Purification and Characterization of an Interleukin-1β-converting Enzyme Family Protease That Activates Cysteine Protease P32 (CPP32)*

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CPP32, a member of the interleukin-1β-converting enzyme (ICE) family of cysteine proteases, cleaves poly(ADP-ribose) polymerase and sterol regulatory element binding proteins during apoptosis. CPP32 normally exists in the cytosol as a 32-kDa inactive precursor and only becomes activated when cells are undergoing apoptosis. The activation is a proteolytic event that generates a 20/11-kDa heterodimer. We report here the identification, purification, and characterization of a hamster CPP32-activating protease (CAP) that cleaves and activates CPP32. The biochemical properties of CAP suggest that it is another member of the ICE family of proteases. Purified CAP consists of two prominent polypeptides of 19 and 13 kDa. Protein sequencing revealed that CAP is derived from the hamster homolog of Mch2, a member of the ICE family recently identified based on the sequence conservation among the ICE family members. CAP activity is inhibited by CrmA, a cowpox virus protein that prevents host cell apoptosis. CAP itself is also activated through proteolytic cleavage. These data are consistent with the idea that the activation of the ICE family of proteases during apoptosis proceeds through a cascade of proteolytic events.

When activated, CPP32 specifically cleaves poly(ADP-ribose) polymerase (PARP) (3, 4) and sterol regulatory element binding proteins (SREBPs) (5, 7). PARP is an enzyme implicated in DNA repair and genome surveillance and integrity. The proteolytic cleavage of PARP during the onset of apoptosis by CPP32 results in the separation of its DNA binding and catalytic domains. This cleavage prevents the catalytic domain of PARP from being recruited to sites of DNA damage and presumably disables the ability of PARP to coordinate subsequent repair and genome maintenance events (3). Furthermore, the Ca2+/Mg2+-dependent endonuclease implicated in the internucleosomal DNA cleavage, a hallmark of apoptosis, is negatively regulated by polyADP-ribosylation, and this inhibition may be relieved when PARP is degraded (8). SREBPs are a family of transcription factors that stimulate transcription of genes involved in cholesterol and fatty acid metabolism, including the low density lipoprotein receptor, 3-hydroxy-3-methyl-glutaryl-CoA synthase, and fatty acid synthase genes (9–12). The amino-terminal halves of SREBPs are bona fide basic helix-loop-helix zipper transcription factors. Unlike any other transcription factors, they are linked to extended carboxy-terminal halves by two trans-membrane domains that anchor the proteins to the membranes of the endoplasmic reticulum and nuclear envelope. In cells starved for cholesterol or undergoing apoptosis, a proteolytic cleavage event between the leucine zipper and the membrane attachment region frees the amino-terminal fragment which enters the nucleus and activates its target genes (5, 13). The CPP32-mediated cleavage of SREBPs during apoptosis is not regulated by cellular cholesterol content and occurs at a different site compared with that of sterol-regulated proteolysis (5). The physiological function of activated SREBPs in apoptotic cells is still obscure. Nevertheless, since the activated SREBPs should have a profound impact on cellular lipid metabolism, it has been speculated that cleavage of SREBPs by CPP32 during apoptosis is involved in preserving the cytoplasmic membrane integrity of apoptotic cells, and/or preparing the membrane for phagocytosis (5).

The ability of activated CPP32 to trigger apoptosis implies that cells must have a tight mechanism to control this activation to prevent unwanted cell death. CPP32 is activated by multiple proteolytic cleavages at aspartic acid residues. The eventual result is cleavage of the 32-kDa precursor into the 20/11-kDa active form (3, 5). The mechanism that triggers CPP32 activation is not known. Partially purified active CPP32 from HeLa cell extracts was able to cleave the CPP32 precursor in vitro (5). This reaction was partially, but not completely, inhibited by a CPP32-specific tetrapeptide inhibitor, suggesting autocatalytic activation as well as the existence of another activating enzyme (5). ICE has also been shown to be able to cleave and activate CPP32 in vitro (4). However, since ICE knockout mice have no general defects in apoptosis (14, 15), ICE does not appear to be a general mediator of apoptosis.
Accordingly, since CPP32 is activated by cleavage at aspartic acid residues, a hallmark of ICE-like proteases (16), a cascade of ICE-like proteolytic cleavages leading to apoptosis has been proposed (4, 5). Such a protease cascade would provide both the regulation and signal amplification necessary for a highly controlled yet rapid and irreversible process of apoptosis.

In this paper, we report the identification and purification of a CPP32-activating protease (CAP) from hamster liver extract. This enzyme specifically cleaves and activates CPP32. The biochemical properties of this protease suggest that it is an ICE-like cysteine protease distinct from ICE and CPP32, the two enzymes that have previously been implicated in the activation of CPP32 (4, 5). The protein sequence of purified CAP revealed that CAP is derived from the hamster homolog of Mc2 (a member of the ICE family (17)). Mc2 may represent the upstream protease acting on CPP32 and may initiate the ICE-like protease cascade leading to apoptosis. We also find that CAP activity is more sensitive to inhibition by CrmA than is CPP32, defining a new and more efficient target for CrmA blockage of the onset of apoptosis.

**EXPERIMENTAL PROCEDURES**

**General Methods and Materials**

We obtained male Golden Syrian hamsters (−150 g) from Sasco (Omaha, NE); [35S]methionine was from Amersham Corp.; N-ethylmaleimide (NEM), iodoacetamide (IAA), phenylmethanesulfonyl fluoride (PMSF), imidazole, and aprophenin were from Sigma; Ac-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO) was from Bachem Bioscience, Inc.; Ac-Asp-Glu-Ala-Asp-aldehyde (Ac-DEAD-CHO) was from Julio C. Medina of Tulark, Inc. (7); molecular weight standards for SDS-PAGE and gel-filtration chromatography were from Bio-Rad. cDNA donors of human SREBP-2 and hamster CPP32 were described in the indicated references as described (13). Protein concentration was determined by the Bradford method. Silver staining was carried out using a Silver Stain Plus kit from Bio-Rad. Plasmids were purified using a Megaprep kit from Qiagen. Asp-Glu-Ala-Asp-aldehyde (Ac-DEAD-CHO) was from Julio C. Medina of Tulark, Inc. (7); molecular weight standards for SDS-PAGE and gel-filtration chromatography were from Bio-Rad. cDNA donors of human SREBP-2 and hamster CPP32 were described in the indicated references as described (13). Protein concentration was determined by the Bradford method. Silver staining was carried out using a Silver Stain Plus kit from Bio-Rad. Plasmids were purified using a Megaprep kit from Qiagen.

**In Vitro Translation of SREBP-2, CPP32, and CrmA**

SREBP-2 was translated in a TNT SP6 transcription/translation kit from Promega as described (7). A PCR fragment encoding amino acids 29–277 of hamster CPP32 (5) was cloned into NdeI and BamHI sites of pET 15b vector (Novagen). The resulting fusion protein of six histidines with hamster CPP32 (amino acids 29–277) was translated in a TNT T7 transcription/translation kit in the presence of [35S]methionine according to the manufacturer’s instructions. The translated protein was passed through a 1-ml nickel affinity column (Qiagen) equilibrated with buffer A (20 mM Hepes-KOH, pH 6.8, 10 mM KCl, 1.5 mM MgCl2, 1 mM Na-EDTA, 1 mM Na-EGTA, 1 mM dithiothreitol (DTT), and 0.1 mM PMSF) after dialysis against the same buffer. After washing the column with 10 ml of buffer A, the translated CPP32 was eluted with buffer A containing 250 mM imidazole. CPP32 disulfide was then cleaved by reduced dithiothreitol and was purified on a Superdex-200 gel-filtration column (Pharmacia, 300 ml) equilibrated with buffer A. The column was eluted with buffer A containing 250 mM NaCl.

**NH2-terminal Sequencing Analysis of CAP**

The CAP peak fractions from Mono Q column (step 7) were subjected to electrophoresis in a 15% SDS gel and transferred onto a poly(vinylidene difluoride) membrane (Millipore). The 19- and 13-kDa subunits were visualized by Coomassie Blue staining and excised for direct NH2-terminal sequencing on a sequencer (Biosystem).

**Western Blot Analysis**

A polyclonal antibody against hamster CPP32 was produced as described (5). A monoclonal antibody against human CPP32 was purchased from Transduction Laboratories. Immunoblot analysis of CPP32 was performed with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G using Enhanced Chemiluminescence Western blotting detection reagents (Amersham Corp.) as described previously (13).

**Expression and Purification of Recombinant His6-tagged CPP32 and CrmA**

The plasmid containing fusion protein of six histidine and hamster CPP32 (amino acid 29–277) was the same as used for the in vitro translation described above. The plasmid was transformed into DE3 competent cells (Novagen). The entire coding sequence of CrmA was PCR-amplified from the plasmid p996 containing cowpox virus crmA gene and cloned into the NdeI and HindIII sites of pQE 30 vector (Qiagen). The plasmid was transformed into the M15 competent cells (Qiagen). The bacteria cultures (1 liter for each plasmid) were grown at 37°C until the density reached A600 reading of 0.6. Isopropyl-1-thio-β-galactopyranoside was then added to the final concentration of 2 mM. After 1-h induction, the bacteria were pelleted and lysed in buffer A containing 1 mM β-mercaptoethanol. After centrifugation, the supernatants were loaded onto a 3-ml nickel-Sepharose (Qiagen) column equilibrated with buffer A. The columns were washed with 10 ml of buffer A, followed by 10 ml of buffer A containing 500 mM NaCl, and again with 10 ml of buffer A. The fusion proteins were eluted with buffer A containing 1 M NaCl imidazole. The peak protein fractions of the nickel column eluates were further purified through a FPLC Superdex-200 16/30 column equilibrated with buffer A, and 1-ml fractions were collected. The column fractions of the recombinant CPP32 were assayed for its enzymatic activity by incubating with [35S]-labeled SREBP-2 as described previously.
of 35S-methionine. The translated CPP32 was purified on a hamster CPP32 mRNA with a six histidine tag in the presence of protease(s) that catalyze this specific cleavage, we translated precursor into the p20/p11 active form (5). To search for the tracts can be specifically activated serine protease related to granzyme (18). However, it is unlikely that CAP is a serine protease since its activity was insensitive to serine protease inhibitors such as PMSF, leupeptin, and aprotinin (data not shown). If CAP is another member of the ICE family, it should be sensitive to cysteine-alkylating reagents such as NEM or IAA (3, 7). As shown in Fig. 3, CAP activity was completely abolished by 1 mM NEM (lane 11) or 100 μM IAA (lane 5), and the inhibiting activity was prevented by an excess of DTT (lanes 7 and 12). ICE and CPP32 can be inhibited specifically by mM concentration of tetrapeptide aldehydes corresponding to the amino acid sequences preceding their cleavage sites (3, 7, 19). The CAP activity was resistant to both ICE and CPP32 inhibitors up to 1 μM (lanes 13 and 14), indicating that CAP is a new ICE-like protease distinct from CPP32 and ICE. Equal amounts of the CPP32-specific inhibitor completely blocked the cleavage of SREBP by CPP32 (Fig. 5, lane 8).

**RESULTS**

Identification of CAP Activity—CPP32 in hamster liver extracts can be specifically activated in vitro by incubating at 30 °C (7). The activation is due to the cleavage of its 32-kDa precursor into the p20/p11 active form (5). To search for the protease(s) that catalyze this specific cleavage, we translated precursor into the p20/p11 active form (5). To search for the tracts can be specifically activated serine protease related to granzyme (18). However, it is unlikely that CAP is a serine protease since its activity was insensitive to serine protease inhibitors such as PMSF, leupeptin, and aprotinin (data not shown). If CAP is another member of the ICE family, it should be sensitive to cysteine-alkylating reagents such as NEM or IAA (3, 7). As shown in Fig. 3, CAP activity was completely abolished by 1 mM NEM (lane 11) or 100 μM IAA (lane 5), and the inhibiting activity was prevented by an excess of DTT (lanes 7 and 12). ICE and CPP32 can be inhibited specifically by mM concentration of tetrapeptide aldehydes corresponding to the amino acid sequences preceding their cleavage sites (3, 7, 19). The CAP activity was resistant to both ICE and CPP32 inhibitors up to 1 μM (lanes 13 and 14), indicating that CAP is a new ICE-like protease distinct from CPP32 and ICE. Equal amounts of the CPP32-specific inhibitor completely blocked the cleavage of SREBP by CPP32 (Fig. 5, lane 8).

Purification of CAP from Hamster Liver Extracts—To complete the purification of CAP, the gel-filtration column fractions containing CAP activity (fractions 16–17) were pooled and loaded onto a Mono Q column. CAP activity was found in the flow-through while the CPP32 activity was bound (data not shown). The Mono Q column flow-through fraction was then heated at 55 °C for 15 min. CAP activity was resistant to this heat treatment (Fig. 4, lanes 1 and 2). After pelleting the heat-denatured proteins, the supernatant was loaded onto a Mono S column and eluted with a 40–440 mM linear NaCl gradient.
gradient. As shown in Fig. 4 (panel A), CAP activity was eluted from the Mono S column around 185 mM NaCl. The same fractions were also subjected to SDS-PAGE followed by silver staining (panel B). Two predominant polypeptides with apparent molecular masses of 19 and 13 kDa eluted precisely with the CAP activity.

Table I shows quantitative estimates of a typical purification procedure, starting with liver cytosol from 25 hamsters (5.1 g of protein). As shown in Fig. 1, the S-100 fraction, the SP-Sepharose flow-through, and bound material did not have a significant amount of CAP activity before preincubation at 30 °C for 2 h. The SP-Sepharose column flow-through fraction was subsequently activated in vitro and passed through the SP-Sepharose column again. About 129 mg of protein was bound and eluted from the SP-Sepharose column with a 49% recovery of CAP activity. The CAP activity from the SP-Sepharose column was precipitated by the addition of 40% ammonium sulfate. After gel filtration, Mono Q chromatography, heat treatment, and finally Mono S chromatography, a 12,000-fold purification was achieved with 6% recovery of the starting activity.

Characterization of CAP—To obtain the amino acid sequence of CAP, the two polypeptides (p19/p13) in the Mono S column peak fractions were isolated from the SDS-PAGE, and the NH₂-terminal sequences from both p19 and p13 were obtained by Edman degradation. As shown in Table II, the NH₂-terminal sequence of purified hamster CAP p19 and p13 subunits, comparison with human Mch2α, was reported by Fernandes-Alnemri et al. (17). Numbers in parentheses denote the amino acid position in the cDNA sequence of human Mch2α. Asterisks (*) denote residues that differed in two sequences.
Purification of CPP32-activating Protease

Fig. 5. CAP activates CPP32 in HeLa cell cytosol. 50 ng of CAP (lanes 1 and 5) and 20 μg of HeLa cell S-100 fraction (lanes 2 and 6) were incubated alone or together (lanes 3 and 7-9) in 20 μl of buffer A at 30°C for 30 min. In panel B, the indicated samples were directly subjected to 15% SDS-PAGE followed by Western blot analysis using a monoclonal antibody against human CPP32. In panel B, the resulting samples were incubated with aliquots (5 μl) of in vitro synthesized, 35S-labeled SREBP-2 for an additional 15 min at 30°C. In lanes 8 and 9, 1 μM final concentration of the indicated tetrapeptide aldehydes dissolved in buffer A containing 3% (v/v) dimethyl sulfoxide was added to the reaction mixture before the addition of 35S-labeled SREBP-2. The samples were subsequently subjected to 12% SDS-PAGE, and the gel was dried and exposed to a film for 12 h at ~80°C.

Fig. 6. Cleavage of CrmA by CAP and CPP32. CAP and recombinant CPP32 were purified as described under “Experimental Procedures.” Aliquots (10 μl) of in vitro translated, 35S-labeled CrmA were incubated with the indicated amount of purified CAP (lanes 2-4) and CPP32 (lanes 5-7) at 30°C for 45 min in 25 μl of buffer A. The samples were subsequently subjected to 12% SDS-PAGE. The gel was dried and exposed to a film for 30 h at room temperature.

Fig. 7. Inhibition of CAP and CPP32 by CrmA. Aliquots of 50 ng of purified CAP (panel A) or CPP32 (panel B) were incubated with the indicated amount of purified CrmA at 30°C for 15 min followed by the addition of 5 μl of in vitro translated, 35S-labeled CPP32 precursor (panel A) or SREBP-2 (panel B). After an additional 15-min incubation at 37°C, the samples were subjected to 15% (panel A) or 8% (panel B) SDS-PAGE. The 15% gel was then transferred to a nitrocellulose filter and the 8% gel was dried. The filter and gel were exposed to film for 12 h at room temperature. The radioactivity in the filter and dried gel was also quantified in a Fuji 1000 phosphorimager, and the results were plotted in panel C.

DISCUSSION

Activation of CPP32 during Apoptosis—Activation of the ICE-like protease CPP32 is associated with several forms of programmed cell death (3–5). The activation is due to proteolysis that cleaves the CPP32 precursor after aspartic acid residues to generate the p20/p11 active form (3, 5). To search for the protease that carries out this cleavage, we identified and purified the CPP32-activating protease from hamster liver extracts. The purified CAP activity contained two prominent protein bands that migrated at 19 and 13 kDa on SDS-PAGE. This is a typical p20/p11 pattern that is shared by other ICE-like proteases (3). However, incubation of HeLa cytosol with purified CAP resulted in the cleavage of CPP32 (lane 3) and activation of CPP32 (lane 7). The activated CPP32 was sensitive to its specific tetrapeptide aldehyde inhibitor Ac-DEAD-CHO (7) (lane 8) but not to ICE inhibitor (lane 9).

Cp11 is Inhibitable by CrmA—Cowpox virus protein CrmA is a potent and specific inhibitor of ICE-like proteases (20) and can protect cells against apoptosis in a variety of systems, including growth factor withdrawal (21), detachment of mammary epithelial cells from the underlying extracellular matrix (22), treatment of cytotoxic T-lymphocytes, and activation of either Fas or the tumour necrosis factor receptor (23, 24). CrmA inhibits ICE-like proteases by serving as a substrate for these enzymes whose cleaved products remain bound to the enzyme after cleavage reaction (2, 6). Fig. 6 shows that the purified CAP was capable of cleaving in vitro translated, 35S-labeled CrmA into one major band of 25 kDa and a minor band of 35 kDa (lanes 2-4). Under the same condition, the same amount of purified recombinant CPP32 had little CrmA cleavage activity, even though it was able to cleave SREBP (see Fig. 7). This result is consistent with the observation that CrmA is a poor inhibitor of CPP32 (3). Fig. 7 shows an experiment in which we tested directly for inhibition of CAP activity by CrmA. Purified CrmA was incubated with purified CAP or CPP32 followed by the addition of 35S-labeled CPP32 or SREBP-2, respectively. About 50% of CAP was inhibited by a 2-fold molar excess of CrmA, while it took five times more CrmA to reach the same level of inhibition of CPP32.

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terminal 10-kDa fragment (19). The p20 and p10 fragments remain associated as a heterodimer, and two heterodimers associate to form the tetrameric active enzyme (28, 29). CAP resembles ICE in several ways: (i) it is sensitive to inhibition by cysteine alkylating reagents; (ii) it cleaves substrates after aspartic acid residues (3); and (iii) it is synthesized as a precursor that becomes activated through cleavage after aspartic acid residues. One interesting note is that CAP behaved as a much smaller protein on the gel-filtration column, even though the size of the two subunits are similar to that of ICE and CPP32. The anomalous behavior of CAP on the gel-filtration column may have been due to an interaction between CAP and the column resin. However, the molecular basis for this behavior remains to be determined.

A cDNA for Mch2α was originally cloned from human Jurkat T lymphocytes using a degenerative PCR strategy based on the sequence conservation among known members of the ICE/CED3 family of proteases (17). Mch2α cDNA encodes a 34-kDa protein that shares 38 and 35% identity with human CPP32 and C. elegans CED-3, respectively. An alternatively spliced version, Mch2β, which is missing residues 15–103 of Mch2α, was also cloned (17). The NH₂-terminal sequence of the CAP p19 subunit corresponds to amino acids 26–49 of human Mch2α and is therefore derived from the hamster homolog of Mch2α. Interestingly, only Mch2α showed protease activity when expressed in bacteria. This protein was also able to induce apoptosis in s9 cells when overexpressed using a baculovirus vector (17).

Autocatalytic Versus CAP-mediated Activation of CPP32—Partially purified CPP32 enzyme from HeLa cell extracts was able to cleave CPP32 precursor (5), indicating that CPP32 can be activated through autocatalysis. A similar mechanism is probably responsible for yielding active enzyme when CPP32 precursor is expressed in large quantity in bacteria (Refs. 2, 7, and “Experimental Procedures”). However, our experimental data suggest that Mch2α is the major protease in hamster liver extracts responsible for cleaving the CPP32 precursor into the p20/p11 active form. As shown in Fig. 2, even though the active CPP32 was present in column fraction 10, there was little CAP activity in this fraction compared with fraction 16, the main peak fraction for CAP. The reason for this observation, whether CPP32 is a preferred substrate for CAP or there is simply more CAP in the hamster liver extracts than CPP32, remains to be determined.

Inhibition of CAP by CrmA—CrmA, a poxvirus-encoded serpin, prevents host cells from undergoing apoptosis by a variety of stimuli that activate CPP32 (4, 5). CrmA was able to directly inhibit CPP32 activity, a property that has been used to explain its prevention of apoptosis (4). However, on a quantitative basis, CrmA is a rather poor inhibitor for CPP32 (2, 3). Our finding that CrmA is a better inhibitor for CAP may reconcile this controversy. By inhibiting CAP, CrmA could prevent the activation of CPP32 and therefore block the onset of apoptosis.

Activating the Activator—Our data also point out that Mch2α itself needs proteolytic processing to become active. The processing includes the removal of a short peptide from the NH₂-terminal of the protein and cleavage at aspartic acid 193 (reference to human sequence). The NH₂-terminal cleavage could be a result of cleavage after TETD followed by the sequential removal of two more amino acid residues (Table I). The NH₂-terminal of p13 kDa subunit is preceded by TEVD. Both of these sequences resemble the recognition site IETD for Mch2α and DEVD for CPP32 (3). It is likely that the Mch2α precursor can be cleaved and activated by autocatalysis and/or by CPP32. In this scenario, the slight activation of Mch2α will trigger a cycle of amplification leading to the irreversible activation of CPP32, which in turn carries out the execution of apoptosis by cleaving death substrates PARP and SREBP3.