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Biochemistry

Purification and cDNA cloning of a second apoptosis-related cysteine protease that cleaves and activates sterol regulatory element binding proteins

[Mch3 protease/CPP32 protease/cholesterol/poly(ADP-ribose) polymerase/programmed cell death]

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ABSTRACT **We have purified from hamster liver a second cysteine protease that cleaves and activates sterol regulatory element binding proteins (SREBPs). cDNA cloning revealed that this enzyme is the hamster equivalent of Mch3, a human enzyme that is related to the interleukin 1β converting enzyme. We call this enzyme Mch3/SCA-2. It is 54% identical to hamster CPP32/SCA-1, a cysteine protease that was earlier shown to cleave SREBPs at a conserved Asp between the basic helix-loop-helix leucine zipper domain and the membrane attachment domain. This cleavage liberates an NH_2-terminal fragment of ≈460 amino acids that activates transcription of genes encoding the low density lipoprotein receptor and enzymes of cholesterol synthesis. Mch3/SCA-2 and CPP32/SCA-1 are synthesized as inactive 30–35 kDa precursors that are thought to be cleaved during apoptosis to generate active fragments of ≈20 and ≈10 kDa. The current data lend further support to the notion that SREBPs are cleaved and activated as part of the program in programmed cell death.**

The interleukin 1β converting enzyme (ICE) is the prototype for a family of cytosolic cysteine proteases that are activated during apoptosis in animal cells (1, 2). These proteases are all synthesized as inactive precursors of 30–35 kDa that are cleaved to active subunits of about 20 and 10 kDa when apoptosis is triggered by a variety of agents, such as tumor necrosis factor, the Fas ligand, or the protein kinase inhibitor staurosporine. These proteases induce apoptosis in cells when overexpressed in an active form. Moreover, they are related in sequence to the product of the *Caenorhabditis elegans* Ced-3 gene, which is required for apoptosis (3). These findings have led to the conclusion that the ICE-related proteases play crucial roles in apoptosis.

All of the ICE-related proteases cleave proteins at aspartic acid. The active sites all contain the sequence QACRG, in which C is the catalytic cysteine (1, 4). The first recognized apoptosis-related substrate was poly(ADP-ribose) polymerase (PARP), a nuclear enzyme that is believed to play a role in DNA repair (5). PARP is cleaved and inactivated by CPP32, an ICE-related protease that was identified as an expressed sequence tag (EST) by virtue of its resemblance to ICE (6, 7). Human CPP32 was also isolated through a biochemical purification that used PARP as a substrate (8).

Our laboratory independently isolated CPP32 from hamster liver in a purification that used another substrate, the sterol regulatory element binding proteins (SREBPs) (9, 10). SREBP-1 and -2 are basic helix-loop-helix transcription factors that are bound to the membranes of the endoplasmic reticulum and nuclear envelope (11, 12). When cells are depleted of cholesterol, a protease cleaves the SREBPs to release NH_2-terminal fragments of ≈500 amino acids that enter the nucleus and activate transcription of genes involved in cholesterol biosynthesis and lipoprotein uptake, thereby replenishing cholesterol. We purified a protease called SREBP cleavage activity 1 (SCA-1), which cleaves both SREBPs to release an NH_2-terminal fragment of ≈500 amino acids. Sequence analysis and cDNA cloning revealed that this protease was the hamster equivalent of human CPP32, and we called it CPP32/SCA-1 (9, 10).

CPP32/SCA-1 cleaves SREBP-1 and -2 at a conserved D in the consensus S/DEPDS (9). The CPP32 cleavage site in PARP also has an acidic residue at the P3 position, and this residue has been shown to be crucial for recognition (8). Cleavage of SREBPs by CPP32/SCA-1 is completely distinct from the sterol-regulated cleavage process. Thus, a D to A substitution at the cleavage site in SREBP-2 abolishes cleavage by CPP32/SCA-1, but it does not affect sterol-regulated cleavage in cells (9, 10). To the contrary, CPP32/SCA-1 cleaves SREBPs only during apoptosis, and this cleavage occurs in the presence or absence of sterols. Three apoptosis-inducing agents (an antibody to the Fas antigen, etoposide, and staurosporine) were shown to activate CPP32/SCA-1 to cleave SREBPs in intact cells (10).

During the purification of CPP32/SCA-1 from hamster liver, we noticed the appearance of another activity that was capable of cleaving SREBPs at approximately the same site as CPP32/SCA-1. In the current studies we have purified this enzyme to homogeneity, determined its partial amino acid sequence, and cloned its cDNA. The enzyme turns out to be the hamster equivalent of Mch3, a human member of the ICE family that was identified recently as an EST (13, 14). We call this enzyme Mch3/SCA-2. It differs from CPP32/SCA-1 in several respects, including pH optimum and behavior on dye affinity chromatography.

METHODS

Materials. We obtained [^{35}S]methionine (>1000 Ci/mmol) from Amersham. cDNA clones for human SREBP-2 (15) and hamster CPP32/SCA-1 (10) were described in the indicated references. Plasmid pBK-CMV-PARP (CMV, cytomegalovirus) encoding human PARP (16) was a gift from Xiaodong Wang (Emory University School of Medicine). Male Golden Syrian hamsters (100–125 g), obtained from Sasco (Omaha, NE), were exposed to a 12-h light/12-h dark cycle and fed a standard mouse/rat chow diet (Teklad, Madison, WI).

***In Vitro* Translation of ^{35}S-SREBP-2 and ^{35}S-PARP.** SREBP-2 cloned into the *Eco*RI site of λExlox(+) vector and

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Abbreviations: EST, expressed sequence tag; ICE, interleukin Iβ converting enzyme; PARP, poly(ADP-ribose) polymerase; SREBP, sterol regulatory element binding protein; SCA, SREBP cleavage activity.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U47332).

PARP cloned into the *Sma*I/*Eco*RI site of pBK-CMV vector (Stratagene) were prepared using plasmid midi kits (Qiagen, Chatsworth, CA) and translated in the TNT SP6 (for SREBP-2) or T7 (for PARP) coupled reticulocyte lysate system (Promega) with a methionine-free amino acid mixture supplemented with 750 μCi/ml [^{35}S]methionine (>1000 Ci/mmol; 1 Ci = 37 GBq). Each coupled transcription-translation reaction contained 5 μg of plasmid DNA in a final volume of 400 μl according to the manufacturer's instructions. After incubation at 30°C for 2 h, each translated SREBP or PARP was purified by passing the transcription-translation mixture through a 10-ml Sephadex G-25 gel filtration column equilibrated with buffer A (50 mM Hepes·KOH, pH 7.5/1.5 mM $MgCl_2$/10 mM KCl/1 mM sodium EDTA/1 mM sodium EGTA/5 mM dithiothreitol/and 0.1 mM Pefabloc). The translated proteins in the exclusion volume of the column were used in the assay described below.

Assay for Cleavage of ^{35}S-SREBP-2 and ^{35}S-PARP. A 5-μl aliquot of the translated ^{35}S-SREBP-2 or ^{35}S-PARP was incubated at 30°C for varying times with enzyme fractions in a final volume of 25 μl of assay solution (1.5 mM $MgCl_2$/10 mM KCl/1 mM sodium EDTA/1 mM sodium EGTA/5 mM dithiothreitol/0.1 mM Pefabloc) containing one of the following buffers: 50 mM Mes·NaOH at pH 6 or 6.5, 50 mM Hepes·KOH at pH 7 or 7.5, or 50 mM Tris·HCl at pH 8. At the end of the incubation, 8.3 μl of 4× SDS sample buffer (17) were added to each tube, after which each sample was boiled for 3 min and then subjected to electrophoresis on SDS/8% polyacrylamide gels. The gels were dried and exposed at room temperature to Reflection film (DuPont/NEN) or to an imaging plate of a Fuji X Bas 1000 Phosphoimager.

Purification of SCA-2 from Hamster Liver. All purification steps were carried out at 4°C. SCA-2 activity was assayed by the standard assay at pH 8 as described above.

Step 1: Preparation of activated S-100 fraction. Livers from 25 hamsters, rinsed once with cold buffer B (same as buffer A except that the concentration of Hepes·KOH was 10 mM) supplemented with a mixture of additional protease inhibitors (5 μg of pepstatin A per ml, 10 μg of leupeptin per ml, and 2 μg of aprotinin per ml), were homogenized for 15 s in the same buffer (0.4 g/ml) in a specially designed Waring blender (18) followed by three strokes of a motor-driven homogenizer. The homogenates were centrifuged at $10^5 \times g$ for 1 h in a Sorvall AH 629 rotor. The resulting supernatant (S-100 fraction) was dialyzed for 16 h against buffer B (not supplemented with the above three protease inhibitors) and then incubated at 30°C for 2 h. This step was necessary to activate the cleavage enzyme.

Step 2: Ammonium sulfate precipitation. The S-100 fraction (15 g protein) was fractionated by ammonium sulfate precipitation. The fraction precipitating between 30% and 50% ammonium sulfate was dissolved in 43 ml of buffer C (50 mM Bis·Tris·HCl, pH 6.5/1.5 mM $MgCl_2$/10 mM KCl/1 mM sodium EDTA/1 mM sodium EGTA/5 mM dithiothreitol/0.1 mM Pefabloc).

Step 3: Heat treatment. The ammonium sulfate fraction from step 2 (3 g protein) was incubated at 60°C for 30 min. The sample was then centrifuged at $10^4 \times g$ in a Sorvall SA 600 rotor for 10 min, after which the supernatant fraction was dialyzed against buffer C overnight.

Step 4: Mono Q chromatography. After filtration through a 0.2 μm filter (Nalgene), the supernatant fraction from step 3 (315 mg protein) was loaded onto a Mono Q 16/10 fast protein liquid chromatography column equilibrated with buffer D (buffer C plus 96 μM Nonidet P-40). The column was washed with 5 column volumes of buffer D and eluted with a 400-ml linear salt gradient (0–0.35 M NaCl) in the same buffer. Fractions of 10 ml were collected. Fractions 16–20 (≈160 mM NaCl) containing SCA-2 activity were pooled.

Step 5: Mono S chromatography. The pooled fractions from step 4 (30 mg protein) were loaded onto a Mono S 5/5 fast protein liquid chromatography column. The column was washed with 20 column volumes of buffer D and eluted with a 40-ml linear salt gradient (0–1 M NaCl) in the same buffer. Fractions of 1 ml were collected. Fractions 16–22 (≈475 mM NaCl) containing SCA-2 activity were pooled.

Step 6: Hydroxylapatite chromatography. The pooled fractions from step 5 in buffer D (0.2 mg protein) were loaded onto a 1-ml hydroxylapatite column (Macro-Prep ceramic hydroxylapatite; Bio-Rad) equilibrated with 10 mM potassium phosphate at pH 6.8. The column was washed with 10 column volumes of 10 mM potassium phosphate at pH 6.8 and eluted with a 20-ml linear gradient (10–400 mM potassium phosphate) followed by a 10-ml elution with 400 mM potassium phosphate. Fractions of 1 ml were collected. Fractions 18–24 (≈400 mM potassium phosphate) containing SCA-2 activity were pooled and concentrated to 1 ml with a Centriprep 10 (Amicon).

Step 7: Superdex 200 gel filtration. The concentrated sample from step 6 (<20 μg protein) was loaded onto a Superdex 200 26/60 gel filtration column equilibrated with buffer D containing 150 mM NaCl and eluted with the same buffer (see Fig. 1). Fractions of 2.5 ml were collected. The peak fractions (numbers 33 and 34) were concentrated with a Centricon 10. These fractions are referred to as purified SCA-2.

Partial Amino Acid Sequence of SCA-2. Purified SCA-2 from hamster liver (step 7) was subjected to electrophoresis in a 12% SDS gel and then transferred onto poly(vinylidene difluoride) membrane (Immobilon-P; Millipore). The 20-kDa and 10-kDa subunits were visualized with Coomassie blue and excised for direct NH_2-terminal sequencing and solid phase tryptic digestion (19). Peptides were isolated and sequenced as described (20).

cDNA Cloning of Hamster SCA-2. An aliquot of phage template DNA from a Syrian hamster liver λZAP cDNA library (21) was amplified with 60 pmol each of two degenerate oligonucleotide primers: primer 1 based on the sequence of peptide 5 (see Table 1), 5′-ACTCTACTAGA(A/G)AA(A/G)CC(G/A/T/C)AA-3′, and primer 2 based on the sequence of peptide 7 (Table 1), 5′-TCGGCCGAA(G/A/T/C)GT(G/A/T/C)A(A/G)(T/C)TC-3′. An aliquot of the amplified PCR product was subjected to a second round of PCR with 60 pmol each of two degenerate oligonucleotides: primer 3 based on the sequence of peptide 6, 5′-TTTATTCAGGC(G/A/T/C)TG-(C/T)(A/C)G(G/A/T/C)GG-3′, and primer 4 based on the COOH-terminal sequence of peptide 2, 5′-TAGGCGAA-GA(A/G)(G/A)AA(G/A)TC(G/A/T/C)GC-3′. The PCR product was cloned into the pNoTA/T7 vector using the Prime PCR Cloner Kit (5 Prime → 3 Prime, Inc). The DNA sequence of the resulting 125-bp PCR product encoded peptides 2 and 6 of SCA-2 (Table 1). The 125-bp PCR product was labeled with [^{32}P]dCTP by PCR (22) using M13 and T7 vector primers. The probe was then used to screen a Syrian hamster liver cDNA library by hybridizing duplicate filters at 65°C for 2 h in Rapid-hyb buffer (Amersham). The filters were washed once with 1× standard saline citrate (SSC)/0.1% SDS at room temperature for 5 min and twice with 0.1× SSC/0.1% SDS at 65°C for 15 min. Of 200,000 plaques screened, 2 positive partial length clones were identified. One of these was recovered using ExAssist Interference-Resistant Helper Phage (Stratagene). The insert of this clone was excised and labeled with [^{32}P]dCTP by the Rediprime Random Primer Labeling Kit (Amersham). The probe was then used to screen 10^6 more plaques as described above. Twenty-eight positive clones were identified, and one of them, clone 11–1, corresponded to a 2.1-kb SCA-2 mRNA, including a 3′ untranslated region of 1.1 kb.

Production of Recombinant Hamster CPP32/SCA-1 and SCA-2. CPP32/SCA-1 and SCA-2 were expressed with six histidine residues and a thrombin cleavage site added to the NH_2-terminus. PCR-amplified hamster CPP32/SCA-1 (10) and SCA-2 cDNAs were subcloned inframe into the *Nde*I/

*Bam*HI sites and *Nde*I/*Xho*I sites, respectively, of the bacterial expression vector pET-15b (Novagen). The primers used for PCR of CPP32/SCA-1 were 5′-AAATCACATATGTCTG-GAATATATCTGGACAGTAGTTACAAAATG-3′ and 5′-CCAATCGGATCCTTAGTGATAAAAGTACAAT-TCTTTTGTGAGC-3′. The primers used for PCR of SCA-2 were 5′-GACGGGCATATGGCTAAGCCCGACCGCTC-CTCC-3′ and 5′-AGGGAACTCGAGTCAACGGCCGAA-GTAGAGCTC-3′. Exponentially growing bacteria BL21 (DE3) carrying the expression plasmids were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h and then lysed in a French press in buffer E (5 mM imidazole/0.5 M NaCl/20 mM Tris·HCl, pH 7.9). The lysates were centrifuged at $3 \times 10^4 \times g$ for 20 min, and the supernatants were subjected to purification on a Ni^{2+}-Sepharose column (2.5 ml) according to the manufacturer's instructions (Novagen). The eluted CPP32/SCA-1 and SCA-2 were dialyzed at 4°C for 16 h against buffers C and A, respectively, and stored at −80°C.

Immunoblot Analysis. Anti-CPP32/SCA-1 antibody was produced by immunizing rabbits (10) with a His(6)-tagged fusion protein containing amino acids 29–175 of hamster CPP32/SCA-1. The fusion protein construct was cloned into the *Nde*I/*Bam*HI sites of pET-15b, expressed in *Escherichia coli*, and purified by Ni^{2+}-Sepharose chromatography. Anti-Mch3/SCA-2 antibody was produced by immunizing rabbits with a synthetic peptide corresponding to amino acids 24–37 of hamster Mch3/SCA-2. The peptide was coupled to keyhole limpet hemocyanin (10). Immunoblot analysis of CPP32/SCA-1 and Mch3/SCA-2 was performed with a 1:1000 dilution of the indicated antiserum, using horseradish peroxidase-conjugated anti-rabbit IgG as the secondary antibody and the enhanced chemiluminescense Western blotting detection kit (Amersham) as described (11).

RESULTS

When partially purified cytosol from hamster liver was fractionated on a green dye column, we observed two peaks of activity that were capable of cleaving *in vitro* translated SREBPs. The activity in the first peak coincided with immunoreactive CPP32/SCA-1. The enzyme in the second peak also cleaved translated SREBPs, but the enzyme did not react with the anti-CPP32/SCA-1 antibody. The two enzymes differed in another respect: CPP32/SCA-1 was maximally active at pH 6.5 and had minimal activity at pH 8. The enzyme in the second peak retained considerable activity at pH 8. We provisionally named this enzyme SCA-2. To follow SCA-2 activity, we measured the cleavage of *in vitro* translated SREBP-2 at pH 8. Using this assay, we purified SCA-2 to homogeneity in a sequential process that included ammonium sulfate fractionation, heat treatment, and four steps of column chromatography (described in detail in *Methods*). Fig. 1 shows the activity-containing fractions from the final Superdex gel filtration column. The peak of SREBP cleavage activity was found in fractions 33 and 34 (Fig. 1*A*). These two fractions contained a pair of proteins with molecular masses of ≈20 and ≈10 kDa as determined by SDS/PAGE followed by silver staining Fig. 1*B*).

We obtained sequences of the NH_2 termini of the 20-kDa and 10-kDa fragments as well as 5 tryptic peptides (Table 1). Five of the sequences were similar, but not identical, to sequences in CPP32/SCA-1. These similarities allowed us to determine the order of the peptides, thereby facilitating the preparation of an authentic probe from PCR of a hamster liver cDNA library. The probe was then used to screen the library to produce a full-length cDNA encoding SCA-2.

Fig. 2 *Upper* compares the predicted amino acid sequence of hamster SCA-2 and CPP32/SCA-1. Overall, the two proteins are 54% identical. They share the QACRG motif that defines the family of ICE-related cysteine proteases. The NH_2-terminal sequence of the P20 fragment begins at residue 24, which follows an Asp, indicating that the precursor had been cleaved at this point. The NH_2 terminus of the P10 fragment occurs at residue 207, which also follows an Asp. Both of these sites are indicated by arrows in Fig. 2.

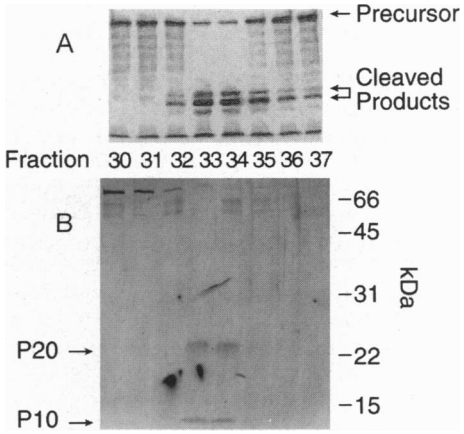

FIG. 1. Superdex 200 chromatography of hamster liver SCA-2. The pooled fractions from the hydroxylapatite column in step 6 were subjected to gel filtration on a Superdex 200 26/60 column as described. (*A*) Aliquots of the indicated fraction (2 μl) were assayed for ^{35}S-SREBP-2 cleavage activity at pH 8 for 10 min at 30°C as described. The dried gel was exposed to a Fuji Phosphoimager for 70 min. (*B*) Aliquots of the same fractions (25 μl) were subjected to SDS/15% PAGE and stained with silver (Bio-Rad) to visualize the protein bands. P20 and P10 denote the 20-kDa and 10-kDa subunits of SCA-2, respectively.

Fig. 2 *Lower* compares the sequence of hamster SCA-2 and a human sequence that was recently obtained from a random EST (13, 14). The hamster and human proteins are 85% identical throughout the length of the proteins. We therefore believe that SCA-2 is the hamster homologue of human Mch3, and we designate this protein as Mch3/SCA-2.

To further characterize the biochemical properties of Mch3/SCA-2, the hamster cDNA was cloned into a bacterial expression vector that adds six histidine residues at the NH_2 terminus of the protein. When the full-length protein was expressed with the His(6) tag adjacent to the initiator methionine, the recov-

Table 1. Sequences of NH_2-terminal and tryptic peptides from hamster SCA-2

Peptide	Amino acid sequence	Amino acid position in cDNA sequence
1	AKPDR--IIS-IL SS S	24-36
2	-NPRYKIPVEAD-LFAY A F	207-223
3	-RVPTYLYR D	53-61
4	-FDKVTGM-VR N D	77-87
5	TLLEKPK	173-179
6	-FFIQACR L	180-187
7	EL-FGR Y	298-303

Sequences were obtained from Edman degradation performed on NH_2-terminal peptides (nos. 1 and 2) and HPLC-purified tryptic peptides (nos. 3–7) isolated from the 20-kDa and 10-kDa subunits of hamster SCA-2, as described. Each tryptic peptide represents a pure species from a single HPLC peak. The (–) denotes a residue that could not be assigned based on peptide sequence analysis. The amino acids at these positions as deduced from the predicted cDNA sequence are indicated below the peptide sequence.

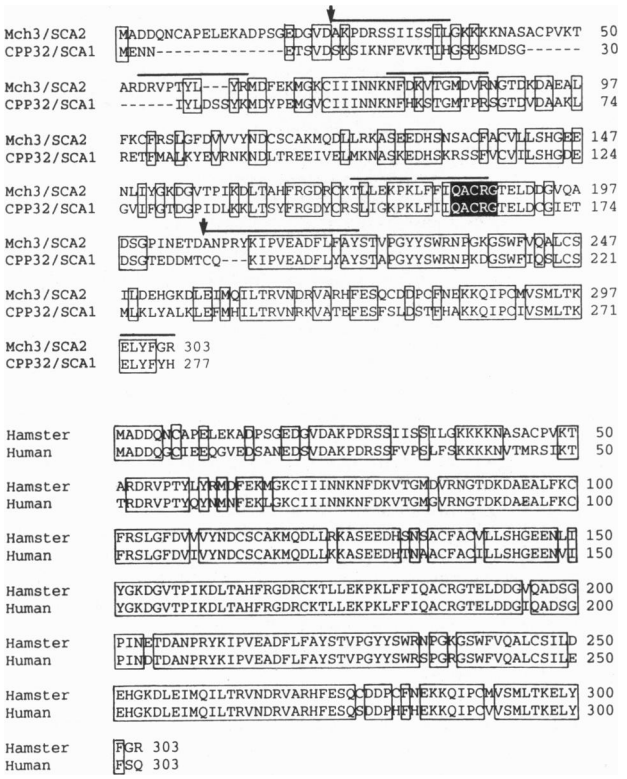

FIG. 2. Amino acid sequence comparisons. (*Upper*) Hamster Mch3/SCA-2 versus hamster CPP32/SCA-1. The deduced sequence from hamster CPP32/SCA-1 was from Wang *et al.* (10). The hamster Mch3/SCA-2 sequence was deduced from cDNA cloning as described. Overlined residues denote peptides obtained from the chemical sequence of the 20-kDa and 10-kDa subunits of SCA-2 (see Table 1). Identical residues are boxed. The black box denotes the QACRG box conserved among all members of the ICE family of proteases. Vertical arrows denote the NH_2-terminal sequences of the purified 20-kDa and 10-kDa subunits of SCA-2. (*Lower*) Hamster Mch3/SCA-2 versus human Mch3. The deduced sequence of human Mch3 was from Fernandes-Alnemri *et al.* (13). The nucleotide sequence of hamster SCA-2 has been deposited in the GenBank data base (accession no. U47332).

ery of protein by nickel affinity chromatography was low. We attributed this to internal cleavage at the NH_2 terminus of P20, which eliminated the His(6) tag. Accordingly, we prepared another construct that initiated at residue 24. The recovery of this protein on the nickel column was high. Approximately two-thirds of the eluted material was in the P20/P10 form, and one-third was still in the intact 30-kDa form. Several fragments of other sizes were also observed. A similar strategy was used to produce recombinant CPP32/SCA-1. In this case we used a construct that initiated at Ser-29, which is the most distal of the NH_2-terminal sequences recorded for the P20 fragment of CPP32/SCA-1 (8). Again, about two-thirds of the enzyme recovered from the nickel column was in the P20/P10 form.

As mentioned above, studies with partially purified preparations indicated that the pH profiles of Mch3/SCA-2 and CPP32/SCA-1 differ. Fig. 3 shows that this difference was observed with the recombinant enzymes. CPP32/SCA-1 had a sharp pH optimum at 6.5 and the activity fell off dramatically at higher pH values. On the other hand, Mch3/SCA-2 had a broad pH optimum between 6.5 and 7. The activity at pH 8 was ≈50% of the maximum.

Mch3/SCA-2 appeared to cleave SREBP-2 at the same site as CPP32/SCA-1 as judged from the sizes of the products. This conclusion was confirmed by the demonstration that replacement of Asp-468 with Ala abolished cleavage of SREBP-2 by both enzymes (Fig. 4).

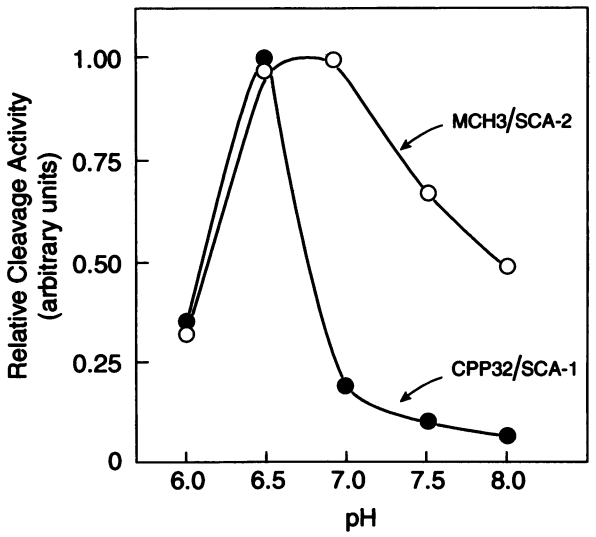

FIG. 3. CPP32/SCA-1 (●) and Mch3/SCA-2 (○) cleave ^{35}S-SREBP-2 with different pH profiles. Aliquots of purified recombinant CPP32/SCA-1 (0.9 ng) and Mch3/SCA-2 (0.7 ng) were assayed for cleavage of translated ^{35}S-SREBP-2 for 10 min at 30°C at the indicated pH as described. The gel was exposed to a Fuji Phosphoimager, and the amount of radioactivity in the cleaved NH_2 fragment of ^{35}S-SREBP was quantified with a Bio-Imaging analyzer. The peak activity at each pH was assigned a value of 1.0 unit.

A characteristic of CPP32/SCA-1 is that it cleaves PARP at a specific site to produce two fragments (7, 8). Fig. 5 shows that Mch3/SCA-2 also cleaves PARP into two fragments of the same approximate size. Thus, the two enzymes share specificity for at least two of the substrates, SREBPs and PARP.

When the two recombinant enzymes were mixed together and subjected to dye interaction chromatography using a green dye, CPP32/SCA-1 emerged earlier than Mch3/SCA-2 (Fig. 6). This result duplicates the result that was earlier observed with the partially purified enzyme.

DISCUSSION

This paper reports the purification, characterization, and cDNA cloning of an ICE-related cysteine protease that cleaves SREBP-1 and -2 at a single Asp to produce a transcriptionally active NH_2-terminal fragment. The enzyme, initially desig-

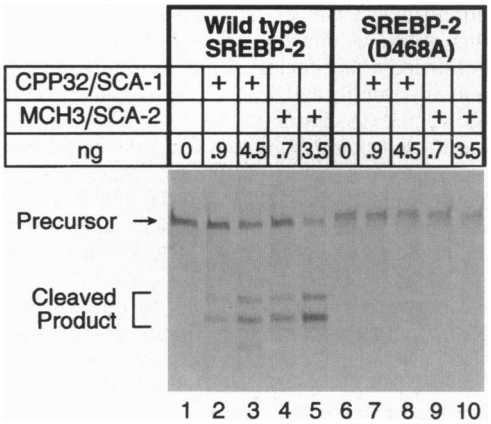

FIG. 4. Abolition of cleavage of ^{35}S-SREBP-2 by replacement of Asp-468. Translated ^{35}S-labeled wild-type SREBP-2 (lanes 1–5) and mutant SREBP-2 with the Asp at position 468 mutated to Ala (lanes 6–10) were incubated at 30°C for 30 min at pH 6.5 with the indicated amount of purified recombinant CPP32/SCA-1 (lanes 2, 3, 7, and 8) or Mch3/SCA-2 (lanes 4, 5, 9, and 10). The gel was exposed to film for 28 h.

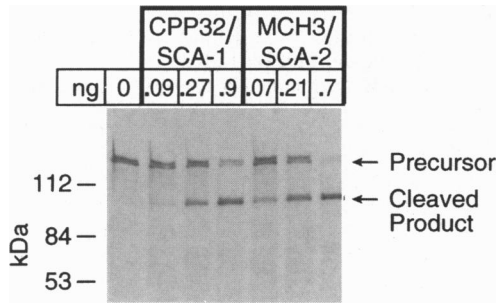

FIG. 5. Cleavage of translated ^{35}S-PARP by purified recombinant CPP32/SCA-1 and Mch3/SCA-2. The indicated amount of the purified recombinant CPP32/SCA-1 or Mch3/SCA-2 was assayed for cleavage of ^{35}S-PARP for 10 min at 30°C as described. The activities of CPP32/SCA-1 and Mch3/SCA-2 were assayed at pH 6.5 and 7.5, respectively. The gel was exposed to film for 3 days.

nated SCA-2, was initially distinguished from CPP32/SCA-1, a closely related cysteine protease, by virtue of its distinct behavior on a green dye column and because of its different pH-activity profile. These properties were confirmed with the recombinant enzymes (Figs. 3 and 6). After the purification and cDNA cloning had been accomplished, a paper appeared that described the sequence of human Mch3 (13), and it was immediately clear that we had purified the hamster equivalent. We therefore call this protein Mch3/SCA-2. Fernandes-Alnemri *et al.* (13) identified the Mch3 cDNA as an EST in a human library by virtue of its resemblance to the ICE family. They showed that the mRNA encoding Mch3/SCA-2, like that encoding CPP32/SCA-1, is expressed in many tissues. They produced recombinant enzyme by expression in bacteria and showed that it was cleaved to an active form that cleaved PARP. They also showed that overexpression of Mch3 in insect Sf9 cells led to apoptosis and that Mch3 was cleaved *in vitro* by CPP32/SCA-1. All of these findings indicate that Mch3/SCA-2, like CPP32/SCA-1, may be a central mediator of apoptosis in a variety of cells.

Our findings confirm and extend those of Fernandes-Alnemri *et al.* (13) by demonstrating, through sequence analysis, the precise NH_2 terminus of the P20 and P10 fragments as they occur in hamster liver. We show that Mch3/SCA-2 can cleave SREBPs at the same site that is cleaved by CPP32/

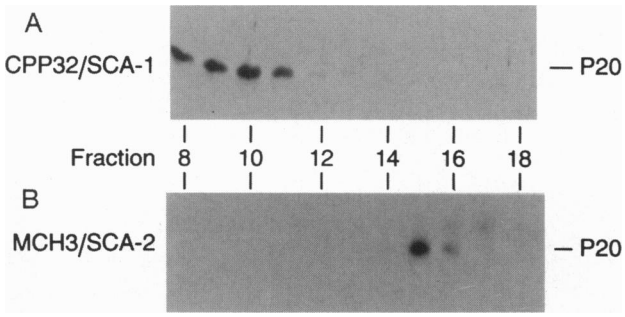

FIG. 6. Separation of CPP32/SCA-1 (*A*) and Mch3/SCA-2 (*B*) by reactive green 19 dye chromatography. Aliquots of the indicated recombinant purified enzyme (20 μg each) were mixed together in 10 ml of assay buffer containing 50 mM Tris·HCl at pH 8 and loaded onto a 1-ml reactive green 19 dye (Sigma) column equilibrated with the same buffer. The column was washed with 8 column volumes of the same buffer and eluted in 1-ml fractions with a 12-ml linear 0–0.9 M NaCl gradient (fractions 1–12), followed by a 2-ml linear 0.9–1.5 M NaCl gradient (fractions 13 and 14), followed by 10-ml of 1.5 M NaCl (fractions 15–24). Aliquots of the indicated fractions (30 μl) were subjected to SDS/15% PAGE followed by immunoblot analysis with antibodies against CPP32/SCA-1 (*A*) and Mch3/SCA-2 (*B*) as indicated. The filters were exposed to film at room temperature for 5 min (*A*) and 1 h (*B*). P20 denotes the 20-kDa subunit of SCA-1 and SCA-2.

SCA-1 and that the two enzymes have different pH optima. The biologic significance of this difference is unclear, but it raises the possibility that the two enzymes may function in different regions of the cell. The reason for the presence of two such similar enzymes in most cells of the body is also not clear. It is possible that they participate in a sequential activation cascade. Alternatively, they might each act independently in response to different inducers of apoptosis.

The sites of cleavage by CPP32/SCA-1 and Mch3/SCA-2 are marked by the presence of acidic residues at the P3 and/or P4 position. This distinguishes these enzymes from ICE, which cleaves substrates with bulky hydrophobic residues at these positions (4). Indeed, ICE does not cleave either the SREBPs or PARP (7–9). ICE also has a more restricted tissue distribution as compared with the other two enzymes (4, 10, 13), suggesting that ICE is not a general mediator of apoptosis in most cells.

The significance of the cleavage of SREBPs during apoptosis is not yet established. Further studies will be necessary to determine whether the liberated NH_2-terminal fragments of SREBPs can activate transcription during apoptosis, at least in the early stages. If so, these findings would suggest that activation of cholesterol-related genes is part of the program in programmed cell death.

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