Interdimer processing and linearity of pro-caspase-3 activation: a unifying mechanism for the activation of initiator and effector caspases

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Caspase activation during apoptosis occurs in a cascade from the initiator caspase(s) (e.g., caspase-8) to the effector caspases (e.g., caspase-3), which ensures the generation of large amounts of active caspases to dismantle cells. However, the mechanism that safeguards against inadvertent caspase activation is not well understood. Previous studies have suggested that activation of procaspase-8 is mediated by cross-cleavage of precursor dimers, formed upon apoptosis induction, which are not only enzymatically competent but also highly susceptible to cleavage, and that procaspase-8 activation is a linear process without self-amplification. Effector procaspases constitutively exist as dimers and their activation is started by trans-cleavage by an initiator caspase followed by auto-cleavage of effector caspases. Here we show that the dimerization of caspase-3 molecules through their protease domains is required for their processing by initiator caspases. The subsequent auto-processing takes place through cleavage between the dimeric intermediates. Moreover, mature caspase-3 fails to process its own precursor. Thus, despite a marked difference in the generation of active intermediates, the activation of initiator and effector caspases share the important features of interdimer cleavage and lack of self-amplification. These features may be important in preventing accidental cell death.

INTRODUCTION
Apoptosis or programmed cell death is executed by caspases, intracellular cysteine proteases that cleave proteins after aspartic acid residues (1-4). The proteases are synthesized as inert precursors comprised of an NH₂-terminal prodomain followed by a characteristic caspase domain that can be further divided into a large (approximately 20 kDa, p20) and a small (p10) subunit. P20 and p10 are found in mature caspases arranged as a homo-dimer of the p20:p10 heterodimer or (p20:p10)₂. The conversion from a procaspase to a mature caspase generally requires two sequential cleavage events with the first one separating p20 and p10 followed by a second cleavage separating p20 from the prodomain. Caspase activation usually occurs as a cascade of initiator and effector caspases. Initiator procaspases, including human caspase-2, -8, -9, and -10, contain relatively long prodomains that facilitate their interaction with death adaptor proteins, and are autoproteolytically activated in large protein complexes. One major caspase-activating complex is the cytosolic apoptosome, which is formed through the binding of cytochrome c to a death adaptor, Apaf-1, in response to various intracellular death stimuli (5). Additionally, the membrane-bound death-inducing signaling...
complex (DISC) is assembled upon binding of death receptors to their ligands or agonistic antibodies (6). The apoptosome and DISC activate procaspase-9 and procaspase-8, respectively (7-9). Once activated, caspase-9 and -8 then process effector caspases, including caspase-3, -6, and -7, which have short prodomains (10-30 amino acids). Effector caspases, especially caspase-3, are responsible for most of the cleavage events observed during apoptosis. The caspase cascade ensures quick generation of large amounts of active caspases to irreversibly damage cells upon apoptosis induction. At the same time, the deadly outcome of this cascade demands that it be tightly regulated. The mechanism whereby this pathway is safeguarded from accidental activation is not clear.

The activation of initiator caspases is induced by their oligomerization. This was originally shown for caspase-8 (10-13). Subsequent studies have shown that the C. elegans caspase CED3 (14), caspase-9 (15-17), and other initiator caspases (18) are activated in a similar way, suggesting that oligomerization is a general mechanism for activation of initiator caspases. Recent studies have shed important light on the mechanism whereby initiator caspases, particularly procaspase-8, are activated by oligomerization. The defining step in the activation of procaspase-8 is the formation of precursor dimers through a stable interaction between the protease domains (19, Boatright, 2003 #984,20). These dimers are structurally similar to the mature caspase-8 and acquire strong proteolytic activity. Importantly, the procaspase-8 dimers are highly susceptible to cleavage themselves by other dimers compared to individual procaspase-8, leading to interdimer processing for the generation of mature caspase-8 (19). Thus, this mechanism requires minimally four procaspase-8 molecules being brought into close proximity to achieve caspase-8 cleavage. The fact that dimeric procaspase-8 rather than individual caspase-8 is the active unit for activation also enables an intriguing mode of regulation through a proteolytically inactive procaspase-8 homologue, c-FLIP L, which induces strong protease activity in procaspase-8 upon their hetero-dimerization (21). Moreover, the activation of procaspase-8 is a linear event: the active procaspase-8 dimers process only themselves and not effector caspases, while mature caspase-8 cleaves effector procaspase but not its own precursor (19). The interdimer processing and the lack of positive feedback, as opposed to cleavage between individual procaspases and a self-amplification loop, minimize the chance of accidental caspase activation.

In contrast to the auto-activation of initiator caspases, the activation of effector caspases is a combination of both trans- and auto-activation (22,23). Different from initiator caspases, which exist as monomers prior to apoptosis induction, effector procaspases constitutively form dimers likely due to the strong hydrophobic nature of the dimer interface in these caspases (24). These precursor dimers, however, are inactive due to the structural constraints imposed by the covalent link between the large and small subunits. Cleavage of this link by an initiator caspase allows for re-ordering of catalytic and substrate-binding residues to form proper active sites, generating an enzymatically competent intermediate (25-27). This intermediate then undergoes autoproteolytic processing to separate the large subunit from the prodomain. In this study, we show that like the activation of procaspase-8, the dimerization of procaspase-3 is required for it to be cleaved. In addition, the second, autocatalytic step takes place between partially processed procaspase-3 dimers. Furthermore, mature caspase-3 does not effectively process its own precursor. Therefore, despite the difference in the generation of the dimeric intermediate, the activation of initiator caspases and effector caspases are similar with regards to autoproteolytic processing between these enzymatically active dimers and the lack of self-
amplification.

**EXPERIMENTAL PROCEDURE**

*Reagents*—Active caspase-3, caspase-6, caspase-7, caspase-8 and caspase-9 (Chemicon International), rabbit heart cytochrome c (Sigma), and EGS [Ethylene glycolbis (succinimidylsuccinate)] (Pierce) were purchased from the indicated sources. AP20187 was kindly provided by ARIAD Pharmaceuticals.

*Plasmids*—All plasmids for in vitro transcription/translation were constructed in pRK5 vector with NH$_2$- or COOH-terminal epitope tags as indicated. All caspase-3 mutations were made by overlap polymerase chain reaction and confirmed by DNA sequence analysis. Primers used for mutagenesis were: 5'-CTGGAGACCCCCAACTTTTTCATTA (M1), 5'-GCCAAGTTCTTGTATGCATACTCCACA (M2), 5'-TTTGACGTCACTTTTCATGCAAAG (M3), 5'-TCAGTGGCTAGCAAATCCATTAAAAATTTG and 5'-TGGATTTGCTAGCCACTGAGTTTTCAGTG (D9A), and 5'-GCGAATCCATGGCCTCTGGAATATCCC TGGAC and 5'-CCAGAGGCCATGGATTCGCTTCCATGTATG (D18A). The caspase-9 expression plasmids were previously described (18).

*Chemical Cross-linking*—$^{35}$S-methionine-labeled wild type and mutant procaspase-3 proteins were made using a coupled *in vitro* transcription/translation system (Promega) according to the manufacturer’s instructions. The caspase-3 proteins were passed through Centricon column YM-10 (Amersham) to change the buffer to 1 x PBS. The EGS stock solutions were made in DMSO. 4.5 μl of the caspase protein sample was mixed with 0.5 μl of 0.25 mM of EGS stock or DMSO and incubated for 45 min at 4 °C. The reaction was stopped by direct addition of the same volume of 2 x SDS-PAGE loading buffer and analyzed by 10% SDS-PAGE and autoradiography.

*Caspase processing reaction*—All caspase processing reactions were done with two microliters of the *in vitro*-translated procaspases and 0.5 μl active caspases for two hours at 37 °C unless otherwise indicated.

**RESULTS**

*Mutations in procaspase-3 that affect its self-association*—Effector procaspases such as caspase-3 constitutively exist as dimers in cells (24). A previous study has shown that induced dimerization of procaspase-8 is required not only for the acquisition of protease activity but also for its processing by other active procaspase-8 dimers (19). To examine whether the dimerization of effector procaspases is requisite for their cleavage by initiator caspases, we generated point mutations that abolished the self-association of procaspase-3. Based on the crystal structure of active caspase-3 (28), we mutated three amino acids located at the interface of the two p17:p12 heterodimers to those of the opposite charges, resulting in interface mutants, K154D(M1), D192K(M2), and K260D(M3) (Fig. 1A). The dimeric state of these mutants was examined by chemical cross-linking using ethylene glycolbis (succinimidylsuccinate) (EGS)$^{1}$ and SDS-PAGE analysis. In agreement with previous studies, wild type procaspase-3 pre-formed dimers as shown by the appearance of a procaspase-3 band of ~60 kDa in the presence but not the absence of EGS (Fig. 1B, lanes 1, 2). In contrast, the M1 and M2 mutants significantly impeded procaspase-3 homodimerization (lanes 3-6), while the M3 mutant had only a minor effect (lanes 7, 8).
Processing of procaspase-3 by initiator caspases requires its dimerization -- We next examined the processing of these mutants by active caspase-8. As expected, wild type procaspase-3 was readily cleaved by active caspase-8, generating fragments corresponding to a processing intermediate (p20, which has both p17 and the prodomain) and the active caspase-3 subunits p17 and p12 (Fig. 2A, lanes 2, 3, and 13). The processing of p20 to p17 happened at Asp28 rather than Asp9 in the prodomain (lanes 15 and 17), consistent with previous results (22,23,29). In comparison, the processing of the M1 and M2 mutants was significantly impaired, and the processing of the M3 mutant was only slightly affected (Fig. 2A). Similar results were obtained when active, recombinant caspase-9 was used to cleave caspase-3 (Fig. 2B). Thus, the processing of these procaspase-3 mutants by caspase-8 and -9 correlated well with ability of these mutants to form dimers.

The recombinant, active caspase-9 was comprised of the large and small subunits. However, during apoptosis, the prodomain of procaspase-9 is not separated from its large subunits and the apoptosome-bound, processed caspase-9 has much higher activity towards caspase-3 than the unbound caspase-9 does (30). We thus tested the processing of procaspase-3 by apoptosome-activated caspase-9. Procaspase-9 in S100 cell extracts can be induced to undergo self-processing upon addition of cytochrome c and dATP or ATP (7). We developed a convenient system to activate procaspase-9 using in vitro-translated, [35S]methionine-labeled procaspase-9. Addition of cytochrome c to the in vitro translation mix led to the processing of procaspase-9 (Fig. 2C), likely mediated by an Apaf-1 homologue in the rabbit reticulocyte lysate. ATP was present in the lysate to allow for transcription and translation to occur; therefore it was not a variable in our experiments. Similar to the processing of procaspase-9 in the S100 cell extracts, the activation of caspase-9 in this system was autoproteolytic in nature because an active site Cys-to-Ala mutant failed to be processed (lane 4), and it occurred at the previously determined amino acid residues (15,31) because mutations of these residues completely abolished procaspase-9 processing (lane 6). The cytochrome c-activated caspase-9 processed procaspase-3 and to a lesser extent, the caspase-3 M3 mutant, but it did not cleave the procaspase-3 mutants M1 and M2 (Fig. 2D). Taken together, these results show that the stable association of procaspase-3 through its protease domain is required for its processing by initiator caspases.

Caspase-3 auto-cleavage occurs between partially processed dimers -- Following the severance of p17 and p12 by initiator caspases, the separation of p17 and the prodomain is carried out by caspase-3 itself. Because the auto-activation of procaspase-8 takes place through interdimer cleavage, we sought to determine whether the auto-activation step during caspase-3 activation proceeds similarly between the p20/p12 dimers. Cleavage of the proteolytically inactive procaspase-3(C/S) by caspase-8 yielded mainly the (p20/p12)2 intermediates due to its inability to self-process (22) (Fig. 3A, lane 8). However, when procaspase-3(C/S) was treated with caspase-8 in the presence of wild type procaspase-3, procaspase-3(C/S) was further processed into p17 and p12 (lane 5), similar to the processing of wild type procaspase-3 (lane 2), suggesting that the wild type caspase-3 p20/12 dimers can process the caspase-3(C/S) p20/p12 dimers. To rule out the possibility that the caspase-3(C/S) p20/12 dimers were cleaved by the fully processed wild type caspase-3, we treated procaspase3(C/S) with active caspase-3 and/or caspase-8. Active caspase-3 did not process its own precursor (Fig. 3B, lane 4, also see below), nor did it process the p20 intermediate generated by caspase-8 cleavage (lanes 2 vs. 1). Therefore, we concluded that the second cleavage event during caspase-3 activation takes place between the p20/p12 dimers.
Mature caspase-3 does not effectively process its own precursor – The processing of procaspase happens at aspartic acid residues, which conform to the substrate recognition consensus of caspase. Thus, caspase activation may be subject to positive feedback. The activation of initiator caspases such as caspase-8, however, does not show such regulation because the mature caspase-8 is not able to process its own precursor. To examine whether this is also the case for effector caspases, we compared the processing of procaspase-3 by mature caspase-3 and by other initiator and effector caspases. As shown in Fig. 4A, procaspase-3 was effectively cleaved by mature caspase-8 as well as by caspase-1, -6, and -9, as predicted. However, mature caspase-3 showed little, if any, activity towards its own precursor even though it effectively cleaved PARP, an established caspase-3 substrate (32) (Fig. 4A and B). Therefore, the activation of procaspase-3, like that of procaspase-8, is a linear process.

DISCUSSION
The caspase proteolytic system fits well with the terminal nature of apoptosis with its abilities to irreversibly affect a large number of substrates and to quickly amplify the death signal through cascades of initiator and effector caspases. As this system is universally present in healthy animal cells, it must be tightly controlled to prevent accidental activation. Effector caspases represent the majority of active caspases in apoptotic cells and are responsible for most of the protein cleavages that give rise to the stereotypic morphological changes during apoptosis (1-4). In this study, we show that like the activation of caspase-8, the cleavage of caspase-3 requires its dimerization and occurs through cleavage between the active intermediates. In addition, mature caspase-3 does not efficiently process its own precursors, and its activation is unlikely to involve a loop of self-amplification. Therefore, the activation of initiator and effector caspases shares important features of interdimer processing and linearity (Fig. 5). These features would significantly enhance the specific activation of effector caspases by initiator caspases and decrease the probability of undesired activation of these caspases.

The interdimer processing of caspase-3 requires at least two caspase-3 dimers to be in close proximity to complete its activation. Although effector procaspases cannot effectively form active sites, an early study has indicated that a small portion of procaspase-3 is enzymatically competent (33). The reason for this activity is not understood, but conceivably could be due to dynamic changes in the structure of procaspase-3 dimers. The interdimer processing mechanism would prevent these scarce active dimers from undergoing self-cleavage. An elusive feature of effector caspases is the presence of the short prodomains (10 to 30 amino acids). In vitro studies have revealed little, if any, difference in enzymatic characteristics of mature effector molecules with and without the prodomain attached to the large subunit (29). However, in many cases, the removal of this prodomain is a requisite step for apoptosis induction (34). The present work suggests an interesting possibility for the function of these prodomains: the presence of the prodomain demands a second cleavage, occurring between partially processed effector caspase dimers, to complete effector caspase activation. Thus, it helps prevent inappropriate activation of these caspases. Moreover, this interdimer processing mechanism also predicts that the activation of procapase-3 is highly sensitive to its concentration, and its processing by initiator caspases during apoptosis may involve its enrichment near the complex where initiator procaspases are activated. To this end, previous studies have shown that procaspase-3 may be recruited to the apoptosome (15,35).

Caspase activation has long been regarded as a self-amplifying process. However, recent studies have indicated that mature caspase-8 does not process its own precursor
Liu et al. (19,36,37). The present work suggests that caspase-3 does not possess this ability to activate its own precursor either. Therefore, the activation of each caspase per se may not be subjected to a positive feedback mechanism. The interdimer processing mechanism for both initiator and effector caspases and the lack of self-amplification for activation of each caspase, in combination with the caspase cascade, allow for effective caspase activation during apoptosis while minimizing inadvertent activation in healthy cells.

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1 The abbreviations used are: EGS, Ethylene glycolbis (succinimidylsuccinate); PAGE, polyacrylamide gel electrophoresis.

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**Figure legends**

Fig. 1. **Mutations that impair the formation of procaspase-3 dimer.**

A. Schematic diagram of procaspase-3 point mutations. The large subunit (p17), small subunit (p12), prodomain (Pro), amino acid substitutions, and amino acids at the domain boundaries are indicated. Asterisk marks the active site cysteine 163. P20 is a processing intermediate comprised of p17 and the prodomain.

B. Interface mutations impair the formation of the procaspase-3 dimer. In vitro-translated, $^{35}$S-methionine-labeled wild type and mutant pro-caspase-3 (Pro-C3) proteins were cross-linked with 0.2
mM EGS or left untreated for 45 min at 4 °C. The reaction mixes were resolved on SDS-PAGE and the procaspase-3 proteins were detected by autoradiography. Molecular weight standards (in kDa) are shown on the right. The bands corresponding to monomeric and dimeric procaspase-3 are indicted. WT, wild type; M1, K154D; M2, D192K; M3, K260D.

Fig. 2. Dimerization of procaspase-3 is required for processing by caspase-8 and caspase-9.
A and B, Procaspase-3 interface mutations inhibit its processing by active caspase-8 and -9. In vitro-translated, 35S-labeled procaspase-3 proteins were treated with the indicated concentration (in 10⁻³ unit/µl) of recombinant active caspase-8 (A, left panel) or caspase-9 (B), or with (+) or without (-) 0.2 unit/µl of caspase-8 (A, right panel). The reaction mixes were then analyzed by SDS-PAGE and autoradiography. FL, full-length procaspase-3.
C, An in vitro procaspase-9 activation system. In vitro-translated, 35S-labeled procaspase-9 (Pro-C9) proteins were incubated with 0.2 µg/µl of cytochrome c for 2h at 37 °C. C/A: the active site Cys287-to-Ala mutation; 3A, the processing site Asp315, Asp330, and Glu306-to-Ala mutation. FL, full length procaspase-9; p35 and p37 are two alternative processing products containing the large subunit and the prodomain, which are generated by caspase-9 auto-cleavage and trans-cleavage by caspase-3, respectively (15).
D, Processing of procaspase-3 and its mutants by apoptosome-activated caspase-9. In vitro-translated, non-radioisotope-labeled procaspase-9 was treated with the indicated concentration of cytochrome c as in C. The reaction mix was then used to process 35S-procaspase-3 proteins.

Fig. 3. Auto-processing of caspase-3 occurs through interdimer cleavage.
A, 35S labeled wild type procaspase-3 or proteolytically inactive caspase-3 (C/S) was mixed with equal volume of the indicated non-radioisotope-labeled protein and treated with 0.2 unit/µl active caspase-8 (+) or left untreated (-) for two hours at 4 °C. Input 35S-labeled proteins (I) are also shown.
B, Active caspase-3 does not process the procaspase-3 intermediate generated by caspase-8 cleavage. 35S-procaspase3(C/S) was treated with the indicated concentration of active caspase-3 and/or caspase-8. 

Fig. 4. Mature caspase-3 does not process its own precursor.
(A) In vitro-translated, 35S-labeled His₆-procaspase-3 was purified on Ni-NTA column and treated with the indicated active caspases. The concentrations of the caspases were 0.2 unit/µl except for that of caspase-8, which was 0.04 unit/µl. 
(B) 35S-PARP made by in vitro transcription/translation was treated with the indicated active caspases as in (A). The reaction mixes were analyzed by SDS-PAGE and auto-radiography.

Fig. 5. Comparison of the activation of procaspase-3 and -8. (I) Initiator procaspases such as procaspase-8 acquire enzymatic activity through adaptor-mediated oligomerization and the subsequent formation of precursor dimers (left), while the effector caspase, caspase-3, becomes enzymatically competent through trans-cleavage by the initiator caspase (right). (II) In both cases, these proteolytically active, dimeric intermediates go on to cleave one another to convert to the mature caspases. Neither mature caspase-8 nor mature caspase-3 cleaves its own precursor.
Fig. 1

A

Procaspe-3

K154D (M1)

D192K (M2)

K260D (M3)

Pro p17 * p12

1 D28 D175

p20

B

| Pro-C3 | WT | M1 | M2 | M3 |
|--------|----|----|----|----|
| EGS    | +  | -  | +  | -  |
|        | -  | +  | -  | +  |

(Pro-C3)$_2$

Pro-C3

1 2 3 4 5 6 7 8

-66

-45

-30

-21
Fig. 2

A

B

C

D
Fig. 3

A

| [35S]Pro-C3 | WT | C/S |
|-------------|----|-----|
| Pro-C3      | -  | -   |
| C8          | I  | +   |

B

| [35S]Pro-C3(C/S) | C3 | .2 | .2 | .2 | Unit/μl |
|------------------|----|----|----|----|---------|
| Pro-C3           | C8 | .2 | .2 | 0  | Unit/μl |

FL, p20, p17, p12
Fig. 4

A

$^{[35S]}$Pro-C3

FL
p20
p17
p12

B

$^{[35S]}$PARP

FL
Cleaved products

1 2 3 4 5 6 7
Fig. 5

**a**

Procaspe → Adaptor → Active intermediate → Mature caspase

**b**

(I) Caspase-8

(II) Caspase-3
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