Cleavage of sterol regulatory element binding proteins (SREBPs) by CPP32 during apoptosis

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Cellular cholesterol homeostasis is controlled by sterol-regulated proteolysis of membrane-bound transcription factors called sterol-regulatory element binding proteins (SREBPs). CPP32, a cysteine protease, was shown previously to cleave SREBP-1 and SREBP-2 *in vitro* at an aspartic acid between the basic helix-loop-helix leucine zipper domain and the first trans-membrane domain, liberating a transcriptionally active fragment. Here, we show that CPP32 exists in an inactive 32 kDa form in Chinese hamster ovary (CHO) cells. When apoptosis was induced with the protein kinase inhibitor staurosporine, CPP32 was cleaved to subunits of 20 and 10 kDa to form the active protease. Under these conditions, membrane-bound SREBP-1 and SREBP-2 were both cleaved, and the transcriptionally active N-terminal fragments were found in nuclear extracts. Similar results were obtained in human U937 cells induced to undergo apoptosis by anti-Fas and etoposide. The apoptosis-induced cleavage of SREBPs was not suppressed by sterols, indicating that apoptosis-induced cleavage and sterol-regulated cleavage are mediated by different proteases. CHO cells expressing a mutant SREBP-2 with an Asp→Ala mutation at the CPP32 cleavage site showed sterol-regulated cleavage but no apoptosis-induced cleavage. These data are consistent with the emerging concept that CPP32 is a central mediator in apoptosis. They also indicate that SREBPs, like poly(ADP) ribose polymerase, are cleaved by CPP32 during programmed cell death.

Keywords: apoptosis/cholesterol/CPP32 protease/sterol regulatory element binding proteins/transcription

Introduction

Two transcription factors designated sterol-regulatory element binding protein-1 and -2 (SREBP-1 and SREBP-2) are bound intrinsically to the endoplasmic reticulum and nuclear envelope of animal cells. In sterol-deprived cells a protease cleaves each of these proteins, releasing an N-terminal fragment of ~500 amino acids that enters the

nucleus and activates transcription of the genes for the low density lipoprotein (LDL) receptor, 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) synthase, and possibly other enzymes of cholesterol biosynthesis (Sato *et al.*, 1994; Wang *et al.*, 1994; Yang, *et al.*, 1994, 1995). This action enhances the uptake of exogenous cholesterol from plasma LDL, and it also increases endogenous cholesterol synthesis from acetyl CoA. When sterols accumulate to high levels, proteolysis of the SREBPs is abolished. The transcription factors remain bound to extranuclear membranes and transcription of the sterol-regulated genes declines.

In searching for the sterol-regulated protease that cleaves SREBPs, we recently identified an enzyme that we designated SCA (SREBP cleavage activity) (Wang *et al.*, 1995). This enzyme has the following properties: (i) it is present in an inactive form in the cytosol of liver and HeLa cells, but it becomes activated when cell extracts are incubated at 30°C *in vitro*; (ii) it is a cysteine protease that resists inhibitors of aspartyl, metallo and serine proteases, but is inactivated by iodoacetic acid or *N*-ethylmaleimide; (iii) it cleaves SREBP-1 and -2 between the D and S residues of the sequences SEPDSP and DEPDSP, respectively; and (iv) it is inhibited by an acidic tetrapeptide aldehyde (Ac-DEAD-CHO) related to the target sequence, but not by a hydrophobic tetrapeptide aldehyde (Ac-YVAD-CHO) that was shown previously to inhibit another cysteine protease, the interleukin-1β converting enzyme (ICE) (Thornberry and Molineaux, 1995).

SCA was purified extensively from hamster liver, and a 20 kDa protein was isolated and subjected to partial sequencing (Wang *et al.*, 1995). This revealed that SCA is the hamster counterpart of human CPP32, a gene product that was identified by Fernandes-Alnemri *et al.* (1994) as an expressed sequence tag in a randomly sequenced human cDNA library. CPP32 was shown to encode a protein that resembles ICE, including the conserved sequence (QACRG), which is the catalytic site of the ICE family of cysteine proteases (Thornberry and Molineaux, 1995).

ICE is the prototype for a family of cysteine proteases that have been implicated in apoptosis, or programmed cell death, in animal cells. Originally identified as a cytosolic protease that activates interleukin-1β prior to secretion, ICE was found subsequently to resemble the product of the *ced-3* gene in *Caenorhabditis elegans* (Yuan *et al.*, 1993). Mutations in this gene prevent the programmed death of certain cells during nematode development (Yuan and Horvitz, 1990). Overexpression of ICE and CED-3 in animal cells leads to apoptosis (Miura *et al.*, 1993).

ICE is synthesized as an inactive precursor of 45 kDa, which undergoes a series of autocatalytic cleavages to yield subunits of 20 and 10 kDa, which associate to form the active enzyme (reviewed in Thornberry and Molineaux,

1995). ICE cleaves proteins at the C-terminal side of aspartic acid residues. The enzyme recognizes Asp-terminated peptides as short as four residues in length, and it has a distinct preference for bulky hydrophobic residues at positions -2 and -3 relative to the Asp. The enzyme is resistant to most protease inhibitors, but it is exquisitely sensitive to sulfhydryl-modifying agents such as iodoacetic acid or *N*-ethylmaleimide.

In addition to CPP32, several other relatives of ICE have been isolated, and all have the conserved sequence, QACRG (Faucheu *et al.*, 1995; Thornberry and Molineaux, 1995). CPP32 is predicted to have a molecular weight of 32 kDa. Because of its resemblance to ICE, Fernandes-Alnemri *et al.* (1994) reasoned that CPP32 might be cleaved to fragments of ~20 and 10 kDa during the course of proteolytic activation. They therefore prepared recombinant baculoviruses that encoded the putative 20 and 10 kDa fragments and used them to infect insect cells. Overproduction of these two components triggered apoptosis. They concluded that CPP32, like ICE, may be involved in apoptosis in animal cells. Interestingly, the internal site of presumed autocatalytic cleavage in CPP32 is a negatively charged peptide, IETDS, which resembles the cleavage sequences in SREBP-1 and -2 (Wang *et al.*, 1995).

Recently, Nicholson *et al.* (1995) purified CPP32 as an enzyme that cleaves poly(ADP-ribose) polymerase (PARP) during apoptosis in human monocytic leukemia cells (THP-1 cells). The active form of the enzyme was composed of subunits of 16.6 and 11.9 kDa. Sequencing showed that these two subunits had been cleaved from a common precursor, namely, CPP32. The enzyme cleaved PARP between the D and G of the sequence DEVDG, which resembles the DEPDS sequence at which CPP32 cleaves SREBP-2 (Wang *et al.*, 1995). Moreover, PARP cleavage was inhibited by the tetrapeptide aldehyde Ac-DEVD-CHO, which resembles Ac-DEAD-CHO, the inhibitor of CPP32-mediated cleavage of SREBP (Wang *et al.*, 1995). *In vitro*, Ac-DEVD-CHO prevented cytosol from apoptotic osteosarcoma cells from inducing apoptotic changes in nuclei isolated from non-apoptotic cells, suggesting that CPP32 plays an important role in apoptosis in these cells (Nicholson *et al.*, 1995). A similar result was obtained by Tewari *et al.* (1995), who showed that CPP32 could be cleaved and activated *in vitro* by ICE and that CPP32, in turn, cleaves PARP.

In the current study, we produce recombinant CPP32 and show that it cleaves SREBP in a reaction that is blocked by Ac-DEAD-CHO. Using antibodies against the N-terminal portion of CPP32, we show that the enzyme is cleaved to produce a subunit of ~20 kDa when apoptosis is induced in Chinese hamster ovary (CHO) cells by treatment with staurosporine, a wide-spectrum protein kinase inhibitor that induces programmed cell death in multiple cell types (Jacobson *et al.*, 1994). This cleavage of CPP32 is accompanied by the cleavage of SREBPs in a fashion that is not inhibited by sterols. Similar results were obtained in U937 cells induced to undergo apoptosis by anti-Fas and etoposide. These data suggest that SREBP-1 and -2, like PARP, are physiologic sites of CPP32 cleavage and that both CPP32 and SREBPs are activated during apoptosis induced by staurosporine.

Human	1	MENTENSVD	1	SKSIKNLEPKIIHGSE	2	SDSGISLDNSYKMDYP	GLCII	I
Hamster	1	---N-T---	1	---F-V-T---	2	---K---	---Y---	S-----V---
Human	51	NNKNFHKSTG	51	MTSRSGTDVDAAN	51	LR	RETFRNLKYEV	RNKNDLTREEIV
Hamster	51	-----P---	51	-----K---	51	-----M---	-----	-----
Human	101	RDVSKEDHSKR	101	SSFVCLLSHGEEGI	101	IFGTNGPVDLKKIT	NFFRGDR	CRS
Hamster	101	KNA-----	101	-----I---	101	-----D---	-----V---	L-SY---
Human	151	LTGKPKLFI	151	IQACRGTELDCGI	151	ETDS	SGVDDDMACH	KIPVEADFLYAYSTA
Hamster	151	-I-----	151	-----S---	151	-----L---	-----H---	-----T-Q---
Human	201	PGYYSWRNSK	201	DGSWFIQSLCAML	201	KQYADKLEF	MHILTRVNR	KVATEFESF
Hamster	201	-----P---	201	-----S---	201	-----L---	-----H---	-----
Human	251	SFDATFHAKKQ	251	IPCIVSMLTKELY	251	FYH	277	
Hamster	251	-L-S-----	251	-----	251	-----	277	

Fig. 1. Amino acid sequences of human and hamster CPP32. The deduced sequence of human CPP32 is from Fernandes-Alnemri *et al.* (1994). The hamster sequence was deduced from cDNA cloning as described in Materials and methods. Dashes denote residues in hamster CPP32 that are identical to human CPP32. Underlined residues denote peptides obtained from the chemical sequence of the 20 kDa subunit of SCA (Wang *et al.*, 1995). The box denotes amino acids conserved among all members of the ICE family of proteases. Asterisks (*) denote the two residues involved in catalysis by ICE-related proteases. Vertical arrows denote sites of cleavage of CPP32 in human THP-1 cells (arrows 2 and 3) and hamster liver (arrows 1 and 3). The nucleotide sequence of hamster pCPP32 has been deposited in GenBank (accession number U27463).

Results

cDNA cloning of hamster CPP32

To clone a cDNA encoding hamster CPP32, we performed PCR on a cDNA library from Syrian hamster liver that was cloned into a bacteriophage λ vector (Garcia *et al.*, 1995). The primer for the 5' end of the cDNA was a degenerate oligonucleotide based upon the peptide sequence at the N-terminus of the 20 kDa peptide that we had purified from hamster liver (Wang *et al.*, 1995). The other primer was derived from the published sequence of the 3' end of the coding region of the cDNA for human CPP32 (Fernandes-Alnemri *et al.*, 1994). The 800 bp amplification product was cloned, radioactively labeled and used to screen a λ phage cDNA library from Syrian hamster brain, yielding a full-length cDNA (see Materials and methods).

Figure 1 compares the predicted amino acid sequences of human and hamster CPP32. Both proteins contain 277 amino acids, with no gaps and only 34 mismatches, most of which are conservative substitutions. Both proteins contain the conserved QACRG sequence that includes the catalytic cysteine of the ICE family of proteases (Thornberry and Molineaux, 1995). The cloned sequence also includes all three peptides that were sequenced from the 20 kDa subunit of SCA/CPP32 that was purified from hamster liver (underlined in Figure 1). The N-terminus of the purified subunit began with the sequence SIKNF, which begins at residue 12 of the encoded protein, indicating that the initial 11 residues had been removed by proteolysis. The sequence preceding the S is NSVDSK. It is possible that this subunit was generated by cleavage between the D and S of this sequence (site 1 in Figure 1) and that a subsequent cleavage occurred at the K, either in the cell or during isolation of the protein.

The active form of CPP32 that was purified previously from human THP-1 cells was cleaved at two Asp-Ser sequences labeled 2 and 3 in Figure 1 (Nicholson *et al.*,

Fig. 2. Purified active CPP32 cleaves translated hamster CPP32.

Aliquots (0.2 μg protein) of partially purified CPP32 from HeLa cells (Wang *et al.*, 1995) were incubated with varying concentrations of the indicated tetrapeptide aldehyde dissolved in Buffer A containing 3% (v/v) DMSO. The final concentration of DMSO in all lanes was 0.3%. After incubation at 30°C for 5 min, aliquots (5 μl) of *in vitro* synthesized, ³⁵S-labeled hamster CPP32 were added to each 20 μl reaction. After an additional 60 min at 30°C, the samples were subjected to SDS-PAGE, and the gel was transferred onto a nitrocellulose filter. The filter was exposed to film for 16 h at room temperature.

1995). This generated an N-terminal subunit of 16.6 kDa and a C-terminal subunit of 11.9 kDa. Cleavage at site 2 could not have occurred in our preparation since the 20 kDa subunit included residues on the N-terminal side of this site (underlined in Figure 1). Thus, CPP32, like ICE, may be cleaved at several alternative sites to generate an active enzyme.

***In vitro* cleavage of SREBP by CPP32**

To determine whether CPP32 can undergo autocatalytic cleavage, we translated the mRNA encoding CPP32 in the presence of [³⁵S]methionine and incubated the protein for 60 min at 30°C. During this time, the CPP32 remained intact at 32 kDa (Figure 2, lane 1). The radiolabeled protein was cleaved to subunits of ~20 and 10 kDa when we added the active two-subunit form of CPP32 that was partially purified from HeLa cells (lane 2). This cleavage was inhibited partially when we pre-incubated the purified enzyme with Ac-DEAD-CHO (lanes 3–5), but not with Ac-YVAD-CHO (lanes 6–8). The inhibition with Ac-DEAD-CHO was not complete, raising the possibility that another protease, in addition to CPP32 itself, can also activate CPP32.

To determine whether the cloned CPP32 is capable of cleaving SREBP, we prepared extracts of human kidney 293 cells that had been transfected with a cDNA encoding CPP32 (Figure 3). The extracts were incubated at 30°C to activate CPP32 and were then incubated with ³⁵S-labeled, *in vitro* translated SREBP-2. At the concentrations that were employed, the extracts of mock-transfected cells did not cleave [³⁵S]SREBP-2 (lanes 2–4). Extracts of cells transfected with pCPP32 cleaved [³⁵S]SREBP-2 (lanes 5–7), and this was inhibited completely by Ac-DEAD-CHO (lanes 8–10), but not by Ac-YVAD-CHO (lanes 11–13).

Activation of CPP32 in CHO cells by staurosporine-induced apoptosis

Figure 4 shows an experiment designed to determine whether CPP32 becomes activated during apoptosis in

Fig. 3. Cleavage of [³⁵S]SREBP-2 by hamster CPP32 in extracts of transfected 293 cells. Cell pellets from eight 60 mm dishes of 293 cells transiently transfected with pCPP32 were lysed in Buffer A containing 1% Triton X-100. After incubation on ice for 30 min, the extracts were spun at top speed in a microcentrifuge for 5 min at 4°C, and each supernatant was incubated at 30°C for 2 h to activate CPP32. The indicated amount of extract from mock (lanes 2–4) or pCPP32-transfected (lanes 5–13) cells was incubated with the indicated tetrapeptide aldehyde in a final volume of 20 μl of Buffer A. After 5 min at 30°C, aliquots (5 μl) of *in vitro* translated, ³⁵S-labeled SREBP-2 were added to each reaction. After 60 min at 30°C, samples were subjected to SDS-PAGE, and the gel exposed to film at room temperature for 16 h.

CHO cells and whether this is associated with the acquisition of SREBP cleavage activity. CHO cells were incubated with staurosporine, a broad-spectrum protein kinase inhibitor that induces apoptosis in a variety of cells (Jacobson *et al.*, 1994). After varying times, the cells were harvested and nuclear DNA was subjected to agarose gel electrophoresis and ethidium bromide staining (Figure 4A). Beginning at 4 h, we observed a ladder of DNA bands, indicating that chromosomal DNA was being cleaved into fragments containing varying numbers of nucleosomes in a fashion that is characteristic of apoptosis. Cytosol from the same cells was subjected to SDS-gel electrophoresis and incubated with an antibody directed against the N-terminal peptide of the SCA/CPP32 that we had purified from hamster liver. At zero time, all of the CPP32 was in the full-length 32 kDa form (Figure 4B). After 4 h of staurosporine treatment, we observed a new band at 20 kDa that corresponds to the large subunit of the catalytically active form of CPP32. The amount of this subunit increased progressively through the 8 h time point. Extracts of the staurosporine-treated cells were also tested for their ability to cleave ³⁵S-labeled SREBP-2 *in vitro* (Figure 4C). The assays were conducted without prior *in vitro* activation of the extracts so as to reflect the *in vivo* SREBP-2 cleavage activity. At zero time, no cleavage of SREBP was observed (lane 1). Beginning at 4 h, we observed an activity that cleaved SREBP-2 into the characteristically sized N- and C-terminal fragments (lanes 3–5). This activity was inhibited by Ac-DEAD-CHO (lanes 6–8), but not by Ac-YVAD-CHO (lanes 9–11).

Staurosporine-induced cleavage of SREBPs in CHO cells undergoing apoptosis

To determine whether staurosporine-induced apoptosis is associated with cleavage of SREBP within the cell, we treated CHO cells with staurosporine in the absence or

Fig. 4. Activation of CPP32 in CHO cells by staurosporine. CHO-7 cells were set up in medium A as described in Materials and methods. After incubation for 3 days, staurosporine at a final concentration of 1 μM was added to the culture medium for the indicated time. The medium in all dishes was adjusted to contain 0.1% (v/v) DMSO, the solvent for staurosporine. Additions were made in a staggered fashion so that all cells could be harvested at the same time, as described in Materials and methods. (A) Genomic DNA (10 μg/sample) from each time point was isolated as described in Materials and methods and loaded onto a 1.3% agarose gel that was subsequently stained with ethidium bromide. (B and C) S-100 fractions were prepared from the harvested cells as described (Wang *et al.*, 1994). (B) Aliquots of the indicated S-100 fraction (5 μg) were subjected to SDS-PAGE and immunoblot analysis with a rabbit antiserum against the N-terminal portion of hamster CPP32 (1/2000 dilution). The film was exposed at room temperature for 2 min. P32 and P20 denote the positions of migration of the CPP32 precursor and its 20 kDa subunit, respectively. (C) Aliquots of the indicated S-100 fraction (5 μg) were incubated in 25 μl of Buffer A with 5 μl of *in vitro* translated ³⁵S-labeled human SREBP-2 (lanes 1–5) at 30°C for 2 h. In lanes 6–12, aliquots of the indicated S-100 fraction (5 μg) from cells treated for 8 h with staurosporine were pre-incubated in 25 μl of Buffer A containing the indicated tetrapeptide aldehyde for 5 min at 30°C, followed by addition of 5 μl of ³⁵S-labeled SREBP-2 and incubation at 30°C for 2 h. The samples in (B) and (C) were then subjected to SDS-PAGE, and the gel was exposed to film at room temperature for 16 h.

presence of sterols (Figure 5). Nuclei were prepared, and the whole nucleus, including the membrane, was extracted with SDS. In the absence of sterols or staurosporine, the antibody against SREBP-1 revealed two cleaved products in the range of 60–70 kDa, as previously described (lane 1). These bands were both suppressed when sterols were added (lane 2). In the presence of staurosporine, we observed two additional bands that migrated slightly faster than the sterol-regulated products (lane 3). When sterols were added, the sterol-regulated bands disappeared but the staurosporine-induced bands persisted (lane 4). Similar results were obtained with SREBP-2 (Figure 5B). Figure 5C shows an immunoblot of the cytosolic fractions from these cells probed with an antibody against the N-terminal peptide of CPP32. In the absence of staurosporine, all of

the immunoreactivity was associated with the 32 kDa band (lanes 9 and 10). In the presence of staurosporine, we observed the expected cleavage to the 20 kDa fragment (lanes 11 and 12).

To confirm that the apoptosis-induced cleavage of SREBP was mediated by CPP32, we stably transfected CHO cells with cDNAs encoding wild-type SREBP-2 and a mutant version in which aspartic acid at the CPP32 cleavage site was changed to alanine (D468A mutant). Both proteins contained a herpes simplex virus (HSV) glycoprotein epitope tag at the N-terminus so that the transfected and endogenous SREBP-2 proteins could be distinguished. The cells were treated with staurosporine in the absence or presence of sterols (Figure 6). Detergent extracts were subjected to SDS-PAGE and immunoblotted with an antibody against the HSV epitope tag. Cells expressing wild-type SREBP-2 showed the sterol-regulated fragment (lanes 1 and 2) and the staurosporine-induced fragment (lanes 3 and 4). In contrast, cells transfected with the D468A mutant showed only the sterol-regulated fragment (lanes 5–8). An identical result was obtained with an independent clone of CHO cells stably transfected with the D468A mutant.

Cleavage of SREBP-2 induced by anti-Fas and etoposide in U937 cells

To determine whether other inducers of apoptosis trigger the cleavage of SREBP-2, we turned to U937 cells, a line of human monoclastic leukemia cells that is known to undergo apoptosis when treated with a monoclonal antibody (mAb) against the cell surface Fas antigen, a natural trigger of apoptosis (Sumimoto *et al.*, 1994). To assess the cleavage of SREBP-2, we used an antibody against the C-terminal fragment. As shown in Figure 7, the C-terminal fragment of SREBP-2 was observed in immunoblots of membrane extracts of U937 cells grown in the absence of sterols (lane 1), and this disappeared after sterol treatment (lane 2). Staurosporine produced a new C-terminal fragment of SREBP-2 that migrated slower than the sterol-regulated fragment (lane 5). This fragment did not disappear after sterol treatment (lane 6). This result is identical to that observed in CHO cells, except that the apoptosis-induced fragment migrated more slowly than the sterol-regulated fragment because it was visualized with the anti-C-terminal antibody. The apoptotic cleavage site, being closer to the N-terminus than the sterol-regulated site, gives a larger C-terminal fragment and a smaller N-terminal fragment. The anti-Fas antibody had the same effect as staurosporine (lanes 3 and 4). An identical effect was achieved by treatment with etoposide (lanes 7 and 8), which damages DNA by blocking topoisomerase activity and thereby induces apoptosis (Solary *et al.*, 1994). All these treatments produced apoptosis in these cells, as monitored by agarose gel electrophoresis of fragmented nuclear DNA (data not shown).

To confirm that the activity that cleaves SREBP-2 in apoptotic U937 cells is CPP32 related, we performed an *in vitro* proteolysis experiment (Figure 8). Extracts of untreated U937 cells failed to cleave *in vitro* translated SREBP-2 (lane 1). Cleavage was observed with extracts of cells treated with anti-Fas (lane 2), staurosporine (lane 5) and etoposide (lane 8). In each case, cleavage was

Fig. 5. Proteolysis of SREBP-1 (A) and SREBP-2 (B) in CHO cells treated with sterols and staurosporine. On day 0, CHO-7 cells were set up as described in Materials and methods. On day 2, cells were refed with medium A containing either no sterols or 0.5 $\mu\text{g/ml}$ 25-hydroxycholesterol plus 5 $\mu\text{g/ml}$ cholesterol (sterols) as indicated. On day 3 staurosporine (stauro) at a final concentration of 1 μM was added to the medium as indicated. The medium in all dishes was adjusted to contain 0.25% (v/v) ethanol and 0.1% (v/v) DMSO, the solvents for sterols and staurosporine, respectively. After incubation at 37°C for 4 h, cells were harvested and lysed as described in Materials and methods and disrupted in hypotonic buffer as described by Wang *et al.* (1994). The cell lysate was centrifuged at 14 000 g for 2 min at 4°C. The supernatant was further spun at $10^5 g$ for 30 min at 4°C to obtain the S-100 fraction. The pellet from the 14 000 g centrifugation was lysed with 3 vol of 1% SDS in 100 mM NaCl and spun at $10^5 g$ for 45 min at 20°C. The supernatant was passed through a 25 gauge needle five times to obtain the nuclear lysate. Aliquots (50 $\mu\text{g/lane}$) of the nuclear lysate (A and B) and the S-100 fraction (C) were subjected to SDS-PAGE and blotted with one of the following antibodies: (A) 5 $\mu\text{g/ml}$ of IgG-2A4, an anti-SREBP-1 mAb directed against the N-terminal domain; (B) 5 $\mu\text{g/ml}$ of IgG-7D4, an anti-SREBP-2 mAb directed against the N-terminal domain; or (C) 1:2000 dilution of polyclonal anti-CPP32 antiserum. The filters were exposed to film at room temperature for 15–60 s.

blocked by Ac-DEAD-CHO (lanes 3, 6 and 9), but not by Ac-YVAD-CHO (lanes 4, 7 and 10).

Tissue distribution of hamster CPP32 mRNA

To determine the tissues in which the CPP32 gene is active, we performed blot hybridization analysis of mRNA from several hamster tissues. These data revealed that CPP32 has a wide distribution, including brain, heart, liver, white fat, spleen and muscle (Figure 9).

Discussion

In the present study we observed that CPP32, a cysteine protease, became activated during apoptosis induced by staurosporine in CHO cells and by anti-Fas and etoposide in U937 cells. Activation was associated with the proteolytic cleavage of SREBPs in a fashion that was insensitive to sterol repression. These data provide evidence to support the emerging hypothesis that CPP32 is a central mediator of apoptosis in animal cells (Fernandes-Alnemri *et al.*, 1994; Nicholson *et al.*, 1995; Tewari *et al.*, 1995). They also indicate that CPP32 has at least three substrates in addition to itself: PARP, SREBP-1 and SREBP-2.

We do not know yet whether apoptotic cells are capable of responding to the N-terminal fragments of the SREBPs by activating transcription of the genes for the LDL receptor or the cholesterol biosynthetic pathway. We also do not know whether such activation plays a role in apoptosis. Previous data indicate that the cholesterol biosynthetic pathway is not necessary for apoptosis when cells have access to cholesterol contained in plasma lipoproteins. On the contrary, inhibition of the synthesis of cholesterol and other isoprenoids with lovastatin, an inhibitor of HMG CoA reductase, induced apoptosis in leukemic HL-60 cells grown in the presence of LDL contained in fetal calf serum (Perez-Sala and Mollinedo, 1994). These cells had access to LDL cholesterol, and it is therefore unlikely that apoptosis resulted from the block in cholesterol synthesis. It is more likely that the apoptosis

Fig. 6. Proteolysis of HSV-tagged wild-type SREBP-2 (A) and mutant SREBP-2 (D468A) (B) in stably transfected CHO-7 cells treated with sterols and staurosporine. On day 0, cells were set up as described in Materials and methods. On day 2, cells were refed with medium A containing either no sterols or 1 $\mu\text{g/ml}$ 25-hydroxycholesterol plus 10 $\mu\text{g/ml}$ cholesterol (sterols) as indicated. On day 3, staurosporine (stauro) at a final concentration of 1 μM was added to the medium as indicated. All dishes received an addition of ALLN at a final concentration of 25 $\mu\text{g/ml}$ at the same time that staurosporine was added. After incubation at 37°C for 4 h, cells were harvested, disrupted in hypotonic buffer and centrifuged at 14 000 g as described in the legend to Figure 5. The 14 000 g pellet was resuspended in 10 mM HEPES-KOH at pH 7.4 containing 1% Triton X-100, 0.42 M NaCl, 2.5% (v/v) glycerol, 1.5 mM MgCl_2 , 0.5 mM EGTA and 0.5 mM EDTA, followed by rotation at 4°C for 30 min and centrifugation at top speed in a microcentrifuge at 4°C for 15 min. Aliquots (100 $\mu\text{g/lane}$) of the nuclear lysate were subjected to SDS-PAGE and blotted with 0.5 $\mu\text{g/ml}$ of HSV-Tag™ antibody. The filters were exposed to film at room temperature for 30 s.

resulted from deprivation of one of the non-sterol isoprenoid products derived from mevalonate, the product of HMG CoA reductase. These include prenylated proteins such as Ras and nuclear lamins as well as dolichol, ubiquinone and isopentenyl adenine (Goldstein and Brown, 1990). These data establish that non-sterol substances are

Fig. 7. Proteolysis of SREBP-2 in U937 cells treated with inducers of apoptosis. On day 0, U937 cells were set up in replicate cultures as described in Materials and methods and harvested 27 h later. Fifteen hours before harvest, each 100 ml culture received a direct addition of 0.1 ml of ethanol with or without 1 μ g/ml 25-hydroxycholesterol (final concentration) as indicated. The cultures in lanes 3 and 4 also received 100 ng/ml anti-Fas monoclonal IgG at the same time. Eight hours before harvest, all cultures received 0.1 ml of DMSO containing one of the following additions: none in lanes 1–4; 1 μ M staurosporine in lanes 5 and 6; or 50 μ M etoposide in lanes 7 and 8. Cells were harvested and lysed, and a S-100 fraction and a 10^5 g pellet were prepared as described in Figure 5. The 10^5 g membrane pellets were lysed with 3 vol of 1% SDS in 100 mM NaCl. Aliquots (100 μ g protein/lane) were subjected to SDS-PAGE and blotted with 4 μ g/ml of IgG-1C6, an anti-SREBP-2 mAb directed against the C-terminal domain. The filters were exposed to film at room temperature for 1 min. The S-100 fractions were used in the experiments of Figure 8.

Fig. 8. *In vitro* proteolysis of SREBP-2 by extracts of U937 cells treated with inducers of apoptosis. Aliquots of the S-100 fraction prepared from cells treated with inducers of apoptosis in Figure 7 (10 μ g protein) were used for assay of cleavage of *in vitro* translated 35 S-labeled human SREBP-2 in the absence and presence of the indicated concentration of tetrapeptide aldehyde as described in the legend to Figure 4C. Incubations were carried out at 30°C for 1.5 h. The samples were subjected to SDS-PAGE, and the gel was exposed to film at room temperature for 16 h. The S-100 fractions in lanes 1, 2–4, 5–7 and 8–10 were obtained from the cells in lanes 1, 3, 5 and 7 of Figure 7, respectively.

not required for apoptosis. Therefore, if the activation of SREBPs has any role in apoptosis, it would seem designed to supply cholesterol rather than a non-sterol isoprenoid.

Why would cells require cholesterol during apoptosis? Apoptosis is tightly regulated and highly organized (Raff,

Fig. 9. Distribution of CPP32 mRNA in hamster tissues. A 32 P-labeled PCR fragment (~800 bp) encoding hamster CPP32 (2×10^6 c.p.m./ml) was hybridized to total RNA (10 μ g/lane) from the indicated hamster tissue. RNA was prepared and blot hybridization was carried out as described (Garcia *et al.*, 1995), except that the hybridization was done at 65°C overnight in Rapid-hyb buffer (Amersham), and the blot was washed twice with $2 \times$ SSC plus 1% (w/v) SDS at room temperature for 30 min each, followed by two washes with the same buffer at 45°C for 30 min each. The blot was exposed to film for 48 h at room temperature. The same filter was subsequently hybridized with a 32 P-labeled rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe and exposed to film for 2 h at room temperature.

1992). Its hallmark is the fragmentation of chromosomal DNA and the nuclear envelope at a time when the cell membrane remains intact, thereby preventing a leakage of cell contents. This contrasts sharply with cell necrosis, in which the plasma membrane becomes permeable to proteins. During apoptosis, cells are also primed for rapid ingestion and degradation by macrophages (Savill *et al.*, 1993). It is possible that the activation of cholesterol synthesis and LDL uptake by SREBP is necessary to supply cholesterol to preserve the integrity of the plasma membrane during apoptosis, or to mark the cells for subsequent degradation by macrophages.

In the current studies, we initially used staurosporine (Jacobson *et al.*, 1994) to induce apoptosis and to activate CPP32 in CHO cells. Staurosporine is a broad-spectrum inhibitor of protein kinases including protein kinase C and cyclic AMP-dependent protein kinase (Rüegg and Burgess, 1989). Data from two other laboratories have indicated that CPP32 is also activated when apoptosis is induced in confluent osteosarcoma cells, in breast carcinoma cells treated with tumor necrosis factor (TNF), or in lymphoma cells treated with anti-Fas (Nicholson *et al.*, 1995; Tewari *et al.*, 1995). In agreement with these findings, we observed that CPP32 was activated in U937 cells treated with anti-Fas or etoposide. This conclusion is based on cleavage of SREBP-2 at the CPP32 site *in vivo* and cleavage of SREBP-2 *in vitro* by a protease that is inhibited by Ac-DEAD-CHO, but not by Ac-YVAD-CHO.

The current experiments indicate that CPP32 and the sterol-regulated protease cleave SREBPs at different sites and are subject to independent regulation. In the experiment of Figure 5A in which an N-terminal domain-specific antibody was used, immunoblots of nuclear extracts from sterol-deprived CHO cells showed two bands corresponding to mature SREBP-1. When staurosporine was present,

two additional bands appeared, both of which migrated more rapidly than the sterol-regulated bands. In experiments to be reported elsewhere, we have found that the upper band of the doublet represents a phosphorylated form of SREBP. The staurosporine-induced bands for SREBP-1 and SREBP-2 appear smaller than the sterol-regulated bands, indicating that the cleavage site for the staurosporine-induced enzyme is closer to the N-terminus of the protein (Figure 5). The cleavage site for CPP32 is at Asp460 in SREBP-1a and Asp468 in SREBP-2 (Wang *et al.*, 1995). It therefore appears that the sterol-regulated cleavage site must be on the C-terminal side of these residues. This conclusion is confirmed by the immunoblots of U937 cells in which a C-terminal domain-specific antibody was used (Figure 7). The C-terminal fragment generated during apoptosis was larger than the fragment generated by sterol deprivation, confirming that the apoptosis-regulated cleavage site is closer to the N-terminus. Independent regulation of the two proteases that act on the SREBPs is indicated by the finding that sterols suppress cleavage at the sterol-regulated site, but not at the staurosporine-induced site, as shown in Figures 5 and 7.

Recent data from Xue and Horvitz (1995) indicate that the protein encoded by the *C.elegans ced-3* gene is a cysteine protease whose substrate specificity resembles that of CPP32. The CED-3 protein cleaves PARP at the same site that is cleaved by CPP32. Inasmuch as CED-3 has been linked genetically to apoptosis (Yuan and Horvitz, 1990), these data reinforce the idea that CPP32, or a related enzyme with similar substrate specificity, is essential to the process of apoptosis in animal cells.

The current data indicate that CPP32, once activated, can undergo autocatalytic cleavage, thus creating the potential for runaway apoptosis. Clearly, the activation of CPP32 must be tightly controlled in order to prevent unwanted cell death. The factor that activates CPP32 is unknown. Partially purified CPP32 from HeLa cell extracts was able to cleave *in vitro* translated CPP32 (Figure 3). This reaction was inhibited partially, but not completely, by the CPP32 inhibitor Ac-DEAD-CHO. This raises the possibility that another protease, in addition to CPP32, is present in our HeLa CPP32 preparation and that this protease is also capable of triggering the activation of CPP32.

Recent *in vitro* data has shown that ICE can cleave and activate CPP32 (Tewari *et al.*, 1995). However, ICE does not appear to be a general mediator of apoptosis since ICE knockout animals fail to show a generalized defect in apoptosis (Kuida *et al.*, 1995; Li *et al.*, 1995). If other proteases, in addition to ICE, can activate CPP32, it would raise the interesting possibility of a cascade of proteases leading to apoptosis. Such a cascade would be similar, in principle, to the proteolytic cascade that leads to another irreversible process, namely, blood clotting. Such cascades offer the potential for exquisite control based on regulation at each of several points in the cascade. It is tempting to speculate that such a protease cascade exists in animal cells, and that this provides both the regulation and the signal amplification necessary for a highly controlled yet rapid and irreversible process.

Materials and methods

Reagents and general methods

We obtained protease inhibitors from Boehringer-Mannheim; *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) from Calbiochem; a mouse mAb (IgM fraction) against human Fas from Upstate Biotechnology, Inc.; a mouse mAb against HSV-Tag™ from Novagen; radioactive amino acids from Amersham; staurosporine and etoposide from Sigma; DNase-free RNase A from Worthington Biochemical Corp; Ac-Tyr-Val-Ala-Asp-CHO (Ac-YVAD-CHO) from BACHEM Bioscience, Inc; and Ac-Asp-Glu-Ala-Asp-CHO (AcDEAD-CHO) from Julio C. Medina of Tularik, Inc. (Wang *et al.*, 1995). Protein concentration was determined by the BCA method (Pierce). Standard molecular biology methods were used as described (Sambrook *et al.*, 1989). Lipoprotein-deficient serum ($d > 1.215$ g/ml) was prepared from newborn calf serum as described (Goldstein *et al.*, 1983). CPP32 was partially purified from HeLa cell extracts as described (Wang *et al.*, 1995).

cDNA cloning of hamster CPP32

An aliquot (10 ng) of purified phage template DNA from a Syrian hamster liver λ ZAP cDNA library (Garcia *et al.*, 1995) was amplified by PCR with two primers: primer 1, 300 pmol of a degenerate oligonucleotide derived from the peptide sequence SIKNFVKT from hamster CPP32 (Wang *et al.*, 1995) [5'-TCIAT(T/C/A)AA(A/G)AA(T/C)TT(T/C)GA(A/G)GTIAA(A/G)AC-3']; and primer 2, 30 pmol of an oligonucleotide encoding the C-terminal peptide sequence ELYFYH from human CPP32 (Fernandes-Alnemri *et al.*, 1994) (5'-TTAGTG-ATAAAATAGAGTTC-3'). The amplified PCR product (~800 bp) was cloned into a pCR™ II cloning vector (Invitrogen) and sequenced with a T7 sequenase kit (United States Biochemical). The sequence confirmed that the PCR product encoded all three of the peptides recovered from the 20 kDa subunit of purified hamster SCA (Wang *et al.*, 1995).

To obtain a full-length cDNA, we used poly(A)⁺ RNA prepared from Syrian hamster brain to construct a size-selected, directional cDNA library with a λ ZAP-cDNA kit as described (Garcia *et al.*, 1995). A total of 0.5×10^6 plaques were transferred to duplicate filters and probed at 42°C in 5× SSPE plus 50% formamide with 2×10^6 c.p.m./ml of the cloned PCR fragment described above that had been uniformly ³²P-labeled by random hexanucleotide priming after excision from the pCR™ II vector by *Eco*RI digestion. After hybridization at 42°C for 16 h, the filters were washed twice with 2× SSC plus 0.1% SDS at room temperature for 30 min each, followed by two additional washes at 45°C for 30 min each. Two rounds of plaque purification yielded nine positive plaques. The pBluescript phagemid were excised from the Uni-Zap vector *in vivo* (Stratagene ExAssit/SOLR system) and sequenced. All nine clones shared the identical open reading frame. Clone 11-1, which encoded a 2.4 kb mRNA including a 3'-untranslated region of 1.5 kb, was further cloned into a eukaryotic expression vector.

Construction of expression vector for CPP32

cDNA clone 11-1 encoding hamster brain CPP32 was excised from the Bluescript vector with *Eco*RI and *Apa*I. The fragment corresponding to the 2.4 kb full-length insert was ligated into a pBK-CMV vector (Stratagene) that had been linearized with *Eco*RI and *Apa*I. The resulting plasmid is designated pCPP32.

In vitro translation of mRNAs for SREBP and CPP32

Human SREBP-2 cDNA was cloned into the *Eco*RI site of λ EXlox (+) vector (Hua *et al.*, 1993), and hamster CPP32 cDNA was cloned in Bluescript SK(+). Plasmids were isolated with a Qiagen Maxiprep kit and translated in the TNT SP6- (for SREBP-2) or T7- (for CPP32) coupled reticulocyte lysate system (Promega). Each coupled transcription-translation reaction contained 5 μ g of plasmid DNA in a final volume of 400 μ l in a methionine-free amino acid mixture supplemented with 750 μ Ci/ml [³⁵S]methionine (>1000 Ci/mmol) according to the manufacturer's instructions. After incubation at 30°C for 2 h, the proteins were isolated by passage through a 10 ml Sephadex G-25 gel filtration column equilibrated with Buffer A (10 mM HEPES-KOH at pH 7.5, 1.5 mM MgCl₂, 10 mM KCl, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol) supplemented with a cocktail of protease inhibitors that consisted of 0.1 mM Pefabloc, 5 μ g/ml pepstatin A, 10 μ g/ml leupeptin, 2 μ g/ml aprotinin and 25 μ g/ml ALLN. The translated proteins emerged in the exclusion volume of the column.

In vitro cleavage of SREBP-2 and CPP32

In vitro translated, ³⁵S-labeled SREBP-2 or CPP32 was prepared as described above. After passage through the G-25 Sephadex column, 5 μ l

of the flow-through fraction was incubated at 30°C for varying times with the indicated protease preparations in a final volume of 25 µl of Buffer A supplemented with the cocktail of protease inhibitors described above. The reaction contained ~10⁵ c.p.m. of [³⁵S]SREBP or [³⁵S]CPP32, which corresponded to ~0.32 or 0.65 fmol, respectively, as estimated from the specific activity of the [³⁵S]methionine. At the end of the incubation, 25 µl of 2× SDS sample buffer was added to each tube, after which each sample was boiled for 3 min and then subjected to electrophoresis on either an 8% SDS gel (for cleavage of SREBP) or a 10–20% gradient SDS gel (for CPP32). The 8% gel was dried and subjected to autoradiography. The proteins in the 10–20% gel were transferred electrophoretically to a nitrocellulose filter. The dried gels and filters were exposed to DuPont-NEF 496 film at room temperature for 16 h.

Transient transfection of 293 cells

On day 0, monolayers of human embryonic kidney 293 cells were set up at 5×10⁵ cells per 60 mm dish in Dulbecco's modified Eagle's medium (DMEM) with low glucose supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin sulfate. After incubation for 48 h at 37°C in a 5% CO₂ incubator, the cells were transfected with 1 µg of control vector (pBK-CMV) or pCPP32 using the calcium phosphate method with a MBS Transfection Kit (Stratagene) according to the manufacturer's instruction. After incubation for 3 h at 35°C in a 3% CO₂ incubator, the cells were washed once with phosphate-buffered saline (PBS), refed with fresh medium and returned to 37°C in a 5% CO₂ incubator. After 24 h, the cells were harvested with the original medium, collected by centrifugation, washed with ice-cold PBS and assayed for CPP32 activity as described in the figure legends.

Stable transfection of CHO-7 cells

pTK-HSV-BP2 is a *neo*-containing expression vector encoding human SREBP-2 in which two copies of the HSV glycoprotein epitope is inserted at the N-terminus of SREBP-2 and in which expression is driven by the HSV thymidine kinase promoter (Hua *et al.*, 1996). Site-specific mutagenesis of the SREBP-2 sequence (Asp468→Ala) in pTK-HSV-BP2 was performed as described by Kunkel *et al.* (1987) using the Muta-gene Phagamid Kit (Bio-Rad).

Stable overexpression of wild-type pTK-HSV-BP2 and mutant pTK-HSV-BP2(D468A) in CHO-7 cells (Metherall *et al.*, 1989) was achieved as follows: CHO-7 cells were plated on day 0 at a density of 5×10⁵ cells per 100 mm dish in medium A [DMEM/Ham's F-12 medium (1:1) supplemented with 5% (v/v) newborn calf lipoprotein-deficient serum, 100 U/ml penicillin and 100 µg/ml streptomycin sulfate]. On day 1, cells were transfected with 5 µg/dish of plasmid using an MBS Transfection Kit. On day 2, medium A containing 700 µg/ml of G418 was added. The medium was changed every second day until well-defined colonies were evident on days 12–14. Clones were obtained by dilutional subcloning (D468A mutant) or cloning cylinders (wild-type) in the presence of 350 µg/ml G418. The resulting stable cell lines were analyzed by immunoblotting with HSV-Tag™ antibody, and the highest expressing clones were selected for further characterization.

Apoptosis in CHO-7 cells

On day 0, CHO-7 cells were set up at 5 or 6×10⁵ cells per 100 mm dish in medium A. After incubation for 48–72 h, steroids and/or staurosporine were added as described in the figure legends. Cells were harvested, collected by centrifugation (1000 g, 10 min, 4°C) and washed with ice-cold PBS. The cell pellets were processed as described in the figure legends.

Apoptosis in U937 cells

Human monocytic leukemia U937 cells were grown in suspension culture (Obeid *et al.*, 1993) in medium B [RPMI 1640 medium with glutamax-1 (GibcoBRL) containing 25 mM HEPES (pH 7.4), 10% newborn calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin]. On day 0, replicate aliquots of cells (100 ml per aliquot) were diluted to a density of 2×10⁵ cells/ml with fresh medium B and incubated at 37°C with 10% CO₂. After incubation for 15 h, additions were made directly to the medium, and the cells were harvested at the indicated time by centrifugation (1000 g, 10 min, 4°C), washed once with PBS and processed as described in the figure legends.

DNA fragmentation assay

DNA was extracted from cell nuclei using a modification of the method of Miller *et al.* (1988). After NaCl was added to a final concentration of 1.5 M, the nuclear debris was spun down for 15 min in a

microcentrifuge at room temperature, and the DNA in the supernatant was extracted with phenol–chloroform and then precipitated with an equal volume of 100% (v/v) ethanol. The DNA precipitates were washed once with 70% ethanol and resuspended in buffer containing 10 mM Tris–HCl, pH 7.5, 1 mM sodium EDTA and 200 µg/ml DNase-free RNase A. After incubation at 37°C for 30 min, ~10 µg of DNA was loaded onto a 1.3% agarose gel in Tris-borate/EDTA. Electrophoresis was conducted at 40 V for 5 h at room temperature. The gel was stained with 2 µg/ml ethidium bromide for 10 min, destained with water for 1 h and visualized under UV light.

Immunoblot analysis

Mouse mAbs against the N-terminal domains of SREBP-1 (IgG-2A4) (Sato *et al.*, 1994) and SREBP-2 (IgG-7D4) (Yang *et al.*, 1995) were described previously in the indicated references. A mouse mAb against the C-terminal domain (amino acids 833–1141) of human SREBP-2 (IgG-1C6) was described by Hua *et al.* (1995). A polyclonal antibody against hamster CPP32 was produced by immunizing rabbits with a synthetic peptide (CSIKNFVEVKT, synthesized by Julio Medina of Tularik) corresponding to amino acids 12–20 of hamster CPP32 (Figure 1). The peptide was coupled to keyhole limpet hemocyanin using *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (Harlow and Lane, 1988). New Zealand white rabbits were immunized with 500 µg of coupled peptide in complete Freund's adjuvant. Immunoblot analysis of SREBP and CPP32 was performed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G using the Enhanced Chemiluminescence (ECL) Western Blotting Detection System Kit (Amersham) as previously described (Wang *et al.*, 1994; Yang *et al.*, 1994). SDS–PAGE was carried out with 8% gels (SREBP) or 15% gels (CPP32). Gels were calibrated with pre-stained high and low range molecular weight markers (Bio-Rad or New England Biolabs). The blots were exposed to DuPont-NEF 496 film at room temperature.

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