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Identification of a Novel Immunosubversion Mechanism Mediated by a Virologue of the B-Lymphocyte Receptor TACI▽

Jason R. Grant,† Alexander R. Moise,‡ and Wilfred A. Jefferies*

The Michael Smith Laboratories, the Biomedical Research Centre, and Departments of Microbiology and Immunology, Medical Genetics, and Zoology, 2222 Health Sciences Mall, University of British Columbia, Vancouver, BC V6T 1Z3, Canada

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TACI (transmembrane activator and calcium modulator and cyclophilin ligand [CAML] interactor) is a part of a novel network of ligands and receptors involved in B-cell survival and isotype switching. The TACI protein mediates its effects through CAML, an endoplasmic reticulum (ER)-localized protein that controls Ca^{2+} efflux. The adenovirus E3-6.7K protein prevents inflammatory responses and also confers resistance from a variety of apoptotic stimuli and maintains ER Ca^{2+} homeostasis; however, the mechanism of action is unknown. Here, we provide evidence that E3-6.7K shares sequence homology with TACI and inhibits apoptosis and ER Ca^{2+} efflux through an interaction with CAML, a Ca^{2+}-modulating protein. We demonstrate a direct interaction between E3-6.7K and CAML and reveal that the two proteins colocalize in an ER-like compartment. Furthermore, the interaction between the two proteins is localized to the N-terminal domain of CAML and to a 22-amino-acid region near the C terminus of E3-6.7K termed the CAML-binding domain (CBD). Mutational analysis of the CBD showed that an interaction with CAML is required for E3-6.7K to inhibit thapsigargin-induced apoptosis and ER Ca^{2+} efflux. E3-6.7K appears to be the first virologue of TACI to be identified. It targets CAML in a novel immunosubversive mechanism to alter ER Ca^{2+} homeostasis, which consequently inhibits inflammation and protects infected cells from apoptosis.

TACI (transmembrane activator and calcium modulator and cyclophilin ligand [CAML] interactor), a member of the tumor necrosis factor (TNF) family, is a receptor involved in B-cell survival and isotype switching pathways. The extracellular ligands of TACI are BAFF (B-cell activating factor belonging to the TNF family) and APRIL (a proliferation-inducing ligand) (62). Both proteins share the ability to bind to TACI and BCMA (B-cell maturation antigen) (42, 49). In addition, BAFF interacts specifically with a third receptor termed BAFF-R (48). Although the role of APRIL is less defined, BAFF plays a very important role in B-cell survival and isotype switching (1, 7). In addition, BAFF has been implicated in the development of autoimmune disorders. Overexpression of BAFF leads to autoimmunity in mice (28, 33), and patients with systemic lupus erythematosus have elevated levels of BAFF (64). While BAFF-R and BCMA are required for B-cell survival, TACI has been shown to be a negative regulator (45). Antibodies to TACI lead to the activation of NF-AT, NF-κB, and AP1. The TACI protein mediates its effects through CAML (58), an endoplasmic reticulum (ER)-localized protein that controls Ca^{2+} efflux (4).

Although it had been implicated in numerous Ca^{2+} signal transduction pathways, the exact function of CAML is unknown. It was initially identified as an important mediator of the Ca^{2+} signal transduction pathway in T cells. Acting downstream of the T-cell receptor, CAML causes an influx of Ca^{2+} leading to the activation of NF-AT (4). CAML has three putative transmembrane domains at the C terminus, the last two of which are necessary and sufficient for mediating the depletion of Ca^{2+} stores (24). In addition, CAML has been implicated in transducing the signal from angiotensin II to NF-AT through an interaction with ATRAP (angiotensin II type I receptor-associated protein) (20). CAML has also been reported to interact with epidermal growth factor receptor (EGFR) (55) and the protein tyrosine kinase $p56^{Lck}$ (54).

Adenovirus is an important etiological agent which causes acute respiratory and gastrointestinal infections worldwide. The virus often establishes a persistent infection with no outward signs of disease (16). Lymphocytes have long been suggested to harbor adenovirus in a latent form (56), with recent evidence pointing towards human mucosal T cells (17). In order to remain persistent, adenovirus must have mechanisms to suppress the host antiviral response that would otherwise act to eliminate the virus. The early transcription region (E3) of species C adenoviruses consists of a cassette of genes involved in the modulation of the host immune response. E3-19K downregulates major histocompatibility complex class I, while other E3 proteins such as 6.7K, 14.7K, and the complex formed by 10.4K and 14.5K, also known as receptor internalization and degradation (RID) protein α (RIDα) and RIDβ, respectively, inhibit death receptor-induced apoptosis (19, 34, 38, 50, 52).

The smallest of the E3 proteins, 6.7K is a 61-amino-acid glycoprotein that exists in three diverse membrane topologies: the type III orientation (N luminal/C cytoplasmic, termed ^{Ntm}E3-6.7K), the opposite type II orientation (C luminal/N cytoplasmic, termed ^{Ctm}E3-6.7K), and the fully translocated form (N and C termini both luminal, termed ^{NC}E3-6.7K) (37).

* Corresponding author. Mailing address: The Biomedical Research Centre, 2222 Health Sciences Mall, University of British Columbia, Vancouver, BC V6T 1Z3, Canada. Phone: (604) 822-6961. Fax: (604) 822-6780. E-mail: wilf@brc.ubc.ca.

† Present address: Department of Agriculture, Food and Nutritional Science, University of Alberta, Edmonton, Alberta T6G 2P5, Canada.

‡ Present address: Department of Ophthalmology, University of Washington, Seattle, WA 98195-6485.

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E3-6.7K appears to have two separate yet not necessarily exclusive roles in inhibiting apoptosis. The first demonstrated function for E3-6.7K was that in conjunction with the RID complex, E3-6.7K is able to down-regulate the TNF-related apoptosis-inducing ligand (TRAIL) receptors. Although localized primarily in the ER (61), a small fraction of E3-6.7K reaches the plasma membrane, where it can interact with RIDβ (2). E3-6.7K was shown to be required for RID-mediated down-regulation of TRAIL-R2 (2, 32). With regard to TRAIL-R1, the requirement for E3-6.7K is less clear. One group showed that E3-6.7K is necessary for the optimal down-regulation of TRAIL-R1 (2), whereas another group showed that the down-regulation of the receptor is independent of E3-6.7K (52). The RID complex also down-regulates Fas (50), TNF-R1 (15), and EGFR (51); however, E3-6.7K was not shown to be required for any of these effects.

The second role of E3-6.7K involves the inhibition of apoptosis independent of other virus proteins. It protects transfected cells against death receptor-mediated apoptosis induced through Fas, TNF receptor, or TRAIL receptors. Cells expressing E3-6.7K also had reduced levels of apoptosis after treatment with thapsigargin (38), a compound that induces apoptosis by mimicking a sustained Ca^{2+} flux. An examination of ER Ca^{2+} flux showed that the presence of E3-6.7K resulted in a reduction of thapsigargin-induced Ca^{2+} release, thereby suggesting a role in events that regulate Ca^{2+} homeostasis.

The mechanism by which E3-6.7K regulates Ca^{2+} homeostasis remains to be elucidated. However, it joins a very small family of other viral proteins that modulate cellular Ca^{2+}. In fact, only two other viral proteins are known to inhibit apoptosis by altering cellular Ca^{2+}: the coxsackievirus 2B protein and the Kaposi's sarcoma-associated herpesvirus K7 protein. The 2B protein is a small (97- to 99-amino-acid) transmembrane protein localized to the ER and Golgi membranes, where it appears to form pores as homomultimers (57). These pores reduce mobilizable Ca^{2+} stores available to induce apoptosis (6). The K7 protein is a small (126-amino-acid) transmembrane protein localized to the mitochondria and to a lesser extent to the ER (59). K7 alters the kinetics and amplitude of cellular Ca^{2+} fluxes and inhibits apoptosis induced by thapsigargin through an interaction with CAML (14).

The small size of E3-6.7K and its lack of sequence homology with 2B or other channel proteins suggest that it is unlikely to form pores on its own. A more likely hypothesis is that E3-6.7K interacts with a cellular protein involved in Ca^{2+} homeostasis.

MATERIALS AND METHODS

Cell lines and culture conditions. The human T-cell leukemia line Jurkat, clone E6-1, and the human cervical carcinoma cell line HeLa were obtained from American Type Culture Collection (Manassas, VA). Jurkat cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 20 mM HEPES. HeLa cells were maintained in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 20 mM HEPES.

Plasmid constructs. Full-length E3-6.7K, truncations of E3-6.7K, and full-length human TACI DNA were cloned into pGBKT7 (Clontech, Mountain View, CA), and DNAs for full-length human CAML or the N-terminal region of CAML (NT-CAML) were cloned into pGADT7 (Clontech) by PCR using forward primers containing an NdeI site and reverse primers containing a BamHI site. For E3-6.7K and its truncations, the template DNA consisted of the EcoRI D fragment of the E3 region of adenovirus type 2 (Ad2) (a kind gift from W. S. M. Wold); for TACI, the template DNA was IMAGE clone ID 5213128 (Incyte Genomics, Wilmington, DE), which contains full-length human TACI cDNA; and for CAML and NT-CAML, the template DNA was IMAGE clone ID 3884754 (Incyte Genomics), which contains the full-length human CAML cDNA. E3-6.7K DNA was also cloned into the green fluorescent protein (GFP) mammalian expression vector pIRES-hrGFP-1a (Stratagene) and in frame with yellow fluorescent protein (YFP) at the 3′ end in pEFYP-C1 (Clontech) to generate piGFP/6.7K and pYFP-6.7K, respectively. The cysteine mutations of E3-6.7K in pGBKT7 and pIRES-hrGFP-1a were introduced by PCR-based mutagenesis (5). CAML and NT-CAML DNA were also cloned into pcDNA 3.1 (Invitrogen) in frame with an N-terminal hemagglutinin (HA) tag to produce pcDNA-HA-CAML and pcDNA-HA-NT-CAML, respectively.

Yeast two-hybrid assay. The yeast two-hybrid assay was performed using the *Saccharomyces cerevisiae* AH109 strain (Clontech), which contains the GAL4-inducible reporter genes *His* and *Ade2* (26). The cells were transformed with appropriate pGBKT7 bait and pGADT7 prey constructs using the polyethylene glycol/lithium acetate method (18) and grown on minimal synthetic dropout (SD) medium in the absence of Leu and Thr (SD-LT). As the vectors pGBKT7 and pGADT7 contain *TRP1* and *LEU2* genes, respectively, growth on SD-LT ensures that both vectors are present. Interactions between bait and prey proteins were tested by plating colonies on SD medium lacking Leu, Thr, Ade, and His (SD-LTAH). Growth on SD-LTAH indicated an interaction between the two proteins. Transformed yeast cells were grown for 72 h at 30°C on selective medium.

Quantification of yeast two-hybrid interactions was measured by yeast growth curve analysis from growth in selective medium, as previously described (10). Briefly, individual colonies were used to inoculate liquid SD-LT medium and were shaken at 200 rpm at 30°C overnight. The overnight cultures were diluted 1,000-fold in SD-LTAH. Cells were also diluted in SD-LT to confirmed viability. The SD-LTAH cultures were incubated at 30°C with continuous shaking at 200 rpm, and the optical density was measured at 595 nm in an Spectronic BioMate 3 spectrophotometer (Thermo Electron Corporation, Waltham, MA) every 24 h for 4 days. Differential optical density values were calculated for each day, and the highest value was divided by the day the measurement was taken to give a growth rate for each culture.

In vitro transcription and translation. Radiolabeled E3-6.7K and CAML were prepared using the TnT T7 quick coupled transcription/translation systems (Promega, Madison, WI) according to the manufacturer's protocol by use of pGBKT7-E3-6.7K and pGADT7-CAML, respectively, as templates. Each reaction was carried out in the presence of 20 μCi of Redivue L-[^{35}S]methionine (Amersham Biosciences, Piscataway, NJ) and 0.6 μl of canine pancreatic microsomal membranes (Promega) for every 40-μl reaction mixture. The pGBKT7 and pGADT7 vectors contain a T7 RNA polymerase promoter and either a c-Myc or an HA epitope tag, respectively. The tag is incorporated at the N terminus of the protein.

Immunoprecipitation and SDS-PAGE analysis. The mixture from the in vitro transcription/translation reaction was diluted in 10 volumes of TNE buffer (50 mM Tris [pH 7.5], 150 mM NaCl, and 10 mM EDTA) and centrifuged for 3 min at 12,000 × *g*. The microsomal pellet was washed once with TNE and then solubilized in lysis buffer (50 mM Tris [pH 7.5], 300 mM NaCl, 0.5% Triton X-100) supplemented with Complete Mini (Roche, Laval, QC), a protease inhibitor cocktail. Samples were precleared for 1 h with Protein A beads (Clontech) and then immunoprecipitated from the supernatant with either a c-Myc monoclonal antibody (mAb) or an HA polyclonal antibody (Clontech) for 1 h at room temperature. The immune complexes were purified with Protein A beads followed by five washes with TNE containing 1% Tween 20. Bead slurries were boiled in sodium dodecyl sulfate (SDS) sample buffer and run on a 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel. After electrophoresis, the gel was fixed and dried onto Whatman 3MM paper before being exposed to a phosphorimaging screen and evaluated using a phosphorimager SI and ImageQuant software (Amersham Biosciences).

Immunofluorescence staining. HeLa cells were transiently transfected with the indicated plasmids by use of FuGENE 6 (Roche). Transfected cells were fixed with 2% paraformaldehyde for 20 min and permeabilized with 0.1% saponin in 2% bovine serum albumin (BSA) in phosphate-buffered saline for 15 min. Cells were then blocked with 2% BSA for 1 h and reacted with 500 ng/ml 3F10 rat anti-HA high-affinity primary antibody (Roche) in 2% BSA for 30 min. After incubation, the cells were washed five times with 2% BSA, incubated with 1:500 diluted Alexa Fluor 568 goat anti-rat secondary antibody (Molecular Probes, Eugene, OR) in 2% BSA for 30 min at room temperature in the dark, and washed five times with 2% BSA. Cells were treated with SlowFade antifade (Molecular Probes) and analyzed by confocal microscopy using a Bio-Rad Radiance 2000 on a Nikon Eclipse TE300 with MaiTia sapphire laser and using Lasersharp software (Bio-Rad, Hercules, CA).

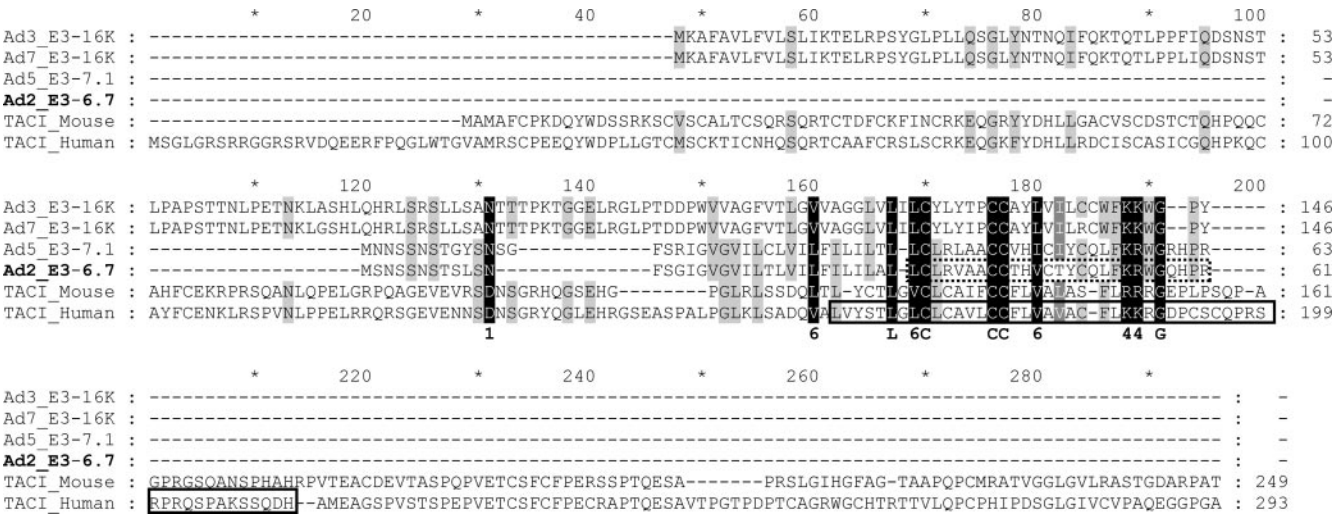

FIG. 1. Sequence comparison of E3-6.7K, E3-16K, and TACI. E3-6.7K from Ad2 and Ad5 (species C) and E3-16K from Ad3 and Ad7 (species B) were aligned with human and mouse TACI by use of CLUSTAL_X (47) and formatted with GeneDoc. Shaded regions show sequence similarity. The consensus sequence is below the alignment (key: 6 = ILV, 4 = KR, 1 = ND). The boxed region indicates the CBD of human TACI. The dotted boxed region indicates CT-E3-6.7K.

Annexin V–Alexa-647 apoptosis assays. Jurkat cells (1×10^7) were transiently transfected with 20 μg of the appropriate plasmid by electroporation with a Bio-Rad Gene Pulser Xcell at 250 V and 950 μF. At 24 h after electroporation, apoptosis was induced in 1.2×10^6 cells with 8 μM thapsigargin (Sigma) for 24 h. Apoptosis was assayed by measuring externalization of phosphatidylserine with annexin V conjugated to Alexa Fluor 647 (Molecular Probes) according the manufacturer's protocol using propidium iodide (PI) as a dead-cell counterstain. Fluorescence-activated cell sorting (FACS) was performed with a FACSCalibur instrument (Becton Dickinson) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Ratiometric intracellular [Ca^{2+}] determination. Jurkat cells (1×10^7) were transiently transfected with 20 μg of the appropriate plasmid by electroporation with a Bio-Rad Gene Pulser Xcell at 250 V and 950 μF. Intracellular Ca^{2+} levels were measured using the ratiometric Ca^{2+} indicator Indo-1 acetoxymethyl ester dye (Molecular Probes) according to the manufacturer's recommendations. In brief, 24 h after electroporation, cells were washed once with Opti-MEM (Invitrogen) and then loaded at 1×10^7 cells/ml with 2 μM Indo-1 for 1 h at 37°C in Opti-MEM. Cells were then washed 2 times with Opti-MEM, resuspended at 1×10^7 cells/ml in Opti-MEM, and kept on ice until analyzed. For each analysis, 100 μl of cell suspension (1×10^6 cells) was added to 1.9 ml of Opti-MEM prewarmed to 37°C. Indo-1-loaded cells were then examined with a FACSVantage SE flow cytometer (BD Bioscience) equipped with a UV laser and appropriate filters for the 405- and 485-nm wavelengths. After the establishment of a stable baseline for the first 2 min, the cells were simulated with 10 nM thapsigargin and monitored for another 6 min. The change in intracellular Ca^{2+} levels was determined through the ratio of emission signals of Indo-1 at 405 nm and 485 nm, representing the ratio of Ca^{2+}-bound to Ca^{2+}-free Indo-1, respectively. The kinetic analysis was performed using FlowJo software.

Statistical analysis. Statistical significance for the quantitative yeast growth curve analysis was determined by the analysis of variance test using GraphPad Prism software (GraphPad Software, San Diego, CA). For all tests, P values of <0.01 were considered to indicate statistical significance. All error bars shown represent standard deviation.

To compare FACS histograms for the analysis of apoptotic cell populations, a probability binning algorithm was used (multisample comparison in FlowJo). This algorithm determines the probability that two or more FACS data distributions are different (43). When the probability binning metric, $T(\chi)$, is >4, the populations differ by more than 4 standard deviations, giving the probability that the two populations are different with a P value of <0.01 (99% confidence).

RESULTS

Determination of E3-6.7K as a viral homologue to TACI. We have shown that E3-6.7K, in the absence of other viral proteins, is able to protect transfected cells from death receptor-induced apoptosis; however, the mechanism of action was unknown. We hypothesized that E3-6.7K may be a viral homologue of a human protein. A BLAST search of E3-6.7K against the human nonredundant protein database revealed no matches, so we expanded our search for distantly related proteins by use of a pattern-hit-initiated BLAST search. Species B adenoviruses encode a 16-kDa homologue of the E3-6.7K protein, termed E3-16K (21). A consensus was obtained from aligning E3-6.7K from Ad2 and Ad5 with its homologue E3-16K from Ad3 and Ad7. We performed a pattern-hit-initiated BLAST search using Ad2 E3-6.7K as the query and the consensus as the pattern against the human nonredundant protein database and got one hit, which was for TACI. TACI, through an interaction with CAML, controls ER Ca^{2+} efflux and the activation of NF-AT (58). E3-6.7K and TACI share sequence similarity within the well-conserved carboxyl terminus of E3-6.7K (Fig. 1).

The CAML-binding domain (CBD) of the 293-amino-acid TACI has not been determined; however, von Bülow and Bram (58) in their initial characterization of the protein showed that residues 162 to 293 are able to bind to CAML. Another group, while demonstrating a TRAF binding domain (BD) for TACI, determined that residues 1 to 212 of TACI are able to bind to CAML (63). Taken together, these findings indicate that the CBD of TACI is localized between residues 162 and 212 (Fig. 1). Interestingly, this domain of TACI overlaps with the region of TACI that shares sequence similarity with E3-6.7K, suggesting that E3-6.7K may also bind to CAML.

E3-6.7K binds to CAML. To investigate whether E3-6.7K binds to CAML, we used a yeast two-hybrid approach. The coding DNA for a bait protein was cloned in frame with the GAL4 DNA-BD of pGBKT7, while the DNA for the prey protein was cloned in frame with the GAL4 DNA activation domain of pGADT7. The yeast strain AH109 was transformed

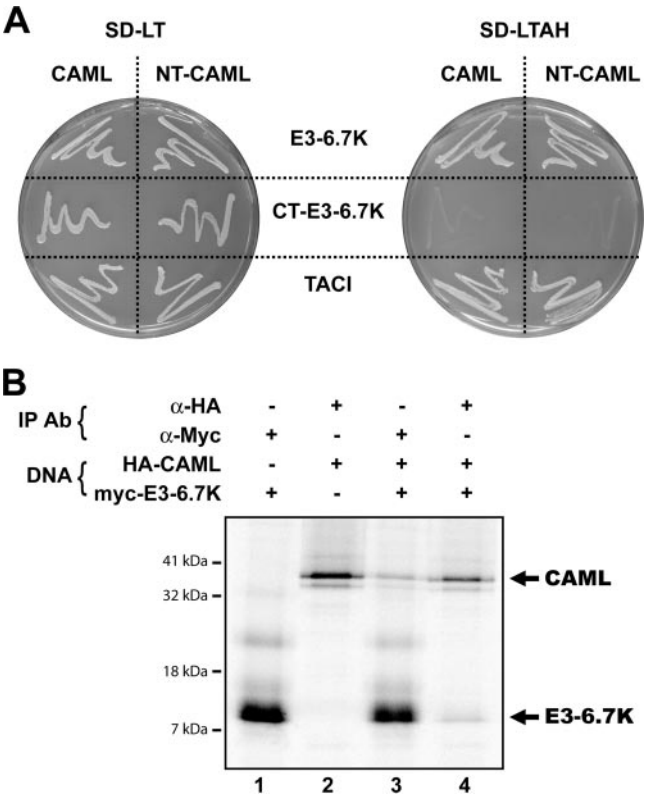

FIG. 2. E3-6.7K interacts with CAML. (A) Yeast two-hybrid assay. AH109 cells were transformed with E3-6.7K, CT-E3-6.7K, or TACI DNA in pGBKT7 and CAML or NT-CAML DNA in pGADT7 and grown on SD-LT to select for the presence of plasmids. Yeast cells were replated on SD-LTAH to select for protein interaction. Growth on SD-LTAH indicates an interaction between the two proteins. (B) Immunoprecipitation (IP) of E3-6.7K and CAML. The indicated DNA was transcribed and translated in vitro in the presence of microsomes and L-[^{35}S]methionine. The newly synthesized proteins were immunoprecipitated with the indicated antibody followed by SDS-PAGE and exposure to a phosphorimager screen. The positions of protein molecular mass markers are shown on the left. OD_{595}, optical density at 595 nm; wt, wild type; α, Ab.

with bait and prey plasmids, and positive interactions were identified based on the activation of the *ADE2* and *HIS3* reporter genes as visualized by growth of the transformants on SD-LTAH selection plates. TACI was originally discovered from a yeast two-hybrid screen using CAML as bait (58). For the current study, TACI was used as a positive control and bound to CAML in our system (Fig. 2A). NT-CAML, consisting of residues 1 to 201, is the TACI-interacting domain (58); therefore, it may also bind to E3-6.7K. We show using the yeast two-hybrid system that E3-6.7 K is able to bind to CAML and NT-CAML (Fig. 2A). The alignment in Fig. 1 shows that the C terminus of E3-6.7K (residues 35 to 61) contains the highest sequence similarity to TACI. This region, termed CT-E3-6.7K, did not interact with CAML or NT-CAML, indicating that additional residues were required for an interaction with CAML (Fig. 2A). BD alone showed no growth with CAML or NT-CAML, and the activation domain alone showed no growth with E3-6.7K, CT-E3-6.7K, or TACI (data not shown).

Unfortunately, no antibodies to E3-6.7K were available; therefore, pull-down assays of E3-6.7K and CAML from virus-infected cells were not possible. Instead, we tested the binding of CAML to E3-6.7K in vitro by generating HA-tagged CAML and c-Myc-tagged E3-6.7K individually or together by in vitro transcription/translation in the presence of L-[^{35}S]methionine and microsomes. The translated products were immunoprecipitated with either anti-c-Myc or anti-HA antibodies. The immune complexes were then purified with Protein A-Sepharose, separated by SDS-PAGE, and examined following exposure to a phosphorimager screen. When made individually, c-Myc-E3-6.7K and HA-CAML are immunoprecipitated with anti-Myc and anti-HA antibodies, respectively (Fig. 2B, lanes 1 and 2). When c-Myc-E3-6.7K and HA-CAML are made together, both proteins are immunoprecipitated independent of the antibody used, indicating that they form a complex (Fig. 2B, lanes 3 and 4). Additional specificity controls were conducted using c-Myc-tagged p53 and HA-tagged simian virus 40 large T antigen provided by the kit supplier. These controls demonstrated that the in vitro system faithfully translated the specific mRNAs and provided specificity controls to ensure that the proteins cross-precipitated using HA-CAML and c-Myc-E3-6.7K were specific and unique (data not shown). This confirms the earlier yeast two-hybrid assay results, verifying that the E3-6.7K and CAML proteins interact.

E3-6.7K colocalizes with CAML. Although a small subset of E3-6.7K reaches the plasma membrane of cells and associates with the adenovirus RID complex (2, 32), the majority is intracellular and localized to the ER membrane (61). CAML is not found on the cell surface but is also a resident of the ER (4). To determine if CAML and E3-6.7K colocalize, HeLa cells were transfected with pYFP or pYFP-6.7K, alone or in combination with pcDNA/HA-CAML. After 72 h, the cells were stained for HA-tagged CAML. First, it was demonstrated that the anti-FLAG mAb did not nonspecifically stain HeLa cells transfected with pYFP or pYFP-6.7K alone (Fig. 3A and B). YFP alone was distributed diffusely throughout the cell (Fig. 3A and C), as has been demonstrated before for YFP in HeLa cells (41). In comparison, YFP-tagged E3-6.7K had a punctate, perinuclear distribution reminiscent of ER localization (Fig. 3B and D). As expected, cells transfected with pcDNA/HA-CAML showed similar perinuclear ER staining with the anti-HA mAb (Fig. 3C and D). In fact, when the fluorescent signals of HA-CAML and YFP-6.7K were superimposed, a white image was uniformly obtained (Fig. 3D). Superposition of the YFP and HA-CAML fluorescent signals resulted in little or no white (Fig. 3C). These results provide evidence that CAML and E3-6.7K localize predominantly in the same subcellular compartments.

To further confirm an interaction between E3-6.7K and CAML, HeLa cells transfected with pYFP or pYFP-6.7K and pcDNA/HA-NT-CAML were analyzed by confocal microscopy. NT-CAML is missing the transmembrane domains found in the C terminus, so this domain is cytoplasmic (25). NT-CAML does contain the TACI BD, and according to the yeast two-hybrid results (Fig. 2A) it binds to E3-6.7K as well. Cells transfected with pcDNA/HA-NT-CAML displayed cytoplasmic staining with the anti-HA mAb (Fig. 3E and F), and although YFP was cytoplasmic as well, there was no white when the images were overlaid (Fig. 3E). In contrast, superposition of the YFP-6.7K and HA-NT-CAML fluorescent sig-

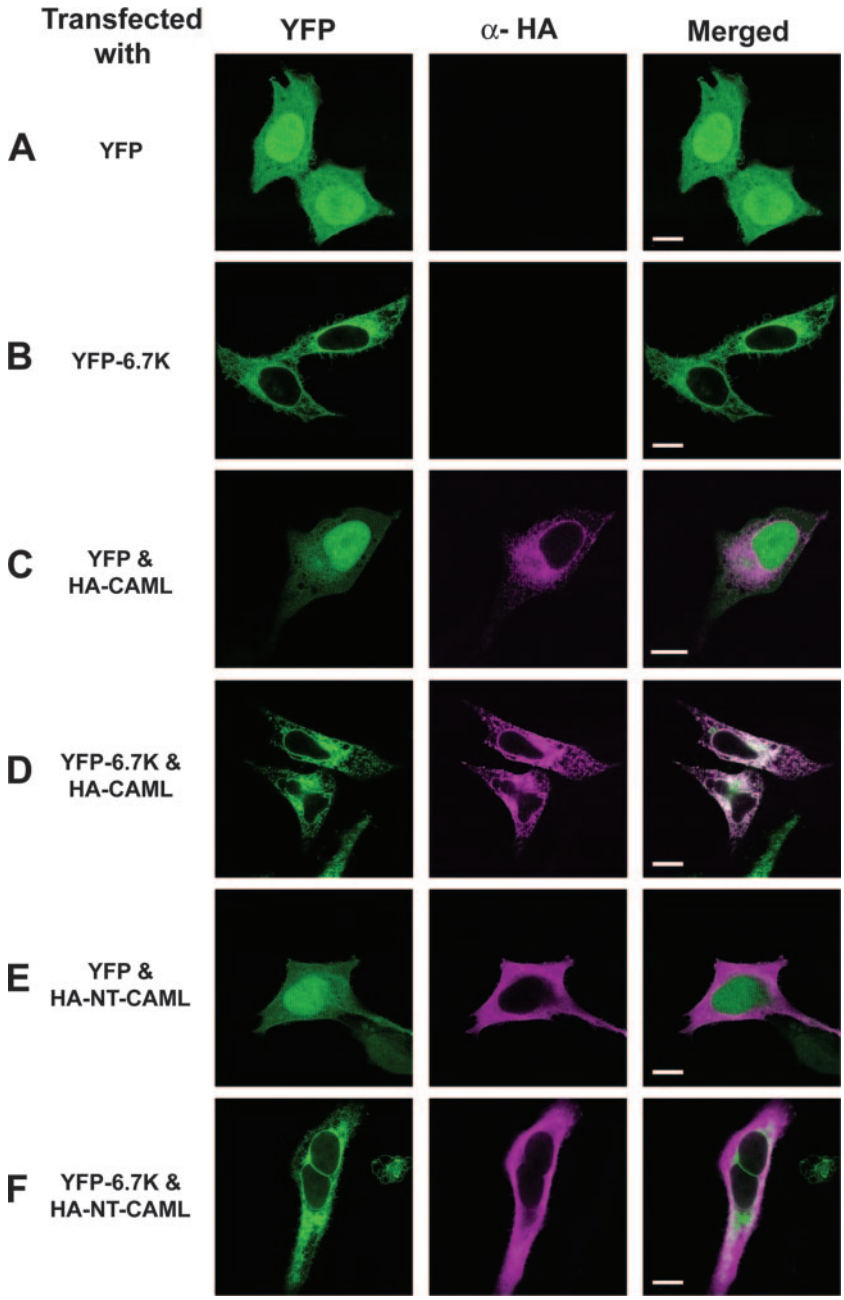

FIG. 3. Colocalization of E3-6.7K with CAML or NT-CAML. (A-F) HeLa cells were transfected with the indicated DNA. Cells were costained with 3F10 anti-HA rat mAb (α-HA) and Alexa 568-conjugated anti-rat secondary antibody (magenta). Immunofluorescence was examined using a Bio-Rad Radiance 2000 confocal microscope, and a representative optical section of each transfection is presented. YFP exhibits green fluorescence. White indicates colocalization. The white scale bar is 10 μm in length. Data were similar in three independent experiments.

nals resulted in bands of white where YFP-6.7K perinuclear staining meets the cytoplasmic staining of HA-NT-CAML (Fig. 3F). This suggests that an interaction between E3-6.7K and NT-CAML is bringing cytoplasmic NT-CAML to the periphery of ER, which explains the colocalization of the two proteins.

Defining the CBD. Although CT-E3-6.7K contains the region of sequence similarity to TACI, it does not interact with CAML (Fig. 2A), suggesting that the CBD of E3-6.7K requires additional N-terminal residues. To localize the CAML-binding region of E3-6.7K, we generated truncations of E3-6.7K from the C terminus in 5-amino-acid increments. In addition, to determine the N-terminal boundary, we added amino acids in 5-amino-acid increments to CT-E3-6.7K. E3-6.7K truncations were assayed for binding to CAML by use of the yeast two-hybrid assay. From the C terminus, 15 residues were capable of being removed and CAML binding still occurred, while 10 additional residues were required at the N terminus of CT-E3-6.7K for an interaction with CAML (Fig. 4A). The resulting domain boundaries suggest a 22-amino-acid region of E3-6.7K

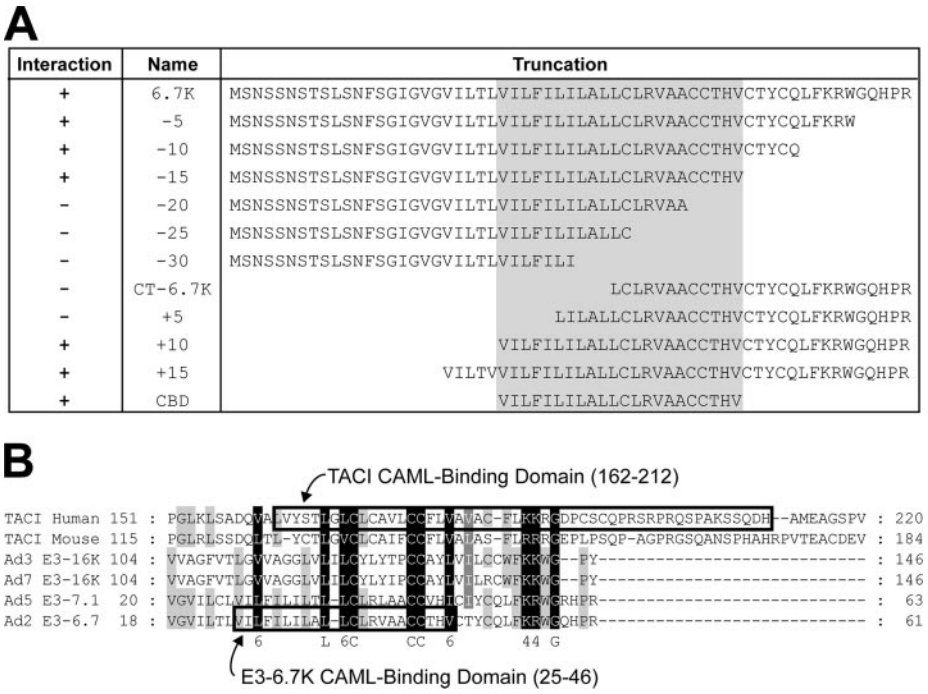

FIG. 4. CBD of E3-6.7K. (A) Determining the CBD. Two sets of E3-6.7K truncations were made: minus (−) truncations removed amino acids in 5-amino-acid increments from the C terminus of the protein, and plus (+) truncations added amino acids in 5-amino-acid increments to the N terminus of CT-E3-6.7K. DNA encoding the truncations was cloned into pGBKT7 and transformed along with pGADT7-CAML into AH109 cells followed by selection for plasmids on SD-LT. An interaction with CAML was selected for by growing the yeast on SD-LTAH. +, growth on SD-LTAH, indicating an interaction with CAML; −, no growth on SD-LTAH, suggesting no interaction with CAML. (B) Comparison of the CBDs of E3-6.7K and TACI. A consensus sequence is below the alignment (key: 6 = ILV, 4 = KR).

responsible for CAML binding. This CBD was generated and found to be able to interact with CAML (Fig. 4A). Interestingly, the CBD of E3-6.7K overlaps with the region of TACI known to bind CAML (Fig. 4B).

Conserved cysteines are required for CAML binding. Amino acid conservation between species is often a good indication of the importance of particular residues in the function of a given protein domain. An examination of the CBD reveals that 7 of

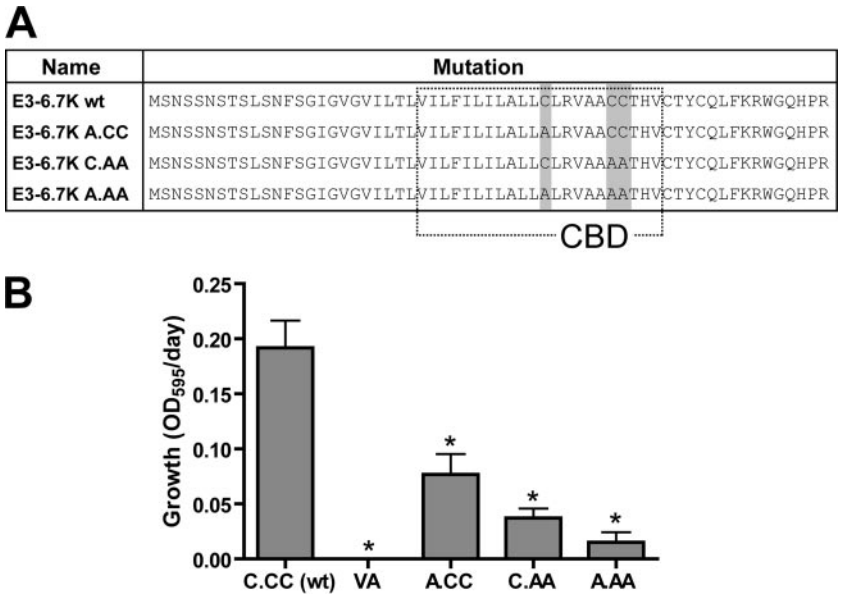

FIG. 5. Cysteine mutational analysis of CBD. (A) Sequence of the wild-type (wt) E3-6.7K and cysteine mutants. The dotted box indicates the CBD. (B) Quantitative growth rate analysis of yeast transformed with the indicated pGBKT7-6.7K mutant or the pGBKT7 vector alone (VA) and pGADT7-CAML grown in liquid SD-LTAH. *, $P < 0.01$ compared to the wild type. Data represent an average of triplicate measurements, and error bars indicate standard deviation. OD_{595}, optical density at 595 nm.

the 22 residues are conserved in E3-6.7K, E3-16K, and TACI (Fig. 4B). Four of the conserved amino acids are aliphatic and therefore very nonreactive and abundant, so attention was turned to the three conserved cysteines. Besides being found in disulfide bonds, cysteines are common in protein active and binding sites. In addition, cysteines are uncommon in proteins, with only tryptophan being more rarely found (3). Therefore, the presence of conserved cysteines in the CBD indicates that they may be important in CAML binding and thus may also affect the functions of E3-6.7K, such as its ability to inhibit apoptosis. To test this, three different mutants of E3-6.7K were generated using site-directed mutagenesis on the pGBKT7-6.7K plasmid, altering the cysteine codon TGC to the alanine codon GCC. Alanine was chosen since it is a neutral substitution (22). E3-6.7K (A.CC) has the Cys36Ala mutation, E3-6.7K (C.AA) has both the Cys42Ala and Cys43Ala mutations, and E3-6.7K (A.AA) has all three cysteines mutated to alanine (Fig. 5A). The yeast strain AH109 was transformed with pGADT7-CAML and either pGBKT7-6.7K or with one of the newly constructed plasmids encoding an E3-6.7K mutant. An interaction between the E3-6.7K mutants and CAML was quantified with a yeast growth curve analysis (Fig. 5B). All the E3-6.7K mutants had significantly reduced growth rates compared to that of wild-type E3-6.7K, indicating a reduced ability to interact with CAML. The cysteines may be involved directly in binding or may alter the structure or topology of E3-6.7K, thus interfering with CAML binding.

E3-6.7K interaction with CAML is necessary for inhibiting apoptosis. The next step was to show that an interaction with CAML is required for E3-6.7K to inhibit apoptosis. In addition to death receptor-induced apoptosis, E3-6.7K confers a similar degree of protection against thapsigargin, a mediator of apoptosis that acts intracellularly by mimicking a sustained Ca^{2+} flux (27). Thapsigargin, a sesquiterpene lactone that selectively inhibits the ER Ca^{2+}-ATPase that directs Ca^{2+} uptake into the ER, has been shown to induce apoptosis in Jurkat cells at high doses (46). The mammalian expression plasmid piGFP/6.7K was mutated by site-directed mutagenesis to produce the three same mutants generated for the binding studies: piGFP/6.7K (A.CC), piGFP/6.7K (C.AA), and piGFP/6.7K (A.AA). Jurkat cells were transiently transfected with the vector piGFP alone, with piGFP/6.7K, or with one of the piGFP/6.7K mutants. After 24 h, the cells were stimulated with 8 μM thapsigargin for a further 24 h before apoptosis was measured with annexin V. One of the signs of apoptosis is a loss of phospholipid asymmetry, leading to the exposure of phosphatidylserine on the outer leaflet of the plasma membrane (13). Annexin V preferentially binds to phosphatidylserine, allowing for a simple assay of apoptosis on a per-cell basis by flow cytometry (29). In addition, annexin V methods have been shown to be as sensitive and specific as terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling apoptotic assays (31). Cells with piGFP alone were 78% apoptotic, while cells containing E3-6.7K were only 52% apoptotic, consistent with earlier findings (Fig. 6) (38). However, in the presence of any of the E3-6.7K mutants, there was no significant reduction in apoptotic cells, implying that an interaction with CAML is necessary for E3-6.7K to inhibit apoptosis.

E3-6.7K interaction with CAML is necessary for inhibiting ER Ca^{2+} flux. E3-6.7K was shown previously to reduce the efflux of Ca^{2+} from the ER in the response to thapsigargin (38). Therefore, it was imperative to test whether non-CAML-binding mutants of E3-6.7K altered intracellular Ca^{2+} kinetics. Jurkat cells were transiently transfected either with the vector piGFP alone, with piGFP/6.7K, or with one of the piGFP/6.7K mutants. After 24 h, the cells were loaded with the Ca^{2+}-sensitive fluorophore Indo-1. Intracellular Ca^{2+} was assayed by FACS and represented using a ratiometric value of the amount of Ca^{2+}-bound Indo-1 to the amount of Ca^{2+}-free Indo-1 per cell. The GFP-positive population was gated, and baseline Ca^{2+} measurements were taken for 2 min, at which time cells were treated with 10 nM thapsigargin. Jurkat cells expressing E3-6.7K had slower kinetics in the elevation of

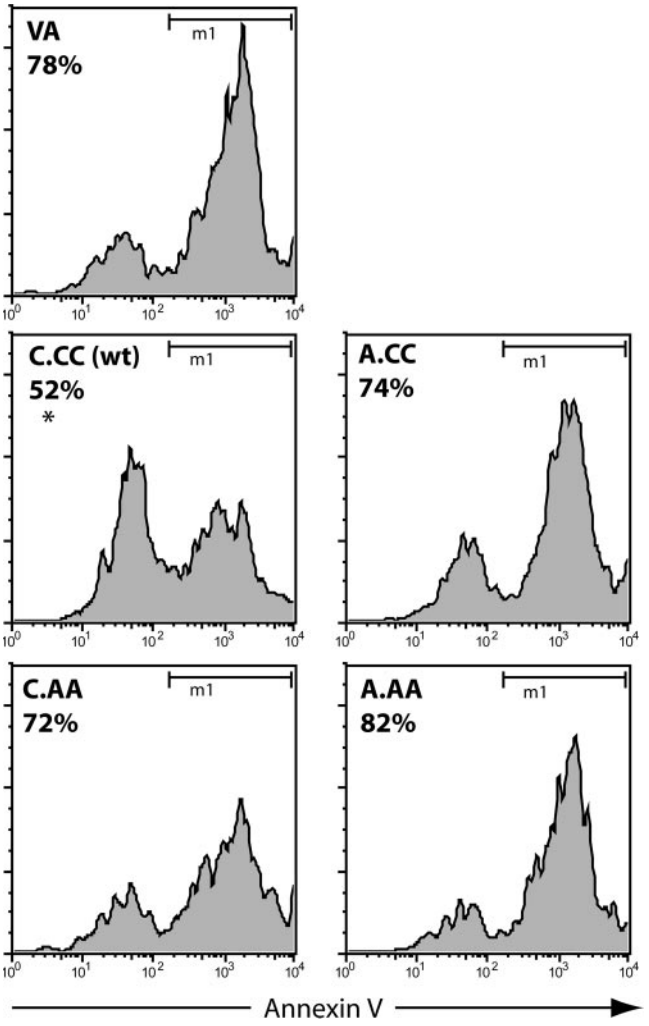

FIG. 6. E3-6.7K cysteine mutants unable to inhibit apoptosis. At 24 h after electroporation with the indicated E3-6.7K construct or with the piGFP vector alone (VA), Jurkat cells were treated with 8 μM thapsigargin for 24 h. Following treatment, cells were stained with annexin V–Alexa-648, indicating the externalization of phosphatidylserine, and with PI as a dead-cell counterstain. Cells were analyzed for annexin V staining by gating on GFP-positive and PI-negative cells. The m1 gate indicates the apoptotic cell population and is shown as the percentage of GFP-positive PI-negative cells. The asterisk indicates that apoptotic population was significantly reduced compared to what was seen for VA ($P < 0.01$). Data were similar in three independent experiments. wt, wild type.

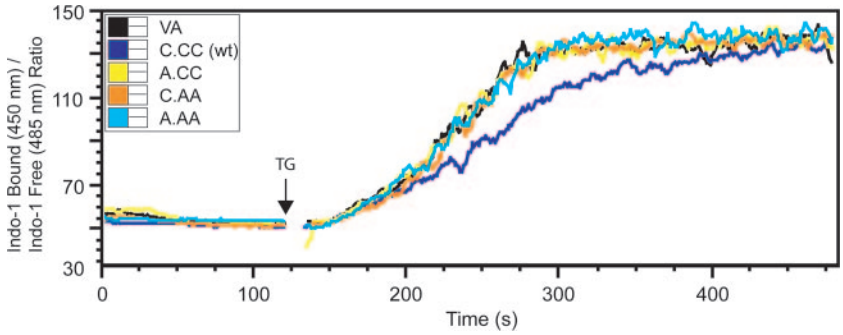

FIG. 7. E3-6.7K mutants do not inhibit thapsigargin-induced Ca^{2+} flux. Jurkat cells were transfected with either the piGFP vector alone (VA), piGFP/6.7K (wt), or one of the piGFP vectors encoding E3-6.7K mutants. After 24 h, the cells were loaded with Indo-1 in the presence of extracellular Ca^{2+}. The GFP-positive population was gated and baseline Ca^{2+} measurements were taken for 2 min, at which point thapsigargin (TG) was added to a final concentration of 10 nM, and then the analysis was immediately resumed. Data were similar in three independent experiments.

cytosolic Ca^{2+} concentration than did cells expressing GFP alone (Fig. 7). In contrast, the expression of any of the E3-6.7K mutants resulted in cytosolic Ca^{2+} kinetics identical to that seen for GFP expression alone. Thus, these results demonstrate that unlike wild-type E3-6.7K, mutants of E3-6.7K that do not interact with CAML have no effect on the kinetics of intracellular Ca^{2+} concentration in response to apoptotic stimuli.

DISCUSSION

It was demonstrated that the adenovirus protein E3-6.7K has sequence similarity to the cellular protein TACI and that E3-6.7K interacts directly with CAML, an important mediator of Ca^{2+} homeostasis to inhibit apoptosis. The E3-6.7K protein was observed to bind to NT-CAML, the same domain shown to interact with TACI. In fact, all known CAML-binding proteins have been demonstrated to interact with NT-CAML. NT-CAML is completely cytoplasmic and is believed to be a regulatory domain. Recently we have shown E3-6.7K to exist in three diverse membrane topologies (Fig. 8) (37). Depending on the topology of E3-6.7K, the CBD can be located either in the cytosol or in the lumen of the ER. E3-6.7K interacts with NT-CAML, indicating that the interaction occurs in the cytosol. Consequently, the likely binding partner of CAML is ^{Ntm}E3-6.7K, as it is the only form to display the CBD in the cytosol (Fig. 8) (36).

The CBD of E3-6.7K was defined to be a 22-amino-acid domain necessary for binding to CAML. Interestingly, this domain overlaps significantly with the CAML-binding region of TACI (Fig. 4B). This suggests that there may be a common domain employed by proteins that bind to CAML; however, a thorough examination of all other proteins known to interact with CAML revealed that none of them contain a similar motif. EGFR and $p56^{Lck}$ have been shown to interact with CAML through their tyrosine kinase domains (54, 55). CAML does not appear to bind indiscriminately to kinases, as no interaction was found with either of two other kinases, $p59^{Fyn}$ and ZAP-70 (54). Clearly CAML is able to bind to numerous proteins through multiple BDs, implying the importance of this ubiquitously expressed protein. While not required for cellular viability, CAML is required for early embryonic development (55).

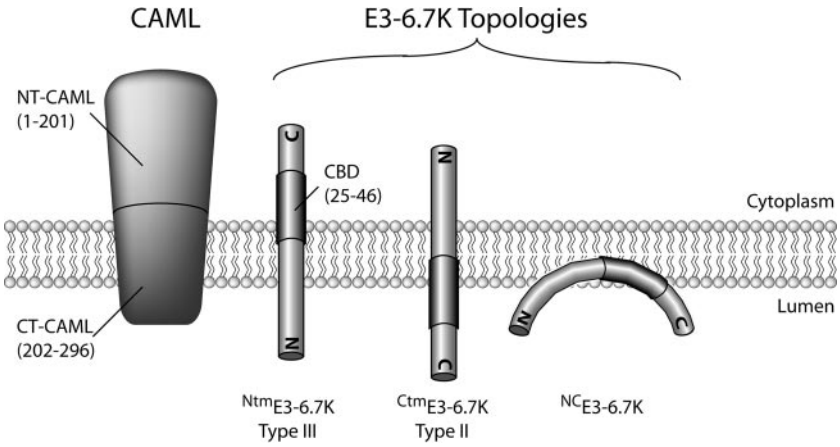

FIG. 8. Schematic of CAML and the different E3-6.7K topologies. The three topologies of E3-6.7K are shown, with the N and C termini labeled as N and C, respectively. The dark gray region on E3-6.7K is the CBD and consists of residues 25 to 46. The N-terminal 201 residues of CAML, labeled NT-CAML, are completely cytoplasmic and contain the E3-6.7K BD.

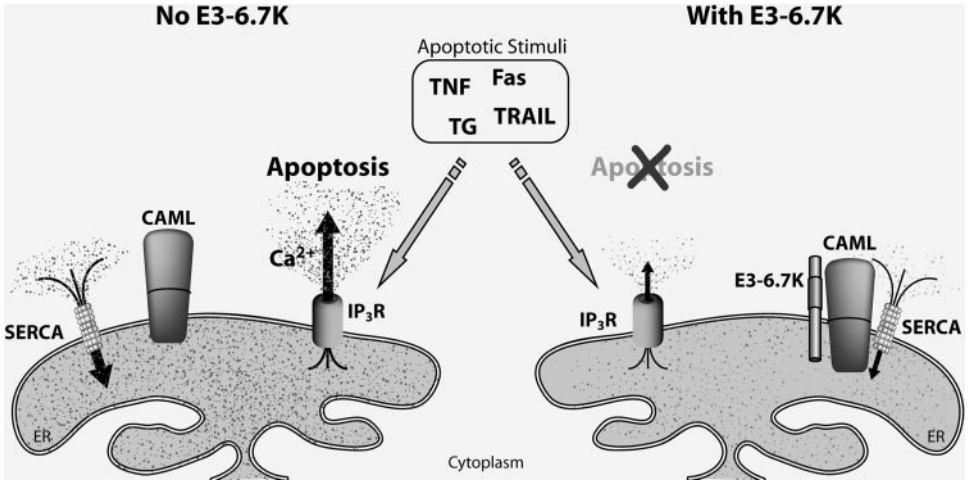

FIG. 9. Proposed model of the E3-6.7K–CAML interaction preventing apoptosis. Ca^{2+}-ATPase pumps such as SERCA are responsible for maintaining high luminal Ca^{2+} concentration in the ER. High steady-state levels of ER Ca^{2+} are required to release proapoptotic Ca^{2+} waves that occur in response to most apoptotic stimuli. In the presence of E3-6.7K, an interaction between E3-6.7K and CAML may result in lower steady-state levels of ER Ca^{2+} by inhibiting a fraction of SERCA Ca^{2+} pumps. The lower ER Ca^{2+} levels would not be high enough to release proapoptotic Ca^{2+} waves, thereby precluding cell death.

The role of CAML in intracellular Ca^{2+} regulation and apoptosis is unresolved. CAML may modulate ER Ca^{2+} by forming Ca^{2+} channels, or it may act directly or indirectly on either preexisting Ca^{2+} release channels or sarcoplasmic/ER Ca^{2+}-ATPase (SERCA) pumps. By comparison, all these Ca^{2+}-modulating mechanisms have been proposed for members of the Bcl-2 family. Bcl-X_L, a Bcl-2 homolog, is very similar in structure to some pore-forming bacterial toxins (39). Both Bcl-2 and Bcl-X_L are able to form ion channels in synthetic lipid membranes (35, 44), although neither has been shown to conduct Ca^{2+}. As CAML has no apparent sequence homology to known Ca^{2+} channels (24), pore formation seems unlikely although not entirely impossible. Recently, much attention has focused on interactions of the Bcl-2-related proteins with Ca^{2+} release channels and pumps. Inositol 1,4,5-trisphosphate receptors (IP_3Rs) are the principal channel for mobilizing Ca^{2+} stores from the ER in almost all cell types. Bcl-2 and Bcl-X_L are both able to interact directly with IP_3Rs (8, 60). The IP_3R–Bcl-2 interaction was increased in the absence of Bax and Bak (40), while tBid and Bax were able to block the interaction between Bcl-X_L and IP_3R (60). The ratio of pro- to antiapoptotic family members may be able to control ER Ca^{2+} levels through an IP_3R-mediated Ca^{2+} leak. CAML is unlikely to have a direct effect on IP_3Rs, as it does not comigrate with these Ca^{2+} release channels in sucrose gradient membrane fractionation (25). CAML does, however, comigrate with SERCA. Furthermore, immunofluorescence staining indicates that CAML and SERCA colocalize (25). Bcl-2 has been shown to interact with SERCA, as demonstrated by coimmunoprecipitation (30). The addition of a truncated form of Bcl-2 to sarcoplasmic reticulum vesicles resulted in the destabilization of SERCA and a reduction in its Ca^{2+}-ATPase activity (11). Dremina et al. (11) have suggested that Bcl-2 may inhibit a fraction of the Ca^{2+} pumps sufficient to decrease ER Ca^{2+} stores to levels below the threshold necessary to induce proapoptotic Ca^{2+} levels in mitochondria. The E3-6.7K–CAML interaction may alter ER Ca^{2+} stores through a similar mechanism. In the end, a CAML–E3-6.7K interaction would achieve the same goal of reducing Ca^{2+} release from the ER whether it inhibited the efflux of Ca^{2+} from the ER or diminished the ER Ca^{2+} stores through a Ca^{2+} leak by inhibiting ER Ca^{2+} uptake. The second scenario seems more likely, as cells overexpressing CAML result in a modest depletion of intracellular Ca^{2+} stores and a reduction in the amplitude of Ca^{2+} waves in response to the agonist ATP (53). CAML may have a natural function in apoptosis that viral proteins may be exploiting, or perhaps CAML normally has no role in apoptosis but its ability to affect Ca^{2+} is subverted by the virus to inhibit cell death. Interestingly, overexpression of CAML in a human B-cell line has been shown to reduce apoptosis in response to thapsigargin (14).

From the available data, a potential model starts to take shape (Fig. 9). Early in the adenovirus infection, the E3-6.7K protein is expressed and localizes primarily to the ER, where it colocalizes with CAML. Through the CBD, E3-6.7K binds to the N-terminal regulatory domain (residues 1 to 201) of CAML, the same region that interacts with TACI. This interaction results in a depletion of intracellular Ca^{2+} stores, possibly by inhibiting a fraction of SERCA Ca^{2+} pumps. The proapoptotic ER-to-mitochondrion Ca^{2+} waves that result in most apoptotic pathways are inhibited as a result of the reduced ER Ca^{2+} steady-state levels. This general inhibition of programmed cell death provides the virus with time to complete viral replication and eventually assist in the maintenance of a persistent infection.

In order to establish a persistent infection, viruses must inhibit host defense mechanisms including apoptosis, interferons, NK cells, and cytotoxic T cells (23). The importance of counteracting the host immune response is underscored by the fact that almost one-third of the adenovirus genome is devoted to the process. Proteins encoded by the E1, E3, and E4 genes, the late protein L4-100K, and virus-associated RNAs all have immune modulating properties. The combination of the antiapoptotic effect of E3-6.7K and the immune modulating abil-

ities of other adenovirus proteins likely contributes to the ability to establish persistence.

The E3-6.7K and K7 proteins are the first two members of a novel class of viral antiapoptotic proteins that target CAML to affect cellular Ca^{2+} signaling. Although their targets and ultimate goals are the same, their localizations and modes of action are different. E3-6.7K is localized primarily to the ER, while K7 is typically present at mitochondria. Even though CAML is predominantly ER bound, this does not appear to be a limitation for K7, as the ER and mitochondria are often in close proximity (9). A major difference between K7 and E3-6.7K is the opposite effects they have on thapsigargin-induced Ca^{2+} release. E3-6.7K reduces the efflux of Ca^{2+}, while K7 expression results in a slight increase in Ca^{2+} release (14). Although sustained high intracellular Ca^{2+} levels contribute to apoptosis, early up-regulation of cytoplasmic Ca^{2+} levels may protect cells against apoptosis (12).

Only three viral proteins are known to inhibit apoptosis by altering cellular Ca^{2+}, and two of these interact with CAML. The study of CAML and the E3-6.7K protein that targets it will ultimately lead to a better understanding of the role of TACI, immune evasion mechanisms, and viral persistence and also provide a novel means to investigate cellular apoptotic pathways.

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