ADENOVIRUS E3-6.7K INHIBITS APOPTOSIS THROUGH AN INTERACTION WITH THE CELLULAR PROTEIN CALCIUM-MODULATING CYCLOPHILIN LIGAND (CAML)

by

JASON ROBERT GRANT

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Abstract

Adenovirus is an important etiological agent which causes acute respiratory and gastrointestinal infections worldwide. The virus encodes several proteins involved in suppressing the host antiviral response. One of these is the E3-6.7K protein which confers transfected cells with resistance from a variety of apoptotic stimuli and maintains endoplasmic reticulum (ER) Ca\(^{2+}\) homeostasis, however, the mechanism of action is unknown. In this study, evidence is provided that E3-6.7K inhibits apoptosis and ER Ca\(^{2+}\) efflux through an interaction with Ca\(^{2+}\)-modulating cyclophilin ligand (CAML), a cellular protein that regulates intracellular Ca\(^{2+}\). E3-6.7K was shown to interact with both mouse and human CAML in a yeast two-hybrid system. A direct interaction with human CAML was confirmed by an in vitro co-immunoprecipitation assay. In addition, immunofluorescence microscopy revealed that the two proteins co-localize in an ER-like pattern in transfected cells. Furthermore, yeast two-hybrid assays indicated that 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. These findings suggest that E3-6.7K targets CAML as a mechanism to alter ER Ca\(^{2+}\) homeostasis which consequently protects cells from apoptosis.

E3-6.7K has been shown to be an integral membrane protein. Most membrane proteins have only one orientation in a membrane bilayer. E3-6.7K can adopt three different topologies in microsomal membranes in a cell-free translation system: a type II orientation (N-cytoplasmic/C-luminal, termed CtmE3-6.7K), the opposite type III orientation (N-
luminal/C-cytoplasmic, termed NtmE3-6.7K) and the possibility of a fully translocated form (N and C termini are both translocated, termed NCE3-6.7K). In this study, the topology of E3-6.7K in cells was explored. Distinct topological forms were found, as both the N and C termini of E3-6.7K were detected on the extracellular surface of transfected cells. Based on the location of the CBD, the most probable binding partner of CAML is NtmE3-6.7K.
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<th>Full Form</th>
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<tbody>
<tr>
<td>Ad</td>
<td>adenovirus</td>
</tr>
<tr>
<td>AD</td>
<td>activation domain</td>
</tr>
<tr>
<td>ADP</td>
<td>adenovirus death protein</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APAF-1</td>
<td>apoptotic protease activating factor-1</td>
</tr>
<tr>
<td>APRIL</td>
<td>a proliferation-inducing ligand</td>
</tr>
<tr>
<td>ARD</td>
<td>acute respiratory disease</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP-ribosylation factor 1</td>
</tr>
<tr>
<td>AT</td>
<td>annealing temperature</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATRAP</td>
<td>angiotensin II type I receptor-associated protein</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-X&lt;sub&gt;L&lt;/sub&gt;/Bcl-2 associated death promoter</td>
</tr>
<tr>
<td>BAFF</td>
<td>B cell activating factor belonging to the TNF family</td>
</tr>
<tr>
<td>BAFF-R</td>
<td>BAFF receptor</td>
</tr>
<tr>
<td>Bak</td>
<td>Bcl-2 antagonist killer</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2 associated x protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell lymphoma 2</td>
</tr>
<tr>
<td>Bcl-X&lt;sub&gt;L&lt;/sub&gt;</td>
<td>long form of Bcl-x</td>
</tr>
<tr>
<td>BCMA</td>
<td>B cell maturation antigen</td>
</tr>
<tr>
<td>BD</td>
<td>binding domain</td>
</tr>
<tr>
<td>BH</td>
<td>Bcl-2 homology</td>
</tr>
<tr>
<td>Bid</td>
<td>BH-3 interacting domain death agonist</td>
</tr>
<tr>
<td>Bik</td>
<td>Bcl-2 interacting killer</td>
</tr>
<tr>
<td>BIR</td>
<td>baculovirus IAP repeat</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>calcium</td>
</tr>
<tr>
<td>CAML</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-modulating cyclophilin ligand</td>
</tr>
<tr>
<td>CAR</td>
<td>coxsackie and adenovirus receptor</td>
</tr>
<tr>
<td>caspases</td>
<td>cysteine-dependent aspartyl-specific proteases</td>
</tr>
<tr>
<td>CBD</td>
<td>CAML binding domain</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>cIAP</td>
<td>cellular inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP responsive element binding protein</td>
</tr>
<tr>
<td>CT-E3-6.7K</td>
<td>C-terminus of E3-6.7K (residues 35-61)</td>
</tr>
<tr>
<td>C-terminus</td>
<td>carboxy-terminus</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>dAMP</td>
<td>deoxyadenosine monophosphate</td>
</tr>
<tr>
<td>DBP</td>
<td>DNA-binding protein</td>
</tr>
<tr>
<td>dCMP</td>
<td>deoxycytidine monophosphate</td>
</tr>
<tr>
<td>DD</td>
<td>death domain</td>
</tr>
<tr>
<td>DED</td>
<td>death effector domain</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded DNA</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded RNA</td>
</tr>
<tr>
<td>dTMP</td>
<td>deoxythymidine monophosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
</tr>
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</table>
EYFP  enhanced yellow fluorescent protein
FACS  fluorescence activated cell sorter
FADD  Fas-associated death domain
Fas    FS-7 cell-associated cell surface antigen, also called CD95 or APO-1
FasL   Fas ligand
FBS    fetal bovine serum
FIP    14.7K interacting proteins
FLIP   FLICE inhibitory protein
GFP    green fluorescent protein
HA     hemagglutinin
HDM2   human double minute 2
HEPES  N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
hrGFP  humanized recombinant GFP
IAP    inhibitor of apoptosis protein
IFN    interferon
IMAGE  Integrated Molecular Analysis of Genomes and their Expression
IP$_3$ inositol 1,4,5-trisphosphate
IP$_3$R  IP$_3$ receptor
IRES   internal ribosomal entry site
ISGF-3 IFN-stimulated gene factor 3
ISRE   IFN-stimulated response elements
ITR    inverted terminal repeat
JAK    Janus family of tyrosine kinase
KSHV   Kaposi’s sarcoma-associated herpesvirus
mAb    monoclonal antibody
mCAML  mouse CAML
MCS    multiple cloning site
MHC    major histocompatibility complex
minCBD minimal CAML binding domain
MLP    major late promoter
mRNA   messenger RNA
NCBI  National Center for Biotechnology Information
NF    nuclear factor
NF-kB nuclear factor kappa beta
NF-AT nuclear factor of activated T cells
NK    natural killer
NPC   nuclear pore complex
NT-CAML N-terminus of CAML (residues 1-201)
N-terminus amino-terminus
NT-mCAML N-terminus of CAML (residues 1-199)
OAS   2'-5' oligoadenylate synthetase
OD    optical density
PAGE  polyacrylamide gel electrophoresis
PBS   phosphate buffered saline
PCR   polymerase chain reaction
PEG   polyethylene glycol
PHI-BLAST pattern-hit initiated-BLAST
PI    propidium iodide
PI-3K phosphotidylinositol-3-OH kinase
PKR   double-stranded RNA-activated protein kinase
pRB   retinoblastoma tumor suppressor protein
PrP   prion protein
PS    phosphatidylserine
pTP   precursor TP
RGD   arginine-glycine-aspartic acid
RID   receptor internalization and degradation
RIP   receptor interacting protein
RNA   ribonucleic acid
RPMI  Roswell Park Memorial Institute
SA    signal-anchor
SD    synthetic dropout
SD-LT SD lacking leucine and tryptophan
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>SD-LTAH</td>
<td>SD lacking leucine, tryptophan, adenine and histidine</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco/endoplasmic reticulum Ca$^{2+}$-ATPase</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>SRP</td>
<td>signal recognition particle</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TACI</td>
<td>transmembrane activator and CAML interactor</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen processing</td>
</tr>
<tr>
<td>tBid</td>
<td>truncated Bid</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-binding protein</td>
</tr>
<tr>
<td>TFIID</td>
<td>transcription factor IID</td>
</tr>
<tr>
<td>TG</td>
<td>thapsigargin</td>
</tr>
<tr>
<td>TMD</td>
<td>tobacco mosaic disease</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TNF-R</td>
<td>TNF receptor</td>
</tr>
<tr>
<td>TP</td>
<td>terminal protein</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR-associated death domain</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNFR receptor-associated factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TRAIL-R</td>
<td>TRAIL receptor</td>
</tr>
<tr>
<td>VA</td>
<td>virus-associated</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
</tr>
<tr>
<td>YPD</td>
<td>yeast extract/peptone/dextrose</td>
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</tbody>
</table>
Acknowledgements

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Special thanks are owed to my family and friends for their love, support and laughs throughout the years. In particular I would like to thank Bonnie Dong and Ashleen Nadan for their motivation and encouragement. I would also like to thank Cheryl, Kyla, Maki and Mei Mei for their company during much needed coffee breaks.
Dedication

I would like to dedicate this work to my parents, Sherran and Daniel Grant. They brought me up to be creative and inquisitive and taught me to not take life too seriously.
Chapter 1: Introduction

1.1 A brief history of viruses

Viruses have had a large impact on human history. The accidental introduction of smallpox by Cortes and his conquistadors to the previously unexposed people of the Aztec Empire in 1520 lead to lethal epidemics and played a major role in the destruction of the Aztec civilization. More recently the influenza pandemic of 1918 resulted in the death of over 20 million people. Evidence of viral disease can be found in ancient records dating back more than 3000 years. For example, an Egyptian hieroglyph from approximately 1500 BC illustrates a man with a withered leg, likely the consequence of a poliovirus infection (1).

Long before the concept of a virus was understood, methods to control viral disease have been practiced. Variolation, the practice of inoculating a healthy individual with material taken from a smallpox pustule, provided immunity to smallpox and was widespread in Asia by the 11th century (2). Between 1 and 2% of those variolated died which although a high fatality rate by today's standards provided a lower fatality rate then the 30% who died when they contracted the disease naturally. In the 1790s, Edward Jenner, an English country physician who had been variolated himself as a boy, discovered a much safer practice. He noted that milkmaids exposed to cowpox were immune to smallpox. Jenner deliberately infected an eight year boy with cowpox and then exposed him to smallpox, which the boy failed to contract (2). Jenner's studies of vaccination (*vacca* = cow in Latin) with cowpox paved the way for the eventual eradication of smallpox.

Almost a hundred years after Jenner developed the smallpox vaccine, the idea that germs were responsible for communicable disease was put forward by Pasteur in his germ
theory (3). Shortly after, German physician Robert Koch developed his criteria, or Koch’s postulates, for the identification of the pathogen responsible for a specific disease. These postulates state that (a) the organism must be present in every case of the disease, (b) the organism must be isolated in pure culture, (c) the disease must be reproduced when a pure culture of the organism is introduced into a susceptible host, and (d) the organism must be recoverable from the experimentally infected host. The first microbes shown to cause disease were all bacteria. In 1876, Koch demonstrated that anthrax was caused by the anthrax bacillus and in 1882, he established the tubercle bacillus as the causative agent of tuberculosis (4).

Researchers soon discovered pathogenic agents with properties unique from bacteria. Pasteur was unable to visualize the pathogen responsible for rabies by light microscopy and in 1892 the Russian scientist Dimitrii Ivanowsky observed that the causative agent of tobacco mosaic disease (TMD) passed through filters that otherwise retained known bacteria (5). At the time, this filterable agent was suspected of being very small bacteria or possibly a toxin. Six years later (1898) in Holland, Martinus Beijerinck independently made the same observation as Ivanowsky. However, he went on to show that the causative agent of TMD multiplied in infected tissue, and therefore could not be a toxin. Furthermore, he established that, unlike bacteria, the agent could not grow in vitro and multiplied only in living and preferable growing tissues (6). This further distinguished this filterable agent as distinct from pathogenic bacteria. Beijerinck concluded that TMD is caused not by a bacterium, but by an agent he termed contagium vivum fluidum, chosen to highlight the infectious nature and fluid physical properties (6). Filterable agents were eventually called viruses from the Latin for “slimy liquid” or “poison”. Although not widely accepted at the time, Beijerinck’s insight
that the plant pathogen he was studying was distinct from bacteria laid the foundation for the field of virology.

Plants are not the only host for viruses as other researchers would soon show. In 1898, the German scientists Friedrich Loeffler and Paul Frosch observed that the causative agent of foot-and-mouth disease was also filterable, thus the discovery of the first animal virus (7). The first human virus discovered was that responsible for yellow fever by a U.S. Army commission headed by Colonel Walter Reed (2). Bacterial viruses, or bacteriophages as they would eventually be called, were first identified by Frederick Twort in 1915 and Felix d’Hérelle in 1917 (7).

The simplicity of bacteriophages and their bacteria host provided an elegant system for the study of viruses. Work with bacteriophages demonstrated that nucleic acid and not protein carries the genetic information (8). In addition, lysogeny, the idea that a virus can coexist with its host without causing lysis was first described in phages. The so called “phage group” led by Max Delbrück at Cold Spring Harbor Laboratory focused on using bacterial viruses as a model for understanding life processes and from their work grew the field of molecular biology (7). Research from the phage group contributed to discoveries of basic processes such as the genetic code, gene regulation, and transcription and translation.

The advent of culturing single cells and the development of the first human cell line, HeLa, derived from a cervical carcinoma (9) allowed the modern era of medical virology to flourish. Routine, reproducible tissue culture allowed the discovery of several new human viruses for which animal hosts were not available, such as rubella virus, measles virus, and adenovirus. The latter of these is the focus of this thesis.
1.2 Adenoviridae

1.2.1 History

In 1953, Rowe and colleagues (10) discovered a previously unidentified virus that was causing spontaneous degeneration of cells derived from human adenoids. They initially named this virus “adenoid-degenerating” agent but upon finding multiple strains from patients with undifferentiated respiratory disease, later proposed the term “adenoidalpharyngeal-conjunctival” agents as a generic designation (11). The following year, Hilleman and Werner isolated a pathogen from army recruits with acute respiratory disease (ARD) that they termed ARD agents (12). The viruses discovered by the two groups were soon shown to have the same complement fixation properties (11) and, in 1956, the current nomenclature of adenoviruses was adopted, after the original source of the virus (13). Today, over 100 serotypes of adenovirus have been identified, of which 51 are of human origin.

In 1962, Trentin and colleagues made the exciting discovery that adenovirus serotype 12 could cause tumours in hamsters (14). Although other non-human viruses, such as the Rous sarcoma virus in chickens (15), had been shown to induce tumours, this was the first description of a human virus inducing malignant tumours in animals. However, there has been no evidence linking adenovirus with cancer in humans; searches of human tumours have failed to find any adenoviral DNA (16, 17).

Due to their ability to produce high titer stocks and the fact that their genome is easily manipulated, adenoviruses have long been used as an experimental system resulting in significant contributions to molecular biology. Most notably was the discovery of messenger RNA (mRNA) splicing. While studying the biogenesis of adenoviral mRNA, it was observed that shorter cytoplasmic mRNAs originated from a large nuclear transcript (18). A
subsequent comparison of the sequence of an adenoviral mRNA and its corresponding DNA revealed the existence of introns (19, 20). More recently adenovirus has shown potential as a vector for gene therapy.

1.2.2 Classification

The adenoviruses comprise the family *Adenoviridae*, which consists of four officially accepted genera: *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, and *Siadenovirus* (21). Until recently, there were only two genera: the *Mastadenovirus* genus included the mammalian viruses, whereas the *Aviadenovirus* genus contained the bird viruses. However, based on the comparison of genome sequence and organization, it became clear that this distinction was too simple and two new genera were established. The genus *Atadenovirus*, named after the high adenosine and thymidine (A + T) content of member viruses, contains certain bovine and ovine adenoviruses (22) as well as snake adenovirus serotype 1 (23). Members of the *Siadenovirus* genus, which include the only known frog adenovirus, have a small genome size (~26 kb) and a putative sialidase gene, hence the genus name (24). It has been theorized that the four adenovirus genera (*Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, and *Siadenovirus*) might correspond to viral lineages that co-evolved with four of the major vertebrate classes (mammals, birds, reptiles, and amphibians) (25). In addition, a fifth genus (*Ichtadenovirus*) has been proposed for a recently analyzed fish adenovirus (25, 26), which would correspond to a fifth vertebrate class. If this co-evolution theory is correct it would mean that the adenovirus family has existed for more than 400 million years, when the divergence of vertebrate classes began (27).
To date there are 51 human adenovirus serotypes (Ad1-Ad51) recognized (28). These are grouped into six species (formally called subgroups or subgenera) based on their hemagglutinating properties, oncogenic potential and genome homologies: species A, B, C, D, E, and F (Table 1-1) (29). Species B is further subdivided into subspecies B1 and B2 based on restriction enzyme cleavage patterns (30). Individual serotypes are assigned on the basis of their immunological distinctiveness, as defined by their ability to resist neutralization by antisera to other known adenovirus serotypes. DNA homology within species is more than 50% and less than 20% between species. Consequently, serotypes from the same species can recombine but the same is generally not true for serotypes from different species.

1.2.3 Epidemiology

Adenovirus is an important etiological agent that is associated with a variety of clinical syndromes including acute respiratory disease, epidemic conjunctivitis, and gastroenteritis. The prevalence of antibodies to one or more adenovirus serotypes is approximately 50% in infants 6 to 11 months of age and nearly 100% in adults over 16 (11). An analysis of the adenovirus serotypes associated with particular diseases results in distinct, yet partially overlapping, disease patterns for the different adenovirus species (Table 1-1). Species A and F cause gastrointestinal infection. Enteric adenoviruses (Ad40 and Ad41 of species F) have been associated with 4-17% of cases of diarrhea in children (31) and are second only to rotavirus as the causal agent of viral gastroenteritis in children (32). Species B1, C, and E are mainly associated with infections of the respiratory tract with clinical symptoms ranging from mild pharyngitis to acute respiratory disease. Roughly 7% of respiratory infections in children younger than 2 years and 3% in adults are associated with
<table>
<thead>
<tr>
<th>Species</th>
<th>Serotypes</th>
<th>Hemagglutination groups†</th>
<th>Tumours in animals</th>
<th>G+C content (%)</th>
<th>Commonly associated diseases</th>
<th>E3-6.7K Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>12, 18, 31</td>
<td>IV</td>
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<td>B</td>
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<td>Respiratory infections</td>
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<td>1, 2, 5, 6</td>
<td>III</td>
<td>Low or none</td>
<td>57-59</td>
<td>Respiratory infections</td>
<td>Yes (61-63 aa)</td>
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<td>8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51</td>
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<td>57-61</td>
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<td>III</td>
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<td>51-52</td>
<td>Gastroenteritis</td>
<td>No</td>
</tr>
</tbody>
</table>

† Hemagglutination groups are based on the ability of the virus to agglutinate monkey or rat erythrocytes

I - complete agglutination of monkey erythrocytes
II - complete agglutination of rat erythrocytes
III - partial agglutination of rat erythrocytes
IV - little or no agglutination

Table 1-1. Classification of human adenoviruses.
The 51 serotypes of human adenovirus are classified into 6 species based on their hemagglutination pattern, oncogenicity, and DNA homology. Species B is further subdividing into subspecies B1 and B2. The E3-6.7K protein is produced by species C adenoviruses and ranges in size from 61 to 63 amino acids (aa). A 16-kDa homologue is produced by species B adenoviruses and ranges in size from 131 to 146 aa. Serotype 2 (bold) is the source of E3-6.7K used in this thesis.
adenovirus (33, 34). Species B2 are usually associated with infections of the kidney and urinary tract (35). Lastly, species D and E are commonly associated with infections of the eye (36), such as conjunctivitis and the more serious epidemic keratoconjunctivitis (37).

Adenovirus often establishes a persistent infection with no outward signs of disease and has been shown to be shed by patients up to 2 years following the initial infection (34). Due to its persistence, adenovirus can resurface in immunocompromised patients; for example, 12% of patients with acquired immunodeficiency syndrome (AIDS) have been reported to shed species B adenovirus in their urine (38, 39). It is unclear how the persistent state of the virus is maintained. There is evidence supporting true latency in which the viral DNA incorporates into the host DNA. Adenovirus serotype 2 DNA has been found in human tonsillar and adenoid tissues, although no infectious virus was recovered (40). The integration of adenovirus DNA into the host DNA of lung tissue has been implicated in chronic obstructive pulmonary disease (41). There is also support for a persistent state that is maintained through a low level of continuous virus production in lymphocytes (42). Lymphocytes have long been suggested to harbor adenovirus in a persistent form (43). Adenovirus DNA has been identified in 1 in $10^4$ to $10^6$ peripheral lymphocytes of healthy individuals (44). It is likely that lymphocytes were the source in the original isolations of adenoviruses from adenoid tissue. A recent study found species C DNA in lymphocytes from 33 of 42 human tonsillar and adenoid tissues tested. Furthermore, viral DNA was enriched in T cell populations but not in B cell populations, suggesting that adenovirus may persist in T lymphocytes (45).
1.2.4 Structure

Adenovirus is a non-enveloped virus with icosahedral symmetry, with the surface of the virion containing 20 facets, each an equilateral triangle, and 12 vertices (46). Virions measure 70-100 nm in diameter (Figure 1-1A) and consist of a DNA-containing core surrounded by a protein shell also called the capsid. Up to 40 polypeptides are encoded by the virus, of which 11 are structural proteins. The proteins of the mature virion are designated by roman numerals (II-X) in order of their decreasing apparent molecular weight on SDS gels (47). The DNA-bound terminal protein is an exception. No polypeptide I exists as the originally designated protein proved to be an artifact.

Most of the structural data comes from studies of human adenovirus serotype 2 (Ad2). The virion capsid is composed of 252 capsomeres: 12 pentons located at the vertices of the capsid and 240 hexons (48). As their names imply, each penton and hexon are surrounded by 5 or 6 neighbors, respectively (Figure 1-1B). The hexon capsomere consists of 3 molecules of polypeptide II (49) and is the major coat protein, accounting for 63% of the virion mass (50). Polypeptides VI, VIII, and IX associate with the hexon and are thought to stabilize the hexon capsomere lattice (51). Two copies of polypeptide IIIa are located at each edge of the capsid and seem to link adjacent facets together (52).

The penton capsomeres which lie at the 12 virion vertices are formed by the penton base and the fiber protein which projects out from the base. The fiber is responsible for attachment of the virus to the host cell while the base is responsible for internalization. Five molecules of polypeptide III associate to form the penton base (50). The fiber is a trimer of polypeptide IV and has three structural domains. The N-terminal tail attaches non-covalently to the base, while the C-terminal globular domain forms a knob that is required for binding to
Figure 1-1. Structure of human adenovirus.
(A) Electron micrograph of a species C human adenovirus. (B) Ad2 cryo-EM reconstruction overlaid with black lines to represent icosahedral structure. A representative penton (○) and hexon (○) are highlighted surrounded by 5 and 6 neighboring capsomeres (●), respectively. Figure adapted from Stewart et al. (53). (C) Schematic view of the adenovirus particle based on current knowledge of its polypeptide components and DNA. Virion components are designated by their number except for the terminal protein (TP). Polypeptide sizes are given in amino acids (aa). Figure adapted from Stewart et al. (54).
host cells (55). The tail and knob are separated by a shaft, characterized by a repeating motif of about 15 residues and the number of repeats varies among adenovirus serotypes (56).

The virion core is made up of a single copy of linear double stranded DNA and four polypeptides: terminal protein (TP), V, VII, and X (also called μ). The TP is covalently linked to the 5' ends of the genome (57). Whereas the role of the free precursor TP (pTP) is to act as a protein primer for DNA replication (58), the significance of the covalently attached mature TP remains to be elucidated. The remaining core polypeptides, V, VII, and μ, interact with the viral DNA (59). Polypeptide VII is the major core protein and complexes tightly with DNA to form compact repeating structures similar to nucleosomes (60). Polypeptide V most likely links the core to the capsid as it is able to bind to the penton base (61) and to polypeptide VI (59). The function of the μ polypeptide is unknown (62). In addition, the virion core contains approximately ten copies of the cysteine protease L3-23K (63). Figure 1-1C shows a schematic view of the adenovirus particle based on current knowledge.

1.2.5 Genome organization

The human adenovirus genome consists of a single molecule of linear double stranded DNA and ranges from 30 to 38 kbp depending on the serotype. All the human adenoviruses sequenced to date have the same general genome organization (Figure 1-2). First, the ends of the genome consist of two inverted terminal repeats (ITR) that each contains an origin for DNA replication. Second, the adenovirus genome can be separated into transcriptional units conventionally classified into early (E) and late (L) regions, referring to the time the region is transcribed relative to the replication of the viral genome. In general
Region | Codes for proteins involved in:
--- | ---
E1A | Regulation of other regions
E1B | Inhibition of apoptosis
E2A and B | DNA Replication
E3 | Modulation of host response to infection
E4 | DNA Replication, inhibition of apoptosis, and mRNA transport
Major Late (L1-L5) | Production and assembly of adenovirus virions

Figure 1-2. Transcription map of adenovirus serotype 2.
Transcripts are diagramed with their exons represented by interrupted arrows. Gaps in the arrows indicate introns. The chronological sequence of transcription of the various regions is color coded. The numbers refer to the relative map units on the adenovirus genome. Functions assigned to the different regions are indicated below the map. The inverted terminal repeats (ITR) contain the origin of replication. The map is based on a figure from Fields' Virology edited by B.N. Fields et al. Lippincott Williams & Wilkins 2001 (64).
early genes are transcribed before DNA replication and code for polypeptides involved in viral genome replication and modulating host cell-virus interactions. The late regions are transcribed after viral replication and code for the structural proteins that make up the capsid. In addition, there are two delayed early genes (pIVa2 and IX) that are turned on during viral replication before the onset of late gene expression (64). The pIVa2 protein is a transcriptional activator of the major late promoter and contributes to the change from the early to late phase of the lytic cycle (65, 66). The IX protein is also a transcriptional activator, although it does not appear to be required for efficient replication (67). The early and late designations are not without exceptions and the distinction between phases is often blurred; for example, E3-11.6K, a protein involved in cell lysis, is expressed early but is amplified in the late phase of infection (68).

1.2.6 Life cycle

The adenovirus lytic life cycle (Figure 1-3) takes approximately 20 to 24 hours and can be defined into two phases: early and late. The early phase includes adsorption, penetration, uncoating, passage of the viral genome to the nucleus, and selective expression of the early genes. Early gene products modulate host cell-virus interactions and facilitate the replication of viral DNA. Upon viral DNA replication, the late phase begins with the expression of late viral genes and assembly of progeny virions, followed by virus release.

Infection begins as the adenovirus particle attaches to target cells via the distal knob domain of the fiber proteins that project from the capsid. The primary receptor for human adenoviruses also happens to be the same receptor for the coxsackie virus (69) and has therefore been termed the coxsackie and adenovirus receptor (CAR). The CAR protein, a
Figure 1-3. Adenovirus life cycle.
A cartoon of the life cycle of adenovirus in a host cell, from adsorption to release of progeny virions. Based on a figure from Fields' Virology edited by B.N. Fields et al. Lippincott Williams & Wilkins 2001 (64).
Adsorption

Plasma Membrane

Block CTL Responses

Modulate cell cycle

DNA replication

Progeny DNA

Late mRNAs

Nucleus

Endosome

Penetration

Uncoating

Virion DNA

Early mRNAs

Nuclear Pore

Block apoptosis

Modulate cell cycle

DNA replication

Progeny DNA

Late mRNAs

Cytoplasm

Cell Lysis

Assembly

Release
member of the immunoglobulin superfamily, serves as a high-affinity attachment receptor for most adenovirus serotypes, except those from species B (70) and some members of species D (71). Recent studies have identified that cluster of differentiation antigen 46 (CD46) is the cellular attachment receptor for most species B serotypes (72, 73) and species D adenovirus serotype 37 (74). CD46, an ubiquitously expressed complement regulatory protein, is also a receptor for both human herpesvirus 6 (75) and measles virus (76).

After attachment, internalization of the virus particle requires a separate interaction between the viral penton base protein and integrins \( \alpha_v \beta_3 \) and \( \alpha_v \beta_5 \) (77). Integrins are a family of heterodimeric cell surface proteins composed of \( \alpha \) and \( \beta \) subunits that bind arginine-glycine-aspartic acid (RGD) motifs. Each polypeptide III that makes up the penton base contains an RGD motif. The tropism observed for the different adenovirus serotypes is undoubtedly affected by the availability of cellular receptors for the fiber and penton proteins.

After adsorption, the penton base-integrin interaction triggers the internalization of the virion in a dynamin-dependant manner (78), indicating that the virus is taken up through clathrin-mediated-endocytosis. Internalization also requires the activation of the phosphotidylinositol-3-OH kinase (PI-3K) pathway which induces rearrangement of the actin cytoskeleton to facilitate endocytosis (79). Internalization of adenovirus triggers a sequential disassembly of virion components (80), starting with the loss of the fiber protein at the cell surface (81). Acidification of the endosome induces partial disassembly of the capsid releasing the penton base, protein IIIa, and VI. In addition, endosomal acidification triggers the escape of the internalized virus to the cytosol prior to endosome fusion with the lysosomal vacuole. Both the penton base-integrin \( \alpha_v \beta_5 \) interaction (82) and the L3-23K
adenoviral cysteine protease (83) have been implicated in viral escape from lysosomal degradation. Recent data also suggests that viral protein VI is crucial for membrane penetration following capsid disassembly (84). Inside the cytosol, the virion rapidly translocates toward the nucleus along microtubules (85). At 30 to 40 minutes post-infection, most virus particles are found in the perinuclear region. The virions dock at the nuclear pore complex (NPC) where further dismantling occurs, followed by passage of the viral DNA-protein VII complex into the nucleus through the NPC (86).

With the viral DNA in the nucleus, early gene expression begins with two main goals: 1) to provide an optimal cellular environment and the viral proteins for viral DNA replication, and 2) to protect the infected cell from the antiviral defenses of the host. Viral defense mechanisms will be discussed later (see Section 1.3 Adenoviral subversion of host defense mechanisms). The first gene to be expressed after viral DNA reaches the nucleus is E1A. E1A is controlled by a constitutively active promoter and directs the synthesis of the other early genes (87). Two major proteins are encoded by alternatively spliced E1A transcripts: 12S and 13S, named after the sedimentation coefficients of the mRNA that encode them. Although the E1A proteins are not transcription factors they activate transcription by binding to cellular regulatory proteins and transcription factors. The 13S E1A protein activates transcription through TATA motifs (88) by interacting with the TATA-binding protein (TBP), a component of the auxiliary transcription factor IID (TFIID) (89). Both E1A proteins are able to bind to the cellular retinoblastoma tumor suppressor protein (pRB) (90), a protein that binds to and inhibits the transcription factor E2F. The resulting activation of E2F not only affects viral gene expression but also induces cell cycle progression into S-phase (91). E1A also activates cell cycle progression through a second
pathway by binding to and inhibiting the highly related proteins, p300 and CREB binding protein (CBP). The CBP/p300 proteins exhibit histone acetylase activity and serve as coactivators for transcription factors, some of which are involved in inhibiting the cell from moving into S-phase. By inducing the infected cell into S-phase, the virus turns on the DNA synthesis machinery thus providing an optimal environment for viral DNA synthesis.

Viral replication begins between 5 to 8 hours after infection and requires the three proteins encoded by the E2 region: precursor terminal protein (pTP), DNA-binding protein (DBP), and DNA polymerase. In addition, three cellular proteins are required: nuclear factor (NF) I (also called CTF-1), NFII and NFIII (also called Oct-1). The pTP protein forms a complex with the viral DNA polymerase (92) and binds to the origin of replication, located within the ITR (93). Both NF1 (94) and NFII (95) stabilize the pTP-polymerase complex at the origin. Initiation begins with the covalent attachment of the nucleotide deoxycytidine monophosphate (dCMP), the first residue at the 5' end of the adenovirus DNA chain, to pTP (96). This is followed by the addition of the deoxyadenosine monophosphate (dAMP) and deoxythymidine monophosphate (dTMP) nucleotides to form the pTP-CAT primer complex (97). Chain elongation begins with the separation of the DNA polymerase from pTP and requires DBP and NFII. After DNA replication and late in the infectious cycle, the covalently attached pTP is processed into the mature form of TP by an adenovirus-encoded protease (98).

When DNA replication begins, viral mRNAs accumulate in the cytoplasm of the infected cell at the expense of host mRNAs (99). This selective export of viral over cellular mRNAs requires the E1B-55K and E4orf6 proteins which form a complex (100). Although the process in not clearly understood, one possible mechanism is that the E1B-55K and
E4orf6 complex relocalize cellular factors required for transport of mRNAs from host cell transcription centers to viral transcription centers (101). This explains the simultaneous inhibition of host and activation of viral mRNA export.

At the onset of DNA replication but before the onset of late gene expression, the two delayed early gene products, proteins IVa2 and IX are produced. The mechanism of the early to late transition is not well understood; however, Lin and Flint (102) have suggested a model based on the current knowledge. In the early phase, a cellular repressor inhibits expression from the IVa2 promoter. With the increase in template number, repression of the IVa2 promoter decreases, leading to the expression of IVa2. In turn, IVa2 binds to and induces the major late promoter (MLP).

The MLP controls the expression of five families of late mRNAs, termed L1 to L5, which code for structural and other proteins required for assembly of virions. In the cytoplasm, trimeric hexon capsomeres are assembled from monomers of polypeptide II (103) and requires the participation of the L4-100K protein (104). Penton capsomeres, composed of polypeptide III and IV, are also assembled in the cytoplasm (103). Hexons, pentons and other structural proteins then accumulate in the nucleus where assembly of progeny virions begins with the formation of the empty procapsid. A viral DNA molecule is then packaged in a polar fashion starting at the left end of the genome (105) where a cis-acting DNA packaging sequence is located (106). Encapsulation of the genome requires the packaging sequence as well as proteins IVa2 and L1-52/55K (107). The L3-23K cysteine proteinase cleaves the precursors of polypeptides VI, VII, VIII, and TP into the mature forms, resulting in the formation of a tightly sealed, stable virion.
The replication cycle ends with the release of progeny virions through the lysis of the infected cell. This process may be mediated by the E3-11.6K protein, also called adenovirus death protein (ADP) (108). Although ADP is expressed at low levels in the early phase, its expression is amplified late in infection (68). The molecular mechanism by which ADP causes cell lysis is unresolved. Another protein implicated in the release of the virus from the cell is the pro-apoptotic E4orf4 protein (109). This protein initiates cell death at the end of the infectious cycle.

1.3 Adenoviral subversion of host defense mechanisms

Viruses are totally dependant upon host cells for replication and therefore to survive must evade the host defense mechanisms including interferons (IFNs), apoptosis, natural killer (NK) cells and cytotoxic T cells (CTLs). 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, E4 genes, the late protein L4-100K, and virus-associated (VA)-RNAs all have immune modulating properties. This large repertoire of immune evasion genes is not only necessary for the survival of the virus during acute infection but undoubtedly also facilitates its persistent phase. To support viral persistence, some immune modulating proteins are expressed in low level infections, in the absence of active viral replication. The E1A proteins and VA-RNAs inhibit cellular responses to IFNs and are constitutively expressed in all cells that have been tested (64). The E3 promoter normally requires E1A for optimal activity during lytic infection. However, the E3 promoter also contains two conserved NF-κB binding sites, facilitating constitutive expression in lymphoid cells (110). In fact, E3 directed transcription increases 100-fold by
activation signals in T cells (111). Thus, adenovirus persistence in lymphoid cells would allow the expression of numerous genes necessary to prevent clearance of the virus.

1.3.1 The IFN response

IFNs are a family of inducible cytokines that play an important role in the innate immune response by inducing transcription of genes involved in antiviral, immunomodulatory and antiproliferative activities (112). The type I IFNs are also known as the viral IFNs and include IFN-α and IFN-β. IFN-α and -β production are often induced early in infected cells, in part by viral double stranded RNA (dsRNA). Adenovirus likely produces dsRNA as a result of symmetric transcription from opposing promoters on the viral chromosome (113). Upon viral infection IFN-α/β bind to specific cell surface IFN receptors and results in the activation of receptor-associated Janus family of tyrosine kinase (JAK) enzymes. Activated JAKs subsequently recruit and phosphorylate the cytoplasmic signal transducers and activators of transcription (STAT) factors. Heterodimers of STAT-1/2 interact with p48 to form a transcription-activating complex known as IFN-stimulated gene factor 3 (ISGF-3). ISGF-3 binds to IFN-stimulated response elements (ISRE) found in IFN-inducible genes. Many IFN-induced genes encode proteins with antiviral effects including 2′-5′ oligoadenylate synthetase (OAS) and the double-stranded RNA-activated protein kinase (PKR) (114). OAS upregulates RNase L to degrade RNA while PKR arrests protein translation by phosphorylating the eIF-2α subunit of the translation initiation complex. Both OAS and PKR are activated by dsRNA. The IFN response forms an early line of defense against viral infection by providing unique and complementary antiviral activities.
1.3.2 Inhibition of the IFN response

Adenoviruses target the IFN response at two levels: the induction phase by blocking the JAK/STAT signaling pathway with E1A and at the execution phase by preventing the activation of PKR with VA-RNAs. As mentioned earlier, E1A is able to bind to and inhibit the CBP/p300 protein. CBP/p300 is a coactivator of STATs; therefore, E1A may compete with STATs for CBP/p300 binding. In addition, E1A is able to block the activation of IFN-response genes by binding to STAT1 directly and inhibiting its activity (115).

VA-RNAs, which are GC-rich and approximately 160bp in length (116), are abundantly expressed late in the viral infection by RNA polymerase III (64). Their stable secondary structure allows them to bind to and inhibit PKR thus allowing continued viral translation (117). Ad2 and Ad5 encode 2 species of VA-RNAs, termed VA-RNA_I and VA-RNA_{II}, however, only VA-RNA_I has been shown to interact with PKR.

1.3.3 Apoptotic pathways

In order to replicate, DNA viruses such as adenovirus must activate the host cell replication machinery. However, in the absence of external signals, the host cell recognizes the initiation of replication as abnormal and triggers apoptotic pathways thereby killing the cell and impeding viral replication. As eukaryotic cells evolved, viruses also evolved and acquired mechanisms aimed to inhibit or avoid apoptosis of host cells. In order to understand how viruses avoid host cell apoptosis, it is essential to understand the many pathways which contribute to apoptosis.

Apoptosis, sometimes referred to as programmed cell death, is a physiological process for killing cells that is critical in the normal development and function of
multicellular organisms. Abnormalities of the apoptotic process can contribute to pathological conditions such as autoimmune disease and cancer. Induction of apoptosis occurs through multiple independent pathways that converge upon the activation of a family of cysteine proteases (caspases) that cleave a number of cellular protein substrates at aspartate residues leading to the apoptotic phenotype (118). Morphological changes that occur during apoptosis include cell shrinkage, chromatin condensation and cellular fragmentation into apoptotic bodies (119). At the molecular level there is a rise in cytosolic Ca\(^{2+}\) (120), internucleosomal DNA cleavage (121), loss of mitochondrial membrane potential (122) and randomization of the distribution of phosphatidylserine (PS) between the inner and outer leaflets of the plasma membrane (123).

There are two principal pathways leading to the activation of caspases (Figure 1-4). One pathway, termed the “intrinsic” pathway, is initiated from events within the cell. A major gatekeeper of the intrinsic apoptosis program is the tumor suppressor p53 (124). The p53 protein is a transcription factor that is activated in response to a variety of stress conditions including UV irradiation, growth factor deprivation, abnormal mitosis or viral infection. Activation of p53 induces the production of pro-apoptotic proteins, such as Bax (125). Translocation of Bax to the mitochondria leads to the release of cytochrome c from mitochondria to the cytosol (126) where it binds to APAF-1 (apoptotic protease activating factor-1) and pro-caspase-9 to form a complex termed the apoptosome (127). Once this complex forms, caspase-9 is activated and continues the caspase cascade leading to cell death. Pro- and anti-apoptotic members of the Bcl-2 family regulate this pathway (Table 1-2) (128). Although the majority of Bcl-2 family members are found at both the mitochondria and ER, their primary role was thought to regulate the release of cytochrome c from the
Figure 1-4. Summary of host apoptotic responses and adenovirus countermeasures.
Intrinsic and extrinsic apoptotic pathways are initiated in virally-infected cells in order to prevent viral replication and therefore the spread of the virus. Viral DNA replication requires forced entry into S-phase which provokes intrinsic apoptosis via activation of p53. The p53 protein induces expression of pro-apoptotic proteins, such as Bax which promotes Ca$^{2+}$ release from the ER and cytochrome c release from mitochondria. This results in activation of the caspase cascade and leads to cell death. Extrinsic apoptosis is induced by CTLs that recognize viral peptides presented on the cell surface by MHC class I molecules. CTLs cause cell death through ligand-death receptor interactions and through the release of perforin and granzymes. Adenovirus expresses several proteins (shown in red and bold) that block these apoptotic pathways.
VIRUS

E1B-19K
L4-100K
Fas-L
TNF
Fas-L
TNF-R
FLICE
Bax
Caspase-8
Apoptosome
E1B-55K
E4orf6
p53
Bax, Fas, etc.
pro-apoptotic genes

Death Receptors

E3-RID (+E3-6.7K)
E3-14.7K

Viral DNA Replication

Mitochondria

Cytochrome C release

E3-gp19K

Plasma Membrane

Effectors Caspases

Caspases-3, -6, -7

Apoptosis

Effector Caspases

Caspases-3, -6, -7

NK

Perforin

TNT

tBid

Proteosome

Peptides

ER

MHC I

Nucleus

Viral DNA Replication
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<th>Bcl-2 Family Members</th>
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</tr>
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<tr>
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<td>ER?</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>ER?</td>
</tr>
<tr>
<td>Boo/Diva</td>
<td>ER?</td>
</tr>
<tr>
<td><strong>Pro-apoptotic Bcl-2 proteins</strong></td>
<td></td>
</tr>
<tr>
<td>Bax</td>
<td>ER, cytosol</td>
</tr>
<tr>
<td>Bak</td>
<td>ER</td>
</tr>
<tr>
<td>Bim</td>
<td>Cytoskeleton</td>
</tr>
<tr>
<td>Bad</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Bik</td>
<td>ER, cytosol</td>
</tr>
<tr>
<td>Bmf</td>
<td>Cytoskeleton</td>
</tr>
<tr>
<td>Bid</td>
<td>Cytosol, ER</td>
</tr>
<tr>
<td>Spike</td>
<td>ER</td>
</tr>
</tbody>
</table>

Table 1-2. Anti- and pro-apoptotic members of the Bcl-2 family.
To be designated as a member of the Bcl-2 family, a protein must contain at least one of the four conserved Bcl-2 homology (BH) domains and have a demonstrable effect on apoptosis. In addition to locations listed in the table, all the Bcl-2 family members localize to mitochondria. Table adapted from Annis et al. (129).
mitochondria. Recent studies have pointed to an equally important role at the ER, where Bcl-2 proteins control the release of Ca^{2+} stores (129). ER to mitochondria Ca^{2+} waves can trigger the opening of the mitochondrial permeability transition pore, thereby releasing cytochrome c and stimulating apoptosis (130).

The second pathway, referred to as the "extrinsic" pathway, is triggered from events outside the cell. The extrinsic apoptosis program utilizes a subfamily of tumour necrosis factor (TNF) receptors, called death receptors (131), that are able to signal cell death via an intracellular death domain (DD) (132). Apoptosis is induced by ligation of the death receptors by ligands of the TNF family. The Fas ligand (FasL) binds to Fas (also known as APO-1 or CD95); TNF binds to its receptor TNF-R; and the TNF-related apoptosis-inducing ligand (TRAIL) binds to its receptors TRAIL-R1 and TRAIL-R2. Fas recruits the adapter protein Fas-associated death domain (FADD) via a DD-DD interaction. FADD, in turn, binds procaspase-8 via a death effector domain (DED) resulting in the aggregation and self-activation of caspase-8. TNF-R can also recruit caspase-8 via the TNF-R-associated death domain (TRADD) protein and FADD. Ligation of TNF-R also signals a pro-survival pathway through the activation of the nuclear factor kappa beta (NF-κB) transcription factor via the DD-containing kinase receptor interacting protein (RIP) (133) and TNF receptor-associated factor (TRAF) 2 (134). NF-κB induces the transcription of genes involved in inflammation, immune cell activation and pro-survival, including the cellular inhibitors of apoptosis (cIAPs) (135). Similar to Fas, the TRAIL receptors lead to activation of caspase-8 through the FADD adapter protein (136). In addition, TRAIL receptors are able to activate NF-κB (137) potentially through a TRAF2-RIP-dependent signaling cascade (138).
Intrinsic and extrinsic apoptotic pathways are not completely independent as some crosstalk does occur between the two pathways. One target of active caspase-8 is the pro-apoptotic Bcl-2 family member Bid. Once cleaved, truncated Bid (tBid) translocates to the mitochondria and promotes release of cytochrome c (139). The p53 protein also induces production of proteins involved in the extrinsic pathway, including Fas (140). The multitude of apoptotic pathways generated in response to viral infection provides a formidable challenge for viruses to overcome if they are to successfully replicate.

1.3.4 E1A induces intrinsic apoptosis pathways

DNA viruses typically infect resting cells and induce cell cycle progression into S-phase. This phase provides an optimal environment for viral replication by providing host nucleotide pools and proteins involved in DNA synthesis. As mentioned earlier, adenovirus enters S-phase through the actions of the E1A proteins. E1A binds to pRB, inducing the release of the transcription factor E2F. However, forced entry into S-phase by E2F activation provokes apoptosis via the cellular tumor suppressor p53 (141). E2F induces production of p14 (also called ARF for ADP-ribosylation factor 1), which inhibits the ubiquitin ligase activity of the human double minute 2 (HDM2) protein that normally promotes rapid degradation of p53 (140). A linear series of events leads to the induction of apoptosis starting with E1A as follows:

E1A → pRB → E2F → p14ARF → HDM2 → p53 → Apoptosis
E1A can also induce apoptosis in a p53-independent manner (142) by transactivation of the E4 transcription unit and synthesis of the pro-apoptotic E4orf4 protein (109). E1A is also responsible for sensitizing infected cells to extrinsic apoptotic stimuli, such as TNF-α (143), FasL (144), or TRAIL (145).

1.3.5 Inhibition of intrinsic apoptosis pathways

The E1A-induced entry into S-phase triggers the cellular machinery for programmed cell death. Therefore, to replicate efficiently adenoviruses have evolved mechanisms to suppress intrinsic apoptotic pathways. The E1B and E4 transcription units encode three proteins that inhibit p53-mediated apoptosis: E1B-55K, E1B-19K, and E4orf6. E1B-55K binds to p53 directly and although it increases p53 affinity for p53-dependent promoters, it represses transcription (146). The E4orf6 protein can also bind to p53 and block transcriptional activation of p53 target genes (147). As discussed previously, E1B-55K and E4orf6 form a complex that late in infection modulate nuclear export of viral and cellular mRNA. In the early phase of infection, the prominent activity of this complex is to inhibit apoptosis by binding to and inducing the rapid degradation of p53 (148).

E1B-19K is a functional homologue of the cellular anti-apoptotic Bcl-2 protein. Similar to Bcl-2, it also associates with pro-apoptotic Bcl-2 family members, Bak (149) and Bik (150); and inhibits apoptotic induction by these proteins. E1B-19K also forms heterodimers with Bax, preventing its translocation to the mitochondria (151) and precluding activation of caspase-9.
1.3.6 Extrinsic apoptosis induced by the immune system

Many effector cells of the immune system, like activated CTLs, macrophages, and NK cells, produce pro-apoptotic cytokines, such as TNF-α, FasL, and TRAIL that are able to bind to death receptors. CTLs are able to induce apoptosis in targets such as virally-infected cells by recognizing viral antigens presented on the cell surface. A fraction of cytosolic proteins are continually degraded by proteasomes to generate a representative sampling of peptides. These are then translocated across the ER membrane by the transporter associated with antigen processing (TAP) (152). Within the ER lumen, these peptides bind to major histocompatibility complex (MHC) class I. Peptide-loaded MHC class I molecules are then transported to the cell surface. Upon recognition of a viral (foreign) peptide-MHC class I antigen complex on the surface of virally-infected cells, CTLs are able to induce apoptosis through ligand-receptor interactions using the FasL-Fas pathway and through the release of cytotoxic granules into the target (153). The principle players in granule-mediated cell death are perforin, a pore-forming protein, and granzymes, a family of serine proteases. Perforin forms pores in the infected cell allowing the entry of granzymes, such as granzyme B (154). Granzyme B, in turn, is able to activate most, if not all, the pro-caspases (155) resulting in massive amplification of the apoptotic pathway.

NK cells, a sub-population of lymphocytes, mediate innate immunity to pathogen-infected cells through direct cell-mediated killing. NK cells typically target cells that do not express MHC class I. Like CTLs, NK cells have the ability to eliminate their target by two principal mechanisms (156). The first is through the release of cytotoxic granules consisting of perforin and granzymes. The second mechanism occurs through death receptor-induced apoptosis. In addition to FasL, NK cells also express TRAIL and TNF-α.
1.3.7 The E3 region and immune evasion

The E3 region of human adenoviruses differs significantly between species, with only the RIDα, RIDβ, and 14.7K proteins conserved in all species (157). References to the E3 region in this thesis will refer to that of species C because most of the functional data reported has been obtained for the proteins of this species. Although not required for virus growth in tissue culture, the E3 region is necessary for efficient evasion of the immune response (158). It codes for seven proteins that have been identified during viral infection, five of which are involved in the subversion of the host immune response (Figure 1-5). E3-14.7K is a non-membrane protein localized in the cytosol that inhibits apoptosis through the TNF-R and Fas death receptors possibly by binding to and inhibiting caspase-8 (159, 160). Using a yeast two-hybrid screen, E3-14.7K was found to interact with three additional cellular proteins named FIPs (14.7K interacting proteins). FIP-1 is a small GTPase whose function is unknown (161). FIP-2 blocks the TNF-α protective effects of 14.7K, however, expression of FIP-2 alone is unable to induce apoptosis (162). Lastly, FIP-3 (also called NEMO or IKKγ) is a pro-apoptotic protein that binds to RIP an inhibitor of NF-κB kinases—components in the TNF-induced activation pathway of NK-κB (163). E3-10.4K and E3-14.5K, also known as Receptor Internalization and Degradation (RID) proteins α and β respectively, form a complex that localizes to the plasma membrane and down-regulates the surface level of Fas (164), TRAIL-R1 (165), TNF-R1 (166), and the epidermal growth factor receptor (EGFR) (167). This complex also prevents the TNF-α-induced release of arachidonic acid (168). In conjunction with the E3-6.7K protein, the RID complex is also
Figure 1-5. E3 region of immunosubversive genes.

Schematic illustration of the mRNA transcripts (labeled a-i) produced from the E3 region of human adenovirus serotype 2. Arrows indicate the splice structure of the mRNAs; solid parts are exons, dashed parts are introns, and the thickness implies relative abundance. The stripped bars above the arrows indicate proteins known to be expressed. Functions assigned to the different proteins are indicated below the transcription map. Figure reproduced from Sparer et al. (169).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp19K</td>
<td>Blocks surface expression of MHC class I</td>
</tr>
<tr>
<td>14.7K</td>
<td>Prevents apoptosis induced by TNF-α</td>
</tr>
<tr>
<td>10.4K (RIDα)</td>
<td>Form complex that down-regulates Fas, TNF-R1, EGFR, and TRAIL-R1. Also down-regulates TRAIL-R2 in conjunction with E3-6.7K</td>
</tr>
<tr>
<td>14.5K (RIDβ)</td>
<td></td>
</tr>
<tr>
<td>11.6K (ADP)</td>
<td>Pro-apoptotic, plays role in virus release</td>
</tr>
<tr>
<td>6.7K</td>
<td>Inhibits death receptor-induced apoptosis and ER Ca$^{2+}$ release</td>
</tr>
<tr>
<td>12.5K</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
able to down-regulate TRAIL-R2 (see Section 1.4.3 E3-6.7K and the RID complex). E3-gp19K, an ER localized membrane glycoprotein, binds to MHC class I and prevents its transport to the cell surface (170) thus blocking the killing of infected cells by CTLs (171). Lastly, there is a non-E3 protein able to inhibit CTL killing. The late protein L4-100K is able inhibit lymphocyte granule-mediated apoptosis by binding to and inhibiting granzyme B (Figure 1-4) (172).

1.4 E3-6.7K

1.4.1 Introduction to E3-6.7K

The E3-6.7K protein is well conserved between Ad2 and Ad5 of species C adenoviruses. There is also a less well conserved homologue in species B, termed E3-16K (173). The E3-6.7K protein used exclusively in this thesis was from Ad2, which at 61 amino acids in length is the smallest protein produced from the E3 region. The E3-6.7K protein is coded bicistronically by the same transcripts (a and c) that code for the E3-gp19K protein (Figure 1-5) (174). Due to an inefficient Kozak consensus at the start site of the E3-6.7K open reading frame, the expression of the E3-6.7K protein is lower than that of the E3-gp19K protein (175). The protein exists in two forms: an unglycosylated form with a molecular weight of 6.7 kDa and a glycosylated form with a molecular weight of 14 kDa. The glycosylated form is modified exclusively with Asn-linked, high mannose oligosaccharides, indicating that it is retained in the ER (176). This was confirmed with immunofluorescence studies (176); however, as will be discussed later at least a fraction of E3-6.7K reaches the
plasma membrane. As with all the E3 proteins, E3-6.7K is not required for replication in cultured cells.

1.4.2 The unique topology of E3-6.7K

The E3-6.7K protein has been shown to be an integral membrane protein (176). Interestingly, all the membrane proteins encoded by the adenovirus genome are exclusively located in the E3 region, where 5 of the 7 encoded proteins are membrane proteins. Sequence analysis of E3-6.7K using the Kyte-Doolittle algorithm (177), predicts a large hydrophobic domain, suggesting a single transmembrane region (Figure 1-6A). As a glycoprotein, it was originally presumed to be a type III transmembrane protein (N-luminal, C-cytoplasmic) because all three possible Asn-linked glycosylation sites are found in the N-terminal region of the protein (Figure 1-6A) (178). Furthermore, the charged residues are all basic and at the C-terminus of the protein and according to the “positive inside” rule, the more positive end of the protein is generally cytosolic (179). In contrast, recent investigations have demonstrated not one but three topologies for the E3-6.7K protein in a cell-free system supplemented with microsomes (Figure 1-6B) (180). The first and already postulated topology has a type III orientation (N-luminal, C-cytoplasmic; termed \( \text{N}^{\text{m}}\text{E3-6.7K} \)). The second form adopts the opposite type II orientation (N- cytoplasmic, C-luminal; termed \( \text{C}^{\text{m}}\text{E3-6.7K} \)) and finally there is the possibility of a fully translocated form (N- and C-luminal; termed \( \text{NC}^{\text{E3-6.7K}} \)). The topologies of E3-6.7K will be discussed in greater detail in chapter 5.
Figure 1-6. Sequence analysis and proposed topologies of E3-6.7K.
(A) Sequence analysis of E3-6.7K. The 61 amino acid protein has three potential Asn-linked glycosylation sites (Δ) and 6 positively charged residues (+). A Kyte-Doolittle plot illustrates the hydrophobicity of this protein. (B) Proposed topologies of E3-6.7K. The E3-6.7K adopts three different topologies in the membrane: a type III orientation (N-luminal/C-cytoplasmic, termed \[^{Ntm}E3-6.7K\]) and a type II orientation (N-cytoplasmic/C-luminal, termed \[^{Ctm}E3-6.7K\]), and a fully translocated form (N and C termini are both translocated, termed \[^{NC}E3-6.7K\]).
1.4.3 E3-6.7K and the RID complex

The first demonstrated function for E3-6.7K was that in conjunction with the RID complex (composed of RIDα and RIDβ) E3-6.7K is able to down-regulate TRAIL receptors. Although localized primarily in the ER (176), a small fraction of E3-6.7K reaches the plasma membrane where it can interact with RIDβ (181). E3-6.7K was shown to be required for RID-mediated down-regulation of TRAIL-R2 (181, 182). With regard to TRAIL-R1, the requirement for E3-6.7K is less clear. One group showed that E3-6.7K is necessary for optimal down-regulation of TRAIL-R1 (181), whereas another group showed that the down-regulation of the receptor is independent of E3-6.7K (165). The RID complex also down-regulates Fas (164), TNF-R1 (166) and EGFR (167); however, E3-6.7K was not shown to be required for any of these effects.

1.4.4 E3-6.7K inhibits apoptosis and ER Ca$^{2+}$ efflux

The E3-6.7K protein has been shown to have additional protective roles independent of other viral proteins. It protects transfected cells against death receptor-mediated apoptosis induced through Fas, TNF receptor, or TRAIL receptors (183). It also reduced the TNF-induced release of arachidonic acid and the presence of E3-6.7K resulted in a reduced inflammatory response in vivo (184). Cells expressing E3-6.7K also had reduced levels of apoptosis after treatment with thapsigargin (183), 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 role of the ER and Ca$^{2+}$ in apoptosis is
becoming increasingly more recognized. By altering Ca\(^{2+}\) homeostasis at the ER, E3-6.7K may act as a general repressor of apoptosis.

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\(^{2+}\) (Table 1-3). 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 (KSHV) K7 protein. The 2B protein is a small (97-99 amino acids) transmembrane protein, localized to the ER and Golgi membranes where it appears to form pores as homomultimers (185). These pores reduce mobilizable Ca\(^{2+}\) stores available to induce apoptosis (186). The K7 protein is a small (126 amino acids) transmembrane protein localized to the mitochondria and to a lesser extent the ER (187). K7 alters the kinetics and amplitude of cellular Ca\(^{2+}\) fluxes and inhibits apoptosis induced by thapsigargin, through an interaction with the cellular Ca\(^{2+}\)-modulating cyclophilin ligand (CAML) (188).

### 1.5 Project rationale and general approach

The focus of this thesis is to add to the basic knowledge of the structure and function of the adenovirus E3-6.7K protein. Experiments were performed in order to examine the mechanism of action as well as the membrane topology of E3-6.7K. This thesis provides data to support three hypotheses, each detailed in a separate chapter. Two chapters are devoted to first looking at the function of E3-6.7K. It has been proposed that E3-6.7K is able to inhibit apoptosis by altering ER Ca\(^{2+}\) homeostasis (183), although how this is done is unknown. Therefore, the hypothesis of Chapter 3 is that E3-6.7K interacts with the cellular protein CAML, a known modulator of cellular Ca\(^{2+}\). This is followed by Chapter 4 in which the
<table>
<thead>
<tr>
<th>Virus</th>
<th>Protein</th>
<th>Localization</th>
<th>Effect on cell</th>
<th>Mechanism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>E3-6.7K</td>
<td>ER, PM</td>
<td>Inhibits apoptosis and ER Ca(^{2+}) flux</td>
<td>Unknown</td>
<td>(183)</td>
</tr>
<tr>
<td>Coxsackievirus</td>
<td>2B</td>
<td>ER, G</td>
<td>Inhibits apoptosis and reduces ER Ca(^{2+}) content</td>
<td>Forms pores in ER membranes draining Ca(^{2+}) stores</td>
<td>(185, 186)</td>
</tr>
<tr>
<td>KSHV</td>
<td>K7</td>
<td>M, ER</td>
<td>Inhibits apoptosis and increases cytosolic Ca(^{2+}) response</td>
<td>Interacts with CAML, an ER Ca(^{2+}) modulator</td>
<td>(188)</td>
</tr>
<tr>
<td>HTLV-1</td>
<td>p12'</td>
<td>ER, G</td>
<td>Releases Ca(^{2+}) from ER to activate infected cell and promote efficient viral infection</td>
<td>Unknown</td>
<td>(189, 190)</td>
</tr>
<tr>
<td>HBV</td>
<td>HBx</td>
<td>M, N</td>
<td>Activates cellular Ca(^{2+}) signaling pathways required for efficient viral replication</td>
<td>Unknown</td>
<td>(191, 192)</td>
</tr>
<tr>
<td>HCV</td>
<td>core</td>
<td>ER</td>
<td>Induce ER Ca(^{2+}) depletion by effecting uptake of Ca(^{2+}) back into ER. Triggers apoptosis</td>
<td>Unknown</td>
<td>(193)</td>
</tr>
</tbody>
</table>

**Table 1-3. Viral proteins that modulate cellular Ca\(^{2+}\).**

Very few viral proteins modulate cellular Ca\(^{2+}\) levels. Of these only three are known to inhibit apoptosis (bold and green). Abbreviations: KSHV, Kaposi’s sarcoma-related herpes virus; HTLV-1, human T leukemia virus-1; HBV, hepatitis B virus; HCV, hepatitis C virus; ER, endoplasmic reticulum; PM, plasma membrane; G, Golgi apparatus; M, mitochondria; N, nucleus. Underlined indicates predominant location.
hypothesis is that an interaction with CAML is required for E3-6.7K to inhibit apoptosis and ER Ca\(^{2+}\) efflux. The focus then changes to the structure of E3-6.7K. A previous report has suggested that E3-6.7K can adopt three alternate topologies in a cell-free system (180). The hypothesis of Chapter 5 is that E3-6.7K forms multiple topologies in cells. Finally, Chapter 6 will discuss the general conclusions and propose future directions.
Chapter 2: Materials and Methods

2.1 Cell lines and culture conditions

The human T cell leukemia line Jurkat, clone E6-1 and the human cervical carcinoma cell line, HeLa, were originally obtained from American Type Culture Collection (ATCC) (Manassas, VA). Jurkat cells were maintained in RPMI (Roswell Park Memorial Institute) 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate and 20 mM N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid (HEPES). HeLa cells were maintained in DMEM (Dulbecco’s Modified Eagle Medium) (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 20 mM HEPES.

2.2 Sequence analysis

All sequencing was performed by the Nucleic Acid Protein Service Unit at the University of British Columbia (Vancouver, BC). Both strands of plasmid inserts were sequenced to ensure accuracy.

2.3 Polymerase chain reaction

Polymerase chain reactions (PCR) were performed with either Platinum Taq or Platinum Pfx (Invitrogen) and were conducted in a Whatman Biometra T3 Thermocycler. Platinum Taq was used for general PCR while Platinum Pfx was used when high fidelity was required, such as when the PCR product was used for cloning. All reactions used 20 pmol of
each primer and a final concentration of 1.5 M Mg\(^{2+}\). The PCR program for Taq reactions consisted of 94°C for 4 min, then 35 cycles of 94°C for 30 s, a specified annealing temperature (AT) for 30 s, and 72°C for 1 min, followed by 1 cycle of a 7 min extension at 72°C. The program for Pfx reactions consisted of 94°C for 2 min, then 35 cycles of 94°C for 30 s, a specified AT for 30 s, and 68°C for 1 min. PCR fragments were visualized on an ethidium bromide stained 1% agarose gel.

2.4 Site-directed mutagenesis

Site-directed mutations were made using double stranded plasmid DNA templates as previously described (194). Briefly, the procedure uses two oligonucleotide primers containing the desired mutation and a double stranded DNA vector with the insert of interest. The primers, each complementary to opposite strands of the vector, are extended by PCR using Pfu DNA polymerase. The PCR reaction consisted of 95°C for 30 s, then 16 cycles of 95°C for 30 s, a specified AT for 1 min, and 68°C for 10 min. Following amplification, the product was treated with 10 U of the endonuclease DpnI for 1 h to digest the methylated parental template DNA. The newly synthesized vector DNA containing the desired mutation was transformed into chemically-competent \textit{Escherichia coli} cells (Invitrogen).
2.5 Construction of plasmids

2.5.1 Vectors, primers and sequencing

The yeast two-hybrid studies used the Clontech (Mountain View, CA) yeast expression vectors pGBK7 (pBD) and pGADT7 (pAD). These vectors require the insert to be cloned in-frame with the GAL4-DNA-Binding Domain (BD) and the GAL4-Activation Domain (AD), respectively (Figure 2-1). The remaining vectors are all mammalian expression vectors. The co-localization studies used pcDNA3.1+ (pcDNA) (Invitrogen) and pEYFP-N1 (pYFP) (Clontech) (Figure 2-2 and Figure 2-3, respectively). Finally, the pIRES-hrGFP-1a (piGFP) (Stratagene, La Jolla, CA) was used in the topology, apoptosis and Ca\(^{2+}\) flux studies (Figure 2-4). In addition, it should be noted that to reduce the size of the names of some the plasmids created, E3-6.7K is sometimes shortened to simply 6.7K.

A list of the linkers and primers used for PCR during the construction of many of the plasmids can be found in Table 2-1. To ensure accuracy of all the created constructs, both strands of plasmid inserts were sequenced.

2.5.2 E3-6.7K constructs

The original source of E3-6.7K DNA was from the EcoRI D fragment of the E3 region of Ad2, a kind gift from W.S.M. Wold (St. Louis University, St. Louis, MO). As previously described, the E3-6.7K gene was amplified from this EcoRI D fragment and cloned into the BPV expression vector pBCMGeno (195) to generate pBCMGeno/6.7K (183); into pEYFP (Clontech) to produce pYFP-6.7K (184); into pIRESpuro2 (Clontech) to
Figure 2-1. Yeast two-hybrid vectors pGBKT7 (pBD) and pGADT7 (pAD).
(A) The pGBKT7 vector (Clontech) expresses a gene of interest cloned into the MCS as a protein fused to amino acids 1-147 of the GAL4 DNA binding domain (DNA-BD). The vector contains the T7 promoter, a c-Myc epitope tag and the TRP1 nutritional marker for selection in yeast. (B) The pGADT7 vector (Clontech) expresses a gene of interest cloned into the MCS as a protein fused to amino acids 768-881 of the GAL4 activation domain (AD). The vector contains the T7 promoter, an HA epitope tag and the LEU2 nutritional marker for selection in yeast. The pGBKT7 and pGADT7 vectors are referred to as pBD and pAD, respectively.
Multiple Cloning Site (MCS)

<table>
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<tr>
<th>Enzyme</th>
<th>Restriction Site</th>
</tr>
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<td>Nhe I</td>
<td>CTGGCTAAACT AGAGAACCCA CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG</td>
</tr>
<tr>
<td>Pme I</td>
<td>3' end of hCMV</td>
</tr>
<tr>
<td>Hind III</td>
<td>T7 promoter primer binding site</td>
</tr>
<tr>
<td>Asp 718 I</td>
<td>BGH polyA</td>
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<tr>
<td>Kpn I</td>
<td>f1 ori</td>
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<tr>
<td>BamH I</td>
<td>SV40 ori</td>
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<td>Nco I</td>
<td>Ampicillin</td>
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<td>EcoR V</td>
<td>Neomycin</td>
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<td>Xho I</td>
<td>Neomycin resistance ORF</td>
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<tr>
<td>Xba I</td>
<td>pUC origin</td>
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<tr>
<td>Dra II</td>
<td>Ampicillin resistance ORF</td>
</tr>
</tbody>
</table>

Figure 2-2. The mammalian expression vector pcDNA3.1.
The pcDNA3.1(+) vector (pcDNA) (Invitrogen) is a mammalian expression vector.
**CMV promoter:** 1-589  
**EYFP ORF:** 613-1410  
**multiple cloning site:** 1330-1417  
**SV40 polyA:** 1550-1600  
**f1 origin:** 1647-2102  
**kanamycin resistance ORF:** 2627-3421  
**pUC origin:** 4006-4649

**Multiple Cloning Site (MCS)**

**EcoO109 I**  
**Nhe I**  
**EcoR II**  
**Age I**  
**BsrG I**  
**MCS**  
**Afl II**

**Figure 2-3. The mammalian YFP fusion expression vector pEYFP-N1 (pYFP).**  
The vector pEYFP-C1 (pYFP) encodes an enhanced yellow-green variant of the *Aequorea victoria* green fluorescent protein (GFP). A gene of interest cloned into the MCS will be expressed as a protein fused to the C-terminus of YFP.
CMV promoter: 1-602  
multiple cloning site: 651-715  
3x FLAG tag: 716-787  
internal ribosome entry site: 823-1397  
hrGFP ORF: 1407-2123  
SV40 polyA: 2188-2571  
f1 origin: 2709-3015  
LoxP sequence: 3178-3211  
ampicillin resistance ORF: 3256-4113  
pUC origin: 4260-4927

Multiple Cloning Site (MCS)  
(sequence shown 651-727)

Sph I  Sal I  Xho I  start of FLAG tag

. . . GC ATG CGT CGA CTC GAG GAC TAC AAG GAT

Figure 2-4. The mammalian GFP expression vector pIRES-hrGFP-1a (piGFP).  
The pIRES-hrGFP-1a vector (piGFP) (Stratagene) contains a dicistronic expression cassette where the MCS is followed by an internal ribosomal entry site (IRES) link to a humanized recombinant GFP (hrGFP) coding sequence. A gene of interest subcloned into the MCS may be fused to three contiguous copies of the FLAG epitope (DYKDDDDK).
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5'→3')</th>
<th>Encoded RE</th>
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<tr>
<td>EcoRI-Sall-link</td>
<td>P-TCGACGAATTTC</td>
<td>EcoRI</td>
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<tr>
<td>CT-E3-6.7K-F</td>
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<td>EcoRI</td>
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<tr>
<td>X-E3-6.7K-R</td>
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<td>XhoI</td>
</tr>
<tr>
<td>6.7K-Del-F</td>
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<td>-</td>
</tr>
<tr>
<td>6.7K-Del-R</td>
<td>CATGCGAATTCTCCCCCTTTGATGGATTCGCCCC</td>
<td>-</td>
</tr>
<tr>
<td>mCAML-F</td>
<td>GGCAGAATTCAAGGATGACCTAGACCCTAGAG</td>
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<td>mCAML-R</td>
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<tr>
<td>NT-mCAML-R</td>
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<td>XhoI</td>
</tr>
<tr>
<td>CAML-F</td>
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<td>NdeI</td>
</tr>
<tr>
<td>CAML-R</td>
<td>GCGGATCCTCATGGTACTTCAGGCCCCCAATAATCC</td>
<td>BamHI</td>
</tr>
<tr>
<td>NT-CAML-R</td>
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<td>BamHI</td>
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<td>HA-CAML-F</td>
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<tr>
<td>TACI-R</td>
<td>ACCTCGAGTTATGCACTTGCGGCCCC</td>
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<td>-</td>
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<tr>
<td>TACI-Mut-R</td>
<td>CACAAAGTTGGGCTCAGGGGGGTCCCAACGCGAGCTGGC</td>
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</tr>
</tbody>
</table>

Table 2-1. Primers and linkers used for cloning and general PCR.
Bold nucleotides correspond to encoded restriction enzyme (RE) sequence. Phosphorylation of the 5' nucleotide is indicated by a “P”. F = Forward primer; R = Reverse primer.
generate pIP-6.7K (183); and into pFLAG a BlueScript vector (Stratagene) that contains a FLAG tag upstream of the insert to produce pFLAG-6.7K (180).

Due to a lack of convenient restriction enzyme sites, pBCMGneo/6.7K required the addition of a new restriction site using a linker. The phosphorylated linker EcoRI-Sall-link, which upon self-annealing provides Sall overhangs bracketing an EcoRI restriction site, was ligated to Sall-digested and calf intestine alkaline phosphatase-treated pBCMGneo/6.7K. The DNA encoding E3-6.7K was excised from the resultant plasmid by digestion with EcoRI and XhoI and subcloned into pAD digested with EcoRI and XhoI and pBD digested with EcoRI and Sall, generating pAD-6.7K and pBD-6.7K. Sall and XhoI restriction sites are complementary although both sites are lost after ligation.

The coding region of the C-terminus of E3-6.7K (CT-E3-6.7K, residues 35-61) was amplified from pBCMGneo/6.7K by PCR (Pfx, AT=50°C) using the EcoRI containing sense primer CT-E3-6.7K-F and the XhoI containing antisense primer X-E3-6.7K-R. After digestion with EcoRI and XhoI, the amplified CT-E3-6.7K DNA was subcloned in-frame with GAL4-DNA-BD into pBD digested with EcoRI and Sall, generating pBD-CT-6.7K.

E3-6.7K DNA was liberated from pIP-6.7K by digestion with ScaI and EcoRI, and subcloned into Smal- and EcoRI-digested piGFP, generating piGFP/6.7K. Both ScaI and Smal produce blunt ends.

For the topology studies, E3-6.7K was required to be FLAG-tagged separately at the N- and C-terminus. DNA coding for E3-6.7K with a FLAG tag at the N-terminus was excised from the bacterial expression vector pFLAG-6.7K with XhoI and ligated into pcDNA digested with the same enzyme to generate pcDNA/FLAG-6.7K. The vector piGFP/6.7K conveniently placed FLAG tag DNA at the 3'-end of E3-6.7K, however, it was not in-frame.
and a stop codon prevented transcription of the tag. To correct for this, a deletion was introduced into the stop codon by site directed mutagenesis (AT = 60°C) using the sense prime 6.7K-Del-F and the antisense primer 6.7K-Del-R, thus eliminating the stop codon and placing the FLAG-tag in-frame. This produced the plasmid, piGFP/6.7K-FLAG.

2.5.3 Mouse CAML constructs

The mRNA sequence of mouse CAML (mCAML) was obtained from the National Center for Biotechnology Information (NCBI) (accession # NM_007596) and used to perform a BLAST (basic local alignment search tool) search of the mouse expressed sequence tag (EST) database. A match was found in a mouse embryo EST library (accession # AI894022; IMAGE clone # 427258). The EST clone (Incyte Genomics, Wilmington, DE), contained in the pT7T3D vector (Pharmacia, Peapack, NJ), was sequenced and the full length clone was confirmed. The coding region of mouse CAML was amplified from pT7T3D/mCAML by PCR (Pfx, AT = 58°C) using the EcoRI containing sense primer mCAML-F and the Xhol containing antisense primer mCAML-R. The amplified mCAML DNA was digested with EcoRI and Xhol before it was subcloned in-frame with the sequence encoding the GAL4-AD in pAD previously digested with the same enzymes, generating pAD-mCAML.

The cDNA encoding the N-terminus of mCAML (residues 1-199) was amplified from pAD-mCAML by PCR (Pfx, AT = 58°C) using the sense primer mCAML-F and the Xhol containing antisense primer NT-mCAML-R. After digestion with EcoRI and Xhol, the PCR product was subcloned into pAD, generating pAD-NT-mCAML.
HA-tagged mCAML was digested from pAD-mCAML with BgIII and XhoI and subcloned into pcDNA digested with BamHI and XhoI to create pcDNA/HA-mCAML. BgIII and BamHI restriction sites are complementary although both sites are lost after ligation.

### 2.5.4 Human CAML constructs

The mRNA sequence of human CAML was obtained from NCBI (accession # NM_001745) and used to perform a BLAST search of the human EST database. A match was found from a large cell carcinoma EST library (accession # BE789495; IMAGE clone # 3884754). The EST clone (Incyte Genomics), contained in the pCMV-SPORT6 vector, was sequenced and confirmed to contain the full length clone. The coding region of human CAML was amplified from pCMV-SPORT6/CAML by PCR (Pfx, AT = 58°C) using the Ndel containing sense primer CAML-F and the BamHI containing antisense primer CAML-R. After digestion with Ndel and BamHI, the amplified CAML DNA was subcloned in-frame with the sequence encoding the GAL4-AD into pAD previously digested with the same restriction enzymes, generating pAD-CAML.

The region coding for the N-terminal 201 amino acids of CAML was amplified from pAD-CAML by PCR (Pfx, AT = 58°C) using the sense primer CAML-F and the BamHI containing antisense primer NT-CAML-R. The PCR product was digested with Ndel and BamHI and subcloned into pAD, generating pAD-NT-CAML.

HA-tagged versions of CAML and NT-CAML were constructed by taking advantage of the sequence encoding an HA tag already present in the pAD vector (Figure 2-1B). The sequence encoding HA-CAML or HA-NT-CAML was amplified from pAD-CAML by PCR (Pfx, AT = 58°C) using the Nhel sense primer HA-CAML-F and either CAML-R or NT-
CAML-R, respectively. The resultant PCR products were subcloned into pcDNA, generating pcDNA/HA-CAML and pcDNA/HA-NT-CAML.

2.5.5 TACI constructs

The mRNA sequence of human TACI was obtained from NCBI (accession # NM_012452) and used to perform a BLAST search of human ESTs. A potential match was found from a leukocyte EST library (accession # BI906503; IMAGE clone # 5213128). TACI DNA was PCR (Pfx, AT = 58°C) amplified from pCMV-SPORT6/TACI (Incyte Genomics) using the EcoRI sense primer TACI-F and the XhoI antisense primer TACI-R. The PCR product was subcloned into the pCR2.1-TOPO vector (Invitrogen). Upon sequencing, the clone was found to contain three point mutations: two silent and one missense. The missense mutation was corrected with site directed mutagenesis (AT = 60°C) using the sense prime TACI-Mut-F and the antisense primer TACI-Mut-R. After digestion with EcoRI and XhoI, the excised TACI DNA was subcloned in-frame with the sequence encoding the GAL4-DNA Binding Domain into the pBD vector.

2.6 Generation of E3-6.7K truncations

Two types of E3-6.7K modifications were made. Minus truncations refer to the number of amino acids removed from the C-terminus, while plus truncations refer to the number of amino acids added to the N-terminus of CT-E3-6.7K. Truncations were first made by PCR in five amino acid increments to localize the CAML binding domain (Figure 2-5A).
**Figure 2-5.** Design strategy and PCR primers used to generate E3-6.7K truncations.

(A) PCR strategy used to generate E3-6.7K truncations. PCR primers were designed to amplify DNA encoding truncations of E3-6.7K as shown. Blue portion of forward primers specifies the Ndel restriction site added during PCR, while the red portion of the reverse primers designates the BamHI site added. (B) Primers used to generate truncations. Bold blue sequence indicates the Ndel restriction site, while bold red italics indicate the BamHI site. The start codon (ATG) is found in the Ndel site at the beginning of the forward oligonucleotides. The underlined TTA sequence indicates the introduced stop codon from the reverse primer. F = Forward primer; R = Reverse primer.
Once localized, the domain was further refined with one amino acid truncations using self-annealing oligonucleotides (Figure 2-6).

2.6.1 Truncations produced by PCR

Minus (-) truncations of E3-6.7K were generated by PCR (Pfx, AT = 58°C) using the Ndel containing sense primer 6.7K-F and one of seven reverse primers (Figure 2-5B). Plus (+) truncations were produced using one of three sense forward primers and the antisense 6.7K-R (Figure 2-5B). PCR products were digested with Ndel and BamHI and ligated into pBD previously digested with the same restriction enzymes.

2.6.2 Truncations produced with oligonucleotides

Complementary forward and reverse oligonucleotides for each truncation were designed so that once annealed they would contain overhangs that corresponded to Ndel and BamHI sticky ends (Figure 2-6). Oligonucleotides were reconstituted to 5 pmol/µl in annealing buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA). Equal volumes of complementary oligonucleotides were mixed in 1.5 ml microfuge tubes and placed in a heatblock preheated to 95°C. The heatblock was removed from the heating apparatus and allowed to cool to room temperature on the workbench (45-60 minutes). The annealed oligonucleotides were ligated into pBD previously digested with Ndel and BamHI.
Figure 2-6. Oligonucleotides used to refine E3-6.7K truncations.
Complementary oligonucleotides were designed to code for truncations of the E3-6.7K amino acid sequence shown. Blue sequence indicates the nucleotides that when annealed together form Ndel sticky ends, while the red sequence represents the nucleotides that form BamHI sticky ends. The start codon (ATG) is found in the NdeI site at the beginning of the forward oligonucleotides. The bold TAA sequence shows the introduced stop codon. F = Forward oligonucleotide; R = Reverse oligonucleotide.
2.7 Yeast two-hybrid analysis

The yeast two-hybrid assay (196) was performed using the *Saccharomyces cerevisiae* AH109 strain (Clontech) which contains the GAL4-inducible reporter genes *His* and *Ade2* (197). The cells were transformed with appropriate plasmids using the polyethylene glycol/lithium acetate method (198). Briefly, 1 ml of YPD (Yeast Extract/Peptone/Dextrose) media was inoculated with freshly grown colonies of AH109 and vortexed to disperse any clumps. Cells were transferred to a flask containing 50 ml of YPD and incubated at 30°C overnight with shaking to stationary phase \( \text{OD}_{600} > 1.5 \). An appropriate volume (approx. 30 ml) of the overnight culture was added to 300 ml of fresh YPD to produce an \( \text{OD}_{600} = 0.2 - 0.3 \). The new culture was incubated at 30°C for ~3 h with shaking until the \( \text{OD}_{600} = 0.5 \pm 0.1 \). Cells were washed once in 25 ml sterile H₂O and resuspended in 1.5 ml freshly prepared sterile 1 x TE/LiAc (10 mM Tris-HCl, 1 mM EDTA, pH 7.5/0.1 M lithium acetate) to generate yeast competent cells. For each transformation 100 ng of the indicated pBD-Bait and pAD-Prey DNA were combined with 100 μg of herring testes carrier DNA (Clontech) and 100 μl of the newly made competent yeast cells. Then 0.6 ml of freshly prepared sterile PEG/LiAc (40% polyethylene glycol 4000 in 1 x TE/LiAc) was added before vortexing at high speed. After incubating at 30°C for 30 min, 70 μl of dimethyl sulfoxide (DMSO) was gently added followed by heat shock for 15 min in a 42°C water bath. Cells were chilled on ice then centrifuged for 5 sec at 14000 rpm. The supernatant was removed and the transformed cells were resuspended in 1 x TE before plating on minimal synthetic dropout (SD) media in the absence of Leu and Thr (SD-LT). As the vectors pBD and pAD (Figure 2-1) contain genes for *TRP1* and *LEU2*, growth on SD-LT ensures that both vectors are present. Interactions between the Bait and Prey were tested by replating colonies on SD
media lacking Leu, Thr, Ade, and His (SD-LTAH). If an interaction occurs between the Bait and Prey the GAL4-inducible reporter genes His and Ade2 will be expressed allowing growth on SD-LTAH. No growth will occur on SD-LTAH if there is no interaction. Transformed yeast were grown for 72 hours at 30°C on selective media.

2.8 Quantification of yeast two-hybrid interactions

Quantification of Bait-Prey interactions was measured by yeast growth curve analysis in selective media, as previously described (199). Briefly, individual colonies were used to inoculate liquid SD-LT media and were shaken at 200 rpm at 30°C overnight. The overnight cultures were 1000-fold diluted in SD-LTAH media. Cells were also diluted in SD-LT media to confirmed viability. The SD-LTAH cultures were incubated at 30°C with continuous shaking at 200 rpm and the optical density (OD) was measured at 595 nm in an Spectronic BioMate 3 Spectrophotometer (Thermo Electron Corporation, Waltham, MA) every 24 h for 4 days. Differential OD 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.

2.9 In vitro transcription and translation

Radiolabeled E3-6.7K and CAML proteins were prepared using the TnT T7 Quick Coupled Transcription/Translation Systems (Promega, Madison, WI) according to the manufacturer protocol using pBD-6.7K and pAD-CAML as templates, respectively. Each reaction was carried out in the presence of 20 μCi of Redivue™ L-[35S] methionine (Amersham Biosciences, Piscataway, NJ) and 0.6 μl of canine pancreatic microsomal membranes (Promega) for every 40 μl reaction mixture. The pBD and pAD vectors contain a
T7 RNA polymerase promoter and either a c-Myc- or HA-epitope tag, respectively. The tag is incorporated at the N-terminus of the protein.

2.10 Immunoprecipitation and SDS-PAGE electrophoretic analysis

The mixture from the \textit{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 x g. The microsomal pellet was washed once with TNE 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 or a HA-Tag polyclonal antibody (Clontech) for 1 h at room temperature. The immune complexes were purified with Protein A beads followed by 5 washes with TNE containing 1% Tween 20. Bead slurries were boiled in SDS sample buffer and run on a 12% SDS-PAGE gel. After electrophoresis, the gel was fixed and dried on to Whatman 3MM paper before being exposed to a phosphorimaging screen and evaluated using a Phosphorimager SI and ImageQuant software (Amersham Biosciences).

2.11 Immunofluorescence staining

In typical confocal microscopy, the images produced from the 488 nm and 568 nm lasers are placed in the green and red channel of imaging software, in this case Adobe Photoshop. The merged image then becomes green if co-localization occurs. However, in the
studies for this thesis, the images from the 568 nm laser were placed into both the red and blue channel to produce a magenta color. This results in a more pronounced color (white) when co-localization occurs. In addition, it makes it possible for those with color vision deficiencies (including the author) to discern a difference.

For the co-localization studies HeLa cells were transiently transfected with the indicated plasmids using 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 were 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 5 times with 2% BSA, incubated with 1:500 diluted Alexa Fluor 568 goat anti-rat secondary antibody (Molecular Probes, Carlsbad, CA) 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).

The topology studies used the same protocol with the following exceptions. Non-permeabilized cells were treated with 2% BSA without saponin for 15 min. The primary antibody was 1:1,000 diluted M2 anti-FLAG (Sigma, St. Louis, MO) and the secondary antibody was 1:10,000 diluted Alexa Fluor 568 goat anti-mouse (Molecular Probes). In addition, cells were analyzed using a Bio-Rad Radiance Plus on an inverted Zeiss Axiovert with DIC optics.
2.12 Generation of E3-6.7K cysteine mutants

Three sets of cysteine mutations were made for the E3-6.7K protein as described in Figure 2-7A. The mutations were generated by changing the cysteine codon TGC to the alanine codon GCC. Alanine was chosen because it is a neutral substitution (200). Mutations were made in both the pBD-6.7K and pGFP/6.7K plasmids by site directed mutagenesis (AT = 50°C) using the primers in Figure 2-7B. For the E3-6.7K A.AA mutants, the C.AA mutant was generated first, followed by mutagenesis with the A.CC primers.

2.13 Annexin V-Alexa-647 apoptosis assay

Jurkat cells (1 x 10⁷) 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 x 10⁶ cells with 8 μM thapsigargin 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 as a dead-cell counter stain. Flow cytometry was performed with a FACS Calibur instrument (Becton Dickinson) and analyzed with FlowJo software (Tree Star, Ashland, OR).
<table>
<thead>
<tr>
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<th>Mutation</th>
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<td>E3-6.7K A.AA</td>
<td>MSNSSNSTSLSNFSGIGGVILTVLILFILLALLCLRVAACCTHVCTYCQLFKRWGQHP</td>
</tr>
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**Figure 2-7. E3-6.7K mutations and PCR primers used for mutagenesis.**

(A) Sequences of wild type E3-6.7K protein and its derived mutants. The mutated regions are highlighted. (B) Primers used to generate mutations in the E3-6.7K DNA sequence of pBD-6.7K and piGFP/6.7K. The nucleotides incorporating the mutations into the template are bold and blue. F = Forward primer; R = Reverse primer.
2.14 Ca\textsuperscript{2+} flux measurements

Jurkat cells (1 x 10\textsuperscript{7}) were transiently transfected with 20 \( \mu \)g of the appropriate plasmid by electroporation with a Bio-Rad Gene Pulser Xcell at 250 V and 950 \( \mu \)F. Intracellular Ca\textsuperscript{2+} levels were measured using the ratiometric Ca\textsuperscript{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 1x10\textsuperscript{7} cells/ml with 2 \( \mu \)M Indo-1 for 1 h at 37°C in Opti-MEM. Cells were then washed 2 times with Opti-MEM, resuspend at 1x10\textsuperscript{7} cells/ml in Opti-MEM and kept on ice until analyzed. For each analysis, 100 \( \mu \)l of cell suspension (1x10\textsuperscript{6} cells) was added to 1.9 ml of Opti-MEM prewarmed to 37°C. Indo-1 loaded cells were then examined with a FACS\textsuperscript{Vantage SE} flow cytometer (BD Bioscience) equipped with an 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\textsuperscript{2+} levels was determined through the ratio of emission signals of Indo-1 at 405 nm and 485 nm, representing the ratio of Ca\textsuperscript{2+}-bound to Ca\textsuperscript{2+} free Indo-1, respectively. The kinetic analysis was performed using FlowJo software.

2.15 Statistical analysis

Statistical significance for the quantitative yeast growth curve analysis and the average of three independent apoptosis assays was determined by the analysis of variance (ANOVA) test using GraphPad Prism software (GraphPad Software, San Diego, CA). For all
tests, P < 0.05 was considered to indicate statistical significance. All error bars shown represent the standard deviation.

To compare FACS histograms for the analysis of apoptotic cell populations a probability binning algorithm was used (Multi-sample Comparison, FlowJo). This algorithm determines the probability that two or more FACS data distributions are different (201). When $T(\chi) > 4$, the populations differ by more than four standard deviations, giving the probability that the two populations are different with a $p < 0.01$ (99% confidence).
Chapter 3: Analysis of CAML as a cellular target of E3-6.7K

3.1 Rationale

Although shown to be expressed in virally infected cells in 1990 (175), the function of E3-6.7K was not thoroughly investigated until 2001. Since then E3-6.7K has been shown to have two separate yet not necessarily exclusive roles in inhibiting apoptosis. The first function takes place at the cell surface where E3-6.7K interacts with the RID complex, more specifically with RIDβ (181) to down-regulate TRAIL-R2 (182). The second role of E3-6.7K involves the inhibition of apoptosis independent of other virus proteins. E3-6.7K inhibits death receptor-induced apoptosis, TNF-induced release of arachidonic acid and ER Ca\textsuperscript{2+} efflux (183). Although seemingly diverse tasks, the effect on Ca\textsuperscript{2+} efflux may be the crucial event. ER Ca\textsuperscript{2+} release is a key step in apoptotic pathways. Moreover, TNF-induced release of arachidonic acid is a Ca\textsuperscript{2+}-dependent process. Therefore by altering ER Ca\textsuperscript{2+} homeostasis E3-6.7K may also affect apoptosis and the release of arachidonic acid.

How E3-6.7K modulates Ca\textsuperscript{2+} flux is unresolved. One possible mechanism is that E3-6.7K is able to form pores in the ER membrane thereby allowing Ca\textsuperscript{2+} to flow down its concentration gradient. This would deplete mobilizable Ca\textsuperscript{2+} from intracellular stores. A similar strategy is used by the enterovirus 2B protein. Multimers of the 2B protein form membrane-integral pores in the ER and Golgi membranes, increasing their permeability for Ca\textsuperscript{2+} (186). However, 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 second, more likely explanation is that E3-6.7K interacts with a cellular protein, potentially a Ca\textsuperscript{2+} channel. This strategy is one of the many used by Bcl-2 which was recently shown to interact with
inositol 1,4,5-trisphosphate (IP$_3$) receptors and inhibit IP$_3$-mediated Ca$^{2+}$ release from the ER (202).

One method to determine how a protein functions is to look for homologues. Viruses are notorious for using viral homologues adopted from host genes. In fact some cellular proteins were discovered only after finding a viral homologue. The discovery of the anti-apoptotic viral FLIPs (203, 204) led to the detection of their cellular counterpart (205). To determine if E3-6.7K is homologous to any human proteins, a search was done with BLAST from NCBI against the human non-redundant protein database (206). However, no matches were found, so the search was expanded for distantly related proteins using a pattern-hit initiated (PHI)-BLAST search (206). PHI-BLAST searches for protein sequences that contain a specified pattern and are similar to the query in the vicinity of the pattern. To obtain a search pattern or a consensus, E3-6.7K from Ad2 and Ad5 was aligned with its homologue E3-16K from Ad3 and Ad7. A PHI-BLAST search was performed using Ad2 E3-6.7K as the query and the consensus as the pattern against the human non-redundant protein database and one match was found: a member of the TNF family, transmembrane activator and CAML interactor (TACI). Expressed on B cells and activated T cells, TACI controls ER Ca$^{2+}$ efflux and the activation of the nuclear factor of activated T cells (NF-AT). The TACI protein mediates its effects by binding to CAML, an ER localized protein that controls Ca$^{2+}$ efflux in T cells (207). E3-6.7K and TACI share sequence similarity within the well-conserved carboxyl terminus of E3-6.7K (Figure 3-1).

The CAML binding domain of the 293 amino acid TACI has not been determined; however, von Bülow et al. (207) in their initial characterization of the protein showed that residues 162-293 are able to bind to CAML. Another group, while demonstrating a TRAF
### Figure 3-1. Alignment 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 using ClustalX (208) and formatted with GeneDoc. Shaded regions show sequence similarity. The consensus sequence is below the alignment (key: 6 = [ILV], 4 = [KR], 1 = [ND]). The dotted boxed region indicates the CAML binding domain of human TACI.

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**Ad3 E3-16K**

**Ad7 E3-16K**

**Ad5 E3-7.1**

**Ad2 E3-6.7**

**TACI Mouse**

**TACI Human**

Figure 3-1. Alignment 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 using ClustalX (208) and formatted with GeneDoc. Shaded regions show sequence similarity. The consensus sequence is below the alignment (key: 6 = [ILV], 4 = [KR], 1 = [ND]). The dotted boxed region indicates the CAML binding domain of human TACI.
binding domain for TACI, determined that residues 1-212 of TACI are able to bind to CAML (209). Taken together this indicates that the CAML binding domain of TACI is localized between residues 162 and 212 (Figure 3-1). 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. CAML is also the target of the KSHV K7 protein which, like E3-6.7K, inhibits apoptosis by altering Ca\(^{2+}\) signaling. This chapter tests the hypothesis that E3-6.7K interacts with cellular CAML, a known Ca\(^{2+}\) modulator.

### 3.2 Results

#### 3.2.1 E3-6.7K interacts with mouse CAML

To investigate whether E3-6.7K binds to CAML, a yeast two-hybrid approach was used as an initial step. In the yeast two-hybrid system, a sequence encoding a bait protein is cloned in-frame with the GAL4 DNA-Binding Domain (BD) in the vector pBD. The sequence encoding the prey protein is cloned in-frame with the GAL4 activation domain (AD) in the vector pAD. The vectors are transformed into a yeast strain containing reporter genes that allow growth on selective media if the BD-Bait and AD-Prey proteins interact.

E3-6.7K was subcloned from the EcoRI D fragment of the E3 region of Ad2 into pBD. Ad2 is a human virus so using human CAML to test for an interaction with E3-6.7K would be the preferred method; however, no clone of human CAML was available at the time so a mouse clone was used instead. Human and mouse CAML have 88% sequence similarity (Figure 3-2) (210). The availability of mouse CAML (mCAML) allowed for a relatively quick test to see if the two proteins interact. Full length mCAML was subcloned
into pAD. All the yeast transformations grew on SD-LT media indicating that they contained both the pBD-Bait and the pAD-Prey plasmids. On SD-LTAH media, growth only occurs if there is an interaction between the bait and prey. In this case, there was growth and therefore an interaction between E3-6.7K and mCAML (Figure 3-3A).

The N-terminal region of human CAML (NT-CAML), consisting of residues 1-201 (Figure 3-2), is the TACI-interacting domain (207), therefore it may also bind to E3-6.7K. NT-mCAML was subcloned by PCR into pAD and found to bind to full length E3-6.7K as well (Figure 3-3B). The alignment in Figure 3-1 shows that the C-terminus of E3-6.7K (residues 35-61) contains the highest sequence similarity to TACI. This region, termed CT-E3-6.7K, did not interact with mCAML or NT-mCAML, indicating that additional N-terminal residues are required for an interaction with CAML (Figure 3-3, A and B). A thorough examination of the E3-6.7K domain necessary to bind to CAML will be presented in chapter 4.

In yeast two-hybrid assays it is important to test for false positives by checking if the AD alone will bind to the bait protein or if the BD alone will bind to the prey protein. In this assay, BD did not bind to AD-mCAML or AD-NT-mCAML (Figure 3-3, A and B) and AD did not bind to BD, BD-6.7K, or BD-CT-6.7K (Figure 3-3C). Therefore, growth on SD-LTAH media occurred because of an interaction between the bait and prey proteins, not through an unintentional interaction between the BD and prey or AD and bait.

### 3.2.2 E3-6.7K co-localizes with mouse CAML

The cellular localization of E3-6.7K and mCAML was investigated next. Although a small subset of E3-6.7K is known to reach the plasma membrane of cells and associate with
Figure 3-2. Protein sequence comparison of mouse and human CAML.

Human and mouse CAML were aligned using ClustalX (208) and formatted with GeneDoc. Shaded regions show sequence similarity. The consensus sequence is shown below the alignment. Human and mouse CAML have 88% sequence identity and 90% sequence positives. The dotted boxed region indicates NT-CAML. Human NT-CAML consists of residues 1-201, while mouse NT-CAML consists of residues 1-199. A consensus sequence is below the alignment (key: 6 = [MILV], 4 = [KR]).
Figure 3-3. Yeast two-hybrid of E3-6.7K with mouse CAML.
(A-C) The indicated pBD-Bait and the pAD-Prey plasmids were transformed into AH109 and grown on SD-LT media to select for the presence of both plasmids. Yeast were replated onto SD-LTAH media to select for protein interactions. Growth on SD-LTAH media indicates an interaction between the Bait and Prey. Prey proteins are indicated on the left. Bait proteins are written in the corners of the plates. Experiments were performed on at least four colonies from each transformation and the data shown here are representative of all trials.
the adenovirus RID complex (181, 182), the majority is intracellular and localizes to the ER membrane (176). CAML is not found on the cell surface but is instead a resident of the ER (211). E3-6.7K cDNA was subcloned into the pYFP mammalian expression vector, tagging the protein with YFP at the N-terminus. This allowed easy visualization of E3-6.7K. No antibody was available for mCAML so a tag was required to determine cellular localization. The vector pAD encodes an HA epitope tag upstream of the multiple cloning site (MCS), therefore, the DNA encoding HA-tagged mCAML was subcloned from pAD-mCAML into the mammalian expression vector pcDNA.

To determine if mCAML and E3-6.7K co-localize in mammalian cells, HeLa cells were transiently transfected with pcDNA/HA-mCAML and either pYFP or pYFP-6.7K. After 72 h, the cells were stained for HA-tagged mCAML. First, it was shown that HA-mCAML does not co-localize with YFP alone (Figure 3-4A). HA-mCAML stained in a perinuclear pattern while YFP alone was distributed diffusely throughout the cell, as expected. YFP-6.7K also displayed perinuclear staining and when the fluorescent signals from YFP-6.7K and HA-mCAML are superimposed, an extensive pattern of co-localization is observed (Figure 3-4B).

This initial characterization, shows that E3-6.7K interacts with mCAML and that the two proteins co-localize. Although encouraging, it would be more practical for future investigations to study an interaction with human CAML. At the time of these confocal studies, a human clone of CAML became available, so studies of mCAML stopped while all future studies were performed with human CAML.
Figure 3-4. E3-6.7K and mouse CAML co-localize.

(A-B) HeLa cells were transfected with the DNA indicated on the side of the panels. Cells were stained with 3F10 anti-HA rat monoclonal antibody 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 co-localization. The white scale bar is 10 µm in length. Data were similar in three independent experiments.
3.2.3 E3-6.7K interacts with human CAML

Through a search of the EST database at NCBI, a clone of human CAML was found (accession # BE789495). The clone was obtained, sequenced and found to contain full length human CAML. Since the remainder of experiments in this thesis use human CAML, it shall be referred to as CAML from now on, while mouse CAML will continue to be referred to as mCAML. As with mCAML the first step was to show an interaction between CAML and E3-6.7K. To this end, the yeast two-hybrid system was used again since all the reagents and constructs were readily available. CAML and NT-CAML DNA were amplified by PCR from the EST and subcloned into pAD.

TACI was originally discovered from a yeast two-hybrid screen using CAML as the bait, so for the current study, TACI was used as a positive control. Another search of the EST database resulted in the acquisition of a human TACI EST clone (accession # BI906503). Upon sequencing however, three point mutations were found: two silent and one missense. The missense mutation was corrected using PCR based site-directed mutagenesis and full length TACI was subcloned into pBD.

All the yeast transformations grew on SD-LT indicating that they contained both the BD-Bait and the AD-Prey plasmids (Figure 3-5, A, B and C). Similar to the results seen with mCAML, CAML and NT-CAML bind to E3-6.7K but not CT-E3-6.7K (Figure 3-5, A and B, respectively). As expected, TACI also interacts with CAML and NT-CAML (Figure 3-5, A and B, respectively). False positives were assayed by checking if the AD will bind to the bait protein or if the BD will bind to the prey protein. BD did not bind to AD-CAML or AD-NT-CAML
Figure 3-5. Yeast two-hybrid of E3-6.7K with human CAML.
(A-C) The indicated pBD-Bait and the pAD-Prey plasmids were transformed into AH109 and grown on SD-LT media to select for the presence of both plasmids. Yeast were replated onto SD-LTAH media to select for protein interactions. Growth on SD-LTAH media indicates an interaction between the Bait and Prey. Prey proteins are indicated on the left. Bait proteins are written in the corners of the plates. Experiments were performed on at least four colonies from each transformation and the data shown here are representative of all trials.
(Figure 3-5, A and B) and AD did not bind to BD, BD-6.7K, or BD-CT-6.7K or BD-TACI (Figure 3-5C).

### 3.2.4 CAML immunoprecipitates with E3-6.7K

Although the yeast two-hybrid data was encouraging, a biochemical approach was used to confirm an interaction between E3-6.7K and CAML. Fortunately, the vectors used for the yeast two-hybrid assay contain a T7 RNA polymerase promoter and either a c-Myc or HA epitope tag (Figure 2-1). This allowed the generation of HA-tagged CAML and c-myc-tagged E3-6.7K proteins by in vitro transcription/translation. Both proteins are membrane proteins so the reaction was done in the presence of microsomes. In addition, the proteins were generated in the presence of L-[³⁵S] methionine. Tagged CAML and E3-6.7K were generated individually or together followed by immunoprecipitation 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 phosphoimager screen. When made individually, myc-E3-6.7K and HA-CAML are immunoprecipitated with anti-Myc and anti-HA antibodies, respectively (Figure 3-6, lanes 1 and 2). When myc-E3-6.7K and HA-CAML are made together, both proteins are immunoprecipitated, independent of which antibody was used indicating that they form a complex (Figure 3-6, lanes 3 and 4). This confirms the earlier yeast two-hybrid results, verifying that the E3-6.7K and CAML proteins interact. Protein complexes of 24 and 32 kDa were seen for E3-6.7K (Figure 3-6, lane 1), possibly representing higher-order structures of E3-6.7K. The possibility that E3-6.7K self-associates will be examined in chapter 5.
Figure 3-6. Immunoprecipitation of E3-6.7K and CAML.
The indicated DNA was transcribed and translated in vitro, in the presence of microsomes and L-[35S] methionine. The newly synthesized proteins were immunoprecipitated (IP) with the indicated antibody followed by SDS-PAGE and exposure to a phosphoimager screen. The blot shown is representative of three independent experiments. E3-6.7K is seen as a doublet with the upper band being the 14-kDa glycosylated form. The white arrows (<<) indicate SDS-resistant multimers of E3-6.7K. The positions of protein molecular mass markers are shown on the left.
3.2.5 E3-6.7K co-localizes with human CAML and NT-CAML

A drawback of the yeast two-hybrid system is that the two proteins have to be directed to the nucleus in order for the assay to be successful. Thus, proteins that normally do not interact due to their cellular localization may interact as they are brought into proximity. The same problem exists with immunoprecipitations. Consequently, a crucial validation is to show that the two proteins exist in the same sub-cellular compartment. To this end, immunofluorescence studies were performed to confirm that E3-6.7K co-localizes with CAML. No antibody was available for CAML so DNA encoding HA-tagged CAML was subcloned from pAD-CAML into pcDNA. HeLa cells were then 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-HA mAb did not non-specifically stain HeLa cells transfected with pYFP or pYFP-6.7K alone (Figure 3-7, A and B). YFP alone was distributed diffusely throughout the cell (Figure 3-7, A and C), as has been demonstrated before for YFP in HeLa cells (212). In comparison, YFP-tagged E3-6.7K had a punctate, perinuclear distribution reminiscent of ER localization (Figure 3-7, B and D). As expected, cells transfected with pcDNA/HA-CAML showed similar perinuclear ER staining with the anti-HA mAb (Figure 3-7, C and D). In fact, when the fluorescent signal of YFP-6.7K and HA-CAML were superimposed, a white image was uniformly obtained (Figure 3-7D). Interestingly, the merged image for YFP-6.7K and HA-CAML (Figure 3-7D) exhibits a more intense white staining pattern then YFP-6.7K and HA-mCAML (Figure 3-4B). This could be due to poor targeting of mCAML in a human cell line or may be due to an increased interaction of E3-6.7K with CAML from the natural host. Superposition of the YFP and HA-CAML fluorescent signal resulted in little or no white (Figure 3-7C). These
Figure 3-7. E3-6.7K and CAML co-localize.

(A-D) HeLa cells were transfected with the DNA indicated on the side of the panels. Cells were stained with 3F10 anti-HA rat monoclonal antibody 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 co-localization. The white scale bar is 10 μm in length. Data were similar in three independent experiments.
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 in cells, 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 and as a result the protein is cytoplasmic (213). NT-CAML does contain the TACI binding domain and according to the results obtained from the yeast two-hybrid assay (Figure 3-5B), NT-CAML binds to E3-6.7K as well. Cells transfected with pcDNA/HA-NT-CAML displayed cytoplasmic staining with the anti-HA mAb (Figure 3-8, A and B) and although YFP was cytoplasmic as well, there was no white when the images were overlaid (Figure 3-8A). In contrast, superposition of the YFP-6.7K and HA-NT-CAML fluorescent signals resulted in bands of white where YFP-6.7K perinuclear staining meets the cytoplasmic staining of HA-NT-CAML (Figure 3-8B). 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 co-localization of the two proteins.

3.3 Discussion

It was demonstrated that E3-6.7K has sequence similarity to the cellular protein TACI, a member of the TNF-R superfamily. TACI is a part of a novel network of ligands and receptors involved in B cell survival and isotype switching. The extracellular ligands of TACI are BAFF (B cell activating factor belonging to the TNF family) and APRIL (a proliferation-inducing ligand) (214). While APRIL is secreted (215), BAFF can be found membrane bound and secreted following cleavage by furin (216). Both proteins are
Figure 3-8. E3-6.7K and NT-CAML co-localize.
(A-B) HeLa cells were transfected with the DNA indicated on the side of the panels. Cells were stained with 3F10 anti-HA rat monoclonal antibody 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 co-localization. The white scale bar is 10 μm in length. Data were similar in three independent experiments.
expressed in monocytes, macrophages, dendritic cells and T cells (216, 217, 218, 219, 220) and share the ability to bind to TACI and BCMA (B cell maturation antigen) (221, 222). In addition, BAFF interacts specifically with a third receptor termed BAFF-R (223). Although the role of APRIL is less defined, BAFF plays a very important role in B cell survival and isotype switching (224, 225). In addition, BAFF has been implicated in the development of autoimmune disorders. Overexpression of BAFF leads to autoimmunity in mice (226, 227) and patients with systemic lupus erythematosus (SLE) have elevated levels of BAFF (228). While BAFF-R and BCMA are required for B cell survival, TACI has been shown to be a negative regulator (229).

Antibodies to TACI lead to the activation of NF-AT, NF-κB and AP1. The TACI protein mediates its effects through CAML (207), an ER-localized protein that controls Ca^{2+} efflux (211). The intracellular domain of TACI has also been shown to interact with TRAF. The CAML and TRAF binding sites are separate domains within the cytoplasmic domain of TACI (Figure 3-9A) (209). E3-6.7K is thought to inhibit apoptosis by altering ER Ca^{2+} homeostasis and, as was shown here, E3-6.7K has sequence similarity to TACI, which is a protein that interacts with a Ca^{2+} modulating protein. Therefore, it became imperative to test for an interaction between E3-6.7K and CAML. In this study, it was found that E3-6.7K is able to bind to both mouse and human CAML. In addition, E3-6.7K and CAML co-localize to the same cellular compartments. This interaction with CAML may be the mechanism by which E3-6.7K can alter Ca^{2+} flux.

Although it had been implicated in numerous Ca^{2+} signal transduction pathways, the exact function of CAML is unknown. TACI is only expressed on B cells and activated T cells, while CAML is ubiquitously expressed. This suggests that CAML has a more extensive
Figure 3-9. TACI and CAML.
(A) TACI is a 293 amino acid protein with one transmembrane domain. The N-terminus is extracellular and binds BAFF and APRIL. The CAML and TRAF binding domains are cytoplasmic. (B) CAML is a 296 amino acid protein with three proposed transmembrane domains, the last two of which are necessary for the modulation of intracellular Ca\(^{2+}\). The N-terminus of the protein is cytoplasmic and is able to interact with all known CAML binding proteins. BD = Binding Domain.
function. CAML appears to be involved in a number of pathways. 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 (211). 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 (Figure 3-9B) (230). In addition, CAML has been implicated in transducing the signal from angiotensin II to NF-AT through an interaction with angiotensin II type I receptor-associated protein (ATRAP) (231). CAML has also been reported to interact with EGFR (232) and the protein tyrosine kinase p56\(^{Lck}\) (233). Lastly and most relevant, the mitochondrial K7 protein of KSHV targets CAML to modulate Ca\(^{2+}\) influx and inhibit apoptosis (188). So not only is CAML an important mediator of Ca\(^{2+}\), it is a demonstrated target of an anti-apoptotic viral protein. Interestingly, E3-6.7K has no sequence similarity to any other CAML binding protein, except TACI.

The E3-6.7K protein was observed to bind to NT-CAML, the same domain shown to interact with TACI (Figure 3-9B). NT-CAML is completely cytoplasmic and is believed to be a regulatory domain. One study demonstrated that NT-CAML can act as a dominant negative. Cells expressing NT-CAML showed reduced activation of NF-AT and to a lesser extent reduced activation of NF-κB and API when treated with an antibody to TACI (207). All the known CAML binding proteins have been demonstrated to interact with NT-CAML.

It seems unlikely that E3-6.7K is a viral homologue of TACI due to the limited sequence similarity. These two proteins may just bind to CAML using similar domains brought about by convergent evolution. Although the C-terminus of E3-6.7K contains the domain with sequence similarity to TACI, CT-E3-6.7K did not interact with CAML. This
implies that the CAML binding region of E3-6.7K requires additional N-terminal residues or perhaps binding can not be localized to one region of the protein. The CAML-E3-6.7K interaction will be examined in detail in the next chapter.
Chapter 4: Functional characterization of the E3-6.7K-CAML interaction

4.1 Rationale

In the previous chapter it was shown that the anti-apoptotic E3-6.7K protein interacts with CAML, a cellular Ca\(^{2+}\)-modulating protein. This chapter will focus on a thorough examination of this interaction and test the hypothesis that E3-6.7K requires an interaction with CAML to inhibit apoptosis and ER Ca\(^{2+}\) efflux. First, a detailed study will map the region of E3-6.7K required for CAML binding, including a mutational analysis of critical binding residues. The characterization of the CAML-binding domain will allow the generation of E3-6.7K mutants that do not interact with CAML. These non CAML-binding mutants will be tested for their ability to inhibit apoptosis and ER Ca\(^{2+}\) efflux.

4.2 Results

4.2.1 Localizing the CAML binding domain

The yeast two-hybrid assay proved very effective in the previous chapter and since many of the necessary constructs were already made, it was the assay chosen to determine the CAML-binding domain of E3-6.7K. The yeast two-hybrid assay has been used to determine binding domains of numerous proteins including the self-association domain of the anti-apoptotic hepatitis B virus X protein (234) and the region of the Hepatitis C virus core protein that interacts with the p53-binding protein, 53BP2 (235).
Although CT-E3-6.7K contains the region of sequence similarity to TACI, it does not interact with CAML (Figure 3-5A), suggesting that the CAML-binding domain of E3-6.7K requires additional N-terminal residues. To localize the CAML-binding region of E3-6.7K, truncations of E3-6.7K were generated from the C-terminus in five amino acid increments (Figure 2-5A). In addition, to determine the N-terminal boundary five amino acid increments were added to CT-E3-6.7K. E3-6.7K truncations were assayed for binding to CAML using the yeast two-hybrid assay. All the transformed yeast grew on SD-LT media, indicating that they contained both the bait and prey plasmids (Figure 4-1). From the C-terminus, 15 residues were able to be 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 (Figure 4-1). The resulting domain boundaries suggest a 22 amino acid region of E3-6.7K responsible for an interaction with CAML. This CAML-binding domain (CBD) was generated and found to be able to bind to CAML and NT-CAML (Figure 4-2). A summary of the truncations and their ability to interact with CAML is shown in Figure 4-3A. Interestingly, the CBD of E3-6.7K overlaps with the region of TACI known to bind CAML (Figure 4-3B).

4.2.2 Conserved cysteines are required for CAML binding

An examination of the CBD reveals 7 of the 22 residues are conserved in E3-6.7K, E3-16K, and TACI (Figure 4-3B). Four of the conserved amino acids are aliphatic which are very non-reactive and abundant, so the 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
Figure 4-1. Yeast two-hybrid of CAML and initial E3-6.7K truncations.
Two sets of E3-6.7K truncations were generated: minus (-) truncations removed five amino acid increments from the C-terminus of the protein; plus (+) truncations added five amino acid increments to the N-terminus of CT-E3-6.7K. The truncations were cloned into pBD and transformed along with pAD-CAML into AH109 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 media indicates an interaction with CAML; no growth on SD-LTAH media indicates no interaction with CAML. The Bait protein is written in the corners of the plates. Experiments were performed on at least four colonies from each transformation and the data shown here are representative of all trials.
Figure 4-2. Yeast two-hybrid of CBD and CAML.
The indicated pAD-Prey plasmid and pBD-CBD were transformed into AH109 and grown on SD-LT media to select for the presence of plasmids. Yeast were replated onto SD-LTAH media to select for protein interactions. Growth on SD-LTAH media indicates an interaction with CBD; no growth on SD-LTAH media indicates no interaction with CBD. The Prey protein is written in the corners of the plates. Experiments were performed on at least four colonies from each transformation and the data shown here are representative of all trials.
Figure 4-3. CAML-binding domain of E3-6.7K.
(A) Summary of the E3-6.7K truncations and their ability to interact with CAML as demonstrated with the yeast two-hybrid system. The shaded region indicates the boundaries of the CBD. +, indicates an interaction with CAML; -, indicates no interaction with CAML.
(B) Comparison of the CAML-binding domains of E3-6.7K and TACI. The black boxed region indicates the CAML-binding domain of human TACI and the red boxed region indicates the CAML-binding domain of Ad2 E3-6.7K. A consensus sequence is below the alignment (key: 6 = [ILV], 4 = [KR]).
rarely found (236). Therefore, the presence of conserved cysteines in the CBD indicates they may be important in CAML binding and thus may also affect the function 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 pBD-6.7K plasmid, altering the cysteine codon TGC to the alanine codon GCC. Alanine was chosen since it is a neutral substitution (200). E3-6.7K (A.CC) has the Cys36Ala mutation; E3-6.7K (C.AA) has both the Cys42Ala and Cys43Ala mutation; and finally E3-6.7K (A.AA) has all three cysteines mutated to alanine (Figure 4-4A). The yeast strain AH109 was transformed with pAD-CAML and either pBD-6.7K or with one of the newly constructed plasmids encoding an E3-6.7K mutant. All transformed yeast grew on SD-LT media (Figure 4-4B). The E3-6.7K mutants were then tested for an interaction with CAML by selection on SD-LTAH media. E3-6.7K (C.AA) and E3-6.7K (A.AA) showed no growth on SD-LTAH indicating that Cys42 or Cys43 or both are essential for a CAML interaction. E3-6.7K (A.CC) did grow although at a reduced rate, so Cys36 may play some role in CAML binding. As the E3-6.7K mutants will be used in further assays, their interaction with CAML was quantified with a yeast growth curve analysis (Figure 4-4C). All the E3-6.7K mutants had significantly reduced growth rates compared to 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.
Table A

<table>
<thead>
<tr>
<th>Name</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>E3-6.7K wt</td>
<td>MSNSSNSTSLNFSIGVGVILTICVTFILILALLLARYCQLFKRWQHPR</td>
</tr>
<tr>
<td>E3-6.7K A.CC</td>
<td>MSNSSNSTSLNFSIGVGVILTICVTFILILALLLARYCQLFKRWQHPR</td>
</tr>
<tr>
<td>E3-6.7K C.AA</td>
<td>MSNSSNSTSLNFSIGVGVILTICVTFILILALLLARYCQLFKRWQHPR</td>
</tr>
<tr>
<td>E3-6.7K A.AA</td>
<td>MSNSSNSTSLNFSIGVGVILTICVTFILILALLLARYCQLFKRWQHPR</td>
</tr>
</tbody>
</table>

Figure 4-4. CAML binding of E3-6.7K cysteine mutants.

(A) Sequence of the wild type E3-6.7K and cysteine mutants. The dotted box indicates the CAML-binding domain. (B) AH109 was transformed with the indicated pBD-E3-6.7K mutant (shown in corners of the plates) and pAD-CAML, and grown on SD-LT media to select for the presence of both plasmids. An interaction with CAML was selected for by growing the yeast on SD-LTAH. Growth on SD-LTAH indicates an interaction. Experiments were performed on at least four colonies from each transformation and the data shown here are representative of all trials. (C) Quantitative growth rate analysis of yeast transformed with the indicated pBD-E3-6.7K mutant or the pBD vector alone (VA) and pAD-CAML grown in liquid SD-LTAH media. *, p < 0.01, as compared to wild type (wt). Data represent an average of triplicate measurements and error bars indicate standard deviation.
4.2.3 Conserved cysteines are required for inhibition of apoptosis

It has now been shown that E3-6.7K interacts with CAML and the binding domain, including critical residues required for binding have been determined. 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 (237). 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 (238). The mammalian expression plasmid piGFP/6.7K was mutated by site-directed mutagenesis to produce the three same mutations 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, 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 measuring apoptosis with annexin V. One of the signs of apoptosis is a loss of phospholipids asymmetry, leading to the exposure of phosphatidylserine on the outer leaflet of the plasma membrane (123). Annexin V preferentially binds to phosphatidylserine allowing for a simple assay of apoptosis on a per-cell basis by flow cytometry (239, 240). Cells with piGFP alone were 78% apoptotic, while cells containing E3-6.7K were only 52% apoptotic, consistent with earlier findings (Figure 4-5) (183). 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. Figure 4-6 shows the average of three independent apoptosis assays. Only cells expressing E3-6.7K had a significant reduction in apoptosis compared to the piGFP vector alone.
Figure 4-5. E3-6.7K mutants do not inhibit thapsigargin-induced 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 externalization of phosphatidylserine and propidium iodide (PI) as a dead-cell counter stain. 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. *, apoptotic population was significantly reduced as compared to VA (p < 0.01). Data were similar in three independent experiments.
Figure 4-6. Summary of apoptosis assays with E3-6.7K mutants.
The percentage of apoptotic cells as measured with Annexin-V after stimulation with thapsigargin. Data represent an average of three independent apoptosis assays as described in Figure 4-5. The data is normalized to the piGFP vector alone (VA) which represents 100% apoptosis for each experiment. *, apoptotic population was significantly reduced as compared to VA (p < 0.05). Error bars indicate standard deviation.
4.2.4 Conserved cysteines are required for reducing ER Ca\(^{2+}\) efflux

E3-6.7K was shown previously to reduce the efflux of Ca\(^{2+}\) from the ER in the response to thapsigargin (183). 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 with either the vector piGFP alone, 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 point cells were treated with 10 nM thapsigargin. Jurkat cells expressing E3-6.7K had slower kinetics in the elevation of cytosolic Ca\(^{2+}\) concentration then cells expressing GFP alone (Figure 4-7). In contrast, the expression of any of the E3-6.7K mutants resulted in identical cytosolic Ca\(^{2+}\) kinetics as 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.

4.2.5 Refining the CAML binding domain

The CBD was determined to be a 22 residue domain by generating truncations of E3-6.7K in 5 amino acid increments. Consequently, this allowed for additional refinement of the CBD through trimming of the CBD one amino acid at a time. To this end, complementary oligonucleotides were designed so that when annealed together they formed double stranded DNA fragments encoding truncations of the CBD. In addition, these fragments contained sticky ends to allow for easy cloning into pBD (Figure 2-6).
Figure 4-7. 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 the analysis was immediately resumed. Data were similar in three independent experiments.
Individual transformations of AH109 with pAD-CAML and one of the pBD vectors encoding refined E3-6.7K truncations all grew on SD-LT media. Interestingly, all the new truncations grew on SD-LTAH media as well (Figure 4-8). This indicates that an additional four amino acids could be removed from both the N- and C-terminus of the previously defined CBD. Indeed, this minimal CBD (minCBD) was subsequently shown to interact with CAML, although the growth rate of transformed yeast was reduced (Figure 4-9). A summary of the refined truncations and their ability to interact with CAML is shown in Table 4-1.

4.3 Discussion

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 (Figure 4-3B). 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 p56Lck have been shown to interact with CAML through their tyrosine kinase domains (232, 233). CAML does not appear to bind indiscriminately to kinases, as no interaction was found with two other kinases p59Fyn or ZAP-70 (233). Clearly CAML is able to bind to numerous proteins through multiple binding domains implying the importance of this ubiquitously expressed protein. While not required for cellular viability, CAML is required for early embryonic development (232). Knocking out the CAML gene in mice results in embryonic lethality.
Figure 4-8. Yeast two-hybrid of CAML and the refined E3-6.7K truncations.
Two sets of E3-6.7K truncations were generated: minus (-) truncations removed single amino acid increments from the C-terminus of E3-6.7K CBD (-15); plus (+) truncations added single amino acid increments to the N-terminus of E3-6.7K CBD (+5). The truncations were cloned into pBD and transformed along with pAD-CAML into AH109 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 media indicates an interaction with CAML; no growth on SD-LTAH media indicates no interaction with CAML. The Bait protein is written in the corners of the plates. Experiments were performed on at least four colonies from each transformation and the data shown here are representative of all trials.
Figure 4-9. Yeast two-hybrid plates of minCBD and CAML.
The indicated pAD-Prey plasmid and pBD-minCBD were transformed into AH109 and grown on SD-LT media to select for the presence of plasmids. Yeast were replated onto SD-LTAH media to select for protein interactions. Growth on SD-LTAH media indicates an interaction with minCBD; no growth on SD-LTAH media indicates no interaction with minCBD. The Prey protein is written in the corners of the plates. Experiments were performed on at least four colonies from each transformation and the data shown here are representative of all trials.
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<td>+</td>
<td>-16</td>
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</tr>
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<td>-17</td>
<td>VILF LIILALLCLRVAAC CT</td>
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<tr>
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</tr>
<tr>
<td>minCBD</td>
<td></td>
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Table 4-1. Summary of CAML binding of refined E3-6.7K truncations
Summary of the E3-6.7K truncations and their ability to interact with CAML as demonstrated with the yeast two-hybrid system. The light gray shaded region indicates the boundaries of the CBD, while the dark gray shaded region indicates the boundaries of the minimal CBD (minCBD). +, indicates an interaction with CAML; -, indicates no interaction with CAML.
Amino acid conservation between species is often a good indication of the importance of particular residues in the function of a given protein domain. The CBD contains three cysteine residues that are conserved in E3-6.7K, E3-16K and TACI. They are essential for an E3-6.7K interaction with CAML. The function of these cysteines is unknown, but they may be involved in a direct interaction with CAML or they may be important for the structure of E3-6.7K, such as in the formation of disulfide bonds. Previous reports have suggested that E3-6.7K may contain intrachain disulfide bonds based on shifts in the electrophoretic mobility between reduced and non-reduced forms of the protein (184). In the cell, disulfide bridges typically only occur in the oxidizing environment of the ER lumen and not in the reducing environment of the cytoplasm. The E3-6.7K-CAML interaction occurs in the cytosol as evident by the ability of E3-6.7K to interact with cytosolic NT-CAML. There are examples of cytosolic disulfide bonds described for viral proteins. Three vaccinia virus proteins, A28L, L1, and F9, have intramolecular disulfide bonds in their cytoplasmic domains (241, 242). The formation of these bonds requires three cytoplasmic thiol oxidoreductases encoded by the virus. Therefore, it seems unlikely that the cytoplasmic cysteines involved in the E3-6.7K-CAML interaction are disulfide bonded since adenovirus does not encode thiol oxidoreductases.

Since it was shown that E3-6.7K interacts with CAML, it was necessary to determine if this interaction is required for the varied functions of E3-6.7K. To this end, apoptosis and Ca\(^{2+}\) assays were performed with CAML-binding wildtype E3-6.7K and non CAML-binding E3-6.7K mutants. In a thapsigargin-induced apoptosis assay, wildtype E3-6.7K was observed to significantly reduce cell death. Conversely, none of the E3-6.7K mutants had any significant effect on cell death as compared to the vector alone control. Similar results were
seen in a Ca\textsuperscript{2+} flux assay. Cells transfected with wildtype E3-6.7K showed a reduced rate of Ca\textsuperscript{2+} release, while cells transfected with any of the E3-6.7K mutants showed rates of Ca\textsuperscript{2+} release comparable to the control. These results indicate that the E3-6.7K interaction with CAML is necessary for inhibiting thapsigargin-induced apoptosis and cellular Ca\textsuperscript{2+} fluxes. Analogous results were seen in studies of the interaction between CAML and the KSHV anti-apoptotic K7 protein. K7 mutants unable to interact with CAML were also unable to prevent apoptosis (188).

Upon further investigation, it was determined that the CBD could be trimmed in size from a 22 to a 14 amino acid domain. This new minCBD was also found to interact with CAML although not as strongly as the full CBD. This reduced ability to interact is not surprising considering the small size. Interestingly, the minCBD is missing Cys43 the last of the three conserved cysteines thought to be important in the E3-6.7K-CAML interaction (Table 4-1). None of the experiments performed looked directly at Cys43. The E3-6.7K (C.AA) mutant has both the Cys42Ala and Cys43Ala mutation. In light of the minCBD being able to bind CAML it would appear that Cys43 is not critical. Therefore, it is likely that Cys36 and Cys42 are the important residues involved in CAML binding.
Chapter 5: Membrane topology of E3-6.7K

5.1 Rationale

In order for membrane proteins to function properly they must insert into the membrane in the proper orientation. Some integral membrane proteins span the membrane once (bitopic), others span the membrane multiple times (polytopic). The E3-6.7K protein has a large hydrophobic domain capable of spanning the membrane once, suggesting the protein is bitopic.

The topology of bitopic membrane proteins is determined by the nature and orientation of the signal sequence (Figure 5-1). Type I membrane proteins (N-luminal/C-cytoplasmic) contain a cleavable transmembrane signal sequence and an additional transmembrane region known as a stop transfer sequence. Signal peptidase cleaves off the signal sequence generating a single membrane-spanning protein with a new luminal N-terminus (243). Type II (N-cytoplasmic/C-luminal) and type III (N-luminal/C-cytoplasmic) integral membrane proteins use their transmembrane domain as both a signal sequence and a stop transfer sequence, classifying them as signal-anchor (SA) proteins. Several factors have been shown to determine the orientation of SA-proteins. The distribution of charged residues in the regions of the polypeptide flanking the transmembrane domain influence orientation: the more positive end is generally cytosolic (179). One reason for this phenomenon, known as the “positive inside” rule, is that the cytosolic positive residues may interact with anionic phospholipids on the cytoplasmic face of the membrane (244). A second determinant affecting the topology of SA proteins is the length and hydrophobicity of the transmembrane domain. Long hydrophobic regions favor the type III orientation, even when the flanking
Figure 5-1. Types of bitopic integral membrane proteins.
Type I membrane proteins (N-luminal/C-cytoplasmic) contain both a cleavable signal sequence and a stop transfer sequence. The signal sequence is recognized by the signal recognition particle (SRP) which directs the nascent protein to the ER. The protein is translocated across the ER membrane until the stop transfer sequence is integrated into the bilayer. Signal peptidases in the ER lumen cleave off the signal sequence generating a new luminal N-terminus for the type I protein. Type II (N-cytoplasmic/C-luminal) and type III (N-luminal/C-cytoplasmic) membrane proteins contain signal-anchors which act as both a signal sequence and a stop transfer sequence. Tail-anchored proteins (N-cytoplasmic/C-luminal) contain a signal-anchor domain at the extreme C-terminus and are post-translationally translocated.
charges are more positive at the N-terminus (245, 246, 247). Lastly, glycosylation of luminal exposed residues in the ER can influence the orientation of proteins (248).

The majority of membrane proteins in eukaryotic cells are cotranslationally integrated into the ER membrane. The hydrophobic signal sequence of type I proteins or the SA of type II and type III proteins is recognized by the signal recognition particle (SRP) as it emerges from the ribosome. Upon binding to the SRP, translation halts while the ribosome/nascent polypeptide/SRP complex is targeted to the ER membrane via an interaction with the SRP receptor. At the ER translation begins again as the new polypeptide is translocated across the ER through the Sec61 complex (249). In contrast, tail-anchored proteins insert in the ER membrane post-translationally, in the absence of the SRP or Sec61 complex (250). Tail-anchored proteins are held in the phospholipids bilayer by a SA close to the C-terminus. The entire functional N-terminus portion of the protein faces the cytosol resulting in a type II orientation. The hydrophobic SA is so close to the C-terminus that it emerges from the ribosome only after termination of translation, making it unlikely to bind to the SRP. Examples of tail-anchored proteins include cytochrome b₅ (251), Bcl-2 (252), and the vaccinia virus F1L protein (253).

The E3-6.7K protein is present in infected cells in two forms, a glycosylated 14-kDa form and an unglycosylated 6.7-kDa form. E3-6.7K was originally presumed to be a type III glycoprotein because all three possible Asn-linked glycosylation sites are found in the N-terminal region of the protein (Figure 1-6A) (176). In addition, the charged residues are all basic and at the C-terminus of the protein. A recent study investigated the topology of E3-6.7K directly using an in vitro translation system supplemented with microsomes followed by proteolysis of exposed domains of the membrane translocated E3-6.7K (180). This study
revealed that in addition to the type III orientation (N-luminal/C-cytoplasmic orientation, termed $^{N_{\text{im}}}$E3-6.7K), E3-6.7K could adopt the opposite type II orientation (N-cytoplasmic/C-luminal, termed $^{C_{\text{im}}}$E3-6.7K) as well as the possibility of a fully translocated form (N and C termini are both translocated, termed $^{N_{\text{C}}}$E3-6.7K). The three possible orientations of E3-6.7K as demonstrated in microsomal membrane in a cell-free translation system can be seen in Figure 1-6B. This chapter tests the hypothesis that E3-6.7K can form alternate topologies in cells.

Potential complexes of E3-6.7K were observed earlier in chapter 3 (Figure 3-6, lanes 1 and 3) suggesting the possibility that E3-6.7K may be able to oligomerize. Therefore, the hypothesis that E3-6.7K may be able to self-associate will also be addressed.

### 5.2 Results

#### 5.2.1 The N-terminus of E3-6.7K is detectable at the cell surface

The majority of E3-6.7K is intracellular and exhibits sensitivity to Endoglycosidase H digestion, indicating that the attached glycans are primarily the ER-specific high mannose type (176). However, in addition to being predominantly ER-localized there is a subset of E3-6.7K proteins which localize to the plasma membrane of cells and associate with the adenovirus RID complex, specifically with RIDβ (181). Benedict et al. (181) made use of an N-terminus VSV-tagged and an N-terminus FLAG-tagged E3-6.7K to demonstrate that the N-terminus of the protein is accessible to exogenous added tag-specific antibodies at the cell surface. Similarly, in this study an N-terminal FLAG-tagged and C-terminal FLAG-tagged E3-6.7K were used to directly study the topology of E3-6.7K in transfected cells.
FLAG-E3-6.7K cDNA was subcloned into the pcDNA vector to generate pcDNA/FLAG-6.7K (Figure 5-2A). HeLa cells were transiently transfected with pcDNA/FLAG-6.7K. After 72 h, the cells were stained for FLAG-6.7K with an anti-FLAG mAb in the presence or absence of saponin. Saponin is a natural surfactant found in quillaja bark that allows penetration of macromolecules through cellular membranes. When HeLa cells were saponin treated, the anti-FLAG mAb labeled intracellular membranes, as expected for a protein primarily localized to the ER (Figure 5-2B). In the absence of saponin, the staining was very specific for the cell surface (Figure 5-2C). This demonstrates that the N-terminus of E3-6.7K is accessible at the cell surface. In addition, this confirms earlier reports that at least a subset of E3-6.7K localizes to the plasma membrane (181).

5.2.2 The C-terminus of E3-6.7K is detectable at the cell surface

Initially, experiments were attempted with untagged E3-6.7K and an anti-sera directed towards the C-termini of E3-6.7K (180); however, the anti-sera stained non-specifically in cells. Therefore, E3-6.7K cDNA was subcloned into the piGFP mammalian expression vector to generate piGFP/6.7K-FLAG (Figure 5-3A). The piGFP vector not only FLAG-tagged the C-termini of E3-6.7K, but also allowed for identification of transfected cells by virtue of the co-cistronically-expressed GFP. HeLa cells were transiently transfected with either piGFP vector alone or piGFP/6.7K-FLAG. After 72 h, the cells were stained with an anti-FLAG mAb, in the presence or absence of saponin. First, it was shown that the anti-FLAG monoclonal does not stain cells transfected with the piGFP vector alone (Figure 5-3B). The staining of piGFP/6.7K-FLAG transfected cells was then examined. When HeLa cells were saponin treated, the anti-FLAG mAb labeled intracellular membrane
Figure 5-2. N-terminus of E3-6.7K is accessible from cell surface.

(A) Schematic representation of the FLAG-tagged E3-6.7K fusion protein encoded by pcDNA/FLAG-6.7K. (B-C) HeLa cells were transfected with pcDNA/FLAG-6.7K, treated with or without saponin as indicated and stained with M2 anti-FLAG mAb, followed by an Alexa 568-conjugated anti-mouse Ab (magenta). Immunofluorescence was examined using a Bio-Rad Radiance Plus confocal microscope and a representative optical section of each transfection is presented. The white scale bar is 10 µm in length. Data were similar in three independent experiments.
Figure 5-3. C-terminus of E3-6.7K is accessible from cell surface.
(A) Schematic representation of the FLAG-tagged E3-6.7K fusion protein encoded by piGFP/6.7K-FLAG. (B-D) HeLa cells were transfected with piGFP or piGFP/6.7K-FLAG, treated with or without saponin as indicated and stained with M2 anti-FLAG mAb, followed by an Alexa 568-conjugated anti-mouse Ab (magenta). Immunofluorescence was examined using a Bio-Rad Radiance Plus confocal microscope and a representative optical section of each transfection is presented. The piGFP vector encodes the GFP protein with emits green fluorescence in transfected cells. The white scale bar is 10 µm in length. Data were similar in three independent experiments.
compartments (Figure 5-3C). In the absence of saponin, it was observed that the staining was specific for the cell surface (Figure 5-3D). These experiments demonstrate for the first time that the E3-6.7K C-terminus is accessible at the cell surface in mammalian cells.

5.2.3 E3-6.7K does not self-associate

Earlier studies have suggested membrane-integrated, higher order multimers of E3-6.7K may exist in vitro (180). Indeed, potential complexes of E3-6.7K can be seen in Figure 3-6 (lanes 1 and 3). This suggests that E3-6.7K may be able to oligomerize. Self-association of E3-6.7K was investigated using the yeast two-hybrid system. E3-6.7K cDNA was subcloned into pAD. Yeast were transformed with pAD-6.7K and either pBD, pBD-6.7K or pBD-CT-6.7K and plated on SD-LT media to confirm expression of both plasmids. Yeast were replated on SD-LTAH to test for an interaction. No growth was seen for any of the transformants, indicating that E3-6.7K does not self-associate.

5.3 Discussion

These studies have shown that for both the N- and C-termini of E3-6.7K the FLAG tags are accessible to exogenously added antibodies. This demonstrates that both the N- and C-terminal domains of E3-6.7K can translocate across the membrane and give rise to proteins that are capable of transport to the cell surface. These data provides support for both N\textsuperscript{Nt}E3-6.7K and C\textsuperscript{Ct}E3-6.7K but do not distinguish between N\textsuperscript{NC}E3-6.7K and the other two forms. However, taken together with the previously reported cell-free experiments (180), there is strong support for three different topologies for E3-6.7K (Figure 1-6B). This challenges the
Figure 5-4. Yeast two-hybrid of AD-6.7K and BD-6.7K.
The indicated pBD-Bait and pAD-6.7K were transformed into AH109 and grown on SD-LT media to select for the presence of plasmids. Yeast were replated onto SD-LTAH media to select for protein interactions. Growth on SD-LTAH media indicates an interaction with E3-6.7K; no growth on SD-LTAH media indicates no interaction with E3-6.7K. The Bait protein is written in the corners of the plates. Experiments were performed on at least four colonies from each transformation and the data shown here are representative of all trials.
current models of membrane protein conformation in several ways. Firstly, the type II \textsuperscript{Cm}E3-6.7K form and the \textsuperscript{NC}E3-6.7K form are exceptions to the “positive-inside” rule because both forms of the protein have their positively charged C-termini in the lumen. More importantly, few membrane proteins exist in multiple topological forms. In general, membrane proteins have one topology and the formation of alternate topologies during biogenesis results in degradation. For example the cystic fibrosis transmembrane conductance regulator (CFTR) has an unstable transmembrane segment that can result in an alternate topology of the protein (254) though, CFTR at the cell surface has been shown to assume only the correct topology (255). Multiple orientations have been observed for polytopic proteins such as ductin (256), P-glycoprotein (257), aquaporin-1 (258) and bitopic proteins such as the prion protein (PrP) (259), microsomal epoxide hydrolase (260) and the Newcastle disease virus fusion protein F (261).

The role alternate topologies play in the function of these unique proteins is ill defined. P-glycoprotein has been hypothesized to alternate between different topologies to facilitate drug transport (262), although this has been disputed (263). Aquaporin-1 is believed to progress through a four transmembrane intermediate at the ER before developing into the mature six-segment-spanning topology at the plasma membrane. Ductin is a four transmembrane protein with two major transport functions, as a subunit of the vacuolar H\textsuperscript{+}-ATPase and a component of the connexon channel of gap junctions. Interestingly, each function utilizes ductin in a different orientation. As part of the proton pump, ductin has both the N- and C-termini vacuolar, while in the gap junctions ductin has the opposite orientation (256). The microsomal epoxide hydrolase is a bifunctional hepatic protein which assumes a type II orientation at the plasma membrane where it mediates bile acid transport (264) and
primarily a type I orientation in the ER where it metabolizes xenobiotics (265). The majority of PrP molecules are fully translocated (\textsuperscript{sec}PrP) into the ER and are the precursor to the glycolphosphatidyl inositol-anchored protein found at the cell surface (266). However, two membrane-embedded conformations also exist: a type I (\textsuperscript{Ntm}PrP) and a type II (\textsuperscript{Ctm}PrP) orientation (267). The normal physiological function of \textsuperscript{sec}PrP is not clear although it may have a role in copper uptake (268). It has been hypothesized that \textsuperscript{Ctm}PrP is involved in the pathogenesis of prion disease (269), while no function has been assigned to \textsuperscript{Ntm}PrP. The Newcastle disease virus fusion protein exists in a classical type I form and a polytopic form with both the N- and C-termini in the lumen. Both forms may play a role in fusion (261).

With the diversity of other proteins with multiple topological forms, it will be interesting to determine where E3-6.7K fits in. Further investigation is needed in order to assess if alternate forms of E3-6.7K have different functions or if only one conformation is functional. Moreover, it will be interesting to see if different forms of E3-6.7K are able to interact with CAML and RIDβ.

It should be noted that E3-6.7K is also unique in terms of its post-translational translocation. It was previously postulated that E3-6.7K may be post-translationally translocated (176). This was subsequently confirmed to occur for both the 6.7-kDa and 14-kDa form of E3-6.7K by demonstrating that \textit{in vitro}-synthesized E3-6.7K translocated across the membrane of microsomes after translation was stopped with cycloheximide (180). This is not surprising considering the small size of E3-6.7K. Since the last ~40 amino acids of a nascent polypeptide are protected within the large ribosomal subunit (270), it is unlikely that the nascent chain would be able to interact with the SRP before translation was complete. The only mammalian proteins which are known to post-translationally translocate are the
tail-anchored proteins. The 6.7-kDa form \( \text{C} \)\text{um}E3-6.7K, with its type II orientation, can be considered tail-anchored. What makes E3-6.7K unique is that the 14-kDa glycosylated forms are able to translocate after translation. The \( \text{N} \)\text{tm}E3-6.7K and \( \text{N} \)\text{c}E3-6.7K form are the first examples of non-tail-anchored proteins that can translocate post-translationally across the membrane of the ER.
6.1 Conclusions

From the results presented, evidence was provided to show that the E3-6.7K protein forms diverse membrane topologies in cells, and is able to inhibit apoptosis and ER Ca$^{2+}$ flux through an interaction with CAML, a cellular Ca$^{2+}$ modulator. This interaction occurs through the 22 amino acid CBD found near the C-terminus of E3-6.7K. Further refinement of the CBD suggests it may be as small as 14 amino acids. Depending on the topology of E3-6.7K, the CBD can be located either in the cytosol or the lumen of the ER. E3-6.7K interacts with cytoplasmic NT-CAML indicating the interaction occurs in the cytosol. Consequently, the likely binding partner of CAML is $^{\text{Nterm}}$E3-6.7K, as it is the only form to display the CBD in the cytosol (Figure 6-1).

With their limited genome, it is beneficial for viruses to encode proteins with multiple functions. The adenovirus E1B-55K and E4orf6 proteins modulate nuclear export of viral and cellular mRNA (271) as well as inhibit apoptosis by inducing the degradation of p53 (148). The CAML-interacting KSHV K7 protein has numerous other roles to provide a favourable environment for viral infection. K7 contains a partial baculovirus IAP (inhibitor-of-apoptosis protein) repeat (BIR) domain that is capable of binding to and inhibiting the activity of caspase-3 (187). K7 also targets ubiquilin, a regulator of the ubiquitin- and proteasome-mediated degradation machinery. One result of this interaction is the rapid degradation of the p53 protein resulting in inhibition of p53-mediated apoptosis (272). Like many viral proteins, E3-6.7K has more than one function, and the two known functions are both involved in inhibiting cell death. At the plasma membrane, it cooperates with the RID
CT-CAML contains three transmembrane domains, two of which are necessary and sufficient for the modulation of intracellular Ca$^{2+}$. NT-CAML is the regulatory domain and is completely cytosolic. All of the known CAML-binding proteins, including E3-6.7K, interact with NT-CAML. E3-6.7K adopts three different topologies in the membrane but only $N_{tm}E3$-6.7K displays the CAML-binding domain (CBD) to the cytosol. This suggests that $N_{tm}E3$-6.7K is the binding partner of CAML.
complex to down-regulate TRAIL-R2, therefore inhibiting TRAIL-induced apoptosis (182). At the ER, through an interaction with CAML, E3-6.7K acts as a general repressor of apoptosis by regulating ER Ca$^{2+}$ release. E3-6.7K is rather unique in that it has more than one membrane topology. The $\text{N}_{\text{tm}}$E3-6.7K form likely interacts with CAML, so it would be of interest to determine whether specific topologies of E3-6.7K are required for its function in downregulating TRAIL-R2 with the RID complex.

It is readily established that Ca$^{2+}$ plays an important role in apoptosis (130). Sustained release of Ca$^{2+}$ can initiate programmed cell death by triggering the opening of the mitochondrial permeability transition pore, thereby stimulating cytochrome c release (273). A rise in cytoplasmic Ca$^{2+}$ also stimulates Ca$^{2+}$-sensitive enzymes, such as calpain and calcineurin, which engage other apoptotic pathways. Upon activation by elevated intracellular Ca$^{2+}$, the cysteine protease calpain translocates from the cytosol to the ER membrane where it cleaves pro-caspase-12 (274). The phosphatase calcineurin dephosphorylates and therefore activates Bad (275), a pro-apoptotic member of the Bcl-2 family. Studies have demonstrated that ER Ca$^{2+}$ steady state levels are an important regulator of apoptosis and there is growing evidence that the Bcl-2 family controls apoptosis by modulating ER Ca$^{2+}$ stores. Overexpression of anti-apoptotic Bcl-2 causes a reduction in the ER steady state levels of mobilizable Ca$^{2+}$ (276, 277, 278). While overexpression of pro-apoptotic Bax and Bak promote ER Ca$^{2+}$ release (279). In addition, cells deficient in Bax and Bak have reduced ER Ca$^{2+}$ levels and are resistant to a variety of apoptotic stimuli (280). With the important role Ca$^{2+}$ plays in apoptotic pathways it is not surprising that viruses have evolved mechanisms to exploit them.
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 pre-existing Ca\(^{2+}\) release channels or sarco/endoplasmic reticulum 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 (281). Both Bcl-2 and Bcl-X\(_L\) are able to form ion channels in synthetic lipid membranes (282, 283), although neither has been shown to conduct Ca\(^{2+}\). As CAML has no apparent sequence homology to known Ca\(^{2+}\) channels (230), 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\(_3\)Rs) 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\(_3\)Rs (202, 284). The IP\(_3\)R-Bcl-2 interaction was increased in the absence of Bax and Bak (285), while tBid and Bax were able to block the interaction between Bcl-X\(_L\) and IP\(_3\)R (284). The ratio of pro- versus anti-apoptotic family members may be able to control ER Ca\(^{2+}\) levels through an IP\(_3\)R-mediated Ca\(^{2+}\) leak. CAML is unlikely to have a direct effect on IP\(_3\)Rs, as it does not comigrate with these Ca\(^{2+}\) release channels in sucrose gradient membrane fractionation (213). CAML does, however, comigrate with SERCA. Furthermore, immunofluorescence staining indicates that CAML and SERCA colocalize (213). Bcl-2 has been shown to interact with SERCA as demonstrated by co-immunoprecipitation (286). 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 (287). Dremina et al. (287) 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 pro-apoptotic 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 did so by inhibiting the efflux of Ca\(^{2+}\) from the ER or by diminishing the ER Ca\(^{2+}\) stores through a Ca\(^{2+}\) leak or 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 reduction in the amplitude of Ca\(^{2+}\) waves in response to the agonist ATP (288). 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 (188).

From the available data, a potential model starts to take shape (Figure 6-2). Early in the adenovirus infection, the E3-6.7K protein is expressed and localizes primarily to the ER where it co-localizes with CAML. Through the CBD, E3-6.7K binds to the N-terminal regulatory domain (residues 1-201) 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 pro-apoptotic ER to mitochondria 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 allow the maintenance of a persistent infection.

Besides inhibiting programmed cell death, modulating ER Ca\(^{2+}\) release would be beneficial for viral infection by preventing the ER-stress response. Many viruses induce an
Figure 6-2. 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 pro-apoptotic 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 pro-apoptotic Ca\(^{2+}\) waves thereby precluding cell death.
ER-overload response (289) through the abundant expression of viral proteins in the ER, as seen for adenovirus E3-19K (290) or influenza hemagglutinin (291). This ER-overload response activates the NF-κB transcription factor (292) which results in the expression of genes important in inflammation and antiviral defense such as the cytokine IFN-β (293) and proteins involved in viral peptide presentation for example, the proteasome subunit LMP2 and TAP1 peptide transporter (294). Messengers of the ER-overload response are ER Ca\(^{2+}\) efflux and reactive oxygen intermediates (295). Interestingly, the induction of adenovirus E3 gene products is enhanced by NF-κB (289). It is therefore possible that by blocking ER Ca\(^{2+}\) efflux, E3-6.7K could affect both the ER-stress response as well as apoptotic pathways.

The E3-6.7K and K7 proteins are the first two members of a novel class of viral anti-apoptotic proteins that target CAML to affect cellular Ca\(^{2+}\) signaling. Although their target and ultimate goal is the same, their localization and mode of action is different. E3-6.7K is primarily localized to the ER, while K7 is typically present at mitochondria. Even though CAML is predominantly ER-bound, this does not pose much of a problem for K7 as the ER and mitochondria are often in close proximity (296, 297). A major difference between K7 and E3-6.7K is the opposite effect 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 (188). Although sustained high intracellular Ca\(^{2+}\) levels contribute to apoptosis, early up-regulation of cytoplasmic Ca\(^{2+}\) levels may protect cells against apoptosis (298).

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 viral proteins that target it will ultimately lead to a better understanding of immune evasion mechanisms and viral persistence as well as provide a novel means to investigate cellular apoptotic pathways.
6.2 Future directions

The results presented here demonstrate that E3-6.7K acts as a general repressor of apoptosis by altering ER Ca\(^{2+}\) homeostasis through an interaction with cellular CAML. While it was shown that E3-6.7K inhibits ER Ca\(^{2+}\) fluxes, it is not known whether it accomplishes this by preventing the release of Ca\(^{2+}\) or by reducing the level of mobilizable Ca\(^{2+}\). One method that could be used to directly determine ER Ca\(^{2+}\) concentration is with ER-targeted Ca\(^{2+}\) sensors, such as the Ca\(^{2+}\)-sensitive photoprotein aequorin or the Ca\(^{2+}\)-sensitive fluorescent cameleon protein (299). Using one of these probes in cells expressing E3-6.7K will allow direct measurements of ER Ca\(^{2+}\) concentration. Similar techniques have been used to determine the effect of Bcl-2 on ER Ca\(^{2+}\) levels (276, 277, 278).

Although overexpression of CAML has been shown to inhibit thapsigargin-induced apoptosis, little has been done to determine its role beyond that. The function of CAML in apoptotic pathways could be examined further by knocking down CAML expression with short interfering RNA (siRNA). Cells expressing reduced levels of endogenous CAML would then be tested for their sensitivity to apoptosis induced by a variety of stimuli, including death receptors. If overexpression inhibits apoptosis, underexpression should result in a greater sensitivity.

With its novel mechanism of inhibiting cell death through its interaction with CAML, E3-6.7K allows the unique opportunity to study apoptosis from a new perspective. In addition, E3-6.7K may interfere with the TACI-CAML pathway which is involved in B and possible T cell development. Future studies will investigate the role of E3-6.7K in apoptotic pathways and lymphocyte development using E3-6.7K-expressing transgenic mice that are currently being generated. This transgenic mouse model contains the E3-6.7K gene under the
control of the tetracycline operator (300). Once this mouse is generated, tissue specific gene expression will be achieved by crossing this transgenic to other transgenics where they express the Tet repressor under the control of various tissue specific promoters. These mice models will allow the examination of the function of the E3-6.7K protein in B cells, T cells and any other tissues. The goal would be to provide evidence that E3-6.7K can modulate parameters of T and B cells function such as differentiation, cytokine production and Ig production.

The E3-6.7K protein characterized in this study is from serotype 2 of species C adenovirus. Species B human adenoviruses encode a homologue of E3-6.7K called E3-16K (173). It would be of interest to determine whether E3-16K is also able to interact with CAML and inhibit apoptosis. The E3-16K gene has been cloned from adenovirus serotype 3, into the pBD vector. Preliminary experiments show that full length E3-16K does not bind to CAML, however, the C-terminal domain (residues 105-146) containing the conserved cysteines does interact with CAML. This suggests that the N-terminus of E3-16K may be a regulatory domain. Once cloned into the piGFP vector, E3-16K will be tested for the ability to inhibit apoptosis and Ca\textsuperscript{2+} flux. These experiments will extend the significance of this CAML-targeting mechanism to other adenovirus species and more clearly establish the functional similarities between these viral anti-apoptotic proteins and host proteins such as TACI.
References


95. van Leeuwen HC, Rensen M, van d, V. 1997. The Oct-1 POU homeodomain stabilizes the adenovirus preinitiation complex via a direct interaction with the priming protein and is displaced when the replication fork passes *J Biol Chem.* 272:3398-405


102. Lin HJ, Flint SJ. 2000. Identification of a cellular repressor of transcription of the adenoviral late IVa(2) gene that is unaltered in activity in infected cells *Virology* 277:397-410


106. Hammarskjold ML, Winberg G. 1980. Encapsidation of adenovirus 16 DNA is directed by a small DNA sequence at the left end of the genome *Cell* 20:787-95


125. Miyashita T, Reed JC. 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene Cell 80:293-9


166. Fessler SP, Chin YR, Horwitz MS. 2004. Inhibition of tumor necrosis factor (TNF) signal transduction by the adenovirus group C RID complex involves downregulation of surface levels of TNF receptor 1 J Virol. 78:13113-21

167. Tollefson AE, Stewart AR, Yei SP, Saha SK, Wold WS. 1991. The 10,400- and 14,500-dalton proteins encoded by region E3 of adenovirus form a complex and function together to down-regulate the epidermal growth factor receptor J. Virol. 65:3095-105


289. Pahl HL. 1999. Signal transduction from the endoplasmic reticulum to the cell nucleus *Physiol Rev.* 79:683-701


