The Ced-3/Interleukin 1β Converting Enzyme-like Homolog Mch6 and the Lamin-cleaving Enzyme Mch2α Are Substrates for the Apoptotic Mediator CPP32

(Received for publication, June 10, 1996, and in revised form, August 6, 1996)

Srinivasa M. Srinivasula§, Teresa Fernandes-Alnemri, James Zangrilli, Noreen Robertson‡, Robert C. Armstrong§, Lijuan Wang‡, Joseph A. Trapani‡, Kevin J. Tomaselli, Gerald Litwack§, and Emad S. Alnemri¶

From the §Center for Apoptosis Research, the Department of Biochemistry and Molecular Pharmacology, and the Kimmel Cancer Institute, Jefferson Medical College, Philadelphia, Pennsylvania 19107, ¶Cellular Cytotoxicity Laboratory, The Austin Research Institute, Heidelberg, Australia, and ¶IDUN Pharmaceuticals, San Diego, California 92121

Recent evidence suggests that CPP32 is an essential component of an aspartate-specific cysteine protease (ASCP) cascade responsible for apoptosis execution in mammalian cells. Activation of CPP32 could lead to activation of other downstream ASCPs, resulting in late morphological changes such as lamin cleavage and DNA fragmentation, observed in cells undergoing apoptosis. Here we describe the identification and cloning of a novel human ASCP named Mch6 from Jurkat T lymphocytes. We demonstrate that the pro-enzymes of Mch6 and the lamin-cleaving enzyme Mch2α are substrates for mature CPP32. Site-directed mutagenesis revealed that CPP32 processes pro-Mch6 preferentially at Asp330 to generate two subunits of molecular masses 37 kDa (p37) and 10 kDa (p10). However, CPP32 processes pro-Mch2α at three aspartate processing sites (Asp23, Asp179, and Asp180) to produce the large (p18) and small (p11) subunits of the mature Mch2α enzyme. The CPP32-processed Mch2α is capable of cleaving the VEIDN lamin cleavage site, indicating that CPP32 can, in fact, activate pro-Mch2α. Granzyme B at a concentration that allows processing and activation of CPP32 failed to process pro-Mch2α. However, incubation of pro-Mch2α with granzyme B in the presence of a cellular extract containing pro-CPP32 resulted in activation of pro-CPP32 and subsequent processing of pro-Mch2α. Interestingly, granzyme B can also process pro-Mch6 but at a site N-terminal to that cleaved by CPP32. These data suggest that Mch2α and Mch6 are downstream proteases activated in CPP32- and granzyme B-mediated apoptosis. This is the first demonstration of a protease cascade involving granzyme B, CPP32, Mch2α, and Mch6 and evidence that the lamin-cleaving enzyme Mch2 is a target of mature CPP32.

Execution of apoptosis in eukaryotic cells is an active biochemical process that depends upon activation of pro-enzymes of the new family of aspartate-specific cysteine proteases (ASCPs)1 (1–4). The prototype of this family is the interleukin 1β converting enzyme (ICE), which exists as a 45-kDa pro-enzyme and is cleaved to form two subunits (p20 and p10), which associate as a tetramer (p20)2/p102 to form the active enzyme (5, 6). ICE bears no resemblance structurally to other known cysteine proteases and is unique in its substrate specificity in that it cleaves after certain aspartates. In light of the homology to Ced-3, an expanded role for ASCPs as positive regulators of apoptosis was hypothesized and subsequently supported when it was shown that overexpression of ICE in fibroblasts resulted in their death by apoptosis (7). This process could be blocked by crmA (a specific viral inhibitor of ICE) or the Bcl-2 proto-oncogene product. Several new members of this family have been described recently, including CPP32 (8), Nedd2/Ich-1 (9, 10), Mch2α and Mch2β (11), Mch3α and Mch3β (12), Mch4 (13), Mch5 (13), TX (ICH-2, ICErel-II) (14–16), ICErel-III (16), and four alternatively spliced isoforms of ICE (17). Like ICE, all of these enzymes require processing to large and small subunits for activity, cleave after specific aspartates, and possess the unique and highly conserved pentapeptide active site containing an essential cysteine. Like Ced-3, overexpression of these ICE homologs is capable of inducing apoptosis in various in vivo models.

Granzyme B is a cytoplasmic granule-associated serine protease expressed by cytotoxic T and natural killer cells and is the only other enzyme with similar substrate specificity to ASCPs (18). Release of granzyme B by these cells has been shown to be essential for rapid degradation of DNA and induction of apoptosis in susceptible target cells (19). It now appears that this activity may be mediated, at least in part, by multiple ASCPs that are activated by granzyme B (13).

Although ASCPs appear to play fundamental roles in inflammation and apoptosis, their individual contribution to the apoptosis mechanism is not yet clear. Additionally, their mechanism of activation, enzymatic specificity, and physiologically relevant apoptotic substrates remain to be established. Recently, we provided evidence for the involvement of multiple ASCPs in apoptosis (13, 20). We also demonstrated that lamin A is cleaved by Mch2α, but not CPP32, at the same site that is cleaved in apoptotic S/M extracts (20). In one study, we demonstrated that the newly identified FADD-like ASCP (Mch4)

1 The abbreviations used are: ASCP, aspartate-specific cysteine protease; ICE, interleukin 1β converting enzyme; PARP, poly(ADP-ribose) polymerase; Mch, mammalian ced-3 homolog; CHAPS, 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; DEVD-CHO, DEVD-aldehyde.
can process and activate CPP32 and Mch3 (13). pro-Mch4 and pro-Mch5 are the only known ASCPs that contain FADD-like death effector domains in their long N-terminal pro-domain, suggesting possible involvement in Fas and other apoptotic pathways (13). It appears that proteases, such as Mch4 and Mch5 with long pro-domains, might be upstream and could interact with the proximal signal transduction machinery of apoptosis. Others with short pro-domains, such as CPP32, Mch3, and Mch2a, are downstream or distal, operating at or near the cell death effector level.

CPP32 appears to be an important central intermediary in the cell death pathway. This is evident from its ability to be activated by granzyme B and by the upstream FADD-like proteases Mch4 and Mch5 (13) to cleave the well characterized cell death substrate poly(ADP-ribose) polymerase (PARP) (11, 12), and induce the morphological changes of apoptosis in isolated nuclei (21). Identification of ASCPs that are targets for activation by mature CPP32 should help us to understand the biochemical events that lead to the apoptotic morphology. The most likely target for CPP32 are those ASCPs that contain a conserved processing site DXDD between their large and small subunits. These include Nedd2/Ich-1, Mch2a, and Mch6 (this study).

Recently, we demonstrated that Nedd2 is a substrate for mature CPP32 and granzyme B (22). In this study, we report the identification and cloning of a new member of the family of mammalian ASCPs that we have designated Mch6 (mammalian ced-3 homolog 6). We provide evidence that pro-Mch6 is a natural substrate for granzyme B and mature CPP32. Furthermore, we show that mature CPP32 can directly process and activate pro-Mch2a by cleaving at three Asp cleavage sites. These data suggest that activation of CPP32 in apoptosis could lead to activation of pro-Mch2a and pro-Mch6.

MATERIALS AND METHODS

Cloning of Human Mch6—To clone Mch6, a 10-μl aliquot of human Jurkat a Uni-ZAP XR cDNA library (8) containing ~10^6 plaque-forming units was denatured at 99°C for 5 min and used as a template for PCR amplification with PCR primer Mch6-pr1 (CTCAACGTACCAGGCCACGCGG) derived from the GenBank-Established Sequence Tag sequence T97582 and the T3 vector-specific primer (Stratagene). A 10-μl aliquot of the primary amplification product was then used as a template for a secondary PCR amplification with primer Mch6-pr2 (CCTGCGGAAAGTAGAGTAGG) derived from the same Established Sequence Tag sequence and the SK-Zap vector-specific primer (11) located downstream of the T3 primer. The secondary amplification products were cloned into a SmaI cut pBluescript II KS vector. The partial cDNA was then excised from the vector, radiolabeled, and used to screen the original Jurkat cDNA library. Positive a clones were purified, rescued into the pBluescript II SK-plus vector, and sequenced.

Northern Blot Analysis—Tissue distribution analysis of Mch6 mRNA was performed on Northern blots (Clontech) using Mch6 cDNA as a probe, as described previously (12, 13).

Expression of CPP32 and Mchs in Bacteria and Assay of Enzyme Activity—Recombinant human CPP32 and Mch2a with a C-terminal His tag were expressed in Escherichia coli and assayed as described recently (11–13). The recombinant proteases were purified on a Ni^{2+}-affinity resin.

Purification of Granzyme B—Granzyme B was purified by immunopurification from human natural killer cell lysates using granzyme B-specific monoclonal antibody and assayed as described previously (23, 24).

Mutagenesis, in Vitro Transcription/Translation, and Cleavage Assays—Potential aspartate processing sites in pro-CPP32, pro-Mch2a, or pro-Mch6 were mutated by site-directed mutagenesis using overlapping PCR mutagenic oligonucleotides. The resulting PCR products were subcloned in pBluescript KS- vector (pro-Mch2a) or pET-21b vector (pro-CPP32 and pro-Mch6) under the T7 promoter, and their sequences were verified by sequence analysis. Wild-type and mutated cDNAs were in vitro transcribed and translated in the presence of ^{35}S methionine using a coupled transcription/translation TNT kit (Promega) according to the manufacturer’s recommendations. Two μl of the translation reactions were incubated with purified enzymes CPP32 (30 ng = 1 pmol) or granzyme B (140 ng = 5 pmol in the case of pro-Mch2a or 280 ng = 10 pmol in the case of CPP32 in ICE buffer from 3 to 4 h at 37°C) with 5 mM EDTA, 5 mM dithiothreitol, and 0.1% CHAPS, pH 7.5) in a final volume of 10 μl. The reaction was incubated at 37°C for various times and then analyzed by Tricine-SDS-PAGE and autoradiography.

Western Blot Analysis of CPP32—Processing of CPP32 in 697 cellular extracts treated with granzyme B was determined by Western blot analysis, using a rabbit polyclonal anti-human CPP32 antibody. This antibody was raised against a recombinant p20 subunit (amino acids 1–175) of human CPP32.

RESULTS AND DISCUSSION

Cloning of Human Mch6—A search of the GenBank Established Sequence Tags for sequences related to CPP32 and Mch2a identified a short established sequence tag with accession number T97582. Based on this sequence information, an ~2-kilobase cDNA was cloned from a human Jurkat T-lymphocyte cDNA library using a similar methodology as described recently (11, 12). This cDNA contains an open reading frame of 1248 base pairs that encodes a 416-amino acid protein, named Mch6, with a predicted molecular mass of ~46.2 kDa (Fig. 1A).

Mch6 Belongs to the Aspartate-specific Cysteine Protease Ced-3 Subfamily—The Ced-3-like ASCP subfamily includes Ced-3, Mch3, Mch4, and Mch5 (13). Sequence alignment of all known human ASCPs and the nematode Ced-3 ASCP. Based on crystal structure of ICE, the lower case letters below the sequences indicate residues that are involved in catalysis (c), binding the substrate-carboxyylate of P1 Asp (a), or adjacent to the substrate P2–P4 amino acids (a). D/X indicates known and potential processing sites between the small and large subunits of ASCPs. The roman numbers on the left indicate the three ASCP subfamilies: the Ced-like subfamily (I), the ICE-like subfamily (II), and the Nedd2/Ich-1 subfamily (III). The asterisk indicates the nonconservative substitution in the active site pentapeptides of Mch4, Mch5, and Mch6. The starting residue of each sequence is numbered to the left of the sequence.
productscanbedetectedwithinthefirst15min(lanes 2 and 3) of molecular masses cleaves pro-Mch6 at one site to generate two cleavage products: a large 35-kDa (p35) product and a small 12-kDa (p12) product. The large residue in its active site pentapeptide (QACGG) is a Gly instead of Arg or Gln (Fig. 1B).

The pro-domain of pro-Mch6 has high homology to the pro-domains of CED-3 and Nedd2/Ich-1. There is evidence suggesting that the pro-domain of some ASCPs could play a role in the activation of the pro-enzyme (25). Interestingly, we discovered recently that the pro-domains of pro-Mch4 and pro-Mch5 contain FADD-like death effector domains (13). These domains could interact with FADD or other cellular FADD-like proteins, thus connecting the proximal apoptotic signal transduction pathways (i.e., Fas pathway) to the downstream ASCP activation cascade.

The crystal structure of ICE revealed that His237, Gly238, and Cys285 are involved in catalysis, whereas Arg179, Gln283, Arg341, and Ser347 are involved in binding the carboxylate side chain of the substrate P1 aspartate (5, 6). These residues are identical in all family members including Mch6, except in Mch5, where there is a Ser to Thr conservative substitution for the residue corresponding to Ser347 of ICE (Fig. 1B). Another Ser to Thr conservative substitution can also be seen in Mch4 in the region corresponding to Ser285 of ICE, which is one of the residues that participates in binding the substrate P2–P4 residues. Other residues that might participate in binding the substrate P2–P4 residues are not widely conserved, suggesting that they may determine substrate specificity.

**Tissue Distribution of Mch6**—The pro-Mch6 riboprobe detected three major mRNA species (~1.0, ~2.4, and ~4.4 kilobases) in most human tissues (Fig. 2). The highest expression is seen in the heart, with moderate expression in liver, skeletal muscle, and pancreas. Lowest expression was observed in the other tissues. The presence of multiple mRNA species was observed previously with ICE mRNA (26) and is suggestive of alternative splicing or polyadenylation.

**pro-Mch6 Is a Substrate of Mature CPP32**—pro-Mch6 contains two potential processing sequences between its large and small subunits (Fig. 1). The site is a potential granzyme B cleavage site because it contains an acidic residue at the P3 position. The site is a potential CPP32 cleavage site, because it is similar to the DEVD6 site in PARP and DNA-PKcs (27) and it contains an Asp residue at the P4 position. To test whether pro-Mch6 is a substrate for CPP32, pro-Mch6 was translated in vitro in the presence of [35S]methionine and then incubated with purified recombinant human CPP32. Time course analysis (Fig. 3A) revealed that CPP32 cleaves pro-Mch6 at one site to generate two cleavage products of molecular masses ~37 kDa (p37) and ~10 kDa (p10). These products can be detected within the first 15 min (lanes 2 and 3).

Longer incubation results in a decrease in the intensity of the full-length 46-kDa band and an increase in the intensity of the p37 and p10 products (lanes 5 and 6). No significant processing of the p37 product was observed upon prolonged incubation, suggesting that CPP32 cleaves preferentially at one site within the pro-Mch6 polypeptide. The sizes of the cleavage products are consistent with cleavage at the DQLDA site, which contains Asp330. This was confirmed by site-directed mutagenesis of Asp315 and Asp330. As expected, CPP32 was unable to process the Asp315 mutant to the p37 and p10 products (Fig. 3C). On the other hand, it was able to process the Asp330 mutant to generate the p37 and p10 products (Fig. 3E). These products are indistinguishable from those obtained with the wild-type pro-Mch6 (Fig. 3A). This establishes that Asp330 is the CPP32 processing site.

**Granzyme B Cleaves pro-Mch6 Preferentially at the PEPDA Site**—One way by which granzyme B can induce apoptosis in target cells is by activation of ASCPs. Recently, we demonstrated that granzyme B is able to process multiple members of the ASCP family (13). To test whether granzyme B can process pro-Mch6, [35S]-labeled pro-Mch6 was incubated with granzyme B and then analyzed by SDS-PAGE and autoradiography. Fig. 3B shows that granzyme B cleaves pro-Mch6 preferentially at one site to generate two cleavage products: a large ~35-kDa (p35) product and a small ~12-kDa (p12) product. The large...
granzyme B-cleaved product migrates faster than the large CPP32-cleaved product, suggesting that the two enzymes cleave at two different sites. The granzyme B cleavage site is N-terminal to the CPP32 cleavage site, and it is most likely to be Asp\textsuperscript{315} within the PEPDA site. This was confirmed using the Asp\textsuperscript{315} and Asp\textsuperscript{330} mutant pro-Mch6 (Fig. 3, D and F). Unlike CPP32, granzyme B was able to cleave the Asp\textsuperscript{330} mutant to generate the p35 and p12 products (Fig. 3 D). Mutation of Asp\textsuperscript{315} prevented granzyme B from cleaving pro-Mch6 at the PEPDA site, as evidenced from the absence of the p35 and p12 products (Fig. 3F). Interestingly, granzyme B could still cleave this mutant at the DQLDA site, but less efficiently than CPP32, to generate faint p37 and p10 products. This establishes that granzyme B processes pro-Mch6 at Asp\textsuperscript{315} and Asp\textsuperscript{330} with preference for Asp\textsuperscript{315} over Asp\textsuperscript{330}. A double mutation of Asp\textsuperscript{315} and Asp\textsuperscript{330} completely blocked granzyme B and CPP32 processing of pro-Mch6 (data not shown).

**Activation of CPP32 by Granzyme B in a Cell Lysate Leads to Processing of pro-Mch6 at Asp\textsuperscript{315} and Asp\textsuperscript{330} Simultaneously—** Because CPP32 and granzyme B process pro-Mch6 preferentially at two different sites, it is possible that activation of pro-CPP32 by granzyme B in cell lysates could result in cleavage of pro-Mch6 at both sites simultaneously. To test this possibility, \textsuperscript{35}S-labeled pro-Mch6 was mixed with cell extract from human 697 lymphocytes and then incubated with granzyme B (lanes 1–6) or granzyme B and DEVD-CHO (lane 7) for the indicated times. The reaction products were then analyzed by SDS-PAGE, followed by Western blotting using rabbit anti-CPP32-p20 antibody (upper panel) or autoradiography (lower panel). B, a schematic diagram illustrating processing of pro-Mch6. pro-Mch6 is processed preferentially after Asp\textsuperscript{315} by granzyme B and after Asp\textsuperscript{330} by CPP32 to generate the large subunit (p35) and small subunit (p10) of mature Mch6.

**Fig. 4.** Processing of pro-Mch6 in cell lysates. A, \textsuperscript{35}S-labeled pro-Mch6 was mixed with cell extract from human 697 lymphocytes and then incubated with granzyme B (lanes 1–6) or granzyme B and DEVD-CHO (lane 7) for the indicated times. The reaction products were then analyzed by SDS-PAGE, followed by Western blotting using rabbit anti-CPP32-p20 antibody (upper panel) or autoradiography (lower panel). B, a schematic diagram illustrating processing of pro-Mch6. pro-Mch6 is processed preferentially after Asp\textsuperscript{315} by granzyme B and after Asp\textsuperscript{330} by CPP32 to generate the large subunit (p35) and small subunit (p10) of mature Mch6.
Activation of Mch6 and Mch2α by CPP32 and Granzyme B

C, large subunit (p18) and small subunit (p11) of mature Mch2 are radiographed. Molecular weight markers are indicated on the left of A, C, and D. A, a schematic diagram illustrating processing of pro-Mch2α. Pro-Mch2α is processed at Asp23, Asp179, and Asp193 to generate the large subunit (p18) and small subunit (p11) of mature Mch2α.

sis (Fig. 5A) revealed that CPP32 cleaves pro-Mch2α at multiple sites to generate four cleavage products of molecular masses 21, 18, 13, and 11 kDa. These products can be detected within the first 15 min (Fig. 5, lanes 2 and 3). Longer incubation results in a decrease in the intensity of the 21- and 13-kDa products and an increase in the intensity of the 18- and 11-kDa products (lanes 5 and 6). This suggests that the 18- and 11-kDa products are derived from the 21- and 13-kDa products, respectively, by further proteolytic processing. No further processing was observed upon prolonged incubation, suggesting that the 18- and 11-kDa products are most likely to be the large and small subunits, respectively, of the mature Mch2α enzyme. Unlike CPP32, an equal amount of recombinant bacterially expressed Mch3 was unable to process pro-Mch2α (data not shown).

Primary Structure of Mch2α—The exact aspartate processing sites in the pro-Mch2α polypeptide chain have not yet been determined. Two potential processing sites between the two subunits of Mch2α (176DVVDN180 and 190TEVDA194) and one in the pro-domain (20TETDA24) are very similar to the CPP32 tetrapeptide substrate DEVD. To determine whether CPP32 can cleave pro-Mch2α at the proposed processing sites, mutant pro-Mch2α variants with a P1 D to A or R substitution in these sites were generated. The wild-type and mutant pro-Mch2α variants were in vitro translated in the presence of [35S]methionine, incubated with purified CPP32, and then analyzed by SDS-PAGE and autoradiography. As expected, CPP32 was able to cleave the Asp23-mutated pro-Mch2α between the two subunits but not within the pro-domain (Fig. 5B). This generated the 21-, 13-, and 11-kDa products but not the 18-kDa product. The 13-kDa product was further processed to the 11-kDa product, indicating that there is an additional processing site between the two subunits of Mch2α. However, the N-terminal 21-kDa product was not further processed to the final 18-kDa product. This establishes that Asp23 is the processing site within the pro-domain and that the 18-kDa product is generated from the 21-kDa product.

Double mutation of Asp179 and Asp193 completely prevented processing between the two subunits of Mch2α (Fig. 5C) but did not affect processing within the pro-domain. CPP32 was able to cleave the 34-kDa double mutant pro-Mch2α at Asp23 to generate a 31-kDa product (Fig. 5C). This establishes that Asp23 and Asp193 are the two processing sites between the two subunits of Mch2α. This was further confirmed using single mutants of Asp179 or Asp193 pro-Mch2α (Fig. 5, D and E). Cleavage of the Asp179-mutated pro-Mch2α by CPP32 generated the 11-kDa product and a 20-kDa product but not the 13- or 18-kDa products (Fig. 5D). This suggests that the 11-kDa and the new 20-kDa products are generated by cleavage at Asp23 and Asp193. Cleavage of the Asp193-mutated pro-Mch2α by CPP32 generated the 21-, 13-, and 13-kDa products but not the 11-kDa product (Fig. 5D). These combined data establish that the 11-kDa product is generated by cleavage at Asp193, and the 18-kDa product is generated by cleavage at Asp23 and Asp193. Furthermore, cleavage at Asp179 appears to be more efficient than cleavage at Asp193. This is because the 21- and 13-kDa products appear earlier than the 18- and 11-kDa products (Fig. 5A, lane 2). In addition, CPP32 requires less time to completely cleave wild-type pro-Mch2α (Fig. 5A) than the Asp179-mutated pro-Mch2α (Fig. 5C). According to these results, the mature Mch2α enzyme is derived from the pro-enzyme by cleavage at Asp23, Asp179, and Asp193 to generate the large (p18) and small (p11) subunits (Fig. 5F).

Cleavage of pro-Mch2α by CPP32 Activates Mch2α—A similar mutation analysis revealed that Mch2α is able to cleave its own precursor pro-Mch2α at Asp23 and Asp193 but not very well at Asp179 (data not shown). Recently, we have shown that the granzyme B-activated CPP32, by auto-catalytically cleaves its own pro-domain once it is activated by granzyme B or Mch4 (13). Therefore, it is possible that once pro-Mch2α is cleaved by CPP32 at Asp179, it becomes active and could act synergistically with CPP32 to process itself at Asp23 and Asp193. However, because both Mch2α and CPP32 can cleave pro-Mch2α at these sites, it is difficult to distinguish between the two activities. To overcome this problem, we introduced the lamin cleavage site (VEIDN) (20) in the wild-type pro-Mch2α and in the Asp179/Asp193 double mutant pro-Mch2α by substitution of...
weight markers. 

The formation of the p20/p19 large subunit of granzyme B in cell extract resulted in activation of endogenous pro-CPP32 by CPP32 and Granzyme B, as shown in Fig. 6B, lane 2. Endogenous CPP32 was processed and activated at Asp179 and Asp193 to generate the 21- to 13-kDa products with 95% cleavage (lane 2). On the other hand, a very weak activity was observed with an equal amount of mature CPP32 (<5% cleavage) (lane 3). Consequently, when pro-Mch2a containing the lamin site was incubated with CPP32, pro-Mch2a was processed at Asp179 and Asp193 to generate the 21-, 13-, and 11-kDa products within the first 15 min (Fig. 6B, lane 2). Processing at Asp23 followed more slowly, and after 2 h of incubation, about 65% of p21 was converted to p18 (lane 5). Since an equal amount of CPP32 generates less than 5% of cleavage at this site (Fig. 6A, lane 3), the remaining 60% of cleavage must be attributed to an autocatalytic activity of the activated Mch2a. Also because the in vitro translated pro-Mch2a is present at a very low concentration in the reaction mixture, the observed autocatalytic activity could be interpreted as evidence for intramolecular processing. Therefore, these data clearly demonstrate that once pro-Mch2a is cleaved at Asp179 and Asp193, it can autocalyze cleavage of its own pro-domain at the artificial lamin site. Thus, cleavage of pro-Mch2a at Asp179 and Asp193 is sufficient to activate Mch2a. Additionally, coexpression of physically separated p21 (amino acids 1–179) and p11 (amino acids 193–293) in bacteria generated active Mch2a enzyme (data not shown), further indicating that Asp179 and Asp193 are the exact processing sites in Mch2a.

**Fig. 6. Cleavage of pro-Mch2a by CPP32 activates Mch2a.** 35S-Labeled pro-Mch2a mutants were incubated with CPP32 or Mch2a at 37°C and then analyzed by SDS-PAGE and autoradiography. In A, Asp179/Asp193 double mutant pro-Mch2a containing the lamin cleavage site at position 20–24 was incubated for 2 h with buffer (lane 1), Mch2a (lane 2), or CPP32 (lane 3). In B, wild-type pro-Mch2a containing the lamin cleavage site at position 20–24 was incubated with CPP32 for the indicated times. The band seen in all lanes between 14 and 11 kDa is the 13-kDa rabbit reticulocyte hemoglobin, which sometimes traps free [35S]methionine and appears as a radioactive band. Left, molecular weight markers. 

Thr20, Thr22, and Ala24 with Val, Ile, and Asn, respectively. Because Mch2a but not CPP32 can cleave the lamin site, it is now possible to distinguish between the activities of the two enzymes. As shown in Fig. 6A, after 2 h of incubation, mature Mch2a was able to efficiently cleave (>95% cleavage) the lamin site (amino acids 20–24), introduced in the Asp179/Asp193 double mutant pro-Mch2a, to generate a 31-kDa product (lane 2). On the other hand, a very weak activity was observed with an equal amount of mature CPP32 (<5% cleavage) (lane 3). Consequently, when pro-Mch2a containing the lamin site was incubated with CPP32, pro-Mch2a was processed at Asp179 and Asp193 to generate the 21-, 13-, and 11-kDa products within the first 15 min (Fig. 6B, lane 2). Processing at Asp23 followed more slowly, and after 2 h of incubation, about 65% of p21 was converted to p18 (lane 5). Since an equal amount of CPP32 generates less than 5% of cleavage at this site (Fig. 6A, lane 3), the remaining 60% of cleavage must be attributed to an autocatalytic activity of the activated Mch2a. Also because the in vitro translated pro-Mch2a is present at a very low concentration in the reaction mixture, the observed autocatalytic activity could be interpreted as evidence for intramolecular processing. Therefore, these data clearly demonstrate that once pro-Mch2a is cleaved at Asp179 and Asp193, it can autocalyze cleavage of its own pro-domain at the artificial lamin site. Thus, cleavage of pro-Mch2a at Asp179 and Asp193 is sufficient to activate Mch2a. Additionally, coexpression of physically separated p21 (amino acids 1–179) and p11 (amino acids 193–293) in bacteria generated active Mch2a enzyme (data not shown), further indicating that Asp179 and Asp193 are the exact processing sites in Mch2a.

**Fig. 7. Granzyme B activates Mch2a in cell lysates.** In A, 35S-labeled wild-type pro-Mch2a was incubated with buffer (lane 1), granzyme B (GraB) (lane 2), CPP32 (lane 2), or CPP32 in the presence of DEVD-CHO (40 nM) (lane 4) at 37°C for 1 h. The products were analyzed by SDS-PAGE and autoradiography. In B, 35S-labeled pro-Mch2a was mixed with cell extract from human 697 lymphocytes and then incubated with granzyme B (DEVD-CHO) counting from left, lanes 1–3) or granzyme B and DEVD-CHO (DEVD-CHO + : lane 6) for the indicated times. The reaction products were then analyzed by SDS-PAGE, followed by Western blotting using rabbit anti-CPP32-p20 antibody (upper panel) or autoradiography (lower panel).

Activation of CPP32 by Granzyme B Leads to Activation of Mch2a—Granzyme B at a concentration that allows processing and activation of CPP32 failed to process pro-Mch2a (Fig. 7A, lane 2). Therefore, it is possible that activation of pro-CPP32 by granzyme B could lead to activation of pro-Mch2a in a protease cascade fashion. To test this possibility, 35S-labeled pro-Mch2a was mixed with cell extract from the 697 lymphocyte cell line and then incubated with granzyme B. The proteins were analyzed at different time points by Western blotting to detect activation of endogenous pro-CPP32 and by autoradiography to detect activation of pro-Mch2a. As shown in Fig. 7B, the addition of granzyme B to the 697 cell extract resulted in activation of pro-CPP32 in less than 15 min (lane 2, upper panel). This is evident from the formation of the p20/p19 large subunit of CPP32. Autoradiographic analysis of the same samples revealed a time-dependent processing of pro-Mch2a (Fig. 7B, lower panel). pro-Mch2a was processed to its individual subunits immediately after activation of the endogenous pro-CPP32 by granzyme B (lanes 3–5). Endogenous CPP32 was unable to process pro-Mch2a in the presence of 40 nM DEVD-CHO inhibitor (lane 6). Similarly, recombinant CPP32 was also unable to process pro-Mch2a in the presence of this inhibitor (Fig. 7A, lane 4). These data suggest that the enzyme responsible for processing of pro-Mch2a in cell extracts is most likely to be CPP32 itself. However, we cannot exclude the possible contribution of other granzyme B-activable, DEVD-CHO-inhibitable ASCPs present in the cell extract that can also process pro-Mch2a. Importantly, these data demonstrate for the first time that granzyme B can indirectly activate members of the ASCP family, like Mch2a, through a protease cascade. Granzyme B could directly activate Mch3, Mch4, Mch5, and Mch6, which may in turn activate ASCPs that are not good substrates for granzyme B, such as ICE and Mch2a. This may depend on the relative abundance of these proteases in different cell types and on the apoptosis signal. Because CPP32 is relatively abun-
Mch2α Can Activate CPP32—Interestingly, we observed that mature Mch2α can also process pro-CPP32 at the 272ETDS276 site between the two subunits of CPP32 (Fig. 8, lane 2). No cleavage was observed with the Asp175-mutated pro-CPP32 (lane 5), indicating that Mch2α processes CPP32 correctly. This processing was accompanied by activation of CPP32, as evident from some autocatalytic conversion of CPP32-p20 to p19 (lane 2). The autocatalytic processing occurs at Asp9, because it is inhibited by a D to A mutation at that site (lane 7). These results are similar to those obtained with Mch4 or granzyme B (13). Also, the autocatalytic processing of CPP32 at Asp9, but not the activity of Mch2α, was inhibited by the DEVD-CHO inhibitor (40 nM) (lane 3), indicating that Mch2α is a non-DEVD-CHO-inhibitable protease. Higher concentrations of DEVD-CHO up to 1 μM had no effect on Mch2α (data not shown).

Therefore, it is possible that once pro-CPP32 is activated in apoptosis by upstream proteases such as Mch4 or Mch5 (13), it could activate pro-Mch2α, which in turn may feed back on pro-CPP32, resulting in a protease amplification cycle (Fig. 9). Similarly, in a parallel apoptotic pathway, pro-Mch2α may be activated first by Mch4 and/or Mch5,5 which then could lead to activation of the same protease amplification cycle. Activation of the upstream proteases Mch4 or Mch5 most likely depends on autocatalysis, which is the slowest step in the protease cascade. Autocatalysis is probably triggered by interaction with FADD or FADD-like death effector proteins that receive the apoptosis signal from the Fas receptor or other signal transduction pathways. We predict that because activation of all ASCPs require cleavage after Asp residues present between their large and small subunits, protease amplification cycles might be common among all members of this family. Once the slow autocatalysis step is completed, feedback from the downstream protease will initiate the protease amplification cycle to ensure efficient processing and activation of the remaining pro-enzymes involved in the cascade.

Conclusions—We have identified and cloned a novel ASCP that could play a role in the apoptotic protease cascade. We demonstrated that pro-Mch6 is a substrate for CPP32 and granzyme B. The mature Mch6 enzyme is composed of a large subunit (p35) and a small subunit (p10) that are derived from a single chain pro-enzyme by cleavage after two aspartate residues. We also demonstrated that the pro-enzyme of the lamin-cleaving enzyme Mch2α is a substrate for CPP32. The mature Mch2α enzyme is made of a large subunit (p18) and a small subunit (p11) that are derived from a single-chain pro-enzyme by cleavage after three aspartate residues. The ability of CPP32 to activate pro-Mch6 and pro-Mch2α suggests that these proteases are downstream proteases that might be responsible for the late morphological changes in apoptotic cells. One of these late morphological changes is lamin cleavage, resulting in collapse of the nucleus into apoptotic bodies. The ability of granzyme B to process/activate pro-Mch2α and pro-Mch6 through CPP32 is the first direct demonstration of an apoptotic protease cascade. These proteases might be active participants in granzyme B-mediated apoptosis.

REFERENCES

1. Martin, S. J., and Green, D. R. (1995) Cell 82, 349–352
2. Henkart, P. A. (1996) Immunity 4, 195–201
3. Earnshaw, W. C. (1995) Curr. Opin. Cell Biol. 7, 337–343
4. Alnemri, E. S. (1996) J. Cell. Biochem. 62, in press
5. Walker, N. P. C., Talanian, R. V., Brady, K. D., Dang, L. C., Bump, N. J., Ferenz, C. R., Franklin, S., Ghayur, T., Hackett, M. C., Hammill, L. D., Herzog, L., Hugunin, M., Houx, W., Mankovich, J. A., McGuiness, L., Orlewicz, E., Paukert, M., Pratt, C. A., Reis, F., Summanni, A., Tannora, M., Welch, J. P., Xiong, L., Moller, A., Tracey, D. E., Kamen, R., and Wang, W. W. (1994) Cell 78, 343–352
6. Wilson, R. P., Black, J. F., Thompson, J. A., Kim, E. E., Griffith, J. P., Navia, M. A., Murcko, M. A., Chambers, S. P., Aldape, R. A., Raybuck, S. A., and Livingston, D. J. (1994) Nature 370, 270–275
7. Miura, M., Zhu, H., Rotello, R., Hartwig, E. A., and Yuan, J. (1993) Cell 75, 653–660
8. Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1994) J. Biol. Chem. 269, 30761–30764
9. Kumar, S., Kinoshita, M., Noda, M., Copeland, N. G., and Jenkins, N. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7464–7469
10. Tomasselli, G., Karakhan, A., Armstrong, R., Krebs, J., Fritz, L., Tomasselli, K. J., Wang, L., Yu, Z., Croce, C. M., Ebert, T., Ebert, W. C., and Alnemri, E. S. (1995) Cancer Res. 55, 2737–2742
11. Fernandes-Alnemri, T., Takahashi, A., Armstrong, R., Krebs, J., Fritz, L., Tomasselli, K. J., Wang, L., Yu, Z., Croce, C. M., Ebert, T., Ebert, W. C., and Alnemri, E. S. (1995) Cancer Res. 55, 6045–6052
12. Fernandes-Alnemri, T., Armstrong, R., Krebs, J., Tomasselli, S. M., Wang, L., Bullrich, F., Fritz, L., Tomasselli, K. J., Litwack, G., and Alnemri, E. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7464–7469
13. Faucheux, C., Diao, A., Chen, A. W., Blanchet, A. M., Miossec, C., Herve, F., Collard-Dutilleul, V., Gu, Y., Aldabe, R. A., Lippke, J. A., Rocker, C., Bue, M. S.-S., Livingston, D. J., Hereund, T., and Lalanne, J.-L. (1995) EMBO J. 14, 1914–1922
14. Camens, J., Paskind, M., Hugunin, M., Talanian, R. V., Allen, H., Banach, D., S.-S., Livingston, D. J., Hercend, T., and Lalanne, J.-L. (1995) Cancer Res. 55, 2737–2742

S. M. Srinivasula, M. Ahmad, T. Fernandes-Alnemri, G. Litwack, and E. S. Alnemri, submitted for publication.
Activation of Mch6 and Mch2α by CPP32 and Granzyme B

Bump, N., Hackett, M., Johnston, C. G., Li, P., Mankovich, J. A., Terranova, M., and Ghayur, T. (1995) J. Biol. Chem. 270, 15250–15256
16. Munday, N. A., Vaillancourt, J. P., Ali, A., Casano, F. J., Miller, D. K., Molineaux, S. M., Yamin, T.-T., Yu, V. L., and Nicholson, D. W. (1995) J. Biol. Chem. 270, 15870–15876
17. Alnemri, E. S., Fernandes-Alnemri, T., and Litwack, G. (1995) J. Biol. Chem. 270, 4312–4317
18. Smyth, M. J., and Trapani, J. A. (1995) Immunol. Today 16, 202–206
19. Shi, L., Kraut, R. P., Aebersold, R., and Greenberg, A. H. (1991) J. Exp. Med. 175, 553–566
20. Takahashi, A., Alnemri, E. S., Lazebnik, Y. A., Fernandes-Alnemri, T., Litwack, G., Mair, R. D., Goldman, R. D., Poirier, G. G., Kaufmann, S. H., and Earnshaw, W. C. (1995) Proc. Natl. Acad. Sci. U.S.A. 93, 8395–8400
21. Enari, M., Talanian, R. V., Wong, W. W., and Nagata, S. (1996) Nature 380, 723–726
22. Harvey, N. L., Trapani, J. A., Fernandes-Alnemri, T., Litwack, G., Alnemri, E. S., and Kumar, S. (1996) Genes Cells 1, in press
23. Trapani, J. A., Browne, K. A., Dawson, M., and Smyth, M. J. (1993) Biochem. Biophys. Res. Commun. 195, 910–920
24. Trapani, J. A., Smyth, M. J., Apostolidis, V. A., Dawson, M., and Browne, K. A. (1994) J. Biol. Chem. 269, 18359–18365
25. Ramage, P., Cheneval, D., Chvei, M., Graff, P., Hemmig, R., Heng, R., Kocher, H. P., Mackenzie, A., Memmert, K., Revesz, L., and Wishart, W. (1995) J. Biol. Chem. 270, 9378–9383
26. Cerretti, D. P., Kozlosky, C. J., Mosley, B., Nelson, N., Van Ness, K., Greenstreet, T. A., March, C. J., Kronheim, S. R., Druck, T., Cannizzaro, L. A., Huebner, K., and Black, R. A. (1992) Science 256, 97–100
27. Song, Q., Lees-Miller, S., Kumar, S., Zhang, N., Smit, G. C. M., Jackson, S., Alnemri, E. S., Litwack, G., and Lavin, M. F. (1995) EMBO J. 15, 3238–3246
28. Martin, S. J., Newmeyer, D. M., Mathias, S., Farschon, D. M., Wang, H.-G., Reed, J. C., Kolesnick, R. N., and Green, D. R. (1995) EMBO J. 14, 5191–5120
29. Enari, M., Hase, A., and Nagata, S. (1995) EMBO J. 14, 5201–5208
30. Lazebnik, Y. A., Cole, S., Cook, C. A., Nelson, W. G., and Earnshaw, W. C. (1993) J. Cell Biol. 123, 7–22
31. Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. (1994) Nature 371, 346–347
32. Lazebnik, Y. A., Takahashi, A., Moir, R. D., Goldman, R. D., Poirier, G. G., Kaufmann, S. H., and Earnshaw, W. C. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 9042–9046
33. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T.-T., Yu, V. L., and Miller, D. K. (1995) Nature 376, 37–43
The Ced-3/Interleukin 1β Converting Enzyme-like Homolog Mch6 and the Lamin-cleaving Enzyme Mch2 α Are Substrates for the Apoptotic Mediator CPP32
Srinivasa M. Srinivasula, Teresa Fernandes-Alnemri, James Zangrilli, Noreen Robertson, Robert C. Armstrong, Lijuan Wang, Joseph A. Trapani, Kevin J. Tomaselli, Gerald Litwack and Emad S. Alnemri

J. Biol. Chem. 1996, 271:27099-27106.
doi: 10.1074/jbc.271.43.27099

Access the most updated version of this article at http://www.jbc.org/content/271/43/27099

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 31 references, 15 of which can be accessed free at http://www.jbc.org/content/271/43/27099.full.html#ref-list-1