A Sequential Two-Step Mechanism for the Production of the Mature p17:p12 Form of Caspase-3 in Vitro*

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The apoptotic cysteine protease, caspase-3, is expressed in cells as an inactive 32-kDa precursor from which 17 kDa (p17) and 12 kDa (p12) subunits of the mature caspase-3 are proteolytically generated during apoptosis. Two amino acid sequences, ESMD S (amino acids 25–29) and IETD S (amino acids 172–176), in the precursor have been defined as the cleavage sites for the production of the p17 and p12 subunits. Using a cell-free assay system, we demonstrate that the caspase-3 precursor appears to be cleaved first at the IETD S site, producing the p12 subunit and a 20-kDa (p20) peptide. Subsequently, the p20 is cleaved at the ESMD S site, generating the mature p17 subunit. The cleavage at the IETD S site required a protease activity that was selectively inhibited by the peptide, Ac-IETD-CHO (acetyl-IETD-aldehyde), and other protease inhibitors, such as the cowpox viral serine protease inhibitor, CrmA, and N-α-tosyl-L-phenylalanine chloromethyl ketone. The protease that catalyzed the cleavage at the ESMD S site was selectively inhibited by another peptide, Ac-ESMD-CHO (acetyl-ESMD-aldehyde). More interestingly, the caspase-3 inhibitor, Ac-DEVD-CHO, but not the caspase-1 inhibitor, Ac-YVAD-CHO, also selectively inhibited the protease activity that cleaves at the ESMD S site. This indicated that the cleavage at the ESMD S site was either autocatalytic or that it required a caspase-3-like activity. In summary, we demonstrate that production of the p17:p12 form of caspase-3 is a sequential two-step process and appears to require two distinct enzymatic activities.

In recent years, evidence has accumulated that the degradation of certain proteins by members of the caspase family is a general biochemical event taking place in cells undergoing apoptosis (for reviews, see Refs. 1–3). One caspase family member, caspase-3 (4–6), has been studied extensively. In particular, several of the cellular protein targets of caspase-3 have been identified. These include the DNA repair enzyme poly-(ADP-ribose) polymerase (PARP) (5, 7), the 70-kDa protein component of the U1 small nuclear ribonucleoprotein (8), and the catalytic subunit of DNA-dependent protein kinase (DNA-PK) (9–11). Interestingly, inhibition of caspase-3 or caspase-3-like proteases in various cells has been shown to block apoptosis (6, 12–17). In addition, the functional inactivation of the caspase-3 gene in knock-out mice results in the profound absence of apoptosis in certain tissues and lethality shortly after birth (18). Therefore, caspase-3 appears to be an extremely biologically relevant apoptotic protease.

All caspase family members are initially synthesized as inactive precursors and require proteolytic processing themselves to generate the two subunits that form the active protease. This suggests that the apoptotic machinery may be regulated, in part, by a proteolytic cascade (for review, see Ref. 19). In the case of caspase-3, the mature enzyme is formed from 17 kDa (p17) and 12 kDa (p12) subunits, which are produced from a 32-kDa precursor (4–6). There are two amino acid sequences, ESMD S (amino acids 25–29) and IETD S (amino acids 172–176), in the 32-kDa caspase-3 precursor protein that have been defined as the cleavage sites for the production of the p17 and p12 subunits (5) (see Fig. 1). At both sites, the cleavage should occur between the aspartic (Asp) and serine (Ser) residues (5). However, the process of how the caspase-3 precursor is converted into the mature caspase-3 remains uncharacterized, and the cellular proteases that cleave these sites have not been identified.

We have recently shown that when the human promyelocytic leukemia HL-60 cell line was induced to undergo apoptosis by exposure to staurosporine (STS), one of the early biochemical events was the conversion of the 32-kDa caspase-3 precursor into the mature protease that was responsible for the subsequent proteolysis of DNA-PK (9). In addition, we have also demonstrated that apoptosis in HL-60 cells triggered by STS was independent of the synthesis of new proteins (20), indicating that HL-60 cells constitutively express all of the factors necessary for apoptosis including the caspase-3 precursor proteases. Therefore, cell extracts from HL-60 cells should provide an ideal system for the identification of the proteases that can catalyze the conversion of the caspase-3 precursor protein into an active apoptotic protease.

A recent study by Liu et al. (21) using a cell-free system has identified cytochrome c and dATP as two essential factors required for the induction of the proteolytic activation of caspase-3 in vitro. In our study, we have modified this in vitro assay system and investigated the process of proteolytic processing of caspase-3. Our results show that this process appears to involve two proteolytic steps and to be catalyzed by two different proteases. Lastly, our study provides insight into why certain protease inhibitors, including the caspase-3 selective inhibitor Ac-DEVD-CHO, can block apoptosis.

DNA-PK, DNA-dependent protein kinase; DNA-PKcs, catalytic subunit of DNA-PK; TPCK, N-α-tosyl-L-phenylalanine chloromethyl ketone; CHO, aldehyde; STS, staurosporine.

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The abbreviations used are: PARP, poly(ADP-ribose) polymerase;
Western blot analysis conditions for DNA-PKcs have been described (9). The proteins were transferred onto nitrocellulose paper by electrophoresis in a Trans-Blot chamber, and proteins were identified by Western detection reagents. The conditions described by Liu et al. (21) were modified for this assay. Briefly, 20 μl of the S-100 extract was mixed with 4 μg of cytochrome c and 7.5 mg EDTA in a final volume of 25 μl. The mixture was incubated at 37 °C for 30–60 min. The reaction was terminated by addition of 5 μl of 6 X SDS sample loading buffer and heated at 100 °C for 5 min. Alternatively, 3 μl (~500 ng) of a purified DNA-PK solution (9) was added to the mixture, and the incubation was continued for another 60 min before it was terminated. When protease inhibitors were used, the extract was first incubated with an inhibitor for 10 min at 20 °C and then mixed with cytochrome c and incubated at 37 °C. Proteins were then subjected to SDS-polyacrylamide gel electrophoresis (12% for caspase-3 analysis and 5% for DNA-PK proteolysis analysis). The proteins were transferred onto nitrocellulose paper by electroelution in a 40-kDa range of 2.5–5 kDa. The purity of these peptides was approximately 98%. The cowpox viral serine protease inhibitor, CrmA, was purchased from Kampe, Biomedical Co. (Seattle, WA). Monoclonal antibodies against human DNA-PKcs have been described (9). Mouse monoclonal anti-human caspase-3 antibodies were purchased from Transduction Laboratories, Inc. (Lexington, KY). The ECL Western blot analysis reagents were purchased from Amersham Life Science, Inc. (Arlington Heights, IL).

Cells—HL-60 cells were originally obtained from the American Type Culture Collection. The cells were cultured in RPMI 1640 medium supplemented with 20% fetal bovine serum, 100 units/ml penicillin and 50 units/ml streptomycin.

Preparation of Cell Extracts—Five liters of HL-60 cells were pelleted and washed in 200 ml of phosphate-buffered saline three times. After the final wash, the cell pellet was resuspended in an equal volume of hypotonic buffer (10 mM HEPES, pH 7.5, 5 mM MgCl₂, 1 mM dithiothreitol, 3 mM phenylmethylsulfonyl, 50 μg/ml each of leupeptin, aprotinin and antipain). The cell suspension was incubated on ice for 15 min. The cells were then broken by passing them 5 times through a syringe with a 25-gauge needle. This resulted in the lysis of over 99% of the cells. The lysate was first centrifuged at 12,500 × g for 10 min at 4 °C. Then, the supernatant was centrifuged at 100,000 × g for 1 h at 4 °C. The 100,000 × g supernatant (S-100) was transferred to another tube, and protein concentration was determined and diluted to a final concentration of 10 mg/ml with the hypotonic buffer. The final preparation also contained 5% glycerol. This preparation was kept at −85 °C.

Processing of Caspase-3, Proteolysis of DNA-PK, and Western Blot Analysis—Conditions described by Liu et al. (21) were modified for this assay. Briefly, 20 μl of the S-100 extract was mixed with 0.4 μg of cytochrome c and 7.5 mg EDTA in a final volume of 25 μl. The mixture was incubated at 37 °C for 30–60 min. The reaction was terminated by addition of 5 μl of 6 X SDS sample loading buffer and heated at 100 °C for 5 min. Alternatively, 3 μl (~500 ng) of a purified DNA-PK solution (9) was added to the mixture, and the incubation was continued for another 60 min before it was terminated. When protease inhibitors were used, the extract was first incubated with an inhibitor for 10 min at 20 °C and then mixed with cytochrome c and incubated at 37 °C. Proteins were then subjected to SDS-polyacrylamide gel electrophoresis (12% for caspase-3 analysis and 5% for DNA-PK proteolysis analysis). The proteins were transferred onto nitrocellulose paper by electrophoresis in a Trans-Blot chamber, and proteins were identified by Western blot analysis using antibodies and ECL Western reagents.

RESULTS

In Vitro Processing of Caspase-3—Liu et al. (21) recently showed that in the presence of dATP, cytochrome c, and in a cytosolic extract from Hela cells, caspase-3 precursors, which were translated in vitro, were converted into active caspase-3. Using a similar approach, we studied the effect of cytochrome c on the status of the endogenous 32-kDa caspase-3 precursor present in cytosolic extracts derived from HL-60 cells. Incubation of the HL-60 S-100 extract with 0.4 μg of cytochrome c at 37 °C for 60 min resulted in the complete loss of the 32-kDa caspase-3 precursor and was accompanied by the appearance of the p17 subunit (which is the only subunit recognized by the antibody), indicating that the 32-kDa precursor was proteolytically processed (Fig. 2A, lane 3). This effect appeared to be specifically induced by cytochrome c since incubation of the S-100 extract alone at 37 °C for either 0 or 60 min did not induce the processing of the 32-kDa caspase-3 precursor (Fig. 2A, lanes 1 and 2, respectively). To confirm these results, the presence of caspase-3 activity was monitored using purified DNA-PK as a substrate (9–11). Thus, purified DNA-PK was incubated with either untreated or cytochrome c-treated HL-60 S-100 extract and subsequently subjected to Western blot analyses. Only the cytochrome c-treated S-100 extract contained an activity that cleaved the DNA-PKcs into a 150-kDa fragment (Fig. 2B, lane 3), which was identical to the size observed in vitro in apoptotic cells (9). Therefore, cytochrome c treatment of HL-60 S-100 extract induced the appearance of an active caspase-3.

The kinetics of cytochrome c-induced proteolytic processing of caspase-3 were determined. Cytochrome c induced the appearance of the p17 subunit within 10 min and complete conversion occurred within 30 min (Fig. 2C). It was interesting to note that during the initial 10–20 min, a small amount of a 20-kDa (p20) peptide was also detected (Fig. 2C, lanes 2 and 3). However, the level of this p20 peptide was far below that of the p17 peptide. According to the reported work on the molecular cloning of the caspase-3 gene (4, 5), the p17 polypeptide is derived from the endogenous precursor caspase-3 into mature p17 and p12 subunits that form the mature caspase-3 (for details, see Ref. 5).

Selective Inhibition of Cleavage at the ESMD ↓ S and IETD ↓ S Sites Reveals a Two-step Processing Mechanism—The predicted cleavage sites in the 32-kDa caspase-3 precursor are defined by the amino acid sequences ESMD ↓ S (amino acids 25–29) and IETD ↓ S (amino acids 172–176), with the cleavage presumed to occur between the Asp and Ser residues at both sites (5). Aldehyde peptide analogs of cleavage site amino acid sequences have been shown to function as highly selective inhibitors of caspase-1 (22) and caspase-3 (5, 7, 9). Therefore, we synthesized acetyl-ESMD-aldehyde (Ac-ESMD-CHO) and acetyl-IETD-aldehyde (Ac-IETD-CHO) peptide analogs and used them as potential site-selective inhibitors in our caspase-3 processing assay. The Ac-ESMD-CHO peptide analog, in a range of 2.5–5 μM, blocked the formation of the p17 subunit and...
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concomitantly induced the accumulation of the p20 peptide (Fig. 3A). In striking contrast, the presence of the Ac-IETD-CHO peptide analog, in a concentration range of 0.5 μM, blocked the formation of the p17 subunit and concomitantly induced the accumulation of the 32-kDa precursor (Fig. 3B). In particular, the Ac-IETD-CHO peptide did not induce accumulation of the p20 product. Thus, the peptide analogs Ac-EMSD-CHO and Ac-IETD-CHO both inhibit caspase-3 processing but they result in the accumulation of different reaction products.

A Caspase-3-selective Inhibitor Preferentially Blocks Cleavage at the ESMD | S Site in the p20 Peptide—To extend the above results, we used two additional well characterized aldehyde peptides, Ac-DEVD-CHO, which is a highly selective inhibitor of caspase-3 (5, 7, 9), and Ac-YVAD-CHO, which is a highly selective inhibitor of caspase-1 (22). Addition of the Ac-DEVD-CHO inhibitor to our assay, in a concentration range of 50–100 nM, completely blocked the production of the p17 subunit and resulted in the concomitant accumulation of p20 (Fig. 3C), indicating that Ac-DEVD-CHO blocked processing of caspase-3 at the ESMD | S site in the p20 peptide. In contrast, the caspase-1 inhibitor, Ac-YVAD-CHO, did not have any effect on the proteolytic processing of caspase-3 (Fig. 3D), consistent with our earlier studies (9). These results suggested that (i) a caspase-3-like proteolytic activity was required for cleavage at the ESMD | S site in the p20 peptide and (ii) that the pattern of inhibition induced by Ac-DEVD-CHO was similar to that observed with Ac-EMSD-CHO.

CrmA and TPCK Selectively Block Cleavage at the IETD | S Site in the 32-kDa Precursor—Two additional inhibitors were found to have a profound inhibitory effect on proteolysis of the caspase-3 precursor. TPCK, which can inhibit serine proteases (30), and, in some studies, apoptosis (25–27) prevented the 32-kDa precursor from being processed at all in a dose-dependent fashion (Fig. 4A). CrmA, which is a cowpox viral protein that has been shown to inhibit the activity of caspase-1 and caspase-3 and block apoptosis (6, 12, 23, 24), also prevented the 32-kDa caspase-3 precursor from being processed at all (Fig. 4B). These findings indicated that (i) both TPCK and CrmA can inhibit caspase-3 activation and (ii) that their pattern of inhibition was similar to that observed with Ac-IETD-CHO.

DISCUSSION

In this study, we have demonstrated that in a cell-free assay system cytochrome c induces the proteolytic conversion of the 32-kDa caspase-3 precursor into the mature caspase-3. Furthermore, our results showed that this process involved two sequential cleavage steps and that each step was catalyzed by a unique proteolytic activity. Finally, our results provide additional insight into the mechanisms by which protease inhibitors block apoptosis.

Proteolytic Processing of Caspase-3—Molecular cloning of the caspase-3 gene and biochemical analysis of the purified, active caspase-3 showed that caspase-3 was initially synthesized as an inactive 32-kDa precursor (4–6), which was subsequently processed by proteolytic cleavage at ESMD | S (amino acids 25–29) and IETD | S (amino acids 172–176) to produce the p12 and p17 subunits that form the mature caspase-3 (5) (see Fig. 1). Our results indicate that this process appears to require two sequential cleavage steps (Fig. 5). Cleavage at the IETD/S site appeared to occur first, yielding a mature p12 subunit and a p20 intermediate product. However, it should be pointed out that since good antibodies that can recognize the p12 subunit are not available now, the exact status of this subunit during the assay period remains speculative. Subsequently, a cleavage occurred at the ESMD | S site in the p20 intermediate, yielding the mature p17 subunit. Together, the p12 and p17 subunits then form the mature caspase-3 (Fig. 5). Thus, blocking the cleavage at IETD | S with Ac-IETD-CHO, CrmA, or TPCK completely abrogated the appearance of any processed products of caspase-3, whereas blocking the cleavage at ESMD | S with Ac-EMSD-CHO or Ac-DEVD-CHO allowed the primary cleavage at IETD | S to occur but not the secondary cleavage at ESMD | S (Figs. 3 and 4). Thus, our data provide strong evidence for a sequential two-step processing mechanism.

The Nature of the Protease That Cleaves the IETD | S Site—
Our results suggest that the protease activity required for the cleavage step at the IETD ↓ S site in the 32-kDa caspase-3 precursor has some novel properties. First, it is sensitive to the sequence-specific peptide inhibitor, Ac-IETD-CHO (Fig. 3B), and it is also sensitive to CrmA and TPCK (Fig. 4). CrmA is a cowpox viral protein that can inhibit several members of the caspase family including caspase-1 (28, 29), caspase-3 (6), caspase-6 (34), and caspase-8 (32). However, it is unlikely that, in our system, CrmA is inhibiting the caspase-1 or caspase-3 activities. First, Northern blot analysis of HL-60 cells found no evidence for the expression of the caspase-1 gene (data not shown). Second, the presence of the highly selective caspase-1 inhibitor, Ac-YVAD-CHO (22), had no effect on the process of proteolytic activation of caspase-3 (Fig. 3D). Third, the cleavage at the IETD ↓ S site proceeded normally in the presence of the caspase-3 inhibitor (Fig. 3C). Therefore, it is unlikely that caspase-1 or caspase-3-like proteases are involved with this event. It remains to be seen if other caspses, such as caspase-6 and caspase 8, might be responsible for cleaving the caspase-3 IETD ↓ S site. This is potentially likely since both activated caspase-6 (33, 34) and caspase-8 (32) have been shown to initiate the proteolytic activation process of caspase-3 in vitro, and the activity of both caspses is inhibitable by CrmA (32, 34).

TPCK is a general inhibitor of chymotrypsin-like serine proteases (30), and while it has not been shown to have an inhibitory effect on caspses, it has been observed, in some studies, to have an inhibitory effect on apoptosis (25-27). Thus, it is possible that TPCK, at the concentrations used in our assay, directly inhibited the protease that cleaves at the IETD ↓ S site, thus inhibiting the processing of the caspase-3 precursor (Figs. 4A and 5). If this is true, then both TPCK and CrmA may be inhibiting the same protease. Alternatively, it is also possible that the TPCK-sensitive protease is different from that of CrmA. This would suggest that the TPCK-sensitive protease acts upstream of the CrmA-sensitive protease. Experiments to test this possibility are underway.

The Nature of the Protease That Cleaves the ESMD ↓ S Site in the p20 Intermediate Peptide—One surprising finding in this study is the observation that the highly selective caspase-3 inhibitor, Ac-DEVD-CHO (5, 7, 9, 14), blocked only the second cleavage step at the ESMD ↓ S site in the p20 peptide (Fig. 3C). This prevented the conversion of the p20 intermediate peptide into the mature p17 subunit. Apparently, the inhibitory effect of Ac-DEVD-CHO was selective upon this cleavage step, since it had no observable effect on the first cleavage step at the IETD ↓ S site in the 32-kDa precursor (Fig. 3C). This suggests that, unlike the protease activity that was required for cleaving the IETD ↓ S site, either caspase-3 itself or, more likely, a caspase-3-like proteolytic activity is specifically required for cleaving the ESMD ↓ S site. It could be argued that the HL-60 cell S-100 extract contained a trace amount of endogenous, active caspase-3, which was responsible for generating the p17 subunit once the p20 product was available. However, this appears to be unlikely for the following reasons. First, we have not been able to detect the presence of p17 in the untreated S-100 extract even in Western blots with overloaded protein samples (data not shown). Second, the effective concentration of Ac-DEVD-CHO (50–100 nM; see Fig. 3C) that was required for the inhibition of this protease activity was relatively high. In assays that have been described for assessing the inhibitor potency of the Ac-DEVD-CHO peptide on purified caspase-3, the K_i values have been estimated to be less than 1 nM (5). The fact that Ac-DEVD-CHO inhibited the protease activity that cleaves the ESMD ↓ S site in a range of 50–100 nM suggests that this activity could not be attributed to the presence of a trace amount of the active caspase-3 in the extract. Rather, it is likely that this activity is simply caspase-3-like.

Alternatively, it could be speculated that p20 and p12 may form a partially active intermediate protease that undergoes autocatalysis at the ESMD ↓ S site, producing the fully active caspase-3 consisting of p17 and p12 (Fig. 5). Unlike the fully active caspase-3, which is extremely sensitive to the peptide inhibitor Ac-DEVD-CHO (5), the partially active p20/p12 intermediate protease may have a much lower affinity for the Ac-DEVD-CHO peptide and thus be less sensitive to it than the fully active p17/p12 protease. Experimentally, it should be possible to prove or disapprove this hypothesis. Introduction of a point mutation into the ESMD ↓ S site that makes it unmeasurable should give rise to the production of only p12 and p20 peptides. If the p20/p12 complex has any proteolytic activity,
then it may be able to quantify this using caspase-3 substrates such as PARP (5, 7) and DNA-PKcs (9–11).

New Aspects to the Mechanisms by which CrmA, TPCK, and Ac-DEVD-CHO Inhibit Apoptosis—It is worthy of note that CrmA, TPCK, and Ac-DEVD-CHO blocked conversion of the inactive 32-kDa caspase-3 precursor into the mature caspase-3 (Figs. 3C and 4). All three inhibitors have been shown, by various investigators, to block apoptosis in a variety of cells under many different treatments (6, 12, 14, 23–27, 31). The reason CrmA and Ac-DEVD-CHO block apoptosis was thought to be due to their direct inhibitory effect on the active apoptotic caspase-3 (5, 6). The results of this study have revealed that both inhibitors are also capable of preventing the conversion of the inactive caspase-3 precursor into the active protease.

Finally, although TPCK has been found to inhibit apoptosis in several studies (25–27), the mechanism by which it mediates this effect was unclear. In particular, it has been well characterized that TPCK has no direct inhibitory effect on caspase-3 (5, 9). Therefore, we suggest that at least one mechanism by which TPCK prevents apoptosis is that it can inhibit a novel protease that participates in the process of the conversion of the caspase-3 precursor to the active apoptotic protease.

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