Oligomerization Is a General Mechanism for the Activation of Apoptosis Initiator and Inflammatory Procaspases

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Proteolytic initiation of procaspases is a crucial step in the cellular commitment to apoptosis. Alternative models have been postulated for the activation mechanism, namely the oligomerization or induced proximity model and the allostERIC regulation model. While the former holds that procaspases become activated upon proper oligomerization by an adaptor protein, the latter states that the adaptor is an allosteric regulator for procaspases. The allostERIC regulation model has been applied for the activation of procaspase-9 by apoptotic protease-activating factor (Apaf-1) in an oligomeric complex known as the apoptosome. Using approaches that allow for controlled oligomerization, we show here that aggregation of multiple procaspase-9 molecules can induce their activation independent of the apoptosome. Oligomerization-induced procaspase-9 activation, both within the apoptosome and in artificial systems, requires stable homophilic association of the protease domains, raising the possibility that the function of Apaf-1 is not only to oligomerize procaspase-9 but also to maintain the interaction of the caspase-9 protease domain after processing. In addition, we provide biochemical evidence that other apoptosis initiator caspases (caspase-2 and -10) as well as a procaspase involved in inflammation (murine caspase-11) are also activated by oligomerization. Thus, oligomerization of precursor molecules appears to be a general mechanism for the activation of both apoptosis initiator and inflammatory procaspases.

Apoptosis is a physiological form of cell death critical for development and the maintenance of homeostasis in multicellular organisms. The central component of the apoptosis machinery is a group of cysteine proteases called caspases that cleave proteins after aspartic acid residues (1–3). These proteases exist in healthy cells as inactive procaspases, which comprise an NH2-terminal prodomain of variable length and a COOH-terminal protease domain that can be further divided into the large and small subunits, the constituent units of mature caspases. Conversion of procaspases to mature caspases involves at least one cleavage event that separates the large and small subunits but often also another cleavage event that separates the procaspase and large subunit. During apoptosis, caspase activation occurs sequentially with long prodomain-containing caspases (initiator caspases, including caspase-2, -8, -9, and -10) being activated first that then cleave and activate short prodomain-containing caspases (effector caspases, including caspase-3, -6, and -7). Mature effector caspases cleave a wide range of intracellular structural and regulatory proteins, leading to a set of stereotypic changes in cell morphology and eventual cell death. Mammalian caspases also play a critical role in inflammation. The maturation of proinflammatory cytokine interleukin-1β, for example, requires both caspase-1 and caspase-11 (4, 5). Similar to initiator caspases, the inflammatory caspases contain a relatively long prodomain.

Activation of the initiator caspases is a critical step in the activation of the caspase cascade and apoptosis. This activation is an autocatalytic process mediated by the interaction of an initiator caspase, via its prodomain, with its adaptor protein. How exactly initiator caspases become activated remains a matter of debate (6, 7). Two alternative models have been described: the oligomerization model (also known as the induced proximity model) and the allostERIC regulation model. The oligomerization model was originally proposed for the activation of caspase-8 (8–10), which is engaged by a group of cell surface death receptors in the tumor necrosis factor receptor family (11). Binding of these receptors to their corresponding trimeric ligands or agonistic antibodies leads to the recruitment, via adaptor proteins such as Fas-associated death domain, of multiple procaspase-8 molecules to a membrane-associated death-inducing signaling complex. The increased local concentration of procaspase-8 in the death-inducing signaling complex likely leads to cross-cleavage among them. The oligomerization model has also been proposed for the activation of CED3 (12), a caspase required for developmental cell death in the nematode Caenorhabditis elegans, and the mammalian counterpart of CED3, caspase-9 (13, 14).

More recently an alternative model has been proposed for the activation of caspase-9 (15). Caspase-9 is the initiator caspase for the mitochondrial apoptosis pathway that is activated by various intracellular lethal signals, including developmental lineage information, oncogenic transformation, and severe DNA damage. These signals lead to the release of mitochondrial cytochrome c to the cytosol where it binds to an adaptor protein Apaf-11 to form an oligomeric complex known as the apoptosome. Using approaches that allow for controlled oligomerization, via adaptor proteins such as Fas-associated death domain, of multiple procaspase-8 molecules to a membrane-associated death-inducing signaling complex. The increased local concentration of procaspase-8 in the death-inducing signaling complex likely leads to cross-cleavage among them. The oligomerization model has also been proposed for the activation of CED3 (12), a caspase required for developmental cell death in the nematode Caenorhabditis elegans, and the mammalian counterpart of CED3, caspase-9 (13, 14).

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Apaf-1 in the apoptosome, which enhances the activity of caspase-9 by about 1,000-fold (15). This observation has led to an \textit{allosteric} regulation model, which postulates that Apaf-1 is a cofactor of procaspase-9 capable of inducing conformational changes that render the zymogen proteolytically competent (15).

In this study, we examine the activation of caspase-9 using approaches that allow for controlled oligomerization independent of the apoptosome. Our data confirm that caspase-9 is a requisite step in caspase-9 activation. We also showed that formation of a stable intermediate by two caspase-9 molecules through their protease domains is a general mechanism for the activation of both initiator and effector caspases.

\textbf{EXPERIMENTAL PROCEDURES}

\textbf{Reagents and Expression Constructs—}HeLa and 293 cells were obtained from ATCC. The following reagents were purchased from the indicated sources: protein \textit{A}-agarose (Invitrogen), glutathione-Sepharose (Amersham Biosciences), and rabbit cytochrome c and anti-FLAG M2-agarose beads (Sigma). AP20187 was kindly provided by ARIAD Pharmaceuticals. Plasmids for mammalian cell expression and \textit{in vitro} translation were made in pcDNA3.1 (Invitrogen). The Fv domain and the protease domain. Expression plasmids for various

\textbf{Production of Recombinant Apaf-1—}Recombinant Apaf-1 with a COOH-terminal His$_{6}$ tag was expressed in insect Sf-9 cells through baculoviral infection and affinity purified with nickel-nitrotriacetic acid-agarose (Qiagen) using 300 mM imidazole for elution as described previously (17). The Apaf-1 protein was further purified on a Mono-Q H5/5 column driven by a fast liquid chromatography system (Amersham Biosciences). The Mono-Q column was equilibrated with CEB buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl$_{2}$, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) and subsequently eluted with a gradient of 0–500 mM NaCl. The peak fractions containing Apaf-1 were collected and concentrated by Centricon-10 Plus (Millipore). The purified Apaf-1 was stored at \textit{\textit{at~80^\circ C}}.

\textbf{In Vitro Transcription/Translation and Caspase Processing—}In \textit{in vitro} transcription and translation were done using the T7 SF6 reticulocyte lysate system (Promega) in the presence of either non-radioisotope-labeled methionine or $^{35}$S-methionine according to the manufacturer’s instruction. Processing of recombinant procaspase fusion proteins and FLAG-tagged caspase-2/9 was performed in the presence of 100 nM AP20187 and the indicated concentration of M2, respectively, at 30 °C for 4–8 h. Processing of full-length caspase-9 proteins by Apaf-1 was performed using the indicated amounts of recombinant Apaf-1 and 1.2 μl of \textit{in vitro} translated, $^{35}$S-labeled caspase-9 proteins in a final volume of 6 μl.

\textbf{Immunoprecipitation Assay—}Three microliters of each \textit{in vitro} translated protein was added to 500 μl of lysis buffer (12). The proteins were precleared with 20 μl of glutathione-Sepharose beads for 1 h and then immunoprecipitated with anti-FLAG antibody conjugated on agarose beads. After washes, the beads were analyzed by SDS-PAGE and autoradiography.

\textbf{Cell Death Assay—}HEK293 and HeLa cells seeded in six-well plates were transfected the 2nd day with the caspase-9 plasmids plus a green fluorescence protein expression plasmid (pEGFP) (Clontech) via the calcium phosphate precipitation method. Cells were fixed at 24 h after transfection. The percentage of apoptosis was determined by the number of fluorescent cells with apoptotic morphology divided by the total number of fluorescent cells. Data shown are averages and standard deviations of three independent experiments, and for each experiment, more than 300 blue cells from randomly chosen fields were counted.
Labeled procaspase-9 proteins were incubated with 0.2 or 0.6 μg of Apaf-1 or without Apaf-1 in the presence of 100 ng/ml cytochrome c for 4 h. Reaction mixtures were then subjected to SDS-PAGE and autoradiography analyses. B, the cell death activity of various procaspase-9 mutants. Two micrograms of each plasmid was transfected into 293 cells together with pEGFP. GFP-positive cells were scored for apoptosis 24 h post-transfection. Data shown (mean ± S.D.) are representative of three independent experiments done in duplicates. Similar results were obtained using HeLa cells (not shown). C9, caspase-9; WT, wild type; C5S, Cys to Ser mutation; M1, D150K; M2, F286A/V298A; M3, K409D/K410E; FL, full-length fusion protein.

RESULTS AND DISCUSSION

Activation of Procaspase-9 by Apoptosome-independent Oligomerization—Given that both the oligomerization and the allosteric regulation models have been proposed for the activation of caspase-9, we wished to examine whether induced oligomerization of caspase-9 outside the apoptosome complex could lead to its activation. Two approaches that allow for controlled oligomerization were used. The first was based on the FK506-binding protein (FKBP) and a divalent FKBP ligand. A derivative of FKBP termed Fv (18) was fused to the protease domain of caspase-9 in place of the protein interaction motif caspase recruitment domain (CARD). A divalent Fv ligand, AP20187, binds to Fv with high affinity but not to endogenous FKBP, thus minimizing the interference from endogenous FKBP and allowing for the effective dimerization of Fv fusion proteins. When Fv-caspase-9 fusion protein was treated with AP20187, the zymogen underwent proteolytic processing within the small subunit and the rest of the protein, a pattern that recapitulates the processing of the full-length caspase-9 in the apoptosome (Fig. 1A, lane 2). To confirm that the cleavage did occur at one of the previously determined cleavage sites (13, 19), we mutated the two major cleavage sites, Asp-315 and Asp-330, as well as a minor cleavage site, Glu-306, to Ala. The resulting mutant failed to undergo processing upon dimerization (Fig. 1A, lane 6). In addition, the processing was autoproteolytic as shown by the corresponding active site Cys-287 to Ala mutant that failed to be processed (Fig. 1A, lane 4).

To ascertain that oligomerization of full-length procaspase-9 is sufficient to trigger its activation independent of Apaf-1, as the second approach, we used antibody-mediated oligomerization of a tagged full-length caspase-9 protein. FLAG-tagged caspase-9 was induced to dimerize/oligomerize by the anti-FLAG monoclonal antibody M2. Notably the processing of the full-length zymogen was evident upon M2 treatment, generating fragments (p10 and p35) characteristic of mature caspase-9 (Fig. 2A, lane 6). The caspase-9 protease domain harboring each of the two precursor molecules through their protease domains is a requisite step in the activation of caspase-8 (20). A previous study also showed that procaspase-9 is mainly an inactive monomer at its physiological concentration but forms a dimer at the high concentration used for crystal formation (21).

Formation of an Intermediate by Stable Interaction of the Protease Domain Is a Requisite Step in Caspase-9 Activation—How may oligomerization lead to caspase-9 activation? We recently found that the formation of an active intermediate by two precursor molecules through their protease domains is a requisite step in the activation of caspase-8 (20). A previous study also showed that procaspase-9 is mainly an inactive monomer at its physiological concentration but forms a dimer at the high concentration used for crystal formation (21). To determine whether the homophilic association of the protease domain is required for the activation of caspase-9 in the apoptosome, we generated mutations in the procaspase-9 protease domain that abolished its self-association and examined the effects of these mutations on Apaf-1-dependent caspase-9 activation. Sequence alignment of caspase-9 with other caspases, revealed amino acids of procaspase-9 that are potentially important for the protease domain interaction. We mutated three single or paired residues to those of opposite charge for charged amino acids and to alanines for the others (Fig. 2A). The caspase-9 protease domain harboring each of the

FIG. 3. Loss of homophilic interaction of the protease domain led to defective caspase-9 activation. A, mutations in the protease domain abolished procaspase-9 processing. In vitro translated, 35S-labeled procaspase-9 proteins were incubated with 0.2 (+) or 0.6 μg (++) of Apaf-1 or without Apaf-1 (−) in the presence of 100 ng/ml cytochrome c for 4 h. Reaction mixtures were then subjected to SDS-PAGE and autoradiography analyses. B, the cell death activity of various procaspase-9 mutants. Two micrograms of each plasmid was transfected into 293 cells together with pEGFP. GFP-positive cells were scored for apoptosis 24 h post-transfection. Data shown (mean ± S.D.) are representative of three independent experiments done in duplicates. Similar results were obtained using HeLa cells (not shown). C9, caspase-9; WT, wild type; C5S, Cys to Ser mutation; M1, D150K; M2, F286A/V298A; M3, K409D/K410E; FL, full-length fusion protein.

FIG. 4. Dimerization-induced autoprocessing of procaspase-2, -10, and -11. A, activation of procaspase-2 by antibody-mediated oligomerization. 35S-Labeled FLAG-tagged procaspase-2 (C2) was treated with increasing concentrations of M2 (8.75, 17.5, and 35 ng/μl) or without M2 (−) for 12 h. The reaction mixtures were analyzed by SDS-PAGE and autoradiography. B, dimerization induces activation of procaspase-10 and -11. In vitro translated, [35S]methionine-labeled Fv-caspase fusions and their corresponding active site Cys to Ser mutants (C5S) were treated with dimerizer AP20187 (100 nm) (+) or left untreated (−) for 4 h. The reaction mixtures were then analyzed as in A. The p12 subunit of Fv-caspase-10 (C10) could barely be detected due to the low content of methionine in this fragment. C11, caspase-11; WT, wild type; FL or F.L., full-length fusion protein; AP, AP20187.
these mutations failed to associate with itself (Fig. 2B, lanes 3–5).

Next we introduced these mutations into full-length procaspase-9 and tested their effect on procaspase-9 activation. When the wild type procaspase-9 protein was treated with recombinant Apaf-1, it became activated in the presence of cytochrome c and dATP as predicted (Fig. 3A, lane 1). However, when the interaction-deficient mutants were treated with Apaf-1 under the same conditions, the processing was either abolished or severely impaired (Fig. 3A, lanes 6–14). Because caspase-9 can become activated without being processed (19), we wished to determine whether these interaction-deficient mutants lost their cell killing activity. When transfected into 293 or HeLa cells, each of these mutants failed to induce cell death (Fig. 3B, lanes 4–6 and data not shown). FLAG-tagged procaspase-9 harboring each of these mutations also failed to be activated upon M2 treatment (data not shown). The correlation between the loss of self-interaction of the prodomain and the loss of caspase-9 activation indicates that the association of the prodomain is a requisite step for the activation of caspase-9.

**Activation of the Other Initiator Caspases and Inflammatory Caspases by Oligomerization**—Besides caspase-8 and -9, the other human initiator caspases include caspase-2 and -10. Procaspase-2 is structurally similar to procaspase-9 in that both contain a CARD motif in their prodomains (22). Recent studies have shown that caspase-2 is an upstream initiator caspase for DNA damage-induced apoptosis and induction of cytochrome c release from the mitochondria (23–25). To examine whether caspase-2 can be activated by oligomerization, we tagged procaspase-2 with a FLAG epitope at the NH2 terminus and treated the protein with M2. Similar to procaspase-9-FLAG, FLAG-procaspase-2 underwent self-processing upon M2 treatment, generating fragments typical of mature caspase-2 (Fig. 4A). Interestingly the processing of caspase-2 mirrored that of caspase-9 with only one cleavage event that separates the small subunit from the rest of the protein.

Procaspase-10 is structurally similar to procaspase-8 with two tandem death effector domains in its prodomain (26) and is engaged by the death receptors to deliver lethal signals to cells. When the prodomain domains of multiple caspase-10 molecules were brought into close proximity through Fv-mediated oligomerization, they underwent self-processing (Fig. 4B).

Murine caspase-11 is required for the activation of procaspase-1 (5), which processes the precursor of proinflammatory cytokine interleukin-1β (4). Our previous study has shown that procaspase-1 is activated by oligomerization (8, 27, 28). To examine whether caspase-11 is activated in a similar mechanism, we used Fv-mediated dimerization. As shown in Fig. 4B, an Fv fusion of the wild type caspase-11 prodomain domain, but not a corresponding fusion of the catalytically inactive mutant, was converted to the mature enzyme by AP20187-induced oligomerization. Thus, inflammatory caspases are also likely to be activated by oligomerization.

Understanding the mechanism of caspase activation is central to our understanding of apoptosis regulation as well as inflammatory responses. Based on this study and previous studies by ourselves and others, we propose that oligomerization is a unifying theme for the activation of long prodomain-containing caspases, including both apoptosis initiator and inflammatory caspases. However, there are notable variations among initiator caspases. For example, while processed caspase-8 appears to be completely functional, processed caspase-9 needs to be associated with Apaf-1 to gain its full potential (15). In light of the fact that caspase-9 activation requires interaction of its prodomain (Fig. 2, 3), it is possible that the processed caspase-9 subunits cannot form stable tetrameric complexes on their own, and the function of Apaf-1 is to hold these subunits in place as opposed to inducing conformational changes in them. This hypothesis is consistent with the observation that processed caspase-9 is mainly a monomer in solution (21). As for procaspase-2, although the protein complex that promotes its activation remains elusive, the similarity in the structure and processing patterns between procaspase-2 and -9 suggest that caspase-2 may also require an association with its adaptor protein to be fully active. Finally, in addition to apoptosis-inducing initiator caspases, we found that dimerization-mediated caspase activation is also applicable to the inflammatory caspases caspase-1 and -11. To date, the regulation of inflammatory caspase activation is not well understood, but upstream adaptor proteins may exist to facilitate the dimerization and autoactivation of these caspases.

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