The Cell Adhesion Molecule CEACAM1-L Is a Substrate of Caspase-3-mediated Cleavage in Apoptotic Mouse Intestinal Cells*

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The CEACAM1 cell adhesion molecule is a member of the carcinoembryonic antigen family. In the mouse, four distinct isoforms are generated by alternative splicing. These encode either two or four immunoglobulin domains linked through a transmembrane domain to a cytoplasmic domain that encompasses either a short 10-amino acid tail or a longer one of 73 amino acids. Inclusion of exon 7, well conserved in evolution, generates the long cytoplasmic domain. A potential caspase recognition site in mouse, rat, and human CEACAM1-L also becomes available within the peptide encoded by exon 7. We used CEACAM1-L-transfected mouse colon carcinoma CT26 cells treated with three different apoptotic agents to study its fate during cell death. We found that CEACAM1-L is cleaved resulting in rapid degradation of most of its 8-kDa cytoplasmic domain. Caspase-mediated cleavage was demonstrated using purified recombinant caspases. The long cytoplasmic domain was cleaved specifically by caspase-3 in vitro but not by caspase-7 or -8. Moreover cleavage of CEACAM1-L in apoptotic cells was blocked by addition of a selective caspase-3 inhibitor to the cultures. Using point and deletion mutants, the conserved DQRD motif in the membrane-proximal cytoplasmic domain was identified as a caspase cleavage site. We also show that once CEACAM1-L is caspase-cleaved it becomes a stronger adhesion molecule than both the shorter and the longer expressing isoforms.

Apoptosis is an essential process for the development of an organism and its homeostasis. A very specific pattern of cell death occurs during embryogenesis leading to viability of the newborn (1). Survival also depends on proper control of apoptosis throughout growth and in aging processes. Indeed tumor development ensues from deregulation of apoptosis due to blockage of cell death. Conversely increased cell death occurs in some neurodegenerative diseases (2).

Hallmarks of apoptosis are cell shrinkage, cell membrane blebbing, formation of apoptotic bodies (3), cleavage of genomic DNA (4), mitochondrial cytochrome c release (5, 6), and activation of caspasas (7). The latter are specific cysteine proteases that either autoactivate themselves or activate other family members in a well defined cascade depending on the type of the apoptotic stimuli (8). Once caspasas are activated, they cleave many different substrates at a specific consensus site always after an aspartic acid residue (Asp). The particular caspase specificity for a substrate resides in the 3 amino acids positioned carboxyl-terminally to the aspartic acid cleavage site (9). Caspase substrates include a number of essential proteins involved in many different types of functions. For example, Gas2 (10), gelsolin (11), spectrin (12, 13), focal adhesion kinase (14, 15), β-catenin (16, 17), plakoglobin (17), and actin (18, 19) are molecules involved in cell-extracellular matrix or cell-cell adhesion that are cleaved by caspasas during apoptosis. In addition, apoptosis activates metalloproteases that are involved in the cleavage and shedding of extracellular domains of the cell adhesion molecules PECAM-1 (20) and vascular endothelial cadherin (17).

CEACAM1 (previously Bgp) (21) is a transmembrane protein of the carcinoembryonic antigen family. It consists of either two or four extracellular Ig-like domains, one membrane-spanning region, and a cytoplasmic tail that can either be short (10 amino acids, CEACAM1-S) or long (73 amino acids, CEACAM1-L). The alternative splicing events responsible for generating the short or the long isoforms are illustrated in Fig. 1 (22). All exons encoding the cytoplasmic tails are found in exons 6–9. To generate the short isoform, exon 6 is alternatively linked to exon 8 that encompasses one TGA stop codon. Exon 7 is only included in the long tail isoform. Introduction of exon 7-encoded sequence in the cytoplasmic domain shifts the open reading frame and makes use of a different TGA stop codon located in exon 9. Exon 7 encodes the most conserved region of the long cytoplasmic tail of the human, mouse, and rat sequences (Fig. 1, gray box). This suggests that important functions rely on the inclusion of this region and might also explain some of the differences between functions of the short versus the long isoform.

CEACAM1 functions as an intercellular homophilic adhesion molecule, binding through its amino-terminal domain (23–26). The cytoplasmic domain influences the adhesion properties of CEACAM1 as the short isoform triggers more efficient adhe-

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The abbreviations used are: PECAM-1, platelet endothelial cell adhesion molecule-1; CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1; CEACAM1-L, long CEACAM1 isoform; CEACAM1-S, short CEACAM1 isoform; Ab, antibody; PARP, poly-(ADP-ribose) polymerase; CHAPS, 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.
Fig. 1. Alternative splicing events in the cytoplasmic tail coding region of the Ceacam1 gene and the resulting amino acid sequence. Exon 7 (boxed) is included only in the long isoform. It encodes the region exhibiting the highest amino acid sequence homology among species. The caspase consensus cleavage site is underlined. Dots indicate amino acid identity to that of the human sequence. Numbering of the amino acids is indicated above the human sequence. TQA (S), stop codon for the short cytoplasmic domain. TQA (L), stop codon for the long cytoplasmic domain. N, A1, B1, and A2 refer to the Ig domains (21). L, leader sequence; TM, transmembrane domain; Cyt, cytoplasmic domain; 5', 3'UT, 5' and 3' untranslated regions, respectively.

EXPERIMENTAL PROCEDURES

Cell Culture—The CT51 mouse colon carcinoma cell line was generously provided by Dr. Michael G. Brattain (Medical College of Ohio, Toledo, OH). Wild-type CT51 and CEACAM1-transduced cells were maintained in α-minimum Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen), 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C in 5% CO2 humidified air. CEACAM1 wild-type or mutant cDNAs were inserted into the pLXSN vector, and viruses were produced as described previously (32). Retroviral mediated infections of CT51 cells with CEACAM1-containing viruses produced by 293 packaging cells and establishment of stably transfected cell populations and clones have been described previously (32). Transfectants were grown in the presence of 750 μg/ml G418 (Invitrogen). NIH 3T3 cells expressing CEACAM1-S, CEACAM1-L, or the mutant CEACAM1-L461 were also generated using retroviral mediated infections; cell surface expression of the proteins was confirmed by fluorescent assays as described previously (32).

Site-directed Mutagenesis—The CEACAM1-L cDNA cloned in the polylinker of the Bluescript SK+ vector (22) was used as a template for PCR. The overlap PCR technique used (34) first involved the generation of two PCR fragments per mutation. The oligo KM4 (5'-AAGGACGGTGAAGCGAGCAGAGCACAAACCC-3') refers to the Ig domains (21). L, leader sequence; TM, transmembrane domain; Cyt, cytoplasmic domain; 5', 3'UT, 5' and 3' untranslated regions, respectively.

In this work, we demonstrate that, upon biochemical induction of apoptosis in mouse CT51 intestinal cells, the cytoplasmic domain of CEACAM1-L is cleaved. As demonstrated using a number of point and deletion mutants, the conserved membrane-proximal DQRD motif, encoded within exon 7, constitutes a caspase-3 cleavage site. This cleavage generates a CEACAM1 protein with a truncated version of the cytoplasmic domain resembling CEACAM1-S but with stronger adhesion properties than both intact isoforms.
Induction of Apoptosis—Apoptosis was induced in CT51 cells by the addition for determined periods of time of either 500 ng/ml actinomycin D, 5 μg/ml camptothecin, or 1 μM staurosporine (Sigma) to tissue culture medium. 8 × 10^6 cells/well of a 6-well dish were plated 1 day prior to addition of the apoptotic agents. At the end of the incubation time, attached cells were mechanically scraped off in the culture medium and counted, and 5 × 10^6 cells were aliquoted as samples for PARP detection. The remaining cell suspension was used for CEACAM1 detection. Both aliquots were centrifuged for 6 min in a microcentrifuge to allow cells and apoptotic bodies to pellet. The pellets were kept at −80°C until all samples were lysed. The PARP samples were lysed in 50 μl of 1× Laemmli sample buffer. The CEACAM1 samples (corresponding to 10^6 cells) were lysed in 80 μl of lysis buffer containing 50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40 detergent, and 10 μg/ml leupeptin, aprotinin, and phenylmethylsulfonyl fluoride as protease inhibitors. Lysis proceeded for 10 min on ice, and lysates were cleared by a 10-min centrifugation. CEACAM1 samples were subjected to deglycosylation with 250 units of peptide N-glycosidase F (New England Biolabs) for 90 min at 37°C according to the manufacturer’s instructions. Reactions were terminated by addition of 3× Laemmli sample buffer. Inhibition of spectrin and CEACAM1-L cleavage was monitored by supplementing the culture medium with 0–64 μM M-826 at the same time as the apoptotic agents for the 24-h treatment.

Antibodies and Immunoblotting—All samples were boiled for 5 min before running SDS-PAGE in precasted polyacrylamide gels (Invitrogen). Proteins were transferred to nitrocellulose membranes. Unspecific sites on the membranes were blocked with 5% electrophoresis grade nonfat milk (Bio-Rad) in 0.1% Tris-buffered saline/Tween 20 from 1 to 18 h. Primary and secondary antibodies were incubated for 1 h in a 1× milk solution. Pierce SuperSignal West Fermo Maximum Sensitivity Substrate (Pierce) was used for detection. An α-PARP monoclonal antibody (from clone C-2-10) used for immunoblotting was purchased from Oncogene Research Products. CEACAM1 was detected using the rabbit polyclonal Ab 2456 raised against the CEACAM1 extracellular domains or the polyclonal Ab 3698 raised against the CEACAM1-L cytoplasmic domain (36). The monoclonal α-spectrin Ab was produced from Chemicon. α-Anti-rabbit antibodies conjugated to horseradish peroxidase (Amersham Biosciences) were used as secondary antibodies.

In Vitro Cleavage Assay—[35S]Methionine-labeled CEACAM1 and PARP were prepared with the coupled transcription/translation assay Tnt kit (Promega). CEACAM1-L and PARP cDNAs cloned into BlueScript SK+ vector were transcribed using the T3 or T7 RNA polymerase, respectively. Transcription reactions, using 0.5 μg of DNA in a 25-μl final reaction volume, were incubated for 60 min at 30°C and then stored at −80°C. Labeled proteins (2.5 μl) were used in cleavage assays together with purified recombinant human caspases. The caspases were prepared as described in Ref. 37 and resuspended in a final volume of 20 μl of ICE III buffer (50 mM HEPES-KOH, pH 7.0, 2 mM EDTA, 0.1% CHAPS detergent, 10% sucrose, and 5 mM dithiothreitol added just prior to use). Cleavage reactions were incubated at 37°C for 1 h and were terminated by the addition of 5 μl of 3× Laemmli sample buffer. Samples were then boiled for 5 min and applied to 10–20% gradient precasted polyacrylamide gels. Gels were fixed for 20 min in 10% acetic acid and 40% methanol and soaked in Enlightning (PerkinElmer Life Sciences) for 10 min. Following exposure to film for 18 h (Biomax, Eastman Kodak Co.), the percentage of cleaved CEACAM1-L was quantified by densitometric scanning (BioRad). The K_m/K_in value was calculated from the relationship k_cat/K_m = e^[−k_cat/k_in] × t where S is the concentration of substrate remaining at time t, S_in is the initial substrate concentration, k_cat is the catalytic rate constant, and k_in is the inhibition constant.

Cell Aggregation Assays—Cell aggregation assays were performed essentially as described in Izzo et al. (25). In brief, single cell suspensions of either wild-type or CEACAM1-transfected NIH 3T3 cells were established. Triplicate samples of 3 × 10^5 cells of each transfectant were incubated in 3 ml of a-minimum Eagle’s medium containing 0.8% fetal bovine serum and DNase I (10 μg/ml) for 3 h at 37°C with constant stirring at 100 rpm. Samples were evaluated by hemocytometer counting for single cells and aggregates at 30–60-min intervals. Standard deviations on the average of five independent experiments were calculated.

RESULTS

Validation of Procedures—CEACAM1 is a highly glycosylated transmembrane protein endogenously expressed in different epithelia. When examined on SDS-polyacrylamide gels, this protein appears as a large smear with different molecular weights due to its variable glycosylation. When expressed in mouse CT51 colon cells, the CEACAM1-L and CEACAM1-S proteins appear as smears with respective molecular masses of ~120 and 112 kDa as shown in Fig. 2, lanes 1 and 2. The 8-kDa difference between the two isoforms is due to the long cytoplasmic peptide. However, the CEACAM1-S and -L are difficult to distinguish when expressed in the same sample even when extensive separations on SDS-polyacrylamide gels are performed (Fig. 2, lane 3). To detect putative apoptotic cleavage within the long cytoplasmic tail, we subjected total cell lysates to enzymatic deglycosylation using peptide N-glycosidase F prior to electrophoresis as described under “ Experimental Procedures.” The deglycosylated CEACAM1-L protein migrates as a sharper band at ~58 kDa (Fig. 2, lane 4), whereas deglycosylated CEACAM1-S was found at 50–52 kDa (Fig. 2, lane 5). A mixture of the two deglycosylated proteins can therefore be separated adequately (Fig. 2, lane 6).

CEACAM1-L Is Cleaved during Apoptosis—Three different apoptotic agents were added to the culture medium to induce apoptosis in a CT51 colon carcinoma cell line stably expressing CEACAM1-L (32). In a time-dependent manner, actinomycin D, camptothecin, or staurosporine treatments resulted in the appearance of a CEACAM1-L cleavage product that, after deglycosylation, migrated as a 52-kDa polypeptide (Fig. 3). The cleavage of PARP, a well characterized early caspase-3 substrate (38), was also monitored as a marker for apoptosis induction. Each of the three agents led to 40–50% cleavage of PARP after 12 h and complete cleavage after 16 h (Fig. 3) as determined by the appearance of an 85-kDa PARP fragment. Compared with results obtained with PARP, the kinetics of CEACAM1-L cleavage were different depending on the apoptotic agent used. Cleaved CEACAM1-L could be detected as early as 4 h after staurosporine treatment (Fig. 3, fifth panel) but could only be detected between 12 and 16 h after actinomycin D and camptothecin treatments (Fig. 3, first and third panels). We also monitored the appearance of the 6–8-kDa fragment released after cleavage following camptothecin apoptotic induction with the polyclonal antibody 836 specific for the cytoplasmic domain of CEACAM1-L (36). Ab 836 detects the intact glycosylated full-length protein (Fig. 4) or the isolated cytoplasmic peptide (data not shown), but it did not detect the corresponding 6–8-kDa cleavage fragment in cytosolic (Fig. 4) or nuclear extracts (not shown), raising the possibility that this fragment was rapidly degraded.
PARP cleavage was detected using an anti-PARP monoclonal antibody. It was recognized by the caspase(s). Mutations were thus introduced that the DQRD membrane-proximal motif might indeed be a caspase cleavage site. This DQRD peptide extension might affect overall conformation or interactions of the shorter cytoplasmic domain. As this peptide extension might affect overall conformation or interactions of the shorter cytoplasmic domain, we questioned how the cleaved adhesion molecule CEACAM1-L would behave in *in vitro* aggregation assays. The cleaved CEACAM1-L fragment is degraded. CT51 cells stably expressing CEACAM1-L were treated with 500 ng/ml actinomycin D (Act), 5 μg/ml camptothecin (Cmp), or 1 μg/ml staurosporine (Str) for the indicated periods of time. Equivalent amounts of total cell lysates were separated on SDS-polyacrylamide gels and transferred to membranes, and blotted with Ab 2456, a polyclonal Ab that detects the extracellular domains. CEACAM1-L was detected with Ab 2456, a polyclonal Ab that detects the extracellular domains.

CT51 cells stably expressing CEACAM1-L were treated with 500 ng/ml actinomycin D (Act), 5 μg/ml camptothecin (Cmp), or 1 μg/ml staurosporine (Str) for the indicated periods of time. Equivalent amounts of total cell lysates were separated on SDS-polyacrylamide gels after deglycosylation, and CEACAM1-L was detected with Ab 2456, a polyclonal Ab that detects the extracellular domains. PARP cleavage was detected using an anti-PARP monoclonal antibody.

CT51 cells was very low despite three optimized transfection experiments, and these mutants could therefore not be studied.

While the three apoptotic agents induced wild-type CEACAM1-L cleavage after 48 h, the D457A,D460A mutant was not cleaved (Fig. 5). Similar results were obtained with three different cell clones. The DQRD motif therefore represents the principal cleavage site within the CEACAM1-L protein during apoptosis.

Caspase-3 Cleaves CEACAM1-L—To identify whether a caspase recognized the DQRD motif, we subjected *in vitro* synthesized [35S]Met-labeled CEACAM1-L proteins to *in vitro* cleavage assays using purified recombinant caspase-3, -7, and -8. Caspase-3 was efficient in cleaving the CEACAM1-L cytoplasmic domain (Fig. 6A, first panel), but caspase-7 (Fig. 6A, second panel) and caspase-8 were not (not shown). Physiological concentrations of purified caspase-3 (8–31 nm) were sufficient to initiate the cleavage. The $k_{cat}/K_m$ value for CEACAM1-L cleavage by caspase-3 was $2.4 \times 10^3$ m$^{-1}$s$^{-1}$. When CEACAM1-L D457A; D460A; D457A,D460A; and ΔDQRDL (full-length protein with only these amino acids deleted) mutant proteins were subjected to similar assays with a 250 nM concentration of purified caspase-3, none were cleaved (Fig. 6B). These experiments demonstrate that Asp$^{457}$ and Asp$^{460}$ are both essential for CEACAM1-L caspase-3-mediated cleavage at the DQRD motif.

A Selective Caspase-3 Inhibitor Abrogates CEACAM1-L Cleavage—To test whether CEACAM1-L caspase-3 cleavage was relevant in cells, we performed the CT51 cells apoptotic induction again with the addition of M-826, a caspase-3-selective inhibitor (39). Fig. 7A shows that CEACAM1-L cleavage following camptothecin treatment is inhibited by M-826 in a dose-dependent manner. As a control, spectrin caspase-3-specific 120-kDa cleavage product was also shown to disappear following M-826 treatment in the same cell extracts. In similar experiments, actinomycin- and staurosporine-induced CEACAM1-L cleavage was inhibited by M-826 after 24 h of treatment (Fig. 7B).

Cleaved CEACAM1-L Has Altered Adhesion Properties—The cleaved CEACAM1-L generates a truncated version of the protein that is very similar to CEACAM1-S except for the inclusion of a DQRD extension. As this peptide extension might affect overall conformation or interactions of the shorter cytoplasmic domain, we questioned how the cleaved adhesion molecule CEACAM1-L would behave in *in vitro* aggregation assays.

Previous studies have shown that CEACAM1-L is less efficient at promoting cell aggregation *in vitro* than CEACAM1-S (25, 26). NIH 3T3 cells stably expressing CEACAM1-S, CEACAM1-L, or the mutant CEACAM1-LΔ461 mimicking the caspase-cleaved CEACAM1-L were prepared as single cell suspensions and monitored for cell aggregation over time. As a negative control, the aggregation properties of NIH 3T3 cells were performed with the anti-CEACAM1-specific antibody (Ab 2456). Actinomycin D, Cmp, camptothecin, Str, staurosporine.

$^{457}$DQRD$^{460}$ Is a CEACAM1-L Cleavage Site—As the apoptotic cleavage product of CEACAM1-L migrated as a 52-kDa band (similar to that of the shorter isoform), this suggested that the DQRD membrane-proximal motif might indeed be recognized by the caspase(s). Mutations were thus introduced into the potential caspase cleavage motif by site-directed mutagenesis. The Asp at positions 457 and 460 was mutated to Ala individually or together. Retroviral mediated infections were performed to generate stably expressing CT51 cell populations. High expressing cells were immunoselected with a CEACAM1 polyclonal antibody, and cell surface expression of CEACAM1 was confirmed by fluorescence-activated cell sorter analysis. The double mutant D457A,D460A was expressed at a high level, and clones were derived by limiting dilution of the cell population. These clones exhibited a 4–9-fold increase in expression relative to background (data not shown). However, expression of the single point mutants D457A or D460A in CT51 cells was very low despite three optimized transfection experiments, and these mutants could therefore not be studied.

$^{a}$ D. W. Nicholson, personal communication.
stably expressing a neomycin cassette were also monitored. As reported before, Fig. 8 shows that CEACAM1-S is a more effective adhesion molecule than CEACAM1-L. Interestingly the CEACAM1-L Δ461 mutant was more efficient in promoting cell aggregation than both known CEACAM1 isoforms as less cells remained as a single cell suspension at the end of the aggregation period. This suggests that exposure of the DQRD motif following apoptotic cleavage is critical for the CEACAM1 adhesion function.

**DISCUSSION**

CEACAM1-L is a highly glycosylated protein expressed in a number of tissues. It is particularly abundant in the intestinal and colonic epithelium. In these tissues, CEACAM1 is found preferentially at the apical plasma membrane of columnar cells lining the luminal surface (40). It is most abundant in the upper portion of the colonic crypts and is completely absent from the bottom of the crypts (40). In this report (40), immunoelectron microscopy has also confirmed expression of CEACAM1 in the glycocalyx and, in particular, in the fuzzy coat of normal colonic epithelium where it is associated with microfilaments linked to the microvillus plasma membrane. This region of the colon is generally associated with apoptosis. We have shown that the cell adhesion molecule CEACAM1-L is cleaved under apoptotic conditions in CT51 mouse colon carcinoma cells. We identified the DQRD motif present only within the long cytoplasmic domain of CEACAM1 as the principal site cleaved in apoptotic cells. This cleavage site lies within the most conserved region of the CEACAM1-L tail. In *in vitro* cleavage assays, we have also shown that caspase-3, but not caspase-7 or caspase-8, mediated CEACAM1-L cleavage at physiological concentrations. The $k_{cat}/K_m$ value of $2.4 \times 10^3$ M$^{-1}$s$^{-1}$ for CEACAM1-L cleavage by caspase-3 is low compared with that of other substrates such as PARP ($15.6 \times 10^5$ M$^{-1}$s$^{-1}$). Although we cannot exclude the possibility that CEACAM1-L is cleaved at the DQRD site by another M-826-sensitive protease in apoptotic cells, we favor the hypothesis that the *in vitro* cleavage assay might underestimate the cleavage kinetics. CEACAM1-L is a transmembrane protein with only 73 of its 521 amino acids exposed in the cytoplasm in cells. When *in vitro* synthesized and not expressed at the cell membrane, CEACAM1-L conformation might possibly be altered, and the caspase-3 cleavage site within the carboxyl-terminal tail might be masked. To address this issue, we used canine microsomal membranes in the *in vitro* transcription/translation assays to allow proper folding of the protein. Unfortunately adding the membranes resulted in inhibition of CEACAM1-L synthesis (data not shown).

CEACAM1-L can be classified as a late apoptotic substrate
Phosphorylation site mutants will have to be conducted to elucidate this hypothesis.

Caspase-3-mediated CEACAM1-L Cleavage

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