Activation of Initiator Caspases through a Stable Dimeric Intermediate*

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Structural and biochemical studies have revealed that procaspases form dimers prior to proteolytic activation. How the two procaspases interact in the dimer is unclear. To study the mechanisms of dimer-dependent caspase activation we used a heterodimeric system so that two caspase molecules can be specifically brought together. Surprisingly, only one caspase partner in the dimer needs to be enzymatically active for caspase processing and activation to occur. Caspase activation is inefficient in the dimer in the absence of intramolecular processing, suggesting that caspase activation is initiated via intramolecular processing. Homodimerization of caspase-8 or caspase-9 leads to the formation of a stable dimeric complex. However, heterodimerization between caspase-8 and caspases-3, -9, or -10 failed to induce stable dimer formation or caspase activation. Our data suggest that the formation of a stable dimeric intermediate initiates caspase activation.

Caspases are a family of cysteine proteases that cleave target proteins at specific aspartate residues (1, 2). The roles of caspases in apoptosis first become evident when a cell death-related gene, ced-3, which is essential for apoptosis in Caenorhabditis elegans, was found to be homologous to the mammalian caspase interleukin-1 β-converting enzyme, caspase-1 (3). It is now clear that caspases are essential effector molecules during apoptosis in eukaryotic cells (4). In addition, caspases are also involved in cytokine maturation, cell growth, and differentiation (5–8).

Caspases exist as zymogens containing a prodomain and a protease domain (2). Caspases that have long prodomains are believed to be the upstream initiator caspases. Among them, caspase-8 and caspase-10 contain two tandem repeats of the “death effector domain” (DED)1 within their prodomains (9–12). Homotypic interaction between the DEDs of caspase-8/-10 and the DED of an adaptor molecule, FADD, induces the recruitment of these caspases to death receptors such as the Fas and TRAIL receptors (9, 10, 13–16). The death receptor, FADD, and caspase-8/-10 form the death-inducing signaling complex (DISC) that leads to the activation of caspase-8 and caspase-10 (13, 14). Another group of caspases that have long prodomains include caspase-1, -2, -4, -5, -9, -11, -12, and -13, each of which contains a caspase recruitment domain (CARD) (17, 18). The CARD of caspase-9 interacts with the CARD-containing adaptor, Apaf-1. Cytochrome c, Apaf-1, and caspase-9 form the apoptosis leading to caspase-9 activation (19–21). By contrast, caspases with short prodomains, including caspase-3, -6, -7, and -14 are believed to be downstream effector caspases that depend on the upstream initiator caspases for full processing and activation (2).

The induced proximity model suggests that adaptor-mediated clustering of initiator caspase zymogens leads to their autoprocessing to form active caspases (22). This model is supported by the evidence that oligomerization of procaspases is sufficient to cause caspase activation (23–25). Moreover, high local concentrations of recombinant caspases in bacteria extracts lead to spontaneous caspase processing (26, 27). Recent studies suggest that procaspases at high local concentrations form a globular dimeric structure (18, 28–31). In contrast to the inactive monomeric procaspases, dimeric procaspases display protease activities (29, 31). Therefore, high local concentrations of caspase-8 and caspase-10 in the DISC and caspase-9 in the apoptosome may lead to caspase activation through dimer formation. Comparisons between the crystal structures of procaspases and active caspases suggest that conformational changes lead to exposure of the protease active sites in active caspases (18, 28–31). Therefore, dimer formation may activate caspases by inducing conformational changes that facilitate proteolytic processing of caspase zymogens into active protease subunits.

Although dimerization is sufficient to activate caspases, the exact mechanism of caspase activation in the dimer has not been resolved. It is possible that one procaspase cleaves an adjacent procaspase by an intermolecular processing mechanism. Alternatively, each procaspase in the oligomer may cleave itself by an intramolecular processing mechanism. In this study, we provide evidence that the formation of a stable dimeric intermediate induces caspase activation by an intramolecular processing mechanism.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—A synthetic rapamycin analog, AP20840 (Ariad Pharmaceuticals), was used as the chemical inducer of dimerization (CID) to induce dimer formation between one FK506-binding protein containing a F36V mutation (Fv) and one FKBP rapamycin-binding (FRB) domain of the FKBP rapamycin-associated protein. Monoclonal and polyclonal anti-HA.1 antibodies were obtained from Covance (Berkeley, CA). Monoclonal anti-FLAG M2 was from Sigma. Protein G-Sepharose beads were from Amersham Biosciences.

Expression Constructs—FKBP containing an Fv or has been described

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(32). The FRB domain used in this study contains FKBP rapamycin-associated protein amino acid residues 2052–2114 with a threonine to leucine mutation at amino acid 2098. A single copy of Fv, or FRB was used to construct fusion proteins to ensure that each fusion protein has only one binding site for the rapamycin analog, AP20880. Part of the prodomain and the entire protease domain of caspase-8 (amino acid residues 207–479), caspase-9 (amino acid residues 210–522), or caspase-10 (amino acid residues 139–410) were fused in frame to the C terminus of Fv, or FRB. The entire coding region of caspase-3, caspase-6, or caspase-7 was fused to the C terminus of Fv, or FRB. An HA or FLAG tag was fused to the N terminus of each Fv, and FRB construct, respectively. The cysteine residues in the QACGQ protease active site of caspase-8 (amino acid residue 380) and caspase-10 (amino acid residue 415) or the QACGQ protease active site of caspase-9 (amino acid residue 315) were mutated to serine by site-directed mutagenesis (Stratagene). The frozen mutant of caspase-8 (caspase-8DDAA) was generated by mutating amino acid residues 374 and 384 from aspartate into alanine by site-directed mutagenesis according to the protocol of the supplier (Stratagene). Plasmids were verified by DNA sequencing, and those free of errors were used for experiments.

**Transfection of HeLa Cells and X-Gal Staining**—HeLa cells in 6-well plates were transfected with wild type and cysteine to serine mutation (C/S) mutant caspase constructs at the indicated amounts plus 1 µg of pcDNA3-lacZ by the FuGENE 6 method (Roche, Indianapolis, IN). 18 h after transfection the cells were fixed with phosphate-buffered saline containing 2% formaldehyde and 0.2% glutaraldehyde at room temperature for 5 min. The cells were then stained by incubation in phosphate-buffered saline containing 1 µg/ml X-gal, 5 mM ferrocyanide, 5 mM ferricyanide, 2 mM MgCl₂, 0.02% Triton X-100, and 0.01% SDS at 37 °C. **Apoptosis Assays**—Jurkat T cells were transfected with 10 µg of both Fv and FRB fusion constructs plus 5 µg of a GFP plasmid by electroporation (Bio-Rad). After an 8-h culture, live cells were purified by Ficol gradient separation, and 200 µl cells (2.5 × 10⁶/ml) were added to 96-well flat-bottom tissue culture plates. The cells were incubated with CID at the indicated concentrations. 24 h later the cells were harvested and stained with phosphatidylethanolamine-conjugated annexin V propidium iodide (Sigma). Propidium iodide, annexin V, and GFP cells were analyzed by flow cytometry. Percentage of cell loss (% apoptosis) was calculated as described previously (33). **Western Blot Analysis**—To detect caspase processing, 293T cells were transfected with 1 µg of different fusion constructs by the FuGENE 6 method (Roche). The cells were cultured at 37 °C for 24 h and treated with 100 nM CID for different periods. The cells were then lysed in lysis buffer (50 mM HEPES, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1:1 protease inhibitor mixture from Roche, and 20 µM benzoylcarbonyl-Val-Ala-Asp) and used for Western blot analysis using anti-HA1.1 (Convance) or anti-FLAG antibody and developed by the chemiluminescent method (Pierce).

**Immunoprecipitation**—Different constructs were transfected into 293T cells by the FuGENE 6 method (Roche). 18 h later the cells were incubated with or without 100 nM CID at 37 °C for 1 h. The cells were then lysed in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 10 µM benzoylcarbonyl-Val-Ala-Asp, and 1:1 protease inhibitor mixture from Roche). The cell lysates were used for Western blot analysis with anti-HA antibodies or for immunoprecipitation with anti-FLAG. Protein G beads (Amersham Biosciences) coated with anti-FLAG M2 (Sigma) were incubated with cell lysates at 4 °C for 2 h. The beads were then washed three times with lysis buffer followed by SDS-PAGE and Western blot analysis with rabbit anti-HA1.1 (Convance). The blots were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences) and developed by the chemiluminescent method (Pierce).

**RESULTS**

**Inefficiency of Protease-deficient Caspases in Dominantly Interfering with Wild Type Caspases**—Crystal structural and biochemical studies suggest that procaspases dimerize prior to proteolytic activation (18, 28–31). A protease-deficient caspase could potentially form a dimer with a wild type caspase and thereby dominantly inhibit the activation of the wild type caspase. To examine the potential dominant negative effects of caspase mutants that contain a mutated protease active site, we examined their interactions with wild type caspases in HeLa cells. As expected, expression of wild type caspase-8, caspase-9, or caspase-10 induced significant apoptosis in HeLa cells (Fig. 1). Protease-deficient mutants with a C/S in the protease active site caused no detectable apoptosis (Fig. 1). We then co-transfected the wild type and C/S mutants of these caspases. However, the caspase-C/S mutants displayed no inhibitory effects on wild type caspases in inducing apoptosis (Fig. 1). We also observed no dominant inhibitory effects when 3-fold more mutant caspases than wild type caspases were used in the experiments (data not shown). By contrast, baculovirus p35 significantly inhibited apoptosis induction by these caspases (Fig. 1). Similar data were observed in MCF-7 cells (data not shown). Therefore, caspases containing mutated active sites are inefficient inhibitors of their wild type counterparts.

**Dimerization-induced Caspase Activation**—The puzzling observations that C/S mutants are incapable of inhibiting the functions of wild type caspases prompted us to study the molecular mechanism of caspase activation. Because caspases can undergo dimer-dependent activation, we set out to examine how enzymatically inactive C/S caspases interact with wild type caspases after their dimerization.

Chemically induced homodimerization has been successfully used to study caspase activation (23–25). In the present study, we adapted a system for chemically induced heterodimerization. Because rapamycin is a bivalent agent that binds to both FK506-binding protein 12 (FKBP12) and the FRB domain of FKBP rapamycin-associated protein simultaneously (34), FKBP12 and FRB domains can be fused to distinct proteins for rapamycin-dependent heterodimer formation. For improved specificity, we used a FKBP12 variant, Fv (32), containing a phenylalanine to valine mutation at amino acid residue 36 and the FRB domain of FKBP rapamycin-associated protein (amino acid residues 2052–2114) containing a threonine to leucine mutation of amino acid 2098. A synthetic rapamycin analog, AP20840, was used throughout this study as a CID. Only one Fv, or one FRB domain was used in each fusion protein so that each fusion protein has only one CID interacting domain.

We replaced the prodomain of caspase-8 with either Fv, or FRB. To test whether the system is suitable for studying caspase activation, we measured apoptosis induction after dimer formation between Fv–caspase-8 and FRB–caspase-8 in Jurkat T cells. CID did not induce apoptosis in cells expressing Fv, or FRB only (Fig. 2A). CID also failed to cause apoptosis in cells expressing Fv and FRB–caspase-8 or Fv–caspase-8 and FRB (Fig. 2A). In contrast, significant apoptosis was induced by dimer formation between Fv–caspase-8 and FRB–caspase-8.

Therefore, two caspase-8 molecules are required for caspase-8

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**Fig. 1. Co-transfection of wild type and protease-deficient caspase in HeLa cells.** HeLa cells were transfected with 1 µg of lacZ reporter construct plus 3 µg of wild type (WT) caspase-8, -9, or -10 plasmid; mutant caspase-8, -9, or -10 constructs containing a cysteine to serine mutation in the QACGQ protease active site (C/S); and the baculovirus p35 as indicated. The cells were stained with X-gal 18 h later.
and the percentage of apoptosis was calculated as in A was calculated.

and propidium iodide, and the percentage of apoptosis of GFP cated concentrations for 18 h. The cells were stained with annexin V with the rapamycin analog of inducer of dimerization (C8c/s) serine mutation at the protease active site (C8) or caspase-8 protease domain containing a cysteine to caspase-8C/S mutant (C8c/s) or caspase-10C/S mutant (C10c/s) and caspase-10 (Fig. 2). Apoptosis induced by dimerization between wild type caspase-8 and caspase-8C/S would lead to processing of the mutant by wild type caspase-8 (Fig. 3A). On the other hand the intramolecular processing model would predict that dimerization between wild type caspase-8 and caspase-8C/S results in autoprocessing of wild type caspase-8 but not caspase-8C/S (Fig. 3A).

Although dimerization is sufficient to activate caspases, the exact model of caspase activation has not been resolved. It is still unclear whether each caspase in the dimer cleaves itself by intramolecular processing or cleaves the other caspase by intermolecular processing. If intermolecular processing were responsible for caspase activation, dimerization between wild type caspase-8 and caspase-8C/S would lead to processing of the mutant by wild type caspase-8 (Fig. 3A). On the other hand the intramolecular processing model would predict that dimerization between wild type caspase-8 and caspase-8C/S results in autoprocessing of wild type caspase-8 but not caspase-8C/S (Fig. 3A).

To distinguish between these models, we examined caspase processing after dimerization between caspase-8 and its C/S mutant. As expected, dimerization between FV-caspase-8 and FRB-caspase-8 resulted in the processing of both caspase-8 molecules (Fig. 3B, panels a and e). Also, CID-induced dimer formation of FV with FRB-caspase-8 or FV-caspase-8 with FRB caused no caspase processing (data not shown). Therefore, two caspase-8 molecules are required for caspase activation to occur. Interestingly, FV-caspase-8 was processed after dimerization with FRB-caspase-8C/S (Fig. 3B, panel b). Therefore, caspase-8C/S contributes to the activation of wild type caspase-8 in a dimer. Similarly, FRB-caspase-8 was processed after dimerization with FV-caspase-8C/S (Fig. 3B, panel g). Because caspase-8C/S does not have a functional protease active site, it cannot cleave itself or the other caspase in the dimer (Fig. 3B, panels d and h). Therefore, it is likely that the wild type caspase-8 had cleaved itself by intramolecular processing (Fig. 3A).

Intramolecular Processing as the Initial Step in Caspase-8 Cleavage—Because caspase-8C/S has no functional proteolytic site, its homodimerization caused no caspase processing (Fig. 3B, panels d and h). However, caspase-8C/S was cleaved following dimer formation with enzymatically active caspase-8 (Fig. 3B, panels c and f). Wild type caspase-8 is likely to be responsible for the processing of caspase-8C/S. Wild type caspase-8 may cleave caspase-8C/S before its intramolecular processing. Alternatively, wild type caspase-8 may undergo intramolecular processing first before acquiring the ability to process other caspases in trans. To distinguish between these two possibilities, we mutated the two aspartate residues that are required for processing between the large (p20) and the small (p10) subunits of the caspase-8 protease domain. Unlike wild type caspase-8, this “frozen” aspartate to alanine (DD/AA) cleavage mutant failed to undergo intramolecular processing between p20 and p10 after dimerization with caspase-8C/S (Fig. 4, panel f versus panel h). We then tested whether the DD/AA mutant retains the ability to confer intermolecular processing after dimerization with another caspase-8. Caspase-8DD/AA can support the processing of a wild type caspase-8 after its dimerization (Fig. 4, panel c). However, caspase-8DD/AA failed to induce the processing of caspase-8C/S after its dimerization (Fig. 4, panel d). Therefore, the caspase-8DD/AA that lacks the potential for intramolecular processing cannot cleave caspase-8C/S following dimerization. This suggests that intramolecular processing is the first step in the cleavage of initiator caspase after dimer formation. In the absence of intramolecular processing, in trans processing does not take place efficiently.
Specific Interaction between the Same Caspases during Dimer-dependent Caspase Activation—Caspase-8 and caspase-10 can both be recruited into the DISC (15, 16, 35), raising the possibility that these two caspases could activate each other in the DISC. However, we found that heterodimerization between caspase-8 and caspase-10 did not induce apoptosis (Fig. 5A). We also failed to observe caspase processing between caspase-8 and caspase-10 after dimerization (Fig. 5B, lane 6). This sug-
expected, FV-caspase-8 was co-precipitated with FRB plus different FV constructs fused to these caspases together with a FRB fused to caspase-8, caspase-9, caspase-10, caspase-3, or caspase-6. In the upper panel (Fig. 6A), we observed no stable dimer formation between FRB-caspase-8 and FRB-caspase-9, caspase-10, caspase-3, or caspase-6. Therefore, caspase-9 can also form stable homodimers but not heterodimers with caspase-8 or caspase-10.

We previously observed that caspase-8 and caspase-10 are independent of each other in inducing apoptosis in human lymphocytes. When caspase-10 is deficient because of inherited genetic mutations, the recruitment and activation of caspase-8 in the DISC are normal (Ref. 36 and data not shown). Likewise, caspase-10 can mediate independent apoptosis signaling when caspase-8 is deficient because of a homozygous inactivating genetic mutation (15, 37). This is consistent with the observations that different caspases are not able to form a stable dimer and activate each other (Fig. 5). Therefore, caspase-8 and caspase-10 likely induce independent apoptosis from the DISC despite the possibility that they may come into close proximity in the DISC.

**DISCUSSION**

The categorization of caspases into either the effector or initiator class is based on both functional and structural features. Caspases that contain one of two related protein interaction motifs, DED or CARD, are believed to be upstream initiator caspases (2). Caspase-8 and caspase-10 have tandem repeats of DEDs in each of their prodomains (9–12). Homotypic interactions between DEDs of caspase-8/9 and FADD lead to the recruitment of caspase-8/10 into the DISC, resulting in autoactivation of these caspases (9, 10, 13–16). Caspases-1,-2, -4, -5, -9, -11, -12, and -13 contain a CARD in each of their prodomains (18). Homotypic interactions between CARDs of caspase-9 and Apaf-1 in the apoptosome contribute to caspase-9 autoactivation (19, 30). Similarly, interactions between the CARDs of caspase-2 and CRADD/RAIDD are likely to trigger caspase-2 activation (36, 37). In contrast, caspases lacking such protein interaction motifs may depend on upstream caspases for activation.

The "induced proximity model" has been postulated to explain the mechanisms for the activation of initiator caspases (22). This model is supported by several observations. High concentrations of caspases in bacterial extracts lead to spontaneous processing that generates large and small subunits of the protease domain (26, 27). Moreover, induced dimer or oligomer formation is sufficient for caspase activation (23–25). Interactions of DEDs in caspase-8/10 with the DED in adaptor FADD in the DISC likely bring two or more of these caspases together, leading to their autoactivation by induced proximity. Similarly, the interaction of the CARD in the caspase-9 prodomain with the CARD in Apaf-1 results in oligomerization and activation of caspase-9.

Recent studies of crystal structures of procaspase-7 and procaspase-9 indicate that procaspases form dimers prior to their activation (18, 28–31). Procaspase-7 and procaspase-9 contain 6 β-sheets in their protease domains (18). During dimerization, the 6-β-sheet protease domain of one caspase lines up in an anti-parallel fashion with the protease domain of the other caspase, forming a globular structure (18). Four protruding loops (L1, L2, L3, and L4) at one end of the 6-β-sheet protease domain form the protease active site, and the cysteine residue...
in the protease active site is located in loop L2 (18). The active sites of the two caspases are on opposite ends of the globular protease structure. The formation of this globular dimer complex of protease domains likely leads to conformational changes and autoprocessing of caspases (18).

To understand how caspases are activated by dimerization of their protease domains, we fused Fv or FRB to the protease domains of different caspases, permitting inducible dimerization. Consistent with previous reports (20, 23–25), we found that homodimer formation of caspase-8, -9, and -10 initiated apoptosis signaling (Fig. 2). Surprisingly, dimerization between these caspases and their protease-inactive C/S mutants also caused apoptosis (Fig. 2). We also examined caspase processing after dimer formation between caspase-8 and inactive caspase-8C/S. Consistent with their ability to trigger apoptosis, we observed that both caspase-8 and caspase-8C/S are processed after their dimerization (Fig. 3B). Because caspase-8C/S does not possess a functional protease active site, the wild type caspase-8 likely undergoes intramolecular processing in this context. In contrast, caspase-8C/S is likely cleaved by the wild type caspase-8 in trans. We then examined whether intramolecular processing of wild type caspase-8 is required before the processing of caspase-8C/S in trans. We observed that caspase-8DD/AA, lacking the ability for intramolecular processing, is inefficient in cleaving caspase-8C/S after dimer formation (Fig. 4). By using inducible dimerization technology, we have demonstrated that intramolecular processing is the first step in the activation of initiator caspases.

Before structural data were available, proteolysis in trans was the simplest model to explain how induced proximity can lead to caspase maturation and activation. However, crystal structures of caspase homodimers are more consistent with intramolecular proteolysis as demonstrated here. The cysteine residue in the protease active site is located in the second loop (L2) (18). Prior to proteolytic cleavage between p20 and p10, the p20/p10 boundary that resides in L2 is adjacent to the protease active site (18). Therefore, it is plausible for each caspase subunit to use its active site to preferentially cleave its adjacent p20/p10 junction by an intramolecular mechanism (Fig. 7). Moreover, in the globular protease structure of a caspase dimer the active sites of the two caspases are diametrically opposed. Because the two protease active sites (QACXG) are located at the opposite sides of the globular structure, it would be sterically more difficult for one caspase active site to cleave the p20/p10 junction of the other caspase that is located closer to the opposite end of the globular caspase structure. However, once the initial cleavage has taken place the fully active caspase subunit may gain the ability to cleave the adjacent subunit via increased flexibility.

In contrast to caspase homodimerization we could not detect caspase activation or apoptosis following heterodimerization between two different caspases. We observed that dimerization between caspase-8 and caspase-9, caspase-10, or downstream caspases caused no caspase activation or apoptosis (Fig. 5). Similarly, dimerization of caspase-9 or caspase-10 with other caspases also induced no apoptosis (Fig. 5A). Therefore, it is likely that different caspases cannot form correct globular caspase dimer structures. In support of this possibility we found that although homodimers of the protease domains of caspase-8 or caspase-9 can be stably immunoprecipitated (Fig. 6), these caspases cannot form stable heterodimers with different caspases (Fig. 6). Therefore, the formation of a stable homodimeric complex between the protease domains of two pro-caspase molecules may be a prerequisite for caspase activation.

Our data also indicate that the protease domains of downstream caspases are less efficient at autoactivation, information that is consistent with previous reports (25, 38). Because downstream effector caspases lack either a DED or CARD that is required for recruitment by adaptors to a signaling complex...
like the DISC or apoptosome, these caspases likely depend on upstream caspases for activation. We observed that homodimerization of effector caspases, including caspase-3, caspase-6, and caspase-7, induced significantly less apoptosis than dimerization of initiator caspases (Fig. 5A and data not shown). It has been reported that the negatively charged Asp-Asp tripeptide next to the cleavage site between p20 and p10 in the caspase-3 protease domain inhibits dimer formation and autoactivation of caspase-3 (39). Caspase-6 and caspase-7 may also have low autoactivation potential like caspase-3. Because of the lack of protein interaction motifs in their prodomains and the lower autoactivation potentials of their protease domains, downstream caspases likely depend on initiator caspases for processing and activation.

Activation of wild type caspase-8 by dimerization with a caspase-8C/S mutant suggests that protease-deficient caspases may also have low autoactivation potential like caspase-3. Be-
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J. Biol. Chem. 2002, 277:50761-50767.
doi: 10.1074/jbc.M210356200 originally published online October 23, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M210356200

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