I,F468V), the initial activity is higher than that of procaspase-8(P466I, F468S) [compare starting values in Fig. 3(C) with those in Fig. 3(B)], which we interpret as the formation of a population of dimerized protein within the dead-time of mixing, ~ 30 s. This result supports the data in Figure 2 which show that the mutations improve dimerization. Following the initial activity, the data demonstrate a lag of ~ 20 min followed by a protein-concentration dependent increase in activity. The maximum activity was obtained at a protein concentration of 67 nM, and the activity decreased at higher protein concentration. This trend is further amplified in procaspase-8 in the presence of four mutations (P466I,F468V,T441H,K473E) [Fig. 3(D)]. In this case, maximum activity was achieved at a protein concentration of 50 nM, and the activity decreased in higher protein concentrations.

We attempted to define a minimal model for association to explain all of the data for the four proteins shown in Figure 3. The data show that activation of procaspase-8 does not result from simple association of two monomers to yield the fully active dimer ($M \leftrightarrow D$). Rather, the activation data show that there are multiple components that participate in activation. We propose the model shown in Scheme 1 as the simplest explanation for the activation data shown in Figure 3. We assume that there is an intermediate dimeric form that exists during activation (I), which does not contain the mature active site. Following the initial second-order reaction of dimer formation, procaspase-8 undergoes structural rearrangements to form the active protein

Scheme 1. Proposed model for assembly of the procaspase-8 dimer. “M” represents procaspase-8 monomer, “I” represents an enzymatically inactive dimeric intermediate, “D” represents the active dimer, and “A” represents aggregation of the monomer.

(D) in a first-order reaction. This simple two-step model explains the protein concentration dependence in the dimerization process followed by the slower increase in activity. The rearrangement of the dimer to yield active protein is supported by previous data that shows citrate not only facilitates dimerization but also assists in active site rearrangement after dimerization.¹⁶ Finally, the two interface mutants containing P446 to Ile and F468 to Val, demonstrate lower activity at higher protein concentrations, indicating that the protein aggregates under these conditions. In the model shown in Scheme 1, the apparent aggregation of procaspase-8 is represented by formation of an enzymatically inactive species (A). We note two caveats to this scheme. First, rather than forming aggregates, it is possible that the procaspase-8 mutants form dimers that do not directly reflect activity (misfolded dimer). Under the conditions of these experiments, we cannot distinguish between procaspase-8 that forms soluble aggregates versus formation of dimeric species that are enzymatically inactive. In either case, the species would result in lower activity in the solution. Second, we cannot rule out more complicated assembly schemes for the procaspase-8 monomers. For example, as is evident from the simulations of the data shown in Figure 3(C), the simple model does not explain the lag phase observed in the P466I,F468V variant [Fig. 3(B)]. These results suggest a pre-existing equilibrium in the monomer ($M^* \leftrightarrow M$), where only one species is competent to dimerize. At present, however, there is insufficient data to support anything more than the simple model presented in Scheme 1. As such, we present the model as a way to compare differences in the wild-type and mutant proteins. According to this scheme for dimer assembly, k_1 and k_{-1} represent the association and dissociation rates in dimerization, respectively ($M \leftrightarrow I$), k_2 and k_{-2} represent the forward and reverse rates in forming activate procaspase-8 ($I \leftrightarrow D$), and k_3 and k_{-3} represent the apparent aggregation step which forms an inactive dimer ($M \leftrightarrow A$).

Overall, the simple model shown in Scheme 1 is adequate to explain all of the data in Figure 3, but it is clear that assembly of the procaspase-8 dimer is more complex than can be explained by following the gain of enzyme activity. Using the model in Scheme 1 and KinSim, we simulated the data, assuming that the reverse reactions (k_{-1} , k_{-2} , k_{-3}) were zero (that is, the reactions were irreversible) because the activation assays shown in Figure 3 provide no information on the dissociation process. The solid lines in Figure 3, Panels A–D, show the results of the simulations, and the apparent rates obtained from the simulations are shown in Table I. For wild-type procaspase-8, dimerization occurs with an apparent rate of $5.8 \times 10^3 M^{-1} s^{-1}$, which is

Table I. Apparent rate constants from simulations of assembly kinetics for wild-type and interface mutants of procaspase-8

Rate	Wild type Procaspase-8	Procaspase-8 (P466I,F468S)	Procaspase-8 (P466I,F468V)	Procaspase-8 (P466I,F468V T441H,K473E)
k_1 ($M^{-1} s^{-1}$)	5.8×10^3	7.5×10^3	1.7×10^4	1.0×10^5
k_2 (s^{-1})	1.7×10^4	1.7×10^4	3.3×10^4	3.3×10^4
k_3 ($M^{-1} s^{-1}$)	1.7×10^4	8.3×10^4	2.5×10^5	5.0×10^5

All proteins were in the background of procaspase-8(D₂A).

consistent with that determined previously for procaspase-8 in the presence of 1.0M citrate, $5.03 \times 10^3 M^{-1} s^{-1}$ (Ref. 16) and is about 100-fold faster than dimerization of procaspase-3 in the absence of citrate ($70 M^{-1} s^{-1}$).²⁹ Each of the mutants demonstrate increases in the apparent second order rate of association ($M \rightleftharpoons I$, Scheme 1) from $7.5 \times 10^3 M^{-1} s^{-1}$ for the P466I,F468S double mutant [Table I, Fig. 3(B)], to $1.7 \times 10^4 M^{-1} s^{-1}$ for the P466I,F468V double mutant [Table I, Fig. 3(C)] to $1 \times 10^5 M^{-1} s^{-1}$ for the quad mutant (P466I,F468S,T441H,K473E) [Table I, Fig. 3(D)]. There was little to no change in the apparent rate of isomerization of the dimer ($I \rightleftharpoons D$, Scheme 1), where the apparent rates were $1.7 \times 10^4 s^{-1}$ to $3.3 \times 10^4 s^{-1}$ regardless of the mutation (Table I). The increase in the rate of dimerization is also consistent with the lower dependence of dimerization on sodium citrate from the steady-state measurements [Fig. 2(C)].

The simulations also showed a competing reaction of aggregation ($M \rightleftharpoons A$, Scheme 1). For simplicity, the aggregation is modeled as formation of an inactive dimer from the monomeric state, although we note that the data in Figure 3 provide no information on the oligomeric state of the aggregate, only that aggregation competes with formation of an enzymatically active dimer. Thus, the apparent rates for aggregation shown in Table I should not be viewed as rate constants for aggregation of the monomer, but rather the simulations show that the mutations increase the aggregation propensity of the procaspase-8 variants. In this regard, while aggregation increases somewhat for the P466I,F468S double mutant compared to wild-type procaspase-8, the P466I,F468V variants (double and quad mutants) show dramatic increases in aggregation. So, while removing the proline and phenylalanine from the dimer interface leads to increased rates of dimerization, the mutations also increase the competing aggregation reactions.

Increased dimerization *in vitro* is not sufficient to trigger apoptosis in cell culture

Although the *in vitro* activity studies show that the two quad mutants, (P466I,F468S/V,T441H,K473E) have lower dependence on sodium citrate, the activity in 0.6M citrate is ~30–40% of that in 0.8M citrate, and below 0.3M citrate the activity is nearly

zero. The cellular environment cannot provide the high kosmotrope conditions of the *in vitro* studies, although molecular crowding in the cytoplasm may facilitate dimerization.³⁰ Thus, it was not clear that increased dimerization *in vitro*, resulting in a lower requirement for citrate, would manifest as constitutive activation *in cellulo*.

To test the effect of the mutated procaspase-8 proteins in human cells, the procaspase-8 genes were cloned into the eukaryotic expression vector pcDNA 3.1(-) to which we added a FLAG tag. The full-length procaspase-8 and the Δ DED procaspase-8 variants were examined in the context of a cleavable versus an uncleavable IL. This resulted in procaspase-8(D₅A), where all five cleavage sites were replaced with alanine: D210, D216, and D223, between the DED regions and the large subunit, and D374 and D384 in the IL [Fig. 2(A)]. In procaspase-8(D₃A), the three sites between the DED regions and the large subunit were mutated, D210A, D216A, and D223A, while the IL remained cleavable [Fig. 2(A)]. Finally, in Δ DED procaspase-8(D₂A) the two sites in the IL, D374, and D384, were mutated to alanine [Fig. 2(A)]. The rationale for using the three backgrounds in the contexts of the interface mutants is to examine whether cleavage of the IL and/or interactions with death receptors were required for efficient apoptosis.

Transfection of HEK293A cells with the Δ DED variants did not result in significant apoptosis regardless of whether the IL was cleavable, where ~10–18% apoptosis was observed compared with ~10% for the empty vector [Fig. 4(A,B)]. These levels of apoptosis likely represent basal levels since removal of the catalytic cysteine, C360S, resulted in apoptosis in the range of ~10–15%. We have shown previously that the constitutively active procaspase-3 variant, V266E, results in robust cell death.³¹ Under the conditions used here, the constitutively active procaspase-3 results in apoptosis levels of ~30–35%. Transfection of cells with the quad mutant—P466I,F468V,T441H,K473E—in the context of D₅A (uncleavable DED, uncleavable IL), D₃A (uncleavable DED, cleavable IL), or D₂A (Δ DED, uncleavable IL) resulted in no appreciable increase in apoptosis unless the DED motifs were included (that is, full-length proteins). The full-length uncleavable procaspase-8 with a wild-type interface demonstrated

Figure 4. Transfection assays of procaspase-8 variants. Uncleavable (Panels A and D) or cleavable (Panels B and E) procaspase-8 variants were transfected into HEK293A (Panels A–C) or NB7 cells (Panels D–F) and levels of apoptosis were measured as described in Methods. Control experiments were performed with empty vector, pcDNA(-)FLAG, and a constitutively active procaspase-3 variant [procaspase-3(D₃A, V266E)]. Western blots (Panels C and F) using antibodies to procaspase-8 or to the FLAG tag show levels of protein production. Error bars represent the standard error of three independent experiments.

robust apoptosis of ~30%, while the quad mutant (P466I, F468V, T441H, K473E) was somewhat lower at ~20% [Fig. 4(A)]. While cleavage of the IL made no difference in efficiency for the wild-type procaspase-8 [that is, procaspase-8(D₃A)], cleavage of the IL in the quad mutant (procaspase-8-D₃A, P466I, F468V, T441H, K473E) resulted in an increase in apoptosis to levels observed for wild-type procaspase-8 and the constitutively active procaspase-3 [compare Fig. 4(A,B)]. In both backgrounds, D₅A and D₃A, removal of the catalytic cysteine in the quad mutant resulted in return to basal levels of apoptosis, demonstrating that the increase in apoptosis was due to the activity of the quad mutant. Western blots of the full-length proteins showed robust protein expression [Fig. 4(C)].

To examine whether the presence of endogenous procaspase-8 affected the activation of the mutants,

we performed transfection studies in NB7 cells, a neuroblastoma cell line that lacks caspase-8. The results of transfection of NB7 cells with the procaspase-8 interface mutants were similar to those observed for the HEK293A cells [Fig. 4(D,E)]. Basal levels of apoptosis were ~15%, and there was little difference in apoptosis for the interface mutants regardless of whether the IL could be cleaved. As in the case of HEK293A cells, robust cell death was observed in the full-length procaspase-8. While we observed an increase in apoptosis in the case of wild-type caspase-8 upon IL cleavage (~30% to ~40%), there was no significant change in the quad mutant with a cleavable IL. Western blots showed low levels of protein translation for the uncleavable quad mutant [Fig. 4(F)], although the mutant provides robust apoptosis. This feature was observed

previously for the constitutively active procaspase-3(V266E) and was interpreted as a toxicity effect of an active caspase.³¹ Consistent with this interpretation, protein levels increased when the catalytic cysteine was removed. Overall, the transfection studies show that the interface mutations do not support increased apoptosis in either HEK293A or NB7 cells. The highest levels of apoptosis are observed when procaspase-8 retains the DED motifs, and a small increase in apoptosis is observed when the IL is cleaved.

Discussion

An unresolved issue in the studies of caspase structure is why procaspase-8 is a stable monomer in the cell while procaspase-3 (and other effector caspases) is a stable dimer. In addition, it is not clear why the procaspase-8 dimer attains enzymatic activity upon dimerization while the procaspase-3 active sites are largely unformed in the dimer. In cellular signaling, formation of the procaspase-8 dimer controls the fate of the cell because it also forms heterodimers with the structurally related protein, FLIP.^{18–20} It is currently thought that the procaspase-8:FLIP heterodimer represents the default pathway in the cell because the heterodimer is more stable than the homodimer *in vitro*,^{25,32} and it prevents programmed necrosis by inactivating the RIPK1-RIPK3 signaling pathway.²¹ High stress loads or activation of death receptors shifts the procaspase-8 oligomer toward the homodimeric state, which then activates procaspase-3 to initiate apoptosis.

Although the protease domains of caspases-3 and -8 are structurally quite similar,^{22,23,33} several regions of the proteins are not conserved. Procaspase-8 contains N-terminal tandem DEDs, which provide interaction surfaces with adapter proteins and death receptors,^{12,34} while procaspase-3 contains a short pro-domain (28 amino acids) in an extended structure.²⁴ The pro-domain of procaspase-3 has been shown to function as an intramolecular chaperone during subunit assembly, and it dramatically stabilizes the protease domains.³⁵ Likewise, the linkers (IL) that connect the large and small subunits in the protease domains have low sequence identity and appear to function differently in maintaining the inactive state. For example, the IL of procaspase-7, a close homologue of procaspase-3, binds in an allosteric site of the dimer interface and prevents rearrangement of the active site loops.^{36,37} In contrast, the IL of the procaspase-8 monomer interacts with active site loop 1 and prevents productive active site formation.¹⁷

In addition to the structural features of the IL and pro-domain regions, the dimer interfaces are very different between caspases-3 and -8. While the interface of caspase-3 contains several backbone hydrogen bond interactions below 3 Å, caspase-8 contains few hydrogen bonds, and the interactions

are at longer distances (>3 Å, Fig. 1). As described by Richardson,²⁶ negative design elements in exposed β -strands prevent indiscriminate association of the strand. The elements present a surface that requires more precise complementarity for the interaction. The dimer interface of the procaspase-8 monomer contains negative design elements due to the presence of P466 and F468. The ring-stacking interactions stabilize the dimer while introducing the requirement for complementarity for homodimer formation. Our results show that replacing the two amino acids with those found in procaspase-3 increase dimerization propensity as evidenced by a lower requirement for the kosmotrope, sodium citrate, and an increase in the apparent rate of dimerization. Maximum dimer formation was obtained by including more optimal charge–charge interactions in α -helix 5, which interact across the interface. Indeed, the enzyme activity obtained for the quad mutant was similar to that for caspase-8 in which the IL was cleaved, even though the IL was uncleaved in the interface variant. As with other studies,^{15,16} the results show that dimerization is sufficient for activation of procaspase-8. The data also suggest that the activity observed in the presence of citrate may be more complicated than a simple dimerization reaction (M $\leftrightarrow$ D). Removing the negative design elements in the interface resulted in increased dimerization, as evidenced by the increase in enzyme activity, but there was also an increase in apparent aggregation of the mutants. Thus, the activity assays in sodium citrate (Fig. 2) do not fully account for the mixture of species (monomer, dimer, aggregate). That is, one should be careful to interpret the results of the assay as equating the changes in enzyme activity with fully dimerized protein. While it is clear that more dimer is present in the citrate-containing buffer, the solution also contains enzymatically inactive species as well. The role of P466 (and possibly F468) may be to inhibit homodimerization as well as aggregation of the monomer.

Our results show that, although removing the negative design elements resulted in increased dimerization *in vitro*, the increased dimerization *in vitro* did not correlate with an increase in apoptosis in cell culture, either in HEK293A cells, which contain endogenous procaspase-8 or in NB7 cells, which lack endogenous procaspase-8. The activity profiles (Fig. 2) showed that the mutants had little or no activity in low sodium citrate concentrations (below ~0.3 M citrate), so the dimer was not sufficiently strengthened to form constitutively in the cell. The results suggest that other negative design elements are present in the interface or that other regions of the protein may be involved in dimerization, or both. The interesting evolution of the procaspase interfaces may have practical applications in treating diseases caused by the dysregulation of apoptosis. For example, understanding how to form a constitutively

active procaspase-8 should provide a new therapeutic avenue for the treatment of cancer as the activated procaspase-8 would initiate apoptosis through cleavage of procaspase-3. Understanding how procaspase-8 is maintained as a monomer will allow development of strategies to shift the populations of procaspase-8:FLIP heterodimer and procaspase-8 homodimer in the cell.

Materials and Methods

Cloning, protein expression, and protein purification

The procaspase-8 and caspase-8 genes without the N-terminal DED domains were first mutated at adenosine 720 (the number follows full-length sequence) to cytosine. This mutation removed an XhoI restriction site without changing the amino acid sequence of caspase-8. The gene was amplified by PCR from pET15b-CP8 (kindly provided by Dr. Guy Salvesen, Sanford-Burnham Medical Research Institute), using primers listed in Supporting Information Table I. The primers introduced an NdeI restriction site at the 5' end and an XhoI site at the 3' end of the gene. The gel-purified product was digested with NdeI and XhoI and ligated into the pET21b vector digested with the same enzymes. The cloning strategy provides a His₆ tag at the C-terminus of the proteins.

Mutagenesis was performed as described previously³⁸ using the Stratagene Quick Change mutagenesis kit and the primers listed in Supporting Information Table I. The nucleotide sequence for the 8-amino acid FLAG tag was synthesized with XhoI and EcoRI sites at the 5' and 3' ends, respectively, and the tag was inserted into pcDNA3.1(-) (Invitrogen) between XhoI and EcoRI sites in the multiple cloning sequence. The genes for full length caspase-8 and Δ DED caspase-8 were ligated into pcDNA3.1(-) between XbaI and XhoI restriction sites (Supporting Information Table I).

Each gene in the pET21b vector was transformed into *E. coli* BL21(DE3) pLysS cells and expressed according to established protocols,¹⁶ except that the expression was conducted at 25°C for 9 h. After purification by Ni-NTA chromatography, the proteins were dialyzed overnight against a buffer containing 50 mM Tris, pH 7.9, 50 mM NaCl (called 50/50 buffer). The proteins were further purified by ion-exchange chromatography (DEAE-sepharose) using a buffer of 50 mM Tris, pH 7.9, and a NaCl gradient between 50 mM and 400 mM. Purified proteins were stored in 50/50 buffer at -80°C. The extinction coefficient used to determine the protein concentration was 23,380 M⁻¹ cm⁻¹.

Activity assays

The enzymatic activities of the procaspase-8 proteins (wild type and mutants) were measured using a

Synergy 2 multi-mode microplate reader. The protein was diluted into a buffer of 20 mM Pipes, pH 7.5, 100 mM NaCl, 0.01% CHAPS, 10 mM DTT, 5% sucrose (activity assay buffer). As described in the figures, sodium citrate was added to the activity assay buffer at final concentrations between 0M and 1M in 0.1 or 0.2M increments. The samples were incubated at 25°C for 4 h. Substrate (Ac-IETD-AFC) was added at final concentrations ranging from 2 μ M to 75 μ M to wells of a 96-well plate, and procaspase-8 was injected into the wells at a final protein concentration of 50 nM. The fluorescence emission of AFC release was measured at 500 nm (20 nm width) after excitation at 400 nm. Data were collected for 120 s. The data were fit to the Michaelis-Menten equation to obtain k_{cat} and K_M values.

Kinetics of procaspase-8 activation

Monomeric procaspase-8 was diluted in a buffer of 1M sodium citrate, 50 mM sodium phosphate, pH 7.5, 10 mM DTT, and 0.01% CHAPS (activation buffer) at final protein concentrations from 27 nM to 300 nM, as indicated in the figures. Samples were incubated at 25°C. At indicated times, protein was added to a 96-well plate that contained activation buffer and substrate, Ac-IETD-AFC (final concentration of 50 μ M), such that the final protein concentration in each well was 20 nM. Enzyme activity was determined at 25°C for 120 s.

The assembly of the active procaspase-8 dimer was simulated using KINSIM40 (Ref. 39) and the kinetic model shown in Scheme 1. In this model, "M" represents monomeric procaspase-8, "I" represents a dimeric intermediate of procaspase-8 that has low activity, "D" represents fully activated dimeric procaspase-8, and "A" represents aggregation of the monomer (no activity). In the simulations, the apparent rates for the back reactions, k_{-1} , k_{-2} , and k_{-3} , were assumed to be zero. Kinetic parameters obtained from the simulations are shown in Table I.

Cell culture and Western blotting

HEK-293A cells were cultivated in Dulbecco's modified Eagle's medium (DMEM). NB7 cells were cultivated in RPMI 1640 medium. The medium was supplemented with 10% heat-inactivated fetal bovine serum. Transfections were carried out with the Nanojuice transfection kit (EMD Millipore). The optimal transfection conditions for HEK293A cells were determined to be 0.5 μ g of DNA per well with 0.5 μ L of core transfection reagent and 1 μ L of transfection booster. The core reagent and booster were mixed with DMEM (50 μ L, not supplemented) and incubated at room temperature for 5 min. Total DNA (0.5 μ g) was then added into the mixture and incubated at room temperature for an additional 15

min. The solution containing DNA was then added to the corresponding well. The optimal transfection conditions for NB7 cells were determined to be 0.5 µg of DNA per well with 0.5 µL of core transfection reagent and 0.25 µL of transfection booster. The empty vector, pcDNA3.1(-)FLAG, was used as a negative control, and the constitutively active procaspase-3(D₃A,V266E) variant, pcDNA3.1(-)3210 (Ref. 31), was used as a positive control. HEK293A cells were harvested 24 h after transfection. For NB7 cells, transfection reagents were removed 4 h after transfection, and cells were cultured in complete RPMI 1640 medium.

For flow cytometry experiments, cells were trypsinized in the well, resuspended in the culture medium, washed twice with cold PBS (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.6, 137 mM NaCl, 2.7 mM KCl) and stained with Annexin V-PE and 7-AAD (Apoptosis detection kit, BD Biosciences). Cells were analyzed by FACS following incubation with Annexin V-PE and 7-AAD in the dark for 15 min at 25°C.

Western blots were performed as described.⁴⁰ The dilution ratios of antibodies were the following: anti-caspase-8 (1:1000), anti-FLAG (1:500), anti-Hsp90 (1:2000), secondary antibody (1:2000).

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