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Caspase-Dependent Cleavage of Carbamoyl Phosphate Synthetase II during Apoptosis

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ABSTRACT

Carbamoyl phosphate synthetase II (CPSII) is part of carbamoyl phosphate synthetase/aspartate transcarbamoylase/dihydroorotase (CAD), a multienzymatic protein required for the de novo synthesis of pyrimidine nucleotides and cell growth. Herein, we identify CAD as a substrate for caspase-3 degradation in both in vitro and in vivo models of apoptosis. Withdrawal of interleukin-3 or incubation with staurosporine (STS) or doxorubicin (Dox) resulted in proteolytic cleavage of CAD in a myeloid precursor cell line (32D) or in a cell line over-expressing CAD. The rapid decline in the CPSII activity paralleled the degradation of CAD and preceded the appearance of Annexin-V–stained apoptotic cells and DNA fragmentation. These events correlated closely with the activation of caspase-3 in these cells and were prevented by the cell-permeable caspase inhibitor *N*-benzyloxycarbonyl-Asp-Glu-Val-Asp fluoromethyl ketone. Moreover, the incubation of purified CAD with recombinant caspase-3 in vitro generated CAD fragments that were similar to those obtained in vivo. Edman sequencing revealed that two of the major caspase-3 cleavage sites occurred at the sequences EAVD ↓ G and VACD ↓ G within the catalytic (B2) and allosteric (B3) domains of CAD, thus providing a potential mechanism for the rapid inactivation of CPSII during apoptosis. Consistent with this, an enhanced loss of the intracellular pyrimidines (UTP and CTP) was observed in response to STS or DOX-induced apoptosis. Therefore, these studies show that CAD is a novel target for caspase-dependent regulation during apoptosis and suggest that the selective inactivation of pyrimidine nucleotide synthesis accompanies the process of apoptosis.

Apoptosis is a highly ordered program of cell death required for the maintenance of the cellular integrity of multicellular organisms (Hengartner, 2000). In addition to a critical role during development, apoptosis, or the lack thereof, may contribute significantly to aberrant cell growth during tumorigenesis (Compagni and Christofori, 2000). The process of apoptosis is characterized by global changes in cellular properties and includes cell shrinkage, membrane blebbing, chromatin condensation, and internucleosomal cleavage of DNA. Concomitant with these changes is the activation of a cascade of intracellular proteolytic events catalyzed by a family of cysteine proteases known as caspases (Thornberry et al., 1997). These proteases are activated in response to multiple apoptotic stimuli and are believed to be responsible for the degradation of key structural and regulatory proteins during apoptosis.

Within this family of enzymes, there are both initiator and effector caspases (Thornberry et al., 1997). Expressed as inactive zymogens, the initiator caspases (2, 8, and 10) interact with the death receptors (tumor necrosis factor and the Fas ligand) and are activated by autoproteolysis as an early event in apoptosis (Thornberry et al., 1997; Hengartner, 2000). Caspase-9 is an initiator caspase that is activated as a result of cytochrome *c* release from the mitochondria (Hengartner, 2000). Increased activity of these enzymes in turn activates the effector caspases (3, 6, and 7), which are believed to be responsible for much of the proteolysis that occurs later in apoptosis (Thornberry et al., 1997). The caspases cleave their substrates on the C-terminal side of aspartic acid residues with slight differences in substrate specificity. Caspases 3 and 7 typically recognize substrates

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ABBREVIATIONS: PARP, poly ADP-ribose polymerase; CAD, carbamoyl phosphate synthetase/aspartate transcarbamoylase/dihydroorotase; CPSII, carbamoyl phosphate synthetase; ATCase, aspartate transcarbamoylase; PRPP, phosphoribosyl pyrophosphate; DHOase, dihydroorotase; Z-DEVD-fmk, *N*-benzyloxycarbonyl-Asp-Glu-Val-Asp fluoromethyl ketone; FBS, fetal bovine serum; PALA, *N*-(phosphonacetyl)-L-aspartate; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; Ac-DEVD-pNA, *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide; Dox, doxorubicin; STS, staurosporine; IL-3, interleukin-3; PI, propidium iodide; wt, wild-type; PR-G9c-WT, PALA-resistant G9c-WT cells; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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containing a DEXD motif, whereas caspases 2, 8, and 10 recognize DXXD or slight variations of this motif (L/V/DXXD) (Thornberry et al., 1997).

A number of targets have been identified as caspase substrates, including DNA repair enzymes, structural proteins, protein kinases, and components of signal transduction or cell cycle regulatory events. Proteolysis of housekeeping or structural proteins is believed to be required for the disassembly of the cell, whereas others may be targeted to prevent replication of damaged cells (Nicholson and Thornberry, 1997). A partial list of these substrates includes poly ADP-ribose polymerase (PARP) (Lazebnik et al., 1994), retinoblastoma protein (Janicke et al., 1996), lamin A (Takahashi et al., 1996), Gas 2 (Brancolini et al., 1995), inhibitor of caspase-activated DNAase (Sakahira et al., 1998), DNA topoisomerase I (Casiano et al., 1998), p21WAF/CIP1 (Gervais et al., 1998), and multiple protein kinases (Krebs and Graves, 2000). Degradation of these proteins is believed to contribute to the cellular changes that occur during apoptosis and to accelerate or coordinate the progression of apoptosis.

Apoptosis and necrosis are considered to be conceptually and morphologically distinct forms of cell death, although there is increasing evidence that these represent only the extreme ends of a wide range of possible biochemical deaths (Nicotera et al., 1999). A hallmark of the apoptotic process is that it is ATP-dependent, and studies demonstrate that ATP is required for completion of the initial stages of apoptosis (Nicotera et al., 2000). In contrast, inadequate ATP levels may favor the development of necrosis (Eguchi et al., 1999; Nicotera et al., 1999). Late in the process of apoptosis, extensive activation of PARP leads to the depletion of β-nicotinamide adenine dinucleotide and ATP (Eguchi et al., 1999; Nicotera et al., 1999). Thus, the balance of intracellular nucleotides may play an important role in deciding the type of cell death that predominates.

Contrary to the studies of ATP, the fate of other nucleotides during apoptosis (or the enzymes that synthesize them) has not been investigated. The de novo synthesis of pyrimidine nucleotide is initiated by CAD, a 240-kDa protein that consists of three enzymatic activities: carbamoyl phosphate synthetase II (CPSII), aspartate transcarbamoylase (ATCase), and dihydroorotase (DHOase). The rate-limiting step in this pathway is catalyzed by CPSII, which is also the site of "feedback" inhibition by uridine nucleotides and activation by the allosteric ligand phosphoribosyl pyrophosphate (PRPP) (Carrey and Hardie, 1988). CPSII activity is increased in tumor cells (Reardon and Weber, 1985) and is a substrate for regulation by the mitogen-activated protein kinase pathway (Graves et al., 2000). CAD activity can also be increased by amplified CAD gene expression, and this enhanced capacity for pyrimidine biosynthesis is believed to provide the uridine nucleotides that are required for rapid cell growth (Smith et al., 1995).

During our studies on the signals that regulate CAD, we observed a rapid decline in CPSII activity during apoptosis. We noticed a selective proteolysis of CAD protein parallel to the loss of this activity that correlated with the activation of caspase-3. In this study, we identified two caspase-3 cleavage sites within the CPSII domain of CAD that could explain the loss of CPSII activity during apoptosis. Concomitant with the loss of CAD protein and activity, we found that cellular pyrimidines are significantly reduced in apoptotic cells. Thus, these studies suggest that selective inactivation of CAD and the uncoupling of the pyrimidine biosynthetic pathway may be important factors in the progression of apoptosis.

Experimental Procedures

Materials. PALA was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutic Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). ATP, UTP, PRPP, RNase A, proteinase K, Nonidet P-40, and staurosporine were purchased from Sigma (St. Louis, MO). Purified active recombinant human caspase-3 (CPP32) was obtained from Invitrogen (Carlsbad, CA). The tetrapeptide caspase substrate DEVD was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Z-DEVD-fmk and Ac-DEVD-CHO were obtained from Calbiochem (San Diego, CA). Annexin-V-Fluos Staining Kit was obtained from Roche Molecular Biochemicals (Summerville, NJ). Bac-To-Baculovirus Expression systems were obtained from Invitrogen. $NaH^{14}CO_3$ (56 mCi/mmol) was obtained from ICN (Costa Mesa, CA). 32D cells were obtained from Dr. Albert Baldwin (Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC).

Cell Lines and Cell Culture. CAD-deficient G9c cells were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium-Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, and 30 μM uridine. Stable transfectants expressing wt-CAD (Wt-G9c) were grown in Dulbecco's modified Eagle's medium-Ham's F12 medium supplemented with 10% FBS and 1% nonessential amino acids without the addition of uridine. The myeloid progenitor cell line (32D) was maintained in RPMI 1640 medium supplemented with 10% FBS, 10% medium conditioned with WEHI3B (a murine myeloid leukemia cell line), 100 μg/ml penicillin, and 100 μg/ml streptomycin.

Establishment of CAD-Overexpressing Cell Line by PALA Selection. The cDNA corresponding to hamster CAD was cloned into pCDNA 3.1His C (Invitrogen), and a stable cell line expressing wt-CAD was created by transfecting CAD-deficient G9c cells as described earlier (Graves et al., 2000). The CAD protein expressed in these cells contained an epitope tag (recognized by the anti-Xpress antibody) fusion with the N terminus of CAD. A PALA-resistant cell line that over-expressed CAD was obtained by stepwise exposure of the wt-CAD–transfected G9c cell line (Graves et al., 2000) to increasing concentrations of PALA for approximately 6 weeks. The IC_{50} value for PALA in the parental wt-CAD-transfected G9c cell line is approximately 50 μM; continuous exposure of this cell line to PALA resulted in a subline that grew in the presence of 1 mM PALA. Because of this selection, the PALA-resistant subline over-expressed the CAD protein by approximately 5- to 10-fold as determined by immunoblotting with anti-CAD antibody or anti-Xpress antibody and CAD activity assays. The PALA-resistant subline (PR-Wt-G9c) was maintained in 500 μM PALA to continue PALA resistance, and all experiments were performed after 7 to 10 days of incubation in drug-free medium.

Analysis of Intracellular UTP by HPLC. 32D (1.7×10^7) cells were treated with or without STS, harvested by centrifugation (5 min at 2000 rpm), and washed twice with ice-cold PBS. The cell pellet was suspended in 1 ml of ice-cold 10% trichloroacetic acid and briefly vortexed. The precipitated protein was removed immediately by centrifugation (2 min at 10,000 rpm), and the supernatant was then extracted four to five times with water-saturated diethyl ether until the pH was greater than 5.0. A portion (500 μl) of the extract was reduced to a volume of approximately 200 to 250 μl by SpeedVac (Thermo Savant, Holbrook, NY). HPLC analyses were performed as described previously (Pogolotti and Santi, 1982). A portion (100 μl) of the filtered sample was injected onto a SAX Partisil 5× HPLC column (Whatman, Clifton, NJ) at a flow rate of 1 ml/min. The running buffer (buffer A) was composed of 7 mM $NH_4H_2PO_4$, pH 3.8,

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and the elution buffer (buffer B) contained 250 mM $NH_4H_2PO_4$, pH 4.5, containing 500 mM KCl. After 6 min of an isocratic period with buffer A, a linear gradient of buffer B was applied for 30 min followed by an additional isocratic period of 10 min of buffer B. Nucleotide standards (ATP, GTP, UTP, CTP) were also run under the same conditions and were used to quantify the amounts of nucleotides obtained from the cell lysates.

Immunoblots. Attached cells were washed with ice-cold PBS and then collected in a lysis buffer containing 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 150 μM Na_3VO_4, 0.25 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 10 nM microcystin-LR. After centrifugation (15,000*g* for 10 min at 4°C), the protein content in the supernatant was assayed using the Bradford method (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. Equal amounts of protein were applied to an SDS-polyacrylamide gel electrophoresis (PAGE) (8% for CAD, 10% for all other separations) and transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore Corporation, Bedford, MA). The membrane was blocked with 3% gelatin in 0.1% Tween 20 in Tris-buffered saline for 1 h and then incubated with primary antibody. Primary rabbit antisera against CAD was used at a dilution of 1:5000 in blocking solution, and the membrane was then washed three times with 0.1% Tween 20 in Tris-buffered saline and incubated in an appropriate secondary antibody (1:5000 dilution of horseradish peroxidase-linked anti-rabbit immunoglobulin (Santa Cruz Biochemicals, Santa Cruz, CA). Detections were performed using an enhanced chemiluminescence system.

DNA Fragmentation Analysis by Agarose Gel Electrophoresis. Both detached and attached cells were harvested by centrifugation (1000 rpm for 5 min), and after three washes with PBS, cell pellets were resuspended at a concentration of 3×10^6 cells/0.3 ml in ice-cold lyses buffer comprising 45 mM Tris-HCl, 1 mM EDTA. pH 8.0, 0.25% Nonidet P-40 and 1 mg/ml RNase A. After incubation for 30 min at 37°C, proteinase K was added at a final concentration of 1 mg/ml and incubated for an additional 30 min at 37°C. The lysate was then spun down at 12,000 rpm for 15 min, an equal volume of phenol-chloroform was added to the supernatant and mixed well, and the mixture was centrifuged at 12,000*g* for 2 min at room temperature. The DNA in the aqueous phases was then precipitated with ethanol and redissolved in Tris/EDTA buffer. The DNA fragments were then separated by the use of 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized by exposure to ultraviolet light.

Expression and Purification of CAD in Insect SF-9 Cells. A cDNA sample encoding the hamster CAD gene was subcloned into HTb donor vector and transposed into DH10 BAC-competent cells, and the recombinant Bacmid DNA obtained from the positive clone was then transfected into SF-9 insect cells by using CELLFECTIN reagent as described in the manufacturer's instructions (Promega, Madison, WI). After several steps of amplification of the CAD-Bacmid DNA virus stock solution, the recombinant fusion protein containing a 6-histidine tag was produced in SF-9 cells. CAD was overexpressed approximately 5- to 10-fold, as determined by immunoblotting with anti-CAD antibody and CPSII activity assays. The expressed CAD protein was purified by the use of Ni-nitrilotriacetic acid resin column according to the manufacturer's instructions.

CPSII Activity Assays. Cells were washed twice with PBS at 4°C and resuspended in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 150 μM Na_3VO_4, 0.25 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 10 nM microcystin-LR. The lysate was cleared by centrifugation (10,000*g* for 10 min at 4°C), and the CPSII activity was measured as described previously (Liu et al., 1994). The reaction mixture contained 87 mM Tris HCl, pH 8.0, 87 mM KCl, 3.3 mM glutamine, 17.5 mM aspartate, 0.8 mM DTT, 6.5% dimethyl sulfoxide, 2.2% glycerol, and 25 mM $MgCl_2$. The final ATP concentration used in these assays varied between 0 and 5 mM. For the observation of allosteric regulation of CPSII by UTP and PRPP, the ATP concentration in assay was 2 mM; the concentration of $MgCl_2$ was always in excess of the nucleotide concentration by 2 mM because the enzyme is sensitive to allosteric effectors using these conditions.

Measurement of Caspase-3 Activity. Cultured cells were washed with PBS twice and then lysed in a buffer containing 50 mM HEPES, pH 7.4, 0.1% CHAPS, 1 mM DTT, and 0.1 mM EDTA. The cell lysates were centrifuged at 13,000*g* for 10 min, and the soluble fraction was assayed for caspase-3 activity with the use of Ac-DEVD-pNA, a colorimetric substrate for caspase-3, as described in the manufacturer's protocol (BIOMOL). The absorbance (A_{405}) was read, and the amount of activity was calculated as picomoles per minute per milligram of protein.

Flow Cytometric Measurement of Annexin-V Binding. Dox- and STS-exposed or IL-3–deprived 32D cells were washed twice with cold PBS and resuspended at a concentration of 1×10^6 cells/100 μl in staining solution containing binding buffer, propidium iodide (PI), and Annexin-V; fluorescence was determined according to the manufacturer's protocol (Roche). Untreated (control) cells were stained with PI or Annexin-V only and PI plus Annexin-V, respectively. After incubation at room temperature for 15 min in the dark, 400 μl of the binding buffer was added, and the cells were analyzed by flow cytometry.

Results

CPSII Activity of CAD Rapidly Inactivated during Apoptosis. Myeloid precursor 32D cells are dependent on the cytokine IL-3 for cell growth; starvation for IL-3 results in the induction of programmed cell death or apoptosis (Antoku et al., 1998). The multienzymatic protein CAD is an essential enzyme in the de novo biosynthesis of pyrimidine nucleotides and is a target for regulation by growth factors that activate the mitogen-activated protein kinase pathway (Graves et al., 2000). While studying the regulation of CAD in 32D cells, we observed a rapid decline in both the CPSII activity of CAD and its allosteric activation by PRPP after IL-3 withdrawal (Fig. 1, A and B). The loss of CPSII activity declined to approximately 50% after 12 h of IL-3 withdrawal and coincided with the increase of apoptotic DNA ladders (Fig. 1C) and Annexin-V positively stained apoptotic cells (Fig. 1D). To further investigate whether CAD was inactivated during the apoptotic process, we examined CPSII activity from cells exposed to STS, a protein kinase inhibitor with broad specificity and a potent inducer of apoptosis (Fabbro et al., 1999). Exposure of 32D cells to STS rapidly induced apoptosis in these cells as observed by the appearance of apoptotic DNA ladders (Fig. 1C) and the increase of Annexin-V positively stained cells (Fig. 1D). As shown in Fig. 1A, STS treatment (1 μM for 1.5–24 h) stimulated a rapid decrease in CPSII activity, with greater than 85% of the activity lost after 24 h. Moreover, activation by PRPP was abolished in response to STS treatment (Fig. 1B), suggesting that both the catalytic activity and the allosteric regulation of CPSII were eliminated during apoptosis.

Loss of CPSII Activity Parallels the Degradation of CAD and the Activation of Caspase-3 in 32D Cells. Next, we examined the fate of CAD protein during apoptosis. As determined by Western immunoblotting, the total amount of CAD protein (native molecular mass, 243 kDa) declined significantly after IL-3 starvation (22 h) of 32D cells (Fig. 2A). Concomitant with this decline was an increase in multiple immunoreactive bands that seemed to be proteolytic products of the CAD protein (Fig. 2A). The major fragments D

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(140 kDa) and E (100 kDa) appeared first (within 6 h of IL-3 withdrawal), whereas maximal fragment G accumulation occurred later (after 16–22 h). Incubation of 32D cells with STS (1 μM) induced a more rapid disappearance of native CAD protein than that observed after IL-3 withdrawal, a result consistent with a more rapid loss of CAD activity after STS treatment (See Figs. 1A and 2B). Comparable with IL-3 withdrawal, fragments D, E, and G accumulated after 6 to 24 h of STS treatment.

Dox is a potent anticancer drug that induces apoptosis through the inhibition of DNA polymerase and nucleic acid synthesis (De Beer et al., 2001). Incubation of 32D cells with 1 μM Dox resulted in the induction of apoptosis, the rapid inactivation of CPSII (data not shown), and the corresponding degradation of CAD protein (Fig. 2C). In response to Dox treatment, E fragments were observed early (within 2 h) whereas D, G, and other fragments accumulated more slowly. To determine whether a similar fragment was obtained from these different treatments, a 15% polyacrylamide gel was used to more accurately resolve the size of this protein. As shown in Fig. 2D, fragment G from STS-treated or IL-3–starved 32D cells comigrated on SDS-PAGE (at approximately 16 kDa).

The caspases family of proteases is responsible for catalyzing highly specific protein degradation during apoptosis (Hengartner, 2000). Caspase-3 recognizes and cleaves substrates containing DXXD or similar motifs (Thornberry et al., 1997). To investigate whether caspase-3 was involved in the inactivation of CAD (CPSII) during apoptosis, caspase-3 activity was determined by assaying the tetrapeptide Ac-DEVD-pNA hydrolyzing activity in 32D cells. Withdrawal of IL-3 from 32D cells increased caspase-3 activity approximately 73-fold after 24 h (Fig. 3A). Similarly, treatment of 32D cells with STS also increased caspase-3 activity (Fig. 3B), albeit more rapidly than that observed with IL-3 withdrawal and consistent with the more rapid induction of apoptosis and degradation of CAD under these conditions. However, to further investigate the involvement of caspase-3 in the degradation of CAD, 32D cells were incubated with the cell-permeable caspase-3 selective inhibitor Z-DEVD-fmk (100 μM). The addition of Z-DEVD-fmk before STS incubation prevented the increase of caspase-3 activity in 32D cells

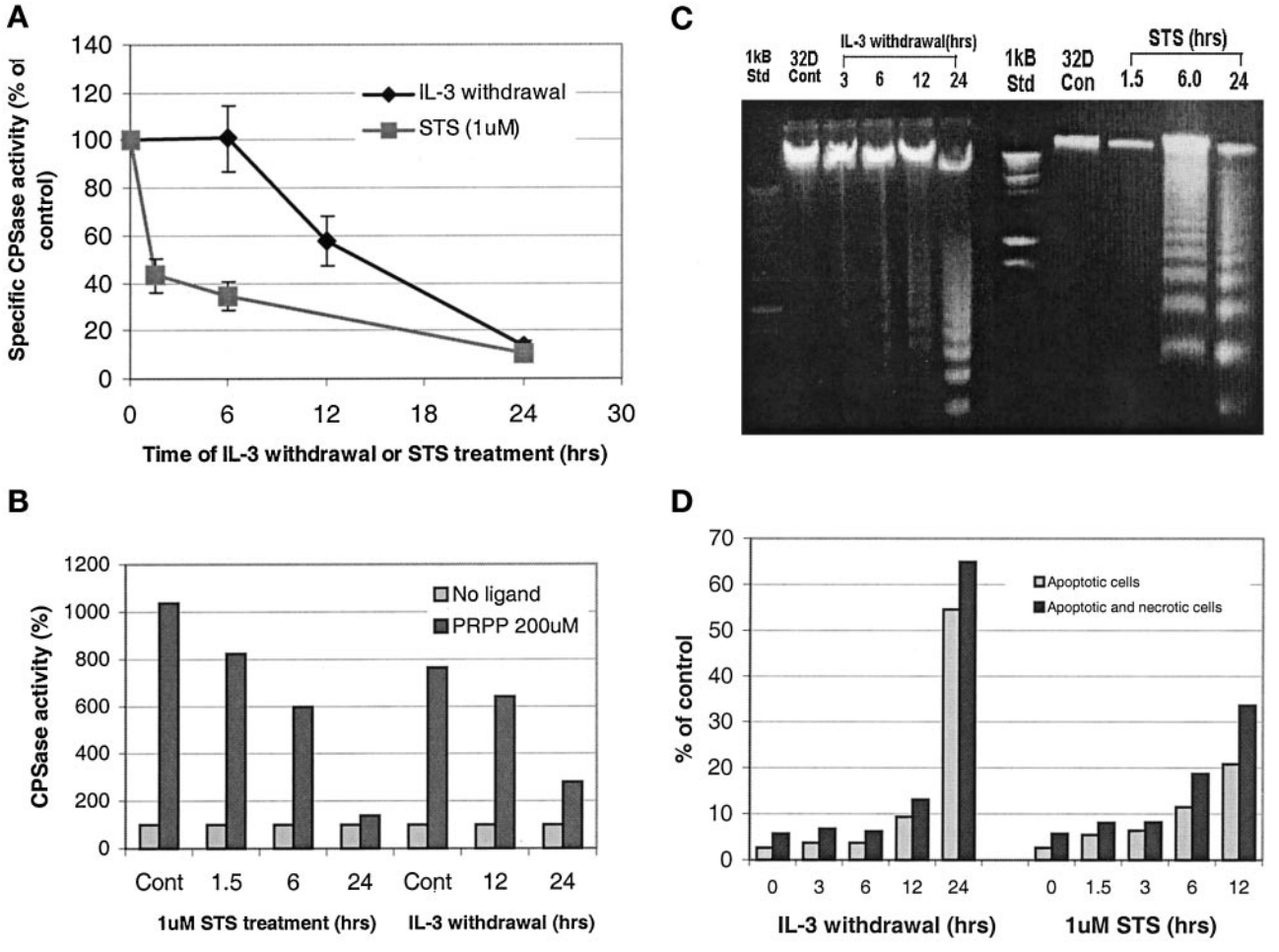

Fig. 1. Reduced activity and allosteric regulation of CPSII during apoptosis induced by IL-3 starvation or treatment of 32D cells with STS. The glutamine-dependent CPSII activity of CAD and the allosteric activation by PRPP were measured in lysates from 32D cells starved for IL-3 or treated with 1 μM STS as indicated. A, specific CPSII activity was plotted as a percentage of the control after adjustment for protein content (n = 4). B, allosteric activity was plotted as the percentage of total activity obtained in the absence of ligand. Shown is a representative experiment of two trials. C, DNA from IL-3 starved or STS-treated 32D was isolated and monitored for DNA fragmentation by electrophoresis in 1.5% agarose gels. Left gel: lane 1, 1-kB DNA marker; lane 2, control DNA; lanes 3 to 6, withdrawal of IL-3 for 3, 6, 12, and 24 h, respectively. Right gel: lane 1, 1-kB DNA marker; lane 2, control DNA; lanes 3 to 5, treatment of 32D cells with STS for 1.5, 6, and 24 h, respectively. D, the percentage of apoptotic and apoptotic-plus-necrotic cells after withdrawal of IL-3 or treatment of 32D cells with 1 μM STS at indicated times was analyzed by fluorescence-activated cell sorter analysis as discussed under *Experimental Procedures*.

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(data not shown) and inhibited the STS-induced degradation of CAD. Moreover, incubation with Z-DEVD-fmk diminished the STS-induced appearance of fragment D (Fig. 3C), further indicating that this proteolytic fragment was derived from caspase-3-dependent cleavage of CAD.

Staurosporine Stimulates the Degradation of CAD in PALA-Resistant Cells and Identification of N-Terminal Fragments of CAD. To further characterize the proteolysis of CAD in vivo, we generated a cell line that over-expressed CAD protein by the repeated exposure of cells (G9c-WT) to increasing concentrations of PALA, as described under *Experimental Procedures*. This treatment resulted in a cell line that increased CAD expression approximately 5- to 10-fold, as determined by SDS-PAGE and Western immunoblotting (data not shown). Exposure of the PALA-resistant G9c-WT cells (PR-G9c-WT) to STS (1 μM for 6–20 h) resulted in a significant increase of caspase-3 activity and apoptosis, similar to that observed in 32D cells (Fig. 4A and data not shown). The degradation of CAD was examined in the PR-G9c-WT cells by Western immunoblotting with a polyclonal antibody against full-length CAD or a monoclonal antibody (anti-Xpress; Invitrogen) against an epitope tag located at the N terminus of the CAD protein expressed in these cells. As shown in Fig. 4A, STS treatment resulted in a concentration- and time-dependent degradation of CAD and the formation of fragments (B-G) similar to those observed in 32D cells. Immunoblotting lysates from these cells with the anti-Xpress antibody reacted with only two fragments of CAD (B, 180 kDa, and G, 16 kDa) after STS treatment (Fig. 4B). Hence, these experiments identify B and G as N-terminal fragments of the CAD protein.

Proteolysis of CAD by Caspase-3 In Vitro and Identification of Caspase-3 Cleavage Sites. To identify where the caspase-dependent cleavage occurred within CAD, we incubated purified CAD protein with caspase-3 and compared the peptide fragments with those obtained in vivo after STS treatment. As shown in Fig. 5, incubation of CAD with caspase-3 in vitro resulted in a series of fragments (B-G) that mirrored those observed in vivo after induction of apoptosis (either in 32D cells or PR-G9c-WT cells) (compare Figs. 4A and 5). The generation of these fragments was almost completely blocked by incubation with the caspase-3 inhibitor Ac-DEVD-CHO (Fig. 5). After electrophoretic transfer of the CAD fragments to a polyvinylidene difluoride membrane, fragments B through G were individually subjected to direct N-terminal Edman sequencing. The results of these experiments revealed two peptide sequences, GVVSAIAISEHV and GECPPQRSILDQ corresponding to fragments D and E, respectively (Fig. 6). These findings demonstrated that caspase-3 cleavage occurred at aspartyl residues 1143 (DAVACD↓GVVSAIAISEHV) and 1371 (EAVD↓GECPPQRSILDQ) of the CAD protein.

Decrease of Intracellular UTP Level in Response to CAD Cleavage during Apoptosis Induced by STS

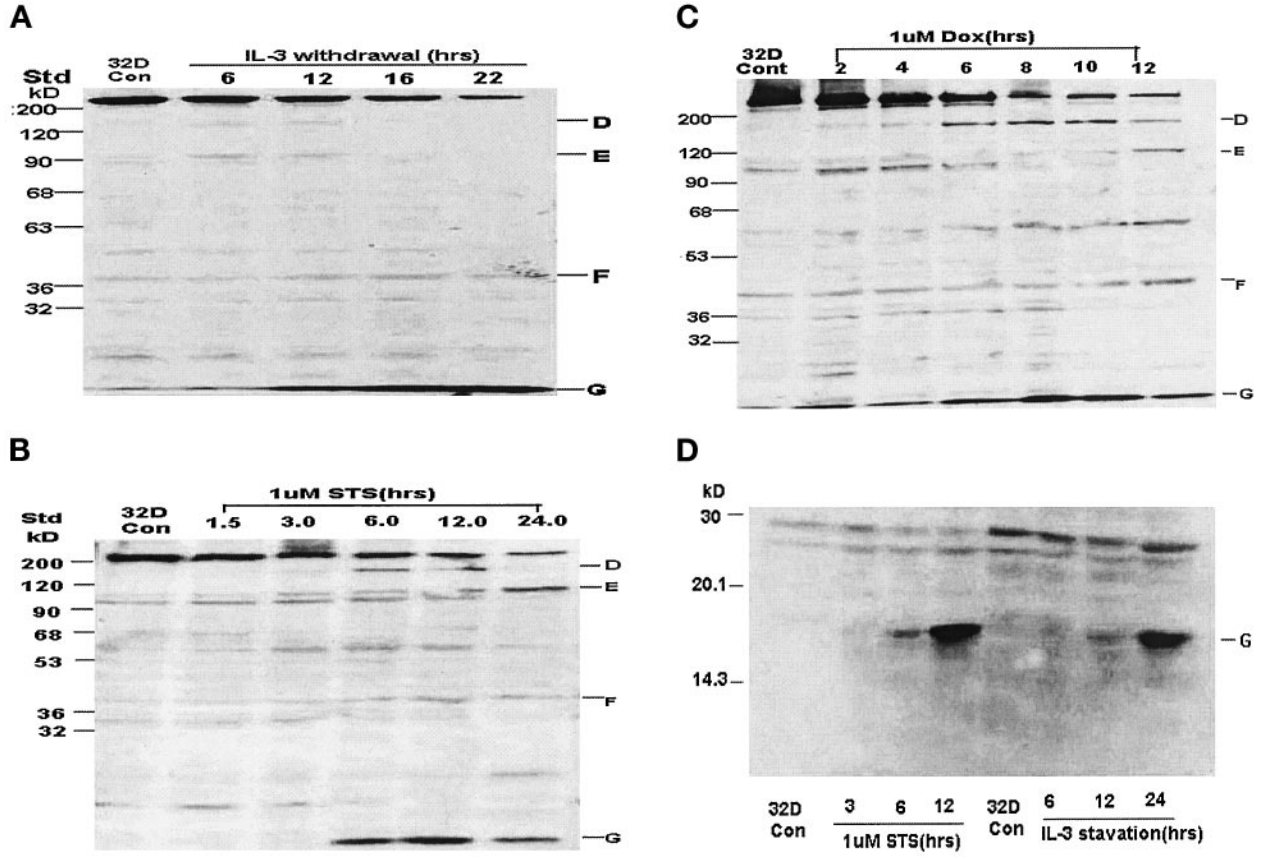

Fig. 2. Induction of CAD cleavage during apoptosis after IL-3 withdrawal or staurosporine or doxorubicin treatment of 32D cells. Lysates from 32D cells were fractionated on 8% SDS-PAGE and immunoblotted with anti-CAD antibody. A, CAD cleavage during apoptosis initiated by IL-3 deprivation. Lane 1, 32D control; lanes 2 to 5, deprivation of IL-3 for the time of IL-3 withdrawal shown. Cell lysates from 32D cells exposed to 1 μM STS for 1.5 to 24 h (B) and 1 μM Dox for 2 to 12 h (C) were fractionated on 8% SDS-PAGE and immunoblotted with anti-CAD antibody. D, cell lysates from 32D cells exposed to 1 μM STS or IL-3 starvation as indicated were fractionated on 15% SDS-PAGE and immunoblotted with anti-CAD antibody.

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Treatment and IL-3 Withdrawal. The CPSII activity of CAD is the rate-limiting step in the de novo biosynthesis of pyrimidine nucleotides in mammalian cells. Because our data suggested that CAD was rapidly inactivated and degraded during apoptosis, we examined the consequence of this event on intracellular UTP and CTP concentrations. 32D cells were treated with STS (1 μM for 1.5–6 h) and the nucleotides were extracted, separated, and quantified as described under *Experimental Procedures*. As shown in Fig. 6A, STS elicited a rapid, time-dependent decline in intracellular UTP and CTP that paralleled the loss of CPSII activity (See Fig. 1A) in these cells. After 1.5 h, the cellular UTP and CTP levels decreased to 59 and 68% of the control value, respectively, and further decreased to 50 and 63%, respectively, after 6 h of exposure to STS. Similarly, Dox treatment resulted in a significant reduction of intracellular pyrimidine levels. After 2 h, the intracellular UTP and CTP levels decreased to 52 and 42% of control, respectively, and further decreased to 27 and 23% of control, respectively, after 8 h of Dox incubation (Fig. 6B). In contrast, intracellular purines (ATP and GTP) declined at a much slower rate under these conditions, suggesting that the caspase-dependent degradation of CAD specifically reduced the cellular concentrations of the pyrimidines UTP and CTP.

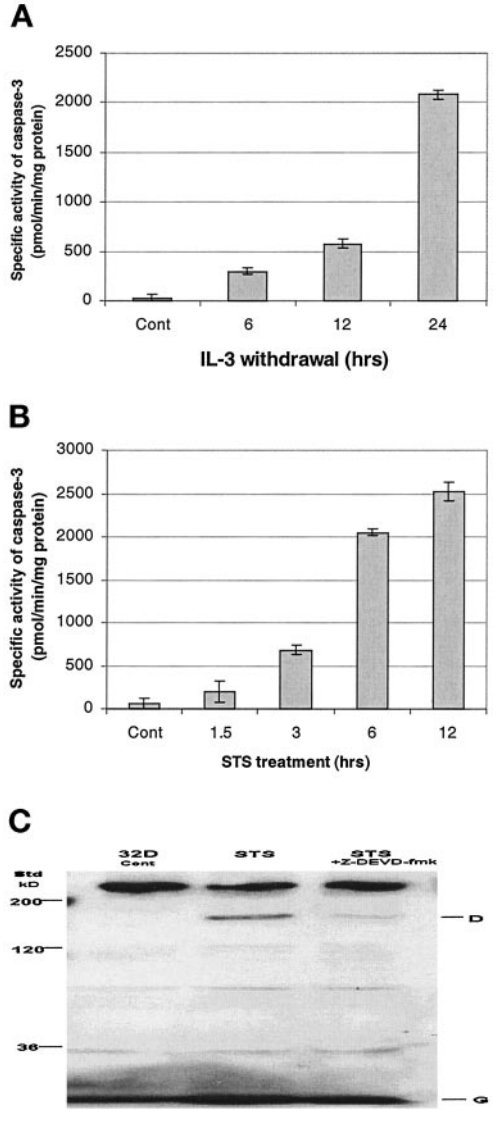

Fig. 3. Activation of caspase-3 by deprivation of IL-3 or treatment of 32D cells with STS and inhibition of CAD degradation by Z-DEVD-fmk. Cell lysates from IL-3-starved (A) or STS-treated (B) 32D cells were assayed for protease activity toward Ac-DEVD-pNA. The absorbance was measured at wavelength 415 nm and plotted as pmol/min/mg of protein. C, cell lysates from 32D cells exposed to 1 μM STS for 6 h with or without pretreatment with 100 μM Z-DEVD-fmk for 1 h were fractionated on 8% SDS-PAGE and immunoblotted with anti-CAD antibody.

Discussion

Apoptosis is a selective process of cell disposal that results from multiple cellular inputs, including exposure to cell death ligands or developmental cues, removal of trophic signals, DNA damage, or viral infection. The completion of cell death requires the highly selective degradation of proteins by caspases, and several important caspase substrates have been identified in recent years (Hengartner, 2000). The results of this study suggest that CAD, the rate-limiting pyrimidine nucleotide biosynthetic enzyme, is a novel target for caspase-mediated degradation during apoptosis. We ob-

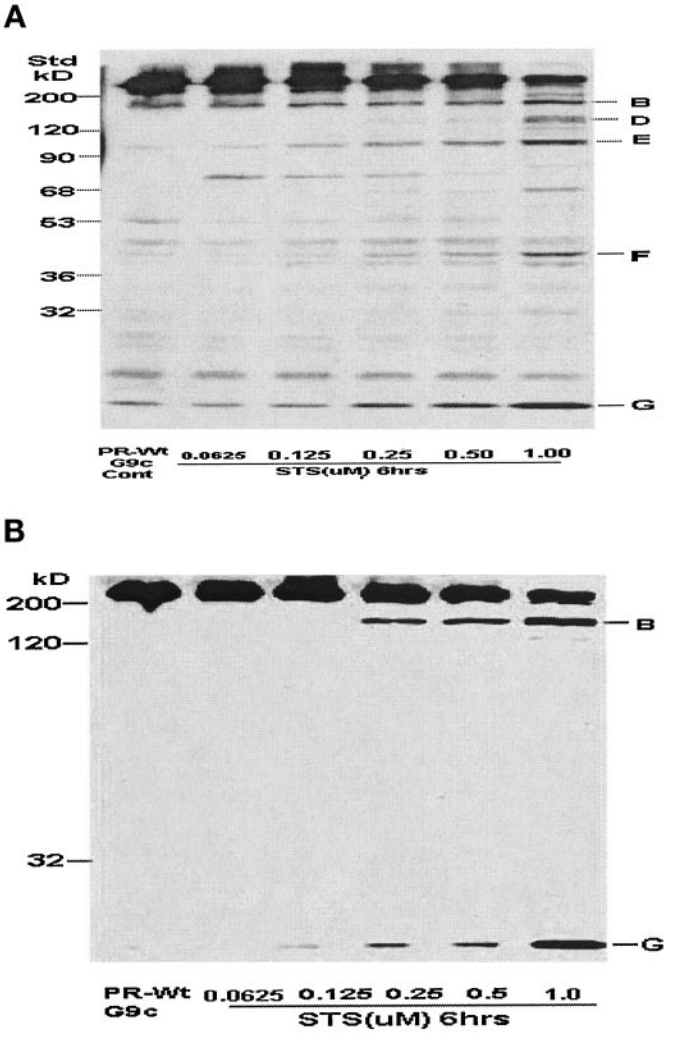

Fig. 4. STS-induced cleavage of CAD during apoptosis in PALA-resistant wt-CAD transfected G9c cells. Cells were treated in the absence or presence of various concentration of STS, and the whole cell lysates were fractionated on 8% SDS-PAGE and immunoblotted with anti-CAD antibody (A) or anti-Xpress antibody (B).

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served a parallel loss of CPSII activity and CAD protein, suggesting that proteolysis was responsible for the inactivation of this enzyme during apoptosis. The degradation of CAD occurred in response to IL-3 withdrawal, STS, or Dox treatment and correlated well with the activation of caspase-3 in cells. Both the loss of CPSII activity and subsequent decline of intracellular pyrimidines preceded the appearance of Annexin-V positively stained cells and apoptotic DNA ladders, indicating that the degradation of CAD was an early event in programmed cell death.

CAD is an extended polypeptide chain composed of three distinct enzymatic activities: CPSII, ATCase, and DHOase (Carrey, 1986) (Fig. 7A). The overall topology of CAD is believed to include multiple compact globular domains connected by exposed polypeptide regions that are sensitive to proteolytic cleavage (Carrey, 1986). Previous studies demonstrated that limited proteolysis with elastase or trypsin resulted in a rapid decrease in the glutamine-dependent CPSII activity and the generation of a 195-kDa "nicked CAD" protein and smaller fragments corresponding to the CPSII, ATCase, or DHOase domains of CAD (Mally et al., 1981; Rumsby et al., 1984; Carrey, 1986). In contrast, our studies suggest that caspase-3 catalyzes the cleavage of CAD within the globular domains of CAD. Two of the caspase-3 cleavage sites (CAD fragments D and E) were identified within the CAD sequences EAVD↓G and VACD↓G, which occur within the catalytic domain (B2) and the allosteric regulatory domain (B3) of CPSII and CAD, respectively. The observation that highly similar CAD fragments were obtained in vitro with purified caspase-3 and in vivo during apoptosis and the finding that this event was blocked by a caspase-3 selective inhibitor Ac-DEVD-CHO (Na et al., 1996) strongly support a role for caspase-3 in the degradation of CAD. However, the pattern of proteolysis induced by STS and Dox was slightly different from that induced by IL-3 withdrawal, suggesting that the activation of additional caspases may also be involved. Moreover, the time or extent of caspase activation induced by different apoptotic stimuli may also affect the degradation pattern of CAD because of the multiple caspase cleavage sites within CAD.

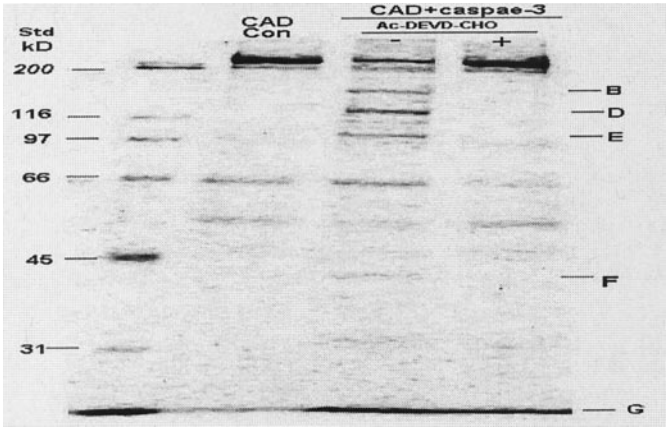

Fig. 5. Cleavage of CAD by caspase-3 and prevention of CAD cleavage by Ac-DEVD-CHO. Purified CAD was incubated with or without caspase-3 in the absence or presence of 1 μM Ac-DEVD-CHO in 100 μl of buffer A (50 mM HEPES, pH 7.4, 5 mM DTT) for 2 h, underwent electrophoresis on an 8% SDS gel, and visualized by Coomassie staining. Lane 1, marker; lane 2, purified CAD before caspase-3 treatment; lane 3, purified CAD treated with 0.25 μg of caspase-3; lane 4, purified CAD treated with 0.25 μg caspase-3 in the presence of 1 μM Ac-DEVD-CHO.

Caspases have a unique requirement for aspartic acid in the P1 position of peptide substrates, with the selectivity being partially dependent on the amino acids at position P4 and, to a lesser extent, at P2 and P3 (Talanian et al., 1997; Thornberry et al., 1997). The typical caspase-3 consensus site is believed to require an aspartate residue at the P4 position (DXXD) (Thornberry et al., 1997); however, recent studies have suggested that alternative substitutions at P4 provide acceptable caspase-3 recognition sites. For example, both topoisomerase I (Samejima et al., 1999) and scaffold attachment factor A (Kipp et al., 2000) are cleaved by caspase-3 at the noncanonical sequences EEED↓G and SALD↓, respectively. Instead, a more important determinant of specificity may be the amino acid immediately following the required aspartic acid, and studies suggest a strong preference for small amino acids such as glycine, alanine, or serine at this position (Stennicke et al., 2000). Our findings that caspase-3 cleaved CAD protein at the sequences EAVD↓G and VACD↓G are consistent with this hypothesis.

Inspection of CAD sequences for other potential caspase-3 cleavage sites, combined with our observations that fragments B (190 kDa) and G (16 kDa) were derived from the N terminus of CAD, allows additional predictions to be made.

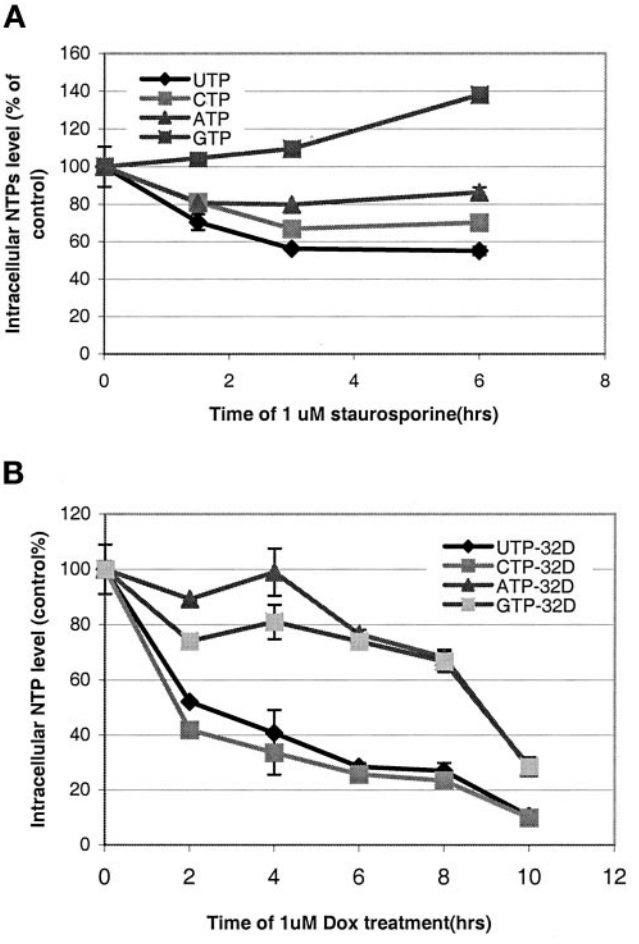

Fig. 6. Intracellular NTP level during apoptosis induced by staurosporine and Dox treatment in 32D cells. Cells (2.0×10^7) were treated with 1 μM STS (A) and Dox (B) for the time as indicated. Intracellular UTP, CTP, GTP, and ATP were extracted and measured as described under *Experimental Procedures* and are shown as the percentage of nucleotide remaining after exposure to STS and Dox. Samples were corrected for cell numbers. The experiments represent duplicate samples.

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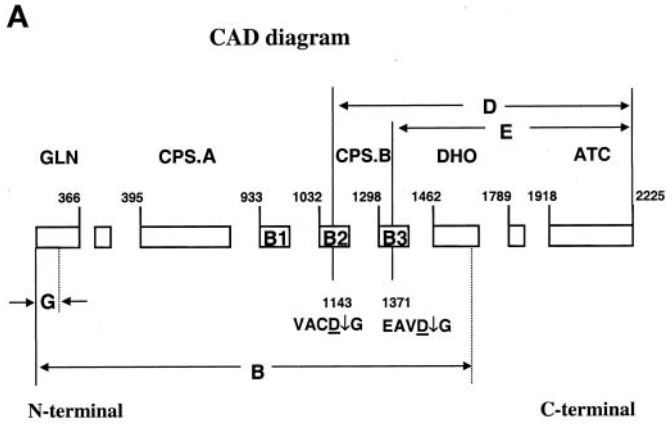
In view of the calculated molecular mass, hypothetical cleavage at amino acid residue 152 (PFVD) or 1753 (VEVD) would result in 16-kDa and 190-kDa N-terminal polypeptides corresponding to the proteolytic fragments G and B, respectively. Cleavage at these sites would occur within the glutaminase domain of CPSII and the DHOase domain of CAD protein and would be predicted to further inactivate the catalytic activity of CAD. Inspection of DNA sequences from multiple species suggests that both of these potential caspase cleavage sites are conserved (Fig. 8); however, the amino acids that follow the aspartate residues 152 and 1753 are not optimal (Pro and Leu, respectively). Thus, whether or not these sites are actual caspase cleavage sites will have to be confirmed by additional Edman sequencing experiments.

Taken together, cleavage of CAD at the two identified caspase-3 cleavage sites or additional sites could explain the rapid loss of both CAD activity and allosteric regulation observed during apoptosis. Because the CPSII activity of CAD is both the rate-limiting step and the locus of allosteric control in this pathway, inactivation of this enzyme would be expected to influence the pyrimidine nucleotide synthesis. Consistent with this hypothesis, our studies indicate that the levels of intracellular UTP and CTP declined rapidly after exposure to STS or Dox treatment. In contrast, a slower decline in the purines (ATP and GTP) was observed during early apoptosis (induced by Dox), supporting previous findings that ATP is maintained to provide for the completion of the apoptotic process (Eguchi et al., 1999). However, in STS-induced apoptosis of 32D cells, cellular ATP was found to decline at a similar rate compared with pyrimidine nucleotide pools. STS, a broad protein kinase inhibitor, was shown in a recent study to result in early disruption of the mitochondrial function. After 1-h exposure of Jurkat T lymphocyte cells to 2.5 μM STS, the mitochondrial membrane potential and ATP/ADP ratio steadily declined in parallel with mitochondrial swelling and outer membrane rupture, indicating that STS treatment results in an irreversible disruption of the mitochondrial function (Scarlett et al., 2000). Thus, these and other studies suggest that STS may have additional effects on the stability of ATP pools in cells (Ha and Snyder, 1999).



B

Sites	Gene	Accession no.	Sequence
1	Golden hamster	A23443	VACD↓GVVSAIAISEHV
	Human	NP-004332	VAS D↓GVVAAIAISEHV
	Spiny dogfish	A57541	VACD↓GVVIAVAISEHV
2	Golden hamster		EAVD↓GECPPQRS ILDQ
	Human		EAVD↓GECPPQRS ILEQ
	Spiny dogfish		EDTD↓NGCPLKERHRNI

Fig. 7. A, diagram of CAD showing the identified and proposed caspase-3 cleavage sites. Purified CAD was incubated with 0.25 μg of caspase-3 for 2 h at 37°C, subjected to an 8.0% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. CAD fragments stained with amido black were excised and subjected to sequential Edman degradation. Sequences of fragment D and E are GVVSAIAISEHO and GECPPQRSILDQ, respectively. B, conserved caspase-3 cleavage sites within CAD sequences: The aspartate residue (underscored) was conserved within sequences of the two identified caspase-3 cleavage sites D and E (the GenBank accession numbers corresponding to the gene products are listed).

Inactivation of pyrimidine synthesis during cell death would be expected to impact numerous biosynthetic processes required for cell viability and growth. Pyrimidine nucleotides provide essential precursors for the synthesis of nucleic acids, RNA, DNA, sugar nucleotides (e.g., UDP-glucose) (Martin et al., 2000), and the energy-rich phospholipid pathway intermediates CDP-diacylglycerol, CDP-ethanolamine, and CDP-choline (Kent and Carman, 1999). Thus, changes in CTP levels would be predicted to influence phosphatidylcholine synthesis, and studies have shown that this process is inactivated during apoptosis (Anthony et al., 1999) and that the inhibition of phosphatidylcholine synthesis induces apoptosis (Allan, 2000). Thus, the uncoupling of CTP synthesis and the corresponding decline in phosphatidylcholine biosynthesis may be important factors in the loss of cell viability.

There are other reasons why the inactivation of CAD may contribute to apoptosis. In normal cells, the balance between deoxyribonucleotide pools is under tight regulation (Slingerland et al., 1995). Four deoxynucleotide triphosphates must be synthesized in appropriate amounts to maintain normal function for cell viability and proliferation. Coordinate regulation of purine and pyrimidine synthesis is essential for maintenance of the multiple biosynthetic processes required for cell viability and growth (Traut, 1994). An imbalance in the synthesis of ribonucleotides can lead to an imbalance between deoxyribonucleotide pools (Traut, 1994). Thus, it is reasonable to predict that stimuli inducing an imbalance between purine and pyrimidine nucleotides could serve as signals to initiate or contribute to the process of apoptosis.

In summary, our studies demonstrate that CAD is a selective target for inactivation and degradation during apoptosis. Given the important role of CAD in providing precursors for cell growth and DNA repair, it may be that CAD, like PARP and DNA-dependent protein kinase, is specifically targeted for inactivation during apoptosis. The respective contributions of this enzyme to growth or cell survival warrant further investigation.

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