# A PROTEIN ADOPTING A UNIQUE MEMBRANE CONFORMATION ALLOWS FOR A NEW VIRAL IMMUNOEVASION STRATEGY

A study of the function, targeting and membrane topology of adenovirus E3/6.7K protein.

by

ALEXANDRU RADU MOISE

B.Sc. Hon., Genetics, The University of British Columbia, 1994

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
in
THE FACULTY OF GRADUATE STUDIES
DEPARTMENT OF ZOOLOGY

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
July 2000 ©Alexandru Radu Moise, 2000

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Zoology

The University of British Columbia
Vancouver, Canada

Date 2nd November 2000

## Abstract

Adenovirus persistently infects the eye, ear and the respiratory and digestive tracts in humans. Its persistence is due to in part to immunosubversive proteins coded by the E3 region of the viral genome. This thesis project started from the enticing prospect that E3/6.7K, one of the two remaining uncharacterized proteins coded by the E3 region, also has a role in virus-host cell interaction. Previously, E3/6.7K was shown to be an endoplasmic reticulum (ER) localized membrane glycoprotein. Our first functional observation was that in the presence of E3/6.7K there was a reduced inflammatory response in the lungs of virally infected mice. Consequently, cells that express E3/6.7K were protected against apoptosis induced by TNF as well as other Death Inducers, such as Fas Ligand or TRAIL. The release of inflammatory mediators by cells stimulated with TNF was also significantly reduced in the presence of E3/6.7K. More importantly, we observed that E3/6.7K prevents the efflux of calcium ions from the ER. The release of calcium ions from the ER is known to be important for the generation of mediators of inflammation and apoptosis. The effect of E3/6.7K on calcium ion homeostasis can explain the protein's protective effects against inflammatory cytokines. In an effort to explore the E3/6.7K protein in further detail, we examined its structure and membrane targeting. In spite of being an ER-localized membrane protein, E3/6.7K lacks the targeting signals that are necessary for a newly synthesized protein to be translocated across the lumen of the ER. We found that E3/6.7K translocated across the ER membrane following complete synthesis in contrast to the signal sequence-dependent mode of targeting characteristic for most other proteins secreted by higher eukaryotes. Analysis of the orientation of E3/6.7K indicated that it can assume two distinct orientations including the first encountered example of a 'hairpin' topology with both its N and C termini in the lumen of the ER. Ultimately, these studies challenged the current model of membrane protein structure and uncovered a new viral immunoevasion mechanism.

Table of contents

## List of Figures

# List of Tables

## List of Abbreviations

| | |
|---|---|
| $^{3}H$ | Tritium |
| $^{35}S$ | Sulphur-35 |
| AAV | Adeno-asssociated virus |
| Ad | adenovirus |
| ATM kinase | ataxia-telangiectasia mutated (ATM) kinase |
| ATP | adenosine triphosphate |
| Bad | Bcl-xL/Bcl-2 associated death promoter |
| BAFF | B cell activating factor belonging to the TNF family (identical to BlyS, TALL-1, THANK, zTNF4) |
| Bak | Bcl-2 antagonist killer |
| Bap31 | B cell accessory protein of 31kDa |
| Bax | Bcl-2 associated x protein |
| BCMA | B cell maturation antigen |
| Bcl-2 | B-cell lymphoma/leukemia -2 proto-oncogene |
| Bcl-xL | Long form of Bcl-x, a member of the Bcl-2 family that inhibits apoptosis |
| Bid | BH-3 Interacting Domain Death agonist that induces ICE-like proteases and apoptosis |
| Bik | Bcl-2 interacting killer. Bik is a BH-3 domain containing protein that is also known as BP4, Bip1 and NBK. Bik is pro-apoptotic |
| Bim | A member of the Bcl-2 family that promotes apoptosis |
| BiP | Immunoglobulin heavy-chain binding protein, lumenal DnaK/hsc70 homologue, known as Kar2p in yeast |
| BLyS | B lymphocyte stimulator (identical to BAFF, TALL-1, THANK, zTNF4) |
| C | Carboxyl terminus of a polypeptide |

| | |
|---|---|
| $Ca^{2+}$ | calcium ions |
| CAD | Caspase-activated DNase in mouse. CAD is homologous to human CPAN or DFF40 |
| CAML | calcium-modulating cyclophilin B ligand |
| CAR | Coxsackievirus and Adenovirus Receptor |
| CBP | CREB binding protein (while CREB is the cAMP-regulated enhancer binding protein) |
| CDC 42 | Rho family GTPase |
| CHX | cycloheximide |
| cIAP | Cellular inhibitor of apoptosis protein |
| CJD | Creutzfeldt-Jakob disease |
| CPE | Cytopathogenic effect |
| cPLA2 | cytosolic phospholipase A2 |
| CTL | Cytotoxic T cell |
| DAD1 | Defender against apoptotic death 1 also known as Ost2p, member of the OST complex |
| DAP kinase | Death associated protein kinase |
| dATP | Deoxyadenine triphosphate |
| DBP | Adenovirus DNA binding protein |
| dCMP | deoxycytidine monophosphate |
| dCTP | deoxycytidine triphosphate |
| DD | death domain as protein motif |
| DED | death effector domain as protein motif |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |

| | |
|---|---|
| DR4, 5 | Death Receptor 4 and 5, respectively, receptors of TRAIL |
| dsRNA | Double stranded RNA |
| DTT | Dithiothreitol |
| dTTP | Deoxy thymidine triphosphate |
| ECM | Extracellular Matrix |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethyleneglycoltetraacetic acid |
| EGF | Epidermal growth factor |
| eIF2 $\alpha$ | Eukaryotic initiation factor 2-alpha |
| ER | endoplasmic reticulum |
| FACS | fluorescence activated cell sorter |
| FADD | Fas-associated Death Domain containing protein |
| Fas | FS-7 cell-associated cell surface antigen, also called CD95 or APO-1 |
| FCS | Fetal calf serum |
| FLASH | FLICE associated huge protein |
| FLICE | Fadd-like ICE. FLICE is also known as Caspase-8 |
| FIP-1 | Adenovirus E3/14.7 interacting protein-1 |
| FIP-2 | Adenovirus E3/14.7 interacting protein-2 |
| FIP-3 | Adenovirus E3/14.7 interacting protein-3 |
| G-418 | Aminoglycoside structurally similar to neomycin, gentamycin and kanamycin blocks protein synthesis in mammalian cells. Resistance to G-418 is mediated by the bacterial aminoglycoside phosphotransferase gene (derived from tn5). |
| GDP | Guanosine diphosphate |
| GPI | Glycophophatidylinositol |

| | |
|---|---|
| GSS | Gerstmann-Straussler-Scheinke disease |
| GTP | Guanosine triphosphate |
| hr | hours |
| hsc70 | 70kDa class heat shock cognate protein |
| Hsp70 | 70kDa class heat shock protein |
| HIV | Human Immunodeficiency Virus |
| IAA | Iodoacetamide |
| ICAD | Inhibitor of caspase-activated DNAse |
| IFN | Interferon |
| Ii | Invariant chain |
| I-κB | Inhibitor of NF-κB |
| IP3 | Inositol 1,4,5-triphosphate |
| ITR | Inverted terminal repeats |
| IU | International units |
| JAK | Janus kinase |
| JNK | c-Jun N-terminal kinase (JNK) protein kinase, also known as stress-activated protein kinase (SAPK) |
| kbp | Kilobase pairs |
| KOac | Potassium acetate |
| MAP | Mitogen activated protein |
| Mdm-2 | Mouse double minute 2 protein (mdm2), and its human homologue (MDM2) associate with p53, also known as p53-associated phosphoprotein p90 |
| MEKK1 | Mitogen-activated protein (MAP) (or Erk kinase)-kinase-1 |
| $Mg(OAc)_2$ | Magnesium acetate |

| | |
|---|---|
| MHC | Major histocompatibility complex |
| MLP | Major late promoter |
| MTOC | Microtubule-organizing center |
| N | Amino terminus of a polypeptide |
| NAC | Nascent-chain associated complex |
| NFI | Nuclear factor I, also known CTF-1 |
| NFII | Nuclear factor II |
| NFIII | Nuclear factor III, also known as Oct-1 |
| NF-AT | Nuclear factor of activated T-cells transcription factor |
| NF-κB | Nuclear transcription factor κB |
| NLS | Nuclear localization sequence |
| NP-40 | Nonidet P-40 also known IPEGAL-CA-630 |
| NPC | Nuclear pore complex |
| OST | Oligosaccharyl-transferase complex |
| PARP | Poly (ADP-ribose) polymerase |
| PI3K | Phosphotidylinositol-3-OH kinase |
| p.i. | Post infection |
| pIH | pIRES-HOOK selectable mammalian expression vector |
| pfu | Plaque-forming units |
| PKC | Protein kinase C |
| PKR | Protein kinase R, double-stranded RNA-dependent protein kinase |
| PMSF | Phenylmethylsulfonylfluoride |
| PNGaseF | Peptide N-glycosidase F |

| | |
|---|---|
| PP2A | Protein phosphatase 2 A |
| ProtK | Proteinase K |
| Prp | Prion protein |
| $Prp^{C}$ | Prion protein, GPI-anchored, cell surface localized |
| $^{Ctm}Prp$ | Prion protein, C-lumenal/N-cytosolic orientation |
| $^{Ntm}Prp$ | Prion protein, N-lumenal/C-cytosolic orientation |
| $Prp^{Sec}$ | Prion protein, secreted form |
| pTP | Precursor of the terminal protein |
| pTP-CAT | pTP attached to an oligonucleotide with the sequence 5'-C-A-T-3' |
| PVDF | Polyvinyldifluoride |
| RAC1 | Rho family GTPase |
| RAIDD | RIP associated Ich-1/CED homologous protein with death domain |
| Rb | Retinoblastane susceptibility gene product |
| RGD | Arginine (R)-Glycine (G)-Aspartic acid (D) motif |
| Rho GTPase | Superfamily of small GTPases |
| RIDα and β | Receptor Internalization and Degradation adenovirus proteins, also known as E3/10.4K and E3/14.5K, respectively |
| RIP | Receptor-interacting protein |
| RyR | Ryanodine receptor |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| Sec | Secretory pore, multisubunit channel composed of proteins identified as secretion impaired mutants (sec) in yeast |
| SPP | Sphingosine-1-phosphate |
| SOCC | Store operated calcium channels |

| | |
|---|---|
| SR | SRP receptor |
| SRP | Signal Receptor Particle |
| STAT | Signal transducer and activator of transcription |
| TACI | Transmembrane activator and CAML-interactor |
| TALL-1 | TNF and ApoL related ligand TALL-1 (identical to BAFF, BLyS, THANK, and zTNF4) |
| TBS-T | Tris-buffered saline with Tween 20 ® detergent |
| TCA | Trichloroacetic acid |
| TEA-HCl | Tris EDTA acetate-hydrocloric acid |
| TfR | Transferrin receptor |
| THANK | TNF homologue that activates apoptosis, nuclear factor-kappaB, and c-Jun NH2-terminal kinase (identical to BAFF, BLyS, TALL-1, and zTNF4) |
| TNE | Tris sodium EDTA |
| TNFα | Tumor Necrosis Factor alpha |
| TNFR | Tumor Necrosis Factor alpha receptor |
| TP | terminal protein |
| TPL | Tripartite leader |
| TRADD | TNFR1-associated Death Domain containing protein |
| TRAF | TNFR- associated factor |
| TRAIL | TNFR related apoptosis inducing ligand |
| TRAM | translocating chain-associating membrane protein |
| zTNF4 | tumor necrosis factor-like protein (identical to BAFF, BLyS, THANK, and TALL-1) |

## Acknowledgements

I am most grateful to my supervisor Dr. Wilfred A. Jefferies for the support, suggestions and advice he provided about life and science throughout my degree. I also acknowledge the guidance of my graduate committee: Dr. Reinhard Gabathuler, Dr. Shirley Gillam, Dr. John Gosline and Dr. Linda Matsuuchi. I am especially grateful to Dr. Tim Vitalis and Izidor Kern for their significant observation of the *in vivo* effects of the E3/6.7K protein (Fig. 13 and 14) and to Dr. Roger Lippe for his early work on E3/6.7K and the observation of the oxidation state of the E3/6.7K protein (Fig. 25B).

I would like to acknowledge the contribution of those who provided reagents used in this project. I thank Dr. W. S. M. Wold for the gifts of polyclonal antiserum to E3/6.7K and especially the dl739-Ad2 (E3/6.7K-deletion) virus and the plasmid bearing the Ad2 E3 region. I am grateful to Dr. F. L. Graham for the gift of wild type Ad2. I am grateful to Dr. G. C. Shore for his gifts of BAP31 cDNA and BAP31 polyclonal antiserum.

I thank those who have taught me special cell biology techniques: Dr Gabathuler for his help with the *in vitro transcription-translation* system and the purification of microsomes. Dr. Sarah Townsend and Geoff Osbourne for their invaluable assistance in analyzing calcium ion fluxes. I am also grateful to Derek Miller for proofreading of the manuscript.

This project would not have been possible without those whose ideas and critical comments illuminated my path through the byzantine complexities of cellular biology. In alphabetical order these contributors are Gaba, Gregor, Ian, Jacob, Jason, Joe, Linda, Maki, Roger, Tim and Wilf. The research was supported by operating grants from the Medical Research Council, Natural Sciences and Engineering Research Council and National Cancer

Institute of Canada to Wilfred Jefferies. I am grateful to the Department of Zoology for the opportunities it created for me during my degree.

Above all, I am grateful to my wife Barbara and my mom for their love and unrelenting support and motivation. In the end, I dedicate this work to the memory of my father.

# Chapter 1. Introduction

## *Why Do We Study Viruses?*

Somewhere at the border between life sciences and organic chemistry lies the force that drives all living things: the maintenance and propagation of self. Virus evolution, like all other living organisms, is motivated by one simple purpose, self-perpetuation. Anything that interferes with this outcome is disadvantageous and a virus takes a minimalist approach to the problem. It replicates, sometimes avoids the immune response and transmits itself to a new host.

For reasons of simplicity and possibly to be undetected by the immune response of the host, a virus relies heavily on its host and on a limited set of its own genes to achieve these goals. The virus is the ultimate parasite; it simply hijacks the machinery of the host cell as an energy source and as a reproductive engine. The virus capsid is built by using large numbers of the product of several genes, packed in a regular fashion around the nucleic acid.

Mechanisms derived from the parallel evolution of the virus and host organism (and often stolen from the host genome) let the virus avoid the immune response. The virus will simply borrow a host gene and modify it to suit its needs as appropriate. Some of these proteins only simulate the structure of host factors not their function. These non-functioning replicas affect the normal workings of the cell.

By studying many viruses, we discover what is truly essential for a cell to function. The survival mechanisms evolved by a virus point out the most effective players of the immune system and the way to avoid them. In fact, virus biology represents the simplicity and perfection of an organism that evolves in very inauspicious circumstances in complete dependence of their host organism. Many early advances in molecular biology were extensions or generalizations of processes first discovered in viruses.

## *1.2 History*

Near the end of the 18th century, Edward Jenner of Gloucestershire, England, performed the first experiments using viruses (Jenner, 1923). His studies of vaccination with cowpox virus paved the way for the eventual eradication of the most devastating of contagious diseases, smallpox. Nearly a century later, several researchers described the existence of pathogens that were unlike any described bacteria. Pathogens invisible by light microscopy were identified in rabies-infected animals by Pasteur in the 1880's (Universite de, 1923) and in rinderpest-infected cattle by Murchinson and Beale in 1866 (Kinnaird, 1866). The next advance was the observation that these agents passed through filters that otherwise retained known bacteria. These filterable agents were discovered in filtered tobacco sap by Ivanovski in 1892 (Lechevalier, 1972), in filtered silkworm extract by von Prowazek in 1907 (Wondrak, 1991), in filtered bacterial culture by Twort and d'Herelle in 1917 (Herelle, 1922)-indeed in nearly all kingdoms of life. Even a human disease, yellow fever, was shown to be transmitted through filterable agents in 1900 by the U.S. Army Yellow Fever Commission headed by Reed (Kelly, 1923).

At the time, these agents were suspected of being very small bacteria, or possibly a soluble bacterial toxin that could not be retained by the existing filters. The idea that the filterable agent undergoes multiplication was advanced by Loeffler and Frosch, who showed in 1898 that the transmission of the agent is not affected by dilution. The idea of extremely small bacteria was gaining credibility (Rott, 1999). However bacteria, small or large, were always absent in the postmortem analysis of infected organisms.

In 1898, Beijerink examined the same findings as Iwanowski and offered a different interpretation that now seems visionary:

"There is another explanation to be considered, namely that the contagion, to reproduce itself, must be incorporated into the living cytoplasm of the cell, into whose multiplication it is, as it were, passively drawn."

Beijerink's paper was published in *Zentralblatt für Bakteriologie* and called "On a *Contagium vivum fluidum* as the cause of mosaic disease of tobacco leaves". The full implications of his hypothesis took another thirty years to become apparent when they brought about the realization that living cells are necessary for the virus to replicate (Beijerinck *et al.*, 1942). This realization yielded the first corollary of virology: that the replication of viruses is completely dependent on the machinery of the cell.

Researchers developed methods for growing viruses in embryonated hen's egg (Woodruff and Goodpasture, 1931) and tissue culture (Parker and Nye, 1925), resulting in the large scale production of viruses for further analysis (Rivers and Amos, 1928). The efforts culminated in the purification of influenza virus by Smith, Andrews and Laidlaw in 1933 (Laidlaw, 1938), the crystallization of tobacco mosaic virus by Stanley in 1935 (Stanley and Valens, 1961) and the development and use of the transmission electron microscope by Ruska and Knoll to produce pictures of tobacco mosaic virus, poxviruses and bacteriophages in the 1930's (Ruska, 1950). Workers in the new field of medical virology started to isolate the etiologic agents for many transmissible diseases while others continued to study the newly characterized life forms from a more basic perspective.

One of the greatest contributions of the early days of virus research was the establishment of international centers of research such as the Phage School at Cold Spring Harbor Laboratory on Long Island. The Phage School contributed such concepts as the description of messenger RNA, the identification of nucleic acid as the genetic material of bacteriophages and the idea that a virus can coexist with its host without causing lysis. The Phage School research also contributed to the discovery of processes such as gene regulation, the genetic code, and transcription and translation. Later, research on animal viruses lead to the discovery of mRNA splicing and polyadenylation and the process of cellular transformation.

Virus research has continued to shed light on very complex mechanisms of cellular biology and is currently at the forefront of the study of the host's immune system. It began by examining the way the virus usurps the host cell to replicate; now it studies how the virus avoids the immune response. The future of virus research may demonstrate new adaptive mechanisms of viruses, as they are relevant to the collective immunity of a population of host organisms. Perhaps, this new knowledge will allow us to understand how genes are expropriated from the host cell and modified to suit the virus's subversive functions.

### *1.3 Adenoviridae*

Adenoviruses are a ubiquitous group of viruses that were first isolated by Rowe et. al. in 1952 (Rowe *et al.*, 1952) from human adenoid tissue, from which the name adenovirus derives. Two years later Hilleman et. al. discovered a pathogen in army recruits with acute respiratory illness (Hilleman and Werner, 1954) which was later shown to have the same complement fixation properties as the virus previously isolated by Rowe et. al.. Over 80 serotypes of adenovirus have been identified since then, of which 49 are of human origin.

The Adenoviridae family is composed of two genera: *Mastadenovirus* (adenovirus with mammal hosts) and *Aviadenovirus* (adenovirus with avian hosts). The haemagglutination pattern obtained by adding the viral isolate to a preparation of red blood cells from rat or rhesus monkey allowed the classification of *Mastadenovirus* in four groups identified by roman numerals (see Table I). The haemagglutination test depends on the assembly of monomeric penton capsomeres into dodecons and the interaction of the assembled penton with the red blood cells. It was observed that some groups like B and D cause complete haemagglutination of rhesus monkey or rat erythrocytes, respectively, while others such as A show a very modest haemagglutination reaction. For groups C, E, F and G, it has been observed that monomeric pentons are made in vast excess over the ones incorporated in the viral capsid. In these groups, monomeric penton units disrupt the complete haemagglutination pattern of the virion resulting in

partial haemagglutination of rat erythrocytes. A complete haemagglutination reaction can be obtained by adding a heterotypic antibody directed against the penton base, which does not recognize the haemagglutinin specific fiber.

As adenovirus was observed to cause tumors in hamsters, the oncogenic potential in mice was used to form another classification scheme. The Human *Mastadenovirus* subgenus is further subdivided in seven groups A-G based on DNA base composition and homology. Table I shows the current classification of human adenovirus subgroups and the various properties, diseases and oncogenic potential in rodents associated with each group. The DNA homology between serotypes found in the same group is usually greater than 85% (except for group A where the homology between the three members is 48-69%). Consequently, serotypes from the same group can recombine but the same is not true for serotypes found in different groups. The parts of the genome that show the greatest degree of sequence divergence are the ones coding for the hexon, the fiber and some of the proteins from the E3 region. It is advantageous for a virus to have a high degree of polymorphism in the genes coding for the major antigenic determinants (the hexon and the fiber). Meanwhile, the E3 region codes for proteins that are not essential for virus growth in tissue culture but are necessary for evasion of the immune response. It is possible that the polymorphism present in the E3 region correlates with the variety of pathological manifestations demonstrated by different serotypes. The expression pattern of the E3/6.7K protein from subgroup C, serotype 2 and related proteins from other serotypes is provided here for reference. The serotypes that are known to code for E3/6.7K or E3/6.7K-like proteins appear to be associated with infections of the upper and lower respiratory tract.

**Table I. Classification of Human Adenovirus Serotypes**

| Sub-group | Serotypes | Hemagglutination groups | Percentage of G+C content | Tumors in animals | Common symptoms of infection in humans | E3/6.7K protein expresssion |
|---|---|---|---|---|---|---|
| A | 12, 18, 31 | IV (low or none) | 48-49 | high | Gastrointestinal or asymptomatic | Not present |
| B | 3, 7, 11, 14, 16, 21, 34, 35 | I (complete of monkey RBC) | 50-52 | moderate | Upper and lower respiratory tract, conjunctivitis, kidney and bladder (acute hemorrhagic cystitis Ad11) | 131-146aa homologue |
| C | 1, 2, 5, 6 | III (partial of rat RBC) | 57-59 | Low or none | Most common Ad infections. Upper respiratory tract in young children, conjunctivitis, kidney, persistent in lymphoid cells | 61-63 aa |
| D | 8, 9, 19, 37, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36, 38, 39 | II (complete of rat RBC) | 57-61 | Low or none | Epidemic keratoconjunctivitis | Not known at present |
| E | 4 | III (partial of rat RBC) | 57-59 | Low or none | Acute respiratory disease (ARD) in military recruits, conjuctivitis | As a 23kDa homologue |
| F | 40 | III (partial of rat RBC) | | Unknown | Pediatric gastroenteritis | Not present |
| G | 41 | III (partial of rat RBC) | | Unknown | Pediatric gastroenteritis | Not present |



TABLE I

### *1.4 Adenovirus Epidemiology*

Adenovirus is a very common human pathogen that results in persistent infections of the respiratory or gastrointestinal tract (Fox *et al.*, 1969; Fox, Hall, and Cooney, 1977). In humans, approximately 5% of the acute respiratory disease in children under the age of five is due to an Adenovirus (Brandt *et al.*, 1969). Adenovirus appeared as a pathogen in military recruits, causing Acute Respiratory Disease (ARD). It can cause infections of the eye such as the epidemic keratoconjunctivitis observed in Germany in the 1920s (Hogan and Crawford, 1942) and can lead to gastrointestinal disease and acute hemorrhagic cystitis. Adenovirus has also been observed in other hosts, causing hepatitis in dogs and other diseases in fowl, pigs, mice, cattle and monkeys.

Adenovirus has been shown to be shed by infected patients up to 24 months following infection (Fox *et al.*, 1969). Due to its persistence adenovirus can resurface and cause disseminated disease in immuno-compromised patients through reactivation (Krilov *et al.*, 1990). For example, over 10% of patients with acquired immunodeficiency syndrome (AIDS) shed adenovirus in their urine (de Jong *et al.*, 1983). In addition to persistent viral infection, adenovirus DNA can be found in a variety of tissues for many years following infection. Its latency in tonsillar tissue has been demonstrated by the presence of viral DNA and the absence of any infectious particles following repeated passage (Neumann, Genersch, and Eggers, 1987). It has been speculated that adenovirus can establish reservoirs in cells of lymphoid origin, in particular B cells (Abken, Butzler, and Willecke, 1987).

Adenovirus is frequently found in mixed infections involving other viral pathogens such as Epstein-Barr virus (Okano, Thiele, and Purtilo, 1990) or bacterial pathogens such as *Haemophilus influenzae* (Korppi *et al.*, 1991) or *Streptococcus pneumoniae* (Korppi *et al.*, 1991) and *Bordetella pertussis* (Nelson *et al.*, 1975).

Human adenovirus was the first virus shown to induce malignant tumors in animals. Fortunately, there have been no direct indications that adenovirus contributes to the generation of

tumors in humans. The transforming ability of adenovirus has been genetically mapped to the left end of the adenovirus (Ad) genome, encompassing a region corresponding to 8% of the Ad genome(Chinnadurai *et al.*, 1976).

Adenovirus infections occur in many species, in a variety of tissues They can result in the establishment of persistent infections particularly in lymphoid tissue such as the tonsils. In most cases, however, these infections do not have serious clinical manifestations.

### *1.5 Structure of adenovirus*

Adenovirus is a non-enveloped virus containing a double-stranded linear DNA genome ranging in size from 30 to 36 kilobasepairs (kbp) encapsulated by a multimeric protein complex. The virus particle ranges in size from 65nm to 80nm. The structural component of the virus is composed of an icosahedral capsid of 252 capsomers of which there are 12 pentons (which have a non-covalently attached fiber) and 240 hexons (Fields, 1985). Figure 1 shows a schematic representation of a cross section through an adenovirus particle.

The self-assembling viral capsid is formed from the products of seven genes. The names hexon and penton refer to the number of planes of symmetry that pass through each subunit. Pentons surrounded by five hexons are found at each of the 12 vertices. Pentons are composed of polypeptides III (85kDa) and IIIa (66kDa) forming the penton base and polypeptide IV (62kDa) forming the fiber. Polypeptide V (48.5kDa) bridges the viral DNA to the penton base and serves to position and package the viral genome. Hexons are surrounded by six identical subunits. A group of nine hexons forms one of 20 triangular surfaces resulting in a regular icosahedron structure. A hexon is a trimer of three identical subunits composed of polypeptide II (120kDa). Polypeptides VI (24kDa), VIII (13kDa) and IX (12kDa) are structural proteins associated with the hexon.

The antigenic determinants of adenovirus are present on the hexon, penton and the fiber. The existence of antigenic determinants is important in the serologic classification of adenovirus and the generation of neutralizing antibodies. Interestingly, the α antigenic determinant, found in all serotypes is present on the inner surface of the hexon capsomere. Consequently the α determinant, hidden within the intact capsid does not give rise to neutralizing antibodies. The α determinant is only accessible in soluble hexons present in infected cells (Fields, 1985). The vast excess of hexons cannot be incorporated in the viral capsid. Other antigenic determinants give rise to neutralizing antibodies. Such is the case for the ε and γ determinants which are present on the surface of the virus, on the hexon and the fiber, respectively.

The nucleocapsid, the replicative component of the virus, is made up of the linear viral genome and a coat of protective proteins. Polypeptide VII (18.5kDa), as the second most abundant protein after polypeptide II, serves as a coat protein that protects the viral genome. It has been shown that polypeptide VII forms a nucleosome-like structure around the viral genome. The ends of the linear genome consist of two inverted terminal nucleotide sequence repeats (ITR) consisting of 52-165bp. A 55kDA terminal protein (TP) is covalently attached to the 5' end of each DNA strand, as shown in Figure 1. Both the ITR and the 55kDa protein serve roles in viral genome replication.

The genome is arranged in functional regions, each region is controlled by the same promoter. The regions are divided into early (E) and late (L) transcription regions referring to the time the region is transcribed relative to the replication of the viral genome (see Table II). The early regions are transcribed before the viral genome has replicated. They code for genes that modulate host cell-virus interactions as well as genes involved in viral genome replication, while the late regions are transcribed after viral replication and they code for the structural proteins that make up the capsid. There are a few cases, though, where late genes are transcribed before viral replication as seen in the case of the L1 mRNA. There are also cases where early genes are transcribed after viral replication under the control of a late promoter as seen in the case of the

E3/11.6K protein. Figure 2 indicates the arrangement of the transcriptional units in the genome of Adenovirus type 2.

In 1977 a team led by Richard J. Roberts at Cold Spring Harbor Laboratory on Long Island and a team led by Phillip A. Sharp at Massachusetts Institute of Technology independently discovered that the genes of adenovirus are discontinuous (Kornblihtt, 1993). Previous studies in bacteria showed that their genes are continuous stretches of DNA that serve as direct templates for mRNA synthesis. In adenovirus, on the other hand, proteins are coded by segments of DNA called exons that are interrupted by non-coding segments of DNA called introns. Later, introns were found in all other eukaryotes.

**Figure 1. Structure of the adenovirus virion.**

Schematic representation of a cross section through an adenovirus particle. Adapted from *Virology* edited by B.N. Fields et. al.. Raven Press 1985 (Fields, 1985). Roman numeral (I-IX) represent the various polypeptides that make up the virus. TP stands for terminal protein, which is associated with the ends of the viral genome.

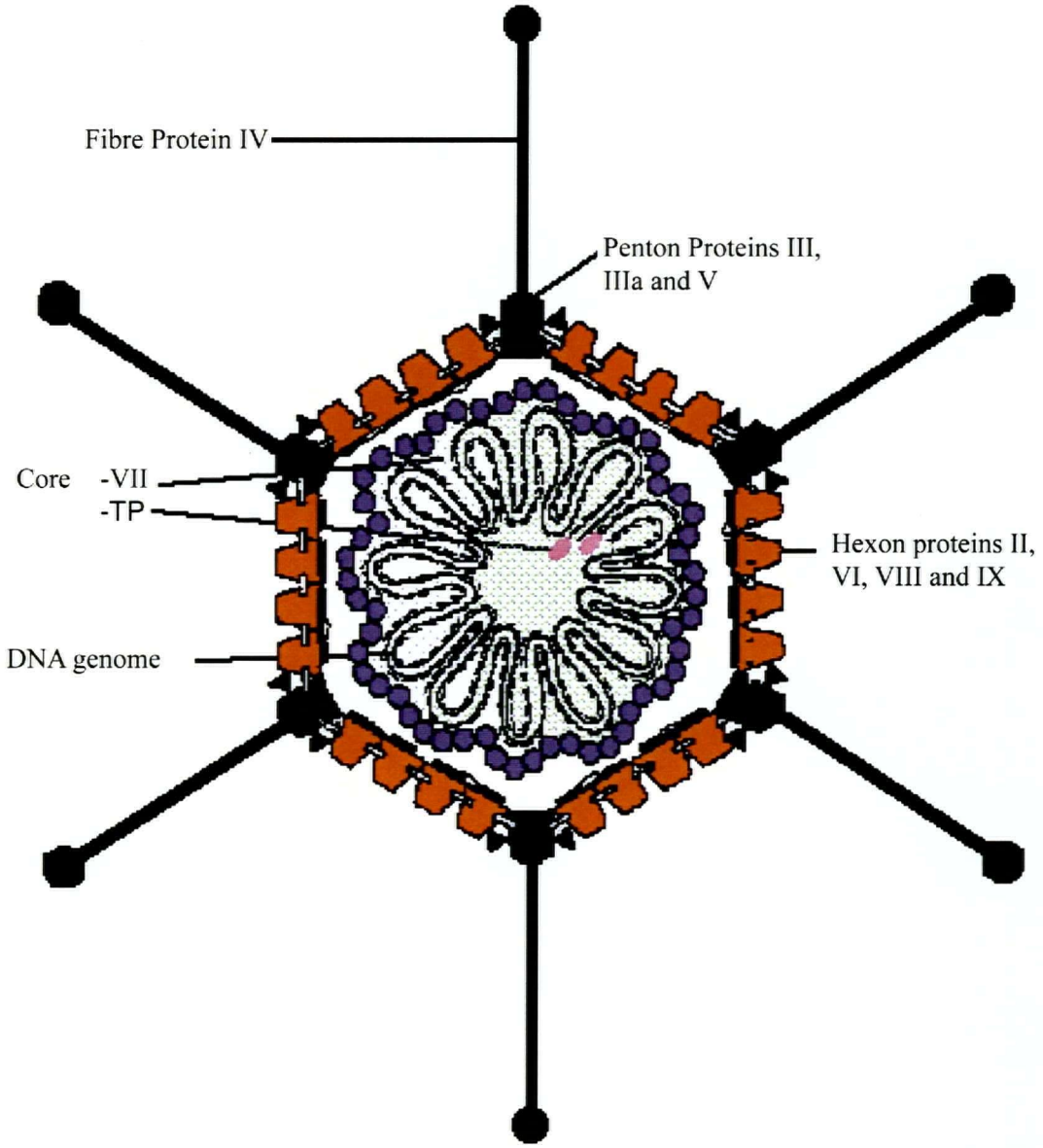
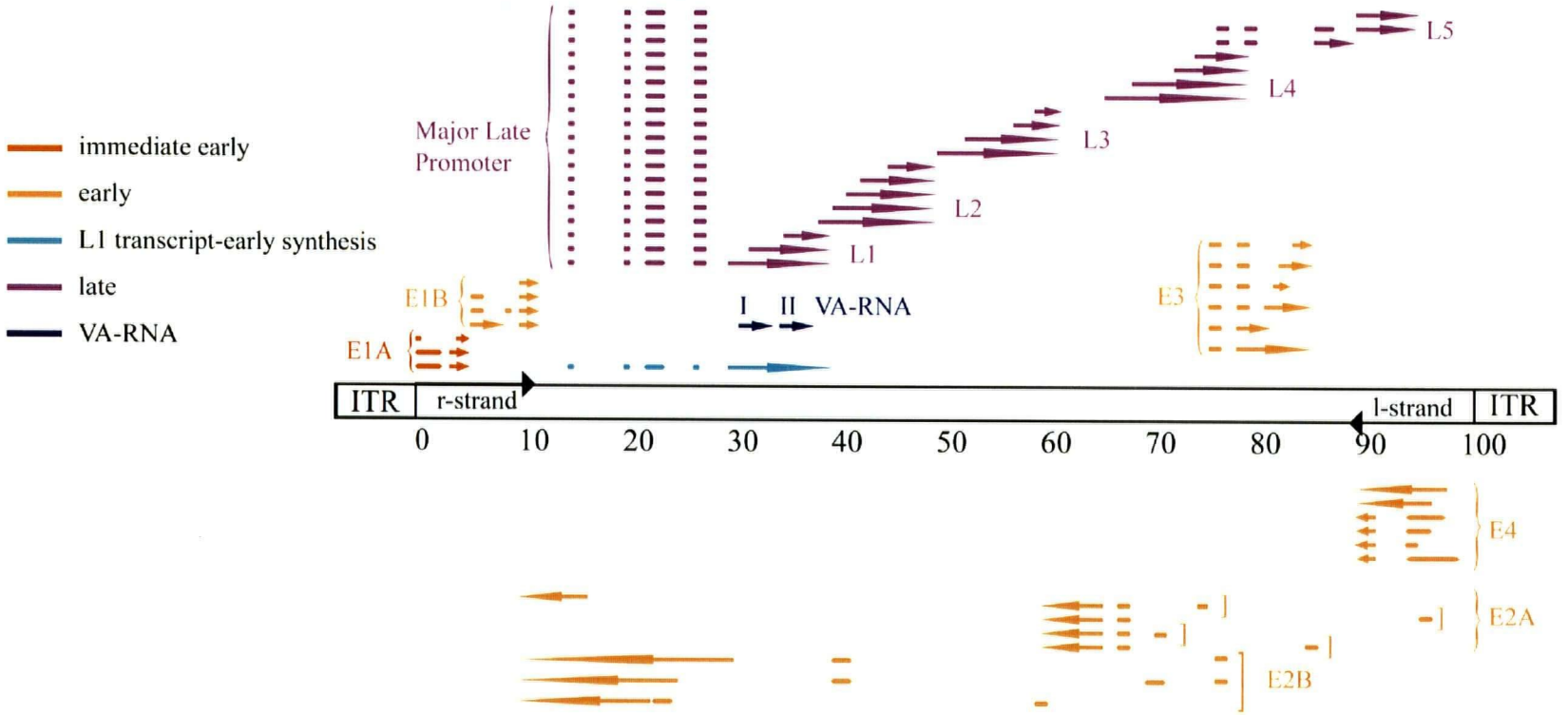

Fibre Protein IV
Penton Proteins III,
IIIa and V
Core -VII
-TP
Hexon proteins II,
VI, VIII and IX
DNA genome

Figure 1

**Figure 2. Transcriptional map of the genome of Adenovirus Type 2.**

Functions assigned to the different regions Ad genome are indicated. Transcripts are indicated by interrupted arrows (indicating the splice structure of a particular transcript). Square brackets at the 5' end of several transcripts refer to the common promoter and start site for the group of transcripts. The chronological sequence of transcription of the various regions is color-coded. The l and r strand denomination refers to the direction in which the transcripts are synthesized. The numbers refer to the relative map units on the Ad genome. The map is adapted from *Virology* edited by B.N. Fields et. al.. Raven Press 1985 (Fields, 1985).




| | |
|---|---|
| E1A | Function in the sequential regulation of all other regions |
| E1B | It is also involved in cell transformation and prevention of host cell apoptosis |
| E2 A and B | Code for proteins involved in DNA replication: |
| | -precursor terminal protein pTP primes DNA replication |
| | -DNA polymerase (Pol) |
| | -DNA binding protein (DBP) |
| E3 | Evasion of host immuno-surveillance, prevention of host cell apoptosis |
| E4 | DNA replication, late gene expression and host cell shutoff |
| L1-L5 | Structural components of the Adenoviral virions |
| ITR | Initiates viral DNA replication |

Figure 2

## *1.6 Life Cycle of Adenovirus*

### 1.6.1 Adenovirus attachment and uptake in the host cell

Adenovirus binds to the target cell via fibers that project from the adenoviral capsid. In this respect, adenovirus is different from other non-enveloped viruses. The receptor binding domain found on most other non-enveloped viruses consists of a depression formed by domains integral to their capsid, as seen in as poliovirus and coxsackie B3 virus (Muckelbauer *et al.*, 1995). Fiber-mediated host-cell attachment is common for enveloped viruses. For example, the gp120 glycoprotein of the enveloped HIV-1 virus binds to CD4 found on host T-cells (Lasky *et al.*, 1987). The cellular receptor for adenovirus has been identified as a membrane protein of 46kDa expressed by most cell types. This protein also happens to be the receptor for coxsackie B virus (Bergelson *et al.*, 1997). This protein, called Coxsackie and Adenovirus Receptor (CAR), a member of the immunoglobulin superfamily, mediates target cell attachment by binding to the adenovirus fiber protein of most adenovirus serotypes except for subgroup B adenovirus (Bergelson *et al.*, 1997).

Internalization of adenovirus requires a separate interaction with the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Wickham *et al.*, 1993). Many different classes of virus (such as hantavirus, coxsackie A9 virus and foot-and-mouth disease virus) employ integrins for cell attachment or as a co-receptor for cell entry. Integrins are large heterodimeric proteins composed of $\alpha$ and $\beta$ subunits that bind arginine-glycine-aspartic acid (RGD) sequence found in various extracellular matrix (ECM) proteins. The integrin-ECM interaction serves essential functions in cell attachment, migration, growth and differentiation and wound repair. Adenovirus exploits this mechanism by using the integrin recognition motif, i.e. the RGD sequence, which is found in the penton base of most adenovirus serotypes except for subgroup F (Albinsson and Kidd, 1999).

Adenovirus binds to integrins via the five conserved RGD motifs found in its penton base, and mediates a signaling event across the plasma membrane. Both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ classes of integrins mediate internalization of the virion into the endocytic compartment. Integrin clustering leads to the generation of an intracellular macromolecular complex known as "focal adhesion" (Miyamoto *et al.*, 1995). Some of the factors recruited to the site of focal adhesion, such as phosphotidylinositol-3-OH kinase (PI3K) and the Rho family GTP-ases RAC1 and CDC42, are required for endocytosis of adenovirus (Li *et al.*, 1998a; Li *et al.*, 1998b), possibly by promoting rearrangement of the actin cytoskeleton. Dynamin is also required for adenovirus uptake indicating that the virus is taken up through clathrin-coated pit-mediated endocytosis (Wang *et al.*, 1998). Once bound to integrins, the internalization process is rapid, occurring within 5 min at 37°C (Greber *et al.*, 1993). The internalized virus escapes from the endocytic pathway prior to endosome fusion with the lysosomal vacuole. Both the penton base-integrin $\alpha_v\beta_5$ interaction (Wang *et al.*, 2000; Wickham *et al.*, 1994) and the 23kDA adenoviral cysteine protease (Greber *et al.*, 1996) have been implicated in viral penetration of the endosomal membrane and escape from lysosomal degradation. However, this process is not clearly understood.

In the cytosol, hexon-associated proteins, protein VI and protein IX are degraded by the adenoviral L3/p23 cysteine protease (Greber *et al.*, 1996). The virion free of pentons and associated cellular proteins moves along microtubules with the aid of the minus-end directed motor, dynein/dynactin, towards the microtubule-organizing center (MTOC) found in the perinuclear region. This is where most virus particles are found 30-40 min post-infection (p.i.) (Suomalainen *et al.*, 1999). The capsid is disassembled in the vicinity of the nuclear pore complex and the viral core is imported into the nucleus through the nuclear pore (Greber *et al.*, 1997). The viral core consists of the genome and the viral polypeptides V, VII and TP.

No adenoviral capsids have ever been found within the nucleus (Fields, 1985) and the mechanism for nuclear import of adenovirus core through the nuclear pore complex (NPC) is not well understood. It is thought that the nuclear localization sequences (NLS) found within the sequence of the covalently attached the terminal protein (TP) are responsible for the targeting of

the adenoviral genome to the nucleus (Greber *et al.*, 1997). Other adenoviral proteins important in transformation, viral gene expression or viral replication, such as E1A, pIVa, DNA binding protein (DBP) and Pol have been shown to contain NLS domains.

### 1.6.2 E1A-The Immediate Early Region

In the nucleus, the viral genome associates with the nuclear matrix through the covalently attached TP. Early gene transcription occurs in five distinct transcription units and is regulated in a sequential fashion. The first gene to be transcribed is E1A, which directs the synthesis of the other early genes prior to replication of the viral genome (Nevins, 1981). Due to its essential role and temporal priority the E1A region is referred to as the "immediate" early region, while the E1B, E2, E3 and E4 are "delayed early regions. The E1A region generates five different transcripts by alternative splicing. The 13S/289 amino acids and the 12S/243 amino acids products are responsible for all currently identified functions attributed to the E1A region. These include transactivation of other early viral genes (Weeks and Jones, 1983). E1A also interacts with cellular proteins such as the p300-CBP, part of the histone acetyltransferase family of transcription factors (Lundblad *et al.*, 1995), and inhibits members of the Retinoblastane susceptibility gene product (Rb) family of proteins (Whyte *et al.*, 1988). Both interactions result in the stimulation of DNA synthesis (Howe *et al.*, 1990) and of cell cycle progression from G(0) to S through activation of the E2F transcription factor (Hiebert *et al.*, 1991). The E1A protein therefore is responsible for the oncogenic, transforming effect that adenovirus infection has on rodent cells (Chinnadurai *et al.*, 1976; Whyte, Williamson, and Harlow, 1989). It is also responsible for the increased susceptibility of infected cells to cytokines through the induction of apoptosis (Cook *et al.*, 1986).

The expression of the E1A protein in the absence of viral anti-apoptotic factors results in apoptosis through the action of the cellular tumor suppressor p53 (Lowe and Ruley, 1993). The cellular protein p53 is a very important tumor suppressor protein that can promote cell cycle

arrest and apoptosis. When a cell's DNA is damaged, the short-lived p53 protein is phosphorylated by the ataxia-telangiectasia mutated (ATM) kinase (Banin *et al.*, 1998; Kastan *et al.*, 1992) and the DNA-dependent protein kinase (DNA-PK) (Lees-Miller, Chen, and Anderson, 1990). Phosphorylation prevents its degradation through mouse double minute 2 (Mdm2) and the ubiquitin/proteasome pathway (Honda, Tanaka, and Yasuda, 1997). This in turn induces growth arrest through cyclin-dependent kinase (Cdk) inhibitor p21 (Xiong *et al.*, 1993) and programmed cell death. During viral infection the p53 pro-apoptotic protein is not degraded in the presence of E1A and it accumulates to toxic levels (Nakajima *et al.*, 1998; Querido, Teodoro, and Branton, 1997).

The region CR1 of E1A is also responsible for sensitizing infected cells to the effects of TNF-α (Shalloway *et al.*, 1987). This later effect of E1A is thought to be mediated by blocking the activation of nuclear factor kappaB (NF-kappaB) (Shao *et al.*, 1999). As discussed later NF-kappaB is part of the cellular survival pathway following TNF-α ligation of the TNF receptor I (TNFRI)-p55kDa. Ultimately, the E1A protein can also induce apoptosis in a p53-independent manner (Teodoro, Shore, and Branton, 1995) through the interaction with adenovirus the E4orf4 protein.

One immunoevassive activity attributed to E1A is the repression of the interferon (IFN) γ-stimulated genes (Leonard and Sen, 1996). The innate antiviral, IFN response inhibits viral protein synthesis. Mice deficient in either the IFN α or β-specific receptor I or the IFN-γ-specific type II receptor are unable to cope with viral infection (Muller *et al.*, 1994). The E1A protein targets the JAK/STAT pathway of IFN signaling by inhibiting the expression of STAT1 and by competing with STAT1 for the available p300/CBP adapter complex, necessary for STAT1 activity (Bhattacharya *et al.*, 1996).

### 1.6.3 The E1B and E4 Regions

The E1B region, downstream of the E1A gene, codes for two proteins, the E1B 55K and the 19K proteins, which are required for the accumulation and transport of viral transcripts (Babiss, Ginsberg, and Darnell, 1985) and the inhibition of apoptosis. The E1B 55K protein regulates viral DNA replication and nucleocytoplasmic RNA transport in infected cells (Kratzer *et al.*, 2000). It also appears to cooperate with the E1A protein in the oncogenic transformation of cells (Jenkins, Rudge, and Currie, 1984). The E1B 55K protein associates with a number of host proteins from which E1B-AP5 is a nuclear RNA-binding protein (Gabler *et al.*, 1998).

Another adenovirus protein, the E4 open reading frame (orf) 6 (E4orf6 also known as E4-34K) appears to form a functional complex with E1B 55K (Sarnow *et al.*, 1984) and localize it to the nucleus (Goodrum, Shenk, and Ornelles, 1996). The complex E1B 55K-E4orf6 is able to shuttle between the nucleus and the cytoplasm mediated by the nuclear export signal found in E4orf6 (Dobbelstein *et al.*, 1997). The E1B 55K-E4orf6 complex relocalizes cellular factors required for proper RNA processing from the host cell transcription centers to the viral transcription centers (Ornelles and Shenk, 1991), which are spatially separated in the nucleus (Pombo *et al.*, 1994). This results in the accumulation of viral mRNA in the cytoplasm of the infected cell at the expense of the host mRNA.

In addition to regulating viral RNA processing both the E1B 55K and the E4orf6 proteins have been shown to be involved in the prevention of apoptosis. The E4orf6 binds and inhibits the cellular tumor suppressor, p53 (Dobner *et al.*, 1996) and cooperates with E1A in the oncogenic transformation of cells (Nevels *et al.*, 1997). Meanwhile, E1B 55K increases the stability and the affinity of p53 for DNA sequences containing p53 binding sites, leading to repression of the activity of p53-modulated genes (Martin and Berk, 1998). Cooperatively, associated as the E1B 55K-E4orf6 complex, the two proteins also maintain low levels of p53 following infection (Querido *et al.*, 1997; Steegenga *et al.*, 1998). It has also been observed that the E1B 55K-E4orf6 complex interacts with another member of the p53 family called p73. This would serve to evade apoptosis induced by additional members of the p53 family (Higashino,

Pipas, and Shenk, 1998; Steegenga *et al.*, 1999). Other studies, though, do not support this contention (Roth *et al.*, 1998; Wienzek, Roth, and Dobbelstein, 2000). Recently, the E4orf3 protein, which is involved in viral RNA processing, has also been shown to be able to shuttle the E1B 55K from the cytoplasm to the nucleus and to be involved in modulating the E1B 55K-E4orf6 interaction with p53 (Konig, Roth, and Dobbelstein, 1999).

The other anti-apoptotic protein associated with the E1 region is the E1B 19K protein (White, 1996; White *et al.*, 1991; White *et al.*, 1992). Similarly to Bcl-2, it associates with the pro-apoptotic Bcl-2 family members Bid and Bik (Han, Sabbatini, and White, 1996) and it heterodimerizes with Bax, preventing its translocation to the mitochondria (Han, Modha, and White, 1998; Han *et al.*, 1996). E1B 19K, like Bcl-2, has been shown to interact with three cellular proteins named Nip1, Nip2 and Nip3 (Boyd *et al.*, 1994). Of these Nip3 has been shown to be a pro-apoptotic mitochondrial protein (Chen *et al.*, 1997).

The E4 region found at the opposite end of the viral genome from E1 codes for proteins that cooperate with E1A and E1B 55K in controlling gene expression. Besides contributing the E4orf6 protein to form the E1B 55K-E4orf6 complex, E4 region proteins have additional roles in regulating gene expression. The E4 region proteins E4orf3 and E4orf6 proteins are required for the proper splicing and stability of Major Late Promoter-(MLP) derived transcripts (Bridge, Hemstrom, and Pettersson, 1991; Nordqvist, Ohman, and Akusjarvi, 1994). Both E4orf3 and E4orf6 proteins operating independently of E1B 55K can be found at the sites of viral replication and transcription inside the nucleus of the infected cell (Ohman, Nordqvist, and Akusjarvi, 1993).

E1A is not the only early region gene product that has been associated with increased apoptosis. The E4orf4 has been shown to be highly apoptotic. The E4orf4 protein appears to be required for p53-independent E1A-induced cell death (Marcellus *et al.*, 1998). However, in the absence of E1A, the E4orf4 protein can still induce apoptosis in a Bcl-2 inhibitable, but caspase- and p53-independent manner (Lavoie *et al.*, 1998). The pro-apoptotic effects of E4orf4 require an interaction with protein phosphatase (PP) 2A (Shtrichman *et al.*, 1999).

Another E4 protein E4orf6/7 binds to the E2F transcription factor and transactivates the promoter for the E2 region (Babiss, 1989; Huang and Hearing, 1989). Ultimately, the Ad E4 region and the E1B 55K are essential in the life cycle of a parvovirus, called Adeno-Associated virus (AAV). This virus is a promising vector for gene therapy (Ferrari *et al.*, 1996) (Samulski and Shenk, 1988).

### 1.6.4 The E2 region-Genome replication

Viral DNA replication begins approximately 7 hr post infection. Adenovirus genome replication relies on three proteins coded by the E2 region: TP, DBP and Pol, as well as on three cellular proteins: nuclear factor (NF) I (also called CTF-1), NFII and NFIII (also called Oct-1). The process starts through the recruitment of the precursor TP (pTP) -Pol complex to the origin of replication, the ITR. This is mediated by NFI and NFIII (Nagata *et al.*, 1982; Pruijn, van Driel, and van der Vliet, 1986). Initiation consists of the attachment of the nucleotide dCTP, which forms a pTP-dCMP covalent complex. Next a dATP and then a dTTP nucleotide are attached forming a pTP-CAT primer complex. The Pol enzyme jumps back and forth to extend the trinucleotide (King and van der Vliet, 1994). Pol-mediated elongation proceeds by extending the protein primed oligonucleotide in the presence of DBP (Dekker *et al.*, 1997). After complete replication, the covalently attached pTP is converted to the mature form TP by an adenovirus-encoded protease. Figure 3 depicts a diagram that outlines this process.

**Figure 3. Diagram illustrating the replication of the adenovirus genome**

Adapted from *Virology* edited by B.N. Fields et. al.. Raven Press 1985 (Fields, 1985).

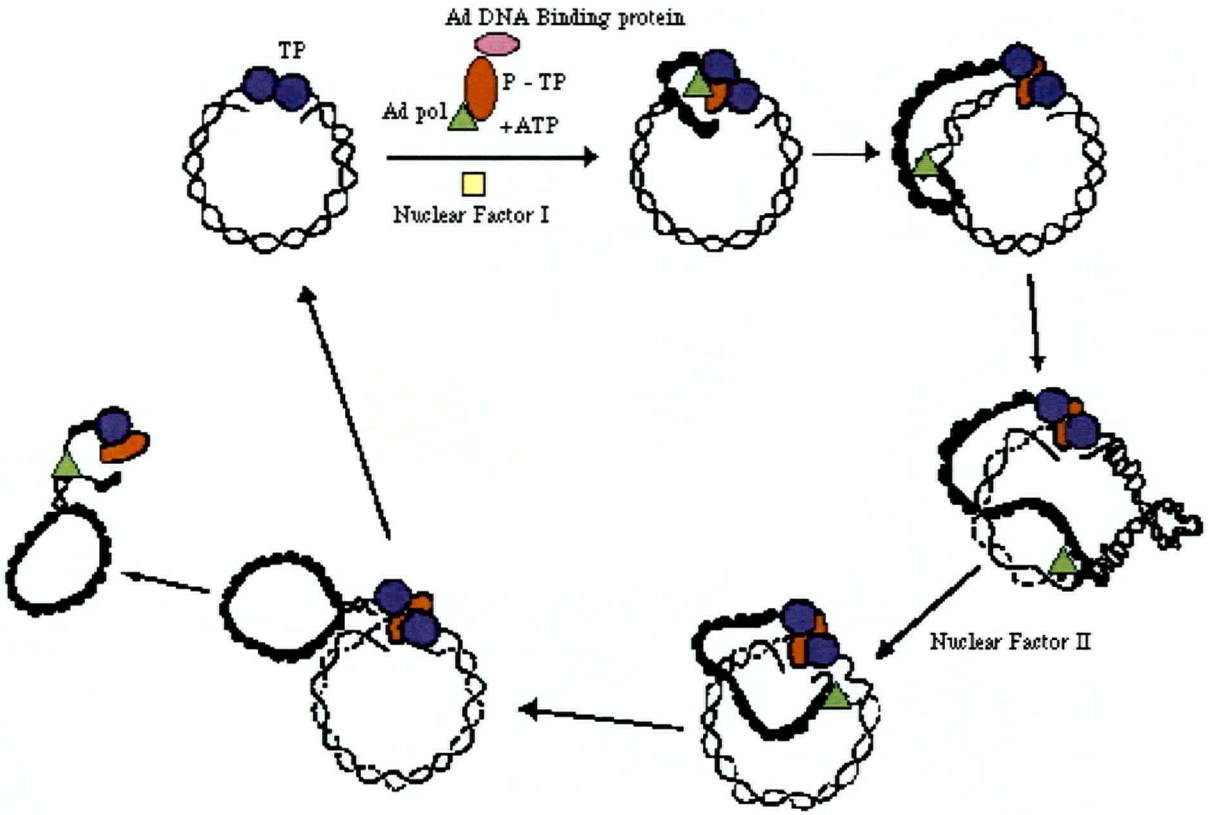

Ad DNA Binding protein
TP
P - TP
Ad pol
+ATP
Nuclear Factor I
Nuclear Factor II

Figure 3

### 1.6.5 The Late phase

Viral gene product pIVa2 contributes to the change from the Early to the Late phase of the lytic cycle. The genes coding for the pIVa2 and the IX polypeptides are turned on during viral replication before the onset of late gene expression. For this reason these two genes are part of the "intermediate" phase of the lytic cycle. Both pIVa2 and IX gene products are transcriptional activators of the major late promoter (MLP). However, the mechanism of the Early-to-Late transition is not well understood. As transcription of genes under the control of the MLP occurs, the genes that are controlled by early promoters are repressed.

MLP controls the expression of the L1 to L5 transcription units. All transcripts generated by the MLP have a tripartite leader (TPL) composed of leader exons 1, 2 and 3, spliced to their 5' termini. Assembly of the infectious particle occurs in the nucleus following translation in the cytoplasm of the late transcripts. Polypeptides VI, VII, VIII and TP are synthesized as larger precursors pVI, pVII, pVIII and pTP, respectively. Three polypeptide II monomeres assemble into trimeric hexon capsomeres. Hexon-associated proteins pVI, pVIII and IX are then incorporated, while polypeptides III, IIIa, and IV form the penton capsomere. The adenovirus' genome is then packed into the capsid resulting in a 5.5 fold length-wise compaction. The L1-coded polypeptide IIIa-55kDa, penton-associated protein and a *cis*-acting DNA sequence located near the left end of the genome both direct the packaging of the viral genome (Grable and Hearing, 1992).

Polypeptides pVI, pVII, pVIII and pTP are cleaved by the viral L3/p23 cysteine protease. This last step results in the formation of a tightly sealed capsid, impermeable to added nucleases. The adenoviral L3/p23 cysteine protease is also responsible for disrupting the cytokeratin network. It cleaves the amino-terminal head domain from cytokeratin K7 and K18 which may result in the disruption of the internal architecture of the cell (Chen, Ornelles, and Shenk, 1993).

Late in the viral cycle (usually 24 hours p.i.) infected cells round up and cluster. The changes that occur before viral release account for the cytopathogenic effect (CPE) of the virus. Inside the nucleus virions start to accumulate in electron-dense aggregates, called nuclear inclusions. In cells infected with group C adenovirus the nuclear inclusions stain Feulgen-negative and eosinophilic and later become Feulgen-positive (due to the viral DNA) and basophilic (Boyer, Denny, and Ginsberg, 1959). At 40 hours p.i. viruses are progressively released from the nucleus to the cytoplasm. It is not clear, however, how viral particles reach the extracellular milieu. It has been proposed that virions are not released through the cell lysis and that productive adenovirus infection is not a true lytic infection (Ginsberg, 1999).

One possibility is that mature virions could be released from the infected cell through apoptosis. This process has been proposed to be mediated by a protein coded by the E3 region, the pro-apoptotic E3/11.6K protein, also called the Adenovirus Death Protein (ADP). Like other E3 proteins, E3/11.6K is expressed early, but it's expression is greatly amplified late in infection (Tollefson *et al.*, 1992). It was observed that virus mutants that do not express the E3/11.6K protein result in much smaller plaques when plated on confluent permissive cells (Tollefson *et al.*, 1996a; Tollefson *et al.*, 1996b). A possible explanation for the small plaque size could be the fact that virions are trapped inside the cell.

Interestingly, even during the late phase of infection, viral mechanisms that allow the virus to evade the immune response are still at work. For example, late in infection RNA polymerase III copiously synthesizes two short transcripts VA-$RNA_I$ and VA-$RNA_{II}$. VA-RNA transcripts prevent the antiviral response triggered in response to ds-RNA or viral infection by protein kinase R (PKR). PKR activates the Nuclear transcription factor κB (NF-κB) by phosphorylating the Inhibitor of NF-κB (I-κB) (Kumar *et al.*, 1994), which induces pro-inflammatory and cytotoxic cytokines (Barnes and Karin, 1997). Other targets of PKR are RNAse L and the eIF2α subunit of the translation initiation complex, which degrade viral RNA and prevent protein synthesis (Williams, 1999), respectively. VA-$RNA_I$ binds and prevents

activation of PKR by ds-RNA (Schmedt *et al.*, 1995) and therefore blocks this form of innate antiviral response.

### 1.6.6 The E3 region

In a manner that is counterproductive to viral infection, the expression of several pro-apoptotic proteins such as E1A, E4orf4 and E3/11.6K, early in infection could compromise the infected cell and lead to its demise. If it were not for the powerful tools evolved by the virus to allow the viral cycle to continue, apoptosis would result in the premature end to adenovirus reproduction. The E3 region, in particular, codes for several proteins that are involved in the evasion of the immune response including the nonspecific response of the infected cell through apoptosis. The E3 region is a cassette of genes dedicated to immune evasion (Wold and Gooding, 1991), which codes for seven proteins that have been identified during viral infection (see Fig. 4 and Table II ). Of these, four are involved in evasion of the immune response. The E3/14.7K protein prevents Tumor Necrosis Factor (TNF) α-induced apoptosis (Zilli *et al.*, 1992) by preventing the activation of FADD-like interleukin-1beta-converting enzyme (FLICE) (Chen *et al.*, 1998) and by interacting with the GTP-binding protein FIP-1 (Li, Kang, and Horwitz, 1997), the TNF-α inducible FIP-2 (Li, Kang, and Horwitz, 1998) and the pro-apoptotic FIP-3 (Li *et al.*, 1999). The E3/10.4K and E3/14.5K (also called RID-α and -β, respectively) proteins form a complex that localizes to the plasma membrane (Stewart *et al.*, 1995) and that downregulates the surface levels of Fas (Elsing and Burgert, 1998; Shisler *et al.*, 1997; Tollefson *et al.*, 1998) and epidermal growth factor (EGF) receptor (Carlin *et al.*, 1989; Tollefson *et al.*, 1991). The complex also protects infected cells from TNF-α induced apoptosis (Gooding *et al.*, 1991) and prevents the TNF-α-induced release of arachidonic acid (Krajcsi *et al.*, 1996). The E3/19K protein localizes to the endoplasmic reticulum (ER) associates with Major Histocompatibility Complex (MHC) Class I (Persson *et al.*, 1980) prevents its transport to the surface (Burgert and Kvist, 1985). The MHC Class I complex is involved of in the presentation of antigenic peptides generated by the degradation of viral proteins (Townsend *et al.*, 1986). These peptides are

exposed on the surface of virally infected cells in the context of MHC Class I and beta-2 microglobulin complex, targeting the infected cell for killing by specific Cytotoxic T cells (CTL). The E3/19K protein retains MHC Class I in the ER, preventing the maturation and secretion of the MHC Class I to the plasma membrane and preventing the killing of the infected cell (Burgert, Maryanski, and Kvist, 1987). In addition, E3/19K also appears to prevent the association of MHC Class I with the chaperone, tapasin (Bennett *et al.*, 1999).

It is important to note that all adenovirus templates used in gene therapy lack the E3 region and are, as a result, more susceptible to the effects of TNF-α. Reincorporating the E3 region in adenovirus vectors restores the persistence and reduces the humoral and cellular immune response to the same level observed in wild-type virus (Ilan *et al.*, 1997). Another illustration of the importance of the E3 region *in vivo*. The E3 promoter has been shown to be upregulated by TNF-α, allowing for expression of adenovirus resistance genes in a timely manner (Deryckere and Burgert, 1996; Deryckere *et al.*, 1995).

**Figure 4. Map of the E3 region of Ad2 genome**

Shown is a schematic illustration of the E3 region from Ad2. Arrows indicate the splice structures of the a-i mRNAs under the control of the E3 promoter; solid parts are exons, dashed parts are introns, the thickness implies the relative abundance. The stripped bars above the arrows indicate proteins known to be expressed. The shaded box indicates the deletion present in the dl739 strain, resulting in the deletion of a region coding for the first 19 amino acid residues from the E3/6.7K open reading frame. Figure reproduced from Sparer et. al. (Sparer *et al.*, 1996).

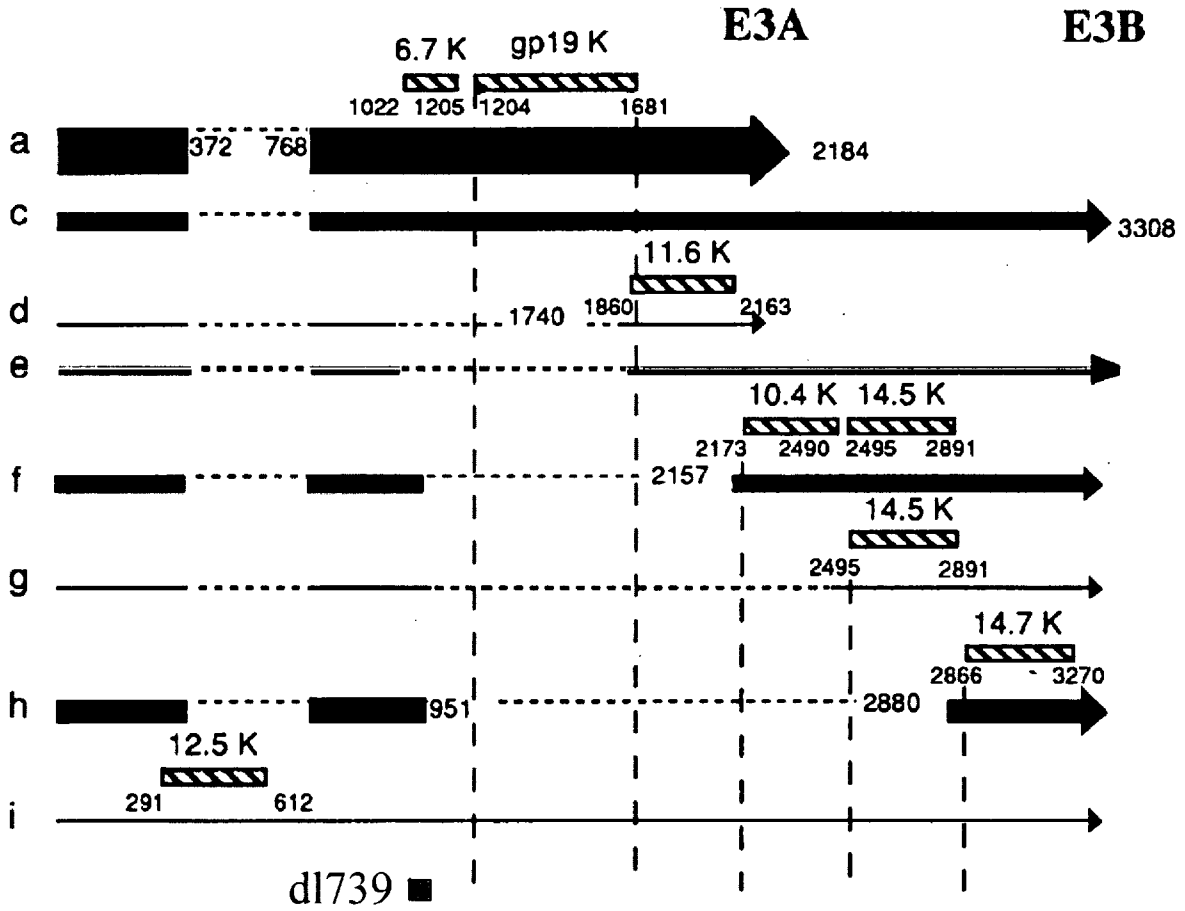

E3A
E3B
6.7 K
gp19 K
1022
1205
1204
1681
a
372
768
2184
c
3308
11.6 K
d
1740
1860
2163
e
10.4 K
14.5 K
2173
2490
2495
2891
f
2157
14.5 K
g
2495
2891
14.7 K
2866
3270
h
951
2880
12.5 K
291
612
i
dl739

Figure 4

**Table II. Functions assigned to the proteins coded by the E3 region of Ad2.**

| | |
|---|---|
| E3/19K | Blocks surface expression of MHC Class I by binding to the heavy chain and retaining the complex in the ER, and by interfering with MHC-tapasin interaction |
| E3/14.7K | Prevents apoptosis induced by TNF-α, to interact with several cellular proteins GTP-binding protein FIP-1, the TNF-α-inducible FIP-2 and the pro-apoptotic FIP-3 |
| E3/ 10.4K (RID-α) | In conjunction with E3/14.5K prevents apoptosis induced by TNF-α and Fas and downregulates Fas and the EGF(epidermal growth factor) receptor |
| E3/14.5K (RID-β) | In conjunction with E3/10.5K prevents apoptosis induced by TNF-α and Fas and downregulates Fas and the EGF(epidermal growth factor) receptor |
| E3/11.6K | It has a pro-apoptotic effect and has been reported to localize to the nuclear membrane following sorting in the Golgi compartment, it is highly expressed late in infection under the control of the late promoter. |
| E3/6.7K | Has been shown to be integral membrane glycoprotein localized to the ER |
| E3/12.5K | This protein is expressed during viral infection but it is still uncharacterized |

### *1.7 The immune response against adenovirus*

The E3/6.7K protein is located within the E3 cassette of immunoevasive genes and is relatively well conserved among different serotypes. The function of the E3/6.7K protein has not been described, yet, it is possible that E3/6.7K is also involved the evasion of the immune response. This formed the starting point of the investigation of the role of E3/6.7K during viral infection. Therefore, a brief review of the current knowledge about the various antiviral mechanisms that operate during adenovirus infection is necessary.

The immune response against adenovirus occurs in two phases. During the initial two days post-infection, there is an increase in polymorphonuclear leukocyte infiltration in lung alveoli. This is accompanied by a significant increase in the secretion of TNF-α, an inflammatory cytokine produced by activated T-cells and activated macrophages (Ginsberg *et al.*, 1991). The importance of TNF-α in the response against adenovirus was highlighted by the use of TNF-α homozygous knock-out (-/-) mice to study the response to intravenous adenovirus administration. These mice show an impaired immune response against adenovirus, demonstrated by the persistent expression of a transgene incorporated by the virus used in these studies (Elkon *et al.*, 1997, Benihoud, 1998 #4384).

In the second phase of the immune response, CD4+, Helper-T-cells respond to the presentation of exogenously derived viral epitopes by specialized Antigen Presenting Cells (APCs) in the context of MHC Class II. This results in the generation of a $Th_1$ and a $Th_2$ response, which strengthen the cytotoxic and the humoral response, respectively (Yang *et al.*, 1995). Following airway administration of adenovirus most antibodies produced are of the IgA isotype (Yang, Trinchieri, and Wilson, 1995). Ad-specific Cytotoxic T-cells (CTL) can be raised in mice infected with UV-crosslinked and psoralen-treated virus (Kafri *et al.*, 1998). This response is mediated by MHC Class I not requiring viral replication or *de novo* protein synthesis. Therefore, at least some of the CTL epitopes present in adenovirus are presented through the MHC Class I exogenous presentation pathway.

## *1.8 Death Receptor-induced apoptosis*

One of our hypotheses is that E3/6.7K dampens the immune response by preventing TNF-α-induced apoptosis, one of the essential components of the immune response against adenovirus. Three proteins coded by the E3 region, E3/10.4K, E3/14.5K and E3/14.7K have already been shown to affect the apoptotic pathway resulting in the extension of the life of the infected cell. Ligation of the receptor for TNF-α on the infected cell results in a plethora of effects whose outcomes are apoptosis and inflammation.

### 1.8.1 Death Receptor ligation

Apoptosis induced by ligation of the members of TNFR superfamily shares common pathways but there are, however, subtle differences which reflect the physiological roles of the individual members.(as outlined in Fig. 5). Ligation of TNFR1 by TNF-α induces concurrent apoptotic, inflammatory and survival pathways. Healthy cells remain unaffected as the apoptotic and survival pathways antagonize each other. The survival pathway does not operate in compromised cells, allowing apoptosis to run its course. This reflects the protective role played by TNF-α in killing compromised or virally infected cells (apoptosis) while recruiting immune cells at the site of infection/damage (inflammation) at the same time leaving normal-healthy cells alone (survival). Ligation of Fas by the FasL results in deletion of immune cells at immune privileged sites such as the eye and the deletion of activated T-cells following an immune response. For this reason, Fas ligation does not involve the induction of a pro-inflammatory or a survival pathway. On the other hand ligation of DR4 and DR5 by TRAIL activates both apoptosis and survival pathways, similarly to TNFR1 ligation (Bodmer *et al.*, 2000; Chaudhary *et al.*, 1997). The wide tissue distribution and the selective effect of TRAIL could suggest a role for this ligand in elimination of cells that have undergone malignant transformation.

Death Receptor-mediated apoptosis begins through the trimerization of the receptor through ligand binding, leading to the recruitment of adapter proteins to the cytoplasmic domain of the oligomerized receptor. There are two different classes of adapter molecules based on the presence of a Death Domain (DD). The first class includes the TNFR1-associated DD protein (TRADD) adapter protein (Hsu *et al.*, 1996) recruited by oligomerized TNFR1, and Fas-associated DD protein (FADD) (Hsu *et al.*, 1996) recruited by oligomerized Fas and TRADD. The second class, not containing a DD motif, is the TNFR- associated factor (TRAF) family of proteins. Recruitment to the receptor complex occurs in the case of TRAF2 through TRADD (Hsu *et al.*, 1996) and the receptor-interacting protein (RIP) (Ting, Pimentel-Muinos, and Seed, 1996).

### 1.8.2 The apoptotic pathway following death receptor ligation

In non-apoptotic cells, caspases are present as inactive zymogens. A procaspase polypeptide is cleaved during apoptosis to form heterodimeric, active enzymes (Nicholson *et al.*, 1995). However, zymogens of initiator caspases such as caspase-8 show considerable enzymatic activity (Muzio *et al.*, 1998). When two zymogen molecules are brought close to one another through their Death Effector Domain (DED), which is also present in FADD, it leads to their self-cleavage and activation (Muzio *et al.*, 1998). The proteolytic pathway begins with the activation of caspase-2 by RIP associated Ich-1/CED homologous protein with death domain (RAIDD) (Duan and Dixit, 1997), and caspase-8 and -10 by FADD (Boldin *et al.*, 1996; Vincenz and Dixit, 1997) and FLICE-associated huge protein (FLASH) (Imai *et al.*, 1999). Adapter-mediated auto-activation of the initiator caspases-8 and -10 is followed by the efflux of $Ca^{2+}$ from the ER to the cytoplasm and production of nitric oxide and reactive oxygen species.

Initiator caspase activation leads to the involvement of the mitochondria and the release of mediators of apoptosis such as cytochrome *c* (Liu *et al.*, 1996), the apoptotic protease activating factor (Apaf-1) (Yoshida *et al.*, 1998) and the Apoptosis Inducing Factor (AIF) (Susin *et al.*,

1999; Susin *et al.*, 1997). The mechanism of release of these factors is not very well understood, but it appears to be controlled by members of the Bcl-2 family of proteins. Pro-apoptotic members of this family such as Bax, Bak, Bim, Bid and Bad, translocate to the mitochondria (Jurgensmeier *et al.*, 1998) following an apoptosis-induced post-translational modification (e.g. dimerization of Bax and Bak (Diaz *et al.*, 1997), phosphorylation of Bad (Zha *et al.*, 1996) and cleavage of Bid (Li *et al.*, 1998c)). The mitochondrial membrane potential is disrupted and the apoptosis mediators are then released. Their release may involve the creation of a permeability transition pore (PTP) (Zamzami *et al.*, 1996) or the formation of multimeric channels by the pro-apoptotic Bcl-2 family members (Antonsson *et al.*, 2000). This process is opposed by the anti-apoptotic members of the family such as Bcl-2, Bcl-xL and Boo/DIVA (Antonsson *et al.*, 1997).

A large cytosolic complex (the apoptosome), composed of procaspase-9, Apaf-1, dATP and cytochome *c* forms. This leads to the activation of the executioner caspases-3, -6 and -7 (Li *et al.*, 1997; Slee *et al.*, 1999). Meanwhile, AIF translocates to the nucleus where it induces large-scale fragmentation of DNA (Daugas *et al.*, 2000; Susin *et al.*, 1999). The cell becomes irreversibly committed to cell death once the substrates of executioner caspases are cleaved. One of these substrates, PARP, is involved in DNA base excision repair as well as DNA replication and transcription and is cleaved *in vivo* by caspase-7 (Germain *et al.*, 1999). Another substrate is the inhibitor of caspase-activated DNAse (ICAD) which releases the terminal endonuclease, caspase-activated DNAse (CAD) that fragments the genome of the apoptotic cell (Enari *et al.*, 1998; Sakahira, Enari, and Nagata, 1998).

### 1.8.3 Calcium ion efflux from the ER during apoptosis

For reasons that will become apparent later in this report, it is important to highlight the role of $Ca^{2+}$ ions release during the process of apoptosis. $Ca^{2+}$ release is implicated in every major biological process, making it very difficult to understand how an apparently similar signal can direct a cell to divide, contract, relax or die. It is speculated that cells use frequency

modulation as a way to vary the intensity and nature of the physiological output. The quantal nature of $Ca^{2+}$ release events allows cellular factors to distinguish a small 'puff' from a 'wave'. The best example is the enzyme $Ca^{2+}$/calmodulin kinase II which recruits additional numbers of calmodulin molecules in relation with increasing frequency of $Ca^{2+}$ fluxes (Hanson *et al.*, 1994). It is also possible that $Ca^{2+}$ release is accompanied by other molecular events, such as protein signaling events, or transcriptional regulation that specify the physiological output.

In the same way that mitochondria respond to cellular injury by releasing apoptosis inducing factors like cytochrome c, Apaf-1 and AIF, the ER responds by releasing a $Ca^{2+}$ wave. The levels of cytosolic free-$Ca^{2+}$ in a resting cell not undergoing any stress are around 0.1μM, representing less than 0.1% of the total cellular $Ca^{2+}$ content. The largest store of $Ca^{2+}$ is the endoplasmic reticulum where concentrations can reach millimolar levels (Montero *et al.*, 1995). The release of $Ca^{2+}$ from the ER not only is it down a steep electrochemical gradient but it also acts as a trigger to induce an additional wave generated by calcium entry through store-operated calcium channels (SOCC) (Hofer, Fasolato, and Pozzan, 1998) from the extracellular milieu. The extracellular levels of free $Ca^{2+}$ range from 0.8-1.0mM (extracellular protein bound $Ca^{2+}$ is also found in a similar range but it is not available for signaling).

It is not clear what triggers the release of $Ca^{2+}$ from the ER during Death Receptor-mediated apoptosis or what channel is involved. There may be more than one mechanism. Several second messengers, such as ceramide, sphingosine-1-phosphate (SPP) (Moore *et al.*, 1999a), inositol 1,4,5-triphosphate (IP3) (Jayaraman and Marks, 2000; Szalai, Krishnamurthy, and Hajnoczky, 1999) and reactive oxygen intermediates (Ridgley, Xiong, and Ruben, 1999) have been implicated in the induction of ER $Ca^{2+}$ efflux during apoptosis.

The passive movement of $Ca^{2+}$ out of the ER, down the concentration gradient can occur through two classes of channels: the inositol 1,4,5-triphosphate receptor (IP3R) triggered by soluble IP3 and $Ca^{2+}$ itself, and the ryanodine receptors (RyR) controlled by cofactors as well as $Ca^{2+}$ itself. Researchers have shown that IP3R-1-deficient T-cells are resistant to apoptosis

induced by Fas (Jayaraman and Marks, 1997; Jayaraman and Marks, 2000), implicating the IP3R-1 receptor, also the isoform with the widest tissue distribution, in apoptosis.

A plume of $Ca^{2+}$ exits from the ER during apoptosis acting as a second messenger to disrupt mitochondrial $Ca^{2+}$ homeostasis (Rizzuto *et al.*, 1998; Szalai, Krishnamurthy, andHajnoczky, 1999) which imports $Ca^{2+}$ through a uniport transporter (Kass, Juedes, and Orrenius, 1992). Calcineurin, a $Ca^{2+}$-regulated, serine-threonine phosphatase, is found immediately downstream of the $Ca^{2+}$ efflux from the ER in several systems (Ankarcrona *et al.*, 1996; Jayaraman and Marks, 2000; Villalba *et al.*, 1999). Calcineurin has been implicated in a variety of processes that result in the induction of apoptosis. The best described target of calcineurin is the nuclear factor of activated T-cells (NF-AT). Dephosphorylation of NF-AT results in its translocation to the nucleus where it transcribes amongst other proteins FasL (Latinis *et al.*, 1997). The pro-apoptotic Bcl-2 family member, Bad is another target of calcineurin. Dephosphorylated Bad translocates to the mitochondria and associates with other Bcl-2 family members to induce apoptosis (Wang *et al.*, 1999). Another proof of the involvement of calcineurin in pro-apoptotic events is the fact that it is directly inhibited by the anti-apoptotic Bcl-2. Bcl-2 bound calcineurin is sequestered away from NF-AT and therefore unable to dephosphorylate it (Srivastava *et al.*, 1999a).

Other proteins that have been shown to be involved in apoptosis downstream of $Ca^{2+}$ efflux from the ER are: the death-associated protein (DAP) kinase (Cohen *et al.*, 1999), protein kinase C (PKC) (Villalba *et al.*, 1999), the calpain class of calcium-regulated cysteine proteases (Vanags *et al.*, 1996), caspase-12 (Nakagawa *et al.*, 2000), the MEF2 transcription factor (Youn *et al.*, 1999) and calcium-regulated cytosolic phospholipase A2 ($cPLA_2$) (Nalefski et al., 1994). DAP-kinase is a calcium/calmodulin regulated serine/threonine kinase that associates with the cytoskeleton (Cohen *et al.*, 1999). MEF-2 is a calcium/calmodulin regulated transcription factor that activates the steroid receptors Nur77 and Nor1, crucial mediators of T cell apoptosis (Liu *et al.*, 1994; Woronicz *et al.*, 1994). The recently discovered caspase-12 is activated by inducers of

the ER-stress response, such as thapsigargin, but not by other inducers of apoptosis, such as TNF-α (Nakagawa *et al.*, 2000).

### 1.8.4 The inflammatory pathway following death receptor ligation

Translocation of $cPLA_2$ to the membrane of the ER is mediated by $Ca^{2+}$ efflux. The TNF-α-inducible $cPLA_2$ is activated by the dual signal of calcium release from the ER stores and phosphorylation by mitogen activated protein (MAP) kinase (Clark *et al.*, 1995; Nalefski *et al.*, 1994). In the presence of a sustained calcium flux $cPLA_2$ translocates preferentially to the ER and nuclear envelope where it releases arachidonic acid from the sn-2 position of various phospholipids (Peters-Golden *et al.*, 1996; Schievella *et al.*, 1995).

The lipase $cPLA_2$ is especially important as it has been shown to mediate the agonist-inducible release of arachidonic acid, leading to the formation of eicosanoids, such as leukotrienes and prostaglandins, important mediators of inflammation. The role of $cPLA_2$ during apoptosis is presently unclear. It has been reported that $cPLA_2$ is a potential substrate for caspase-1, -8 (Luschen *et al.*, 1998) as well as -3 and -7 (Adam-Klages *et al.*, 1998). The different proteolytic products generated according to the different cleavage sites used by various caspases, appear to be enzymatically inactive. Moreover, it has been suggested that it is a $Ca^{2+}$-independent phospholipase and not $cPLA_2$ which may be involved in Fas-induced apoptosis (Atsumi *et al.*, 1998). Despite the above evidence, failure to activate $cPLA_2$ results in resistance of the myeloid cell lines U937 and HL-60 to the effects of TNF-α (Wu *et al.*, 1998). In addition, the activity of $cPLA_2$ correlated well with the sensitivity of various melanoma cell lines and freshly explanted melanoma tumor tissue to TNF-α (Voelkel-Johnson, Thorne, and Laster, 1996). Even more importantly, the activity of $cPLA_2$ appears to be necessary for the killing of adenovirus-infected cells by TNF-α (Thorne *et al.*, 1996). The three proteins coded by the E3 region, E3/10.4K, E3/14.5K and E3/14.7K that prevent apoptosis have also been shown to prevent the release of arachidonic acid following stimulation with TNF-α.

### 1.8.5 The pro-survival pathway following death receptor ligation

A cell survival pathway is triggered by RIP and TRAFs, the second set of TNFR1/TRADD adapters following TNF-α- or TRAIL-induced apoptosis. Activation of TRAF2 leads to the activation of NF-κB through NIK (Malinin *et al.*, 1997). Another pathway activated by oligomerized TRAF2 is the mitogen-activated protein (MAP)/Erk kinase-kinase-1 (also called MEKK1) and the activation of downstream effectors, such as the p38-MAP kinase and the stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) protein kinase pathway. More importantly, it activates NF-κB through I-κB kinase (IKK) (Baud *et al.*, 1999). The family of cellular inhibitors of apoptosis (cIAP) have also been implicated in the activation of JNK1 (Sanna *et al.*, 1998). The JNK pathway has been ambiguously implicated in both pro-apoptotic (Ichijo *et al.*, 1997) and anti-apoptotic pathways (Yujiri *et al.*, 1998), depending on the cell type and the method used to induce apoptosis (Leppa and Bohmann, 1999). Apoptosis induced through TNFR1 is augmented by the addition of inhibitors of protein synthesis, such as cylcloheximide in our study. Steady-state levels of short-lived protein factors controlled probably by activated NF-κB, are reduced in the presence of cycloheximide favoring the apoptotic pathway versus the survival pathway following TNFR1 induction. Ligation of Fas by the FasL has been shown to induce a pro-apoptotic amplification loop through the caspase-mediated cleavage of survival mediators such as RIP (Martinon *et al.*, 2000) and TRAF1 (Irmler *et al.*, 2000). Therefore the pro-apoptotic pathway predominates following ligation of Fas by FasL.

**Figure 5. Diagram illustrating Death Receptor induced signaling pathways.**

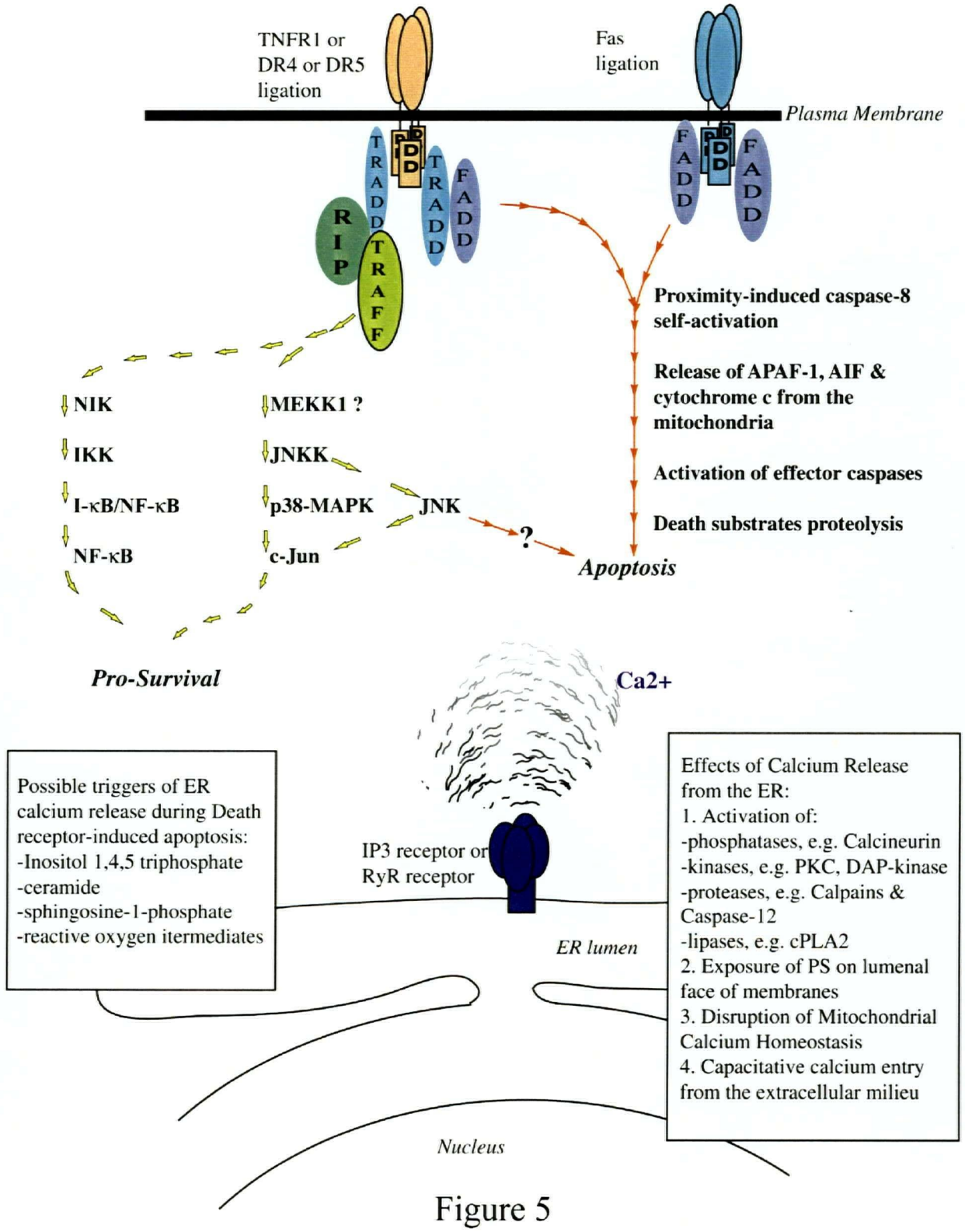

TNFR1 or
DR4 or DR5
ligation
Fas
ligation
Plasma Membrane
TRADD
DD
TRADD
FADD
RIP
TRAFF
FADD
DD
FADD
Proximity-induced caspase-8
self-activation
Release of APAF-1, AIF &
cytochrome c from the
mitochondria
Activation of effector caspases
Death substrates proteolysis
NIK
IKK
I-κB/NF-κB
NF-κB
MEKK1 ?
JNKK
p38-MAPK
JNK
c-Jun
?
Apoptosis
Pro-Survival
Ca2+
Possible triggers of ER
calcium release during Death
receptor-induced apoptosis:
-Inositol 1,4,5 triphosphate
-ceramide
-sphingosine-1-phosphate
-reactive oxygen itermediates
IP3 receptor or
RyR receptor
ER lumen
Effects of Calcium Release
from the ER:
1. Activation of:
-phosphatases, e.g. Calcineurin
-kinases, e.g. PKC, DAP-kinase
-proteases, e.g. Calpains &
Caspase-12
-lipases, e.g. cPLA2
2. Exposure of PS on lumenal
face of membranes
3. Disruption of Mitochondrial
Calcium Homeostasis
4. Capacitative calcium entry
from the extracellular milieu
Nucleus

Figure 5

### *1.9 Viral Immunoevasion mechanisms*

Viral infection often results in a cellular injury, and induces programmed cell death of the host cell. Apoptosis can be induced by the members of TNFR superfamily such as Fas (APO-1 or CD95), p55-TNF Receptor 1 (TNFR1) and the death domain-containing receptors 3, 4 and 5 (DR3, DR4 and DR5, respectively). Many viruses, particularly persistent DNA viruses, modify the apoptotic response of a cell to allow continued virus replication. The intracellular mediators of apoptosis are highly conserved across species and are the target of viral inhibitors found in different classes of virus.

Viral inhibitors of apoptosis show a convergent evolution as proteins from unrelated virus species have evolved to mimic the same cellular target. Therefore, they can be classified according to the common cellular target or the common mechanism of action, as shown in Table III. For example, adenovirus E1B 55K (Debbas and White, 1993), SV40 Large T antigen (Levine, 1997; Lill *et al.*, 1997) and human papilloma virus E6 (Levine, 1997) inhibit p53-mediated lysis. The cellular survival factor Bcl-2 is mimicked by adenovirus E1B 19K (White, 1996), Epstein-Barr virus BHRF1 (Henderson *et al.*, 1993) and African swine fever virus LMW5-HL (Neilan *et al.*, 1993). Members of the Interleukin 1b Converting Enzymes (ICE)-family of terminal proteolytic enzymes, also known as caspases, are blocked by baculovirus p35 (Clem, Fechheimer, and Miller, 1991; Xue and Horvitz, 1995) and crmA, the cowpox serpin protein (Tewari and Dixit, 1995; Zhou *et al.*, 1997). The Inhibitor of Apoptosis (IAP) family of baculovirus and its mammalian homologues interact with the TNF-α receptor-associated factors (TRAFs), therefore blocking the signaling cascade that leads to the recruitment of caspases (Liston *et al.*, 1996). The activation of FADD-like interleukin-1beta-converting enzyme (FLICE), also known as caspase-8, through Fas is blocked by viral-FLICE-inhibitory proteins (vFLIPS), found in the genomes of various types of herpes virus (Thome *et al.*, 1997). Figure 6 illustrates the multiple apoptotic pathways that are the target of viral inhibitor of apoptosis.

## Table III. Viral immunoevasion factors.

Adapted from Tortorella et. al. (Tortorella *et al.*, 2000) and Roulston et. al. (Roulston, Marcellus, and Branton, 1999). Abbreviations used in this table: ASFV, african swine fever virus; BHV, bovine herpesvirus; CSFV, classical swine fever virus; EBV, Epstein-Barr virus; EHV, equine herpesvirus; HCMV, human cytomegalovirus; HCV, Hepatitis C virus; HHV, human herpesvirus; HIV, human immunodeficiency virus; HPV, human papillomavirus; HSV, human herpesvirus; HTLV, human T-cell leukemia virus; HVS, Herpesvirus saimiri; MCV, molluscum contagiosum virus; MHV, murine gammaherpesvirus; SFV, Shope fibroma virus; SV40, simian virus 40; TMEV, Theiler's murine encephalomyelitis virus.

| Cellular Target | Virus | Gene | Description |
|---|---|---|---|
| Fas/TNF prevent the interaction between the Death Receptor and its ligand | Myxoma | MT-2 | TNFR homolog:(Upton *et al.*, 1991)<br>-secreted form blocks TNF-α (Schreiber, Rajarathnam, and McFadden, 1996)<br>-intracellular form prevents apoptosis through an unknown mechanism (Schreiber, Sedger, and McFadden, 1997; Sedger and McFadden, 1996) |
| | Adenovirus | E3/10.4/14.5K (RID) | Multimeric complex forces internalization and degradation of Fas, also prevents TNF-α induced apoptosis (Elsing and Burgert, 1998; Gooding *et al.*, 1991; Shisler *et al.*, 1997; Tollefson *et al.*, 1998) |
| | Shope Fibroma Virus | SFV-T2 | Neutralizes TNF-α (Smith *et al.*, 1991) |
| | Cowpox | CrmB | Neutralizes TNF-α and LT (Hu, Smith, and Pickup, 1994) |
| | | CrmC | Neutralizes TNF-α but not LT (Smith *et al.*, 1996) |
| | | CrmD | Secreted complex prevents LT and TNF-α activity of vitro (Loparev *et al.*, 1998) |
| vFLIPS | HHV8 | K13 | VFLIPS contain two DED and prevent recruitment of caspase-8 by Death Receptors (Thome *et al.*, 1997; Tschopp, Irmler, and Thome, 1998) |
| | HVS | orf71 | |
| | EHV-2 | E8 | |
| | MCV | MC159, MC160 | |
| | BHV4 | BORFE2 | |
| Caspase inhibitors | Cowpox | crmA | Serpin family, inhibits caspase -1, -8 and granzyme B (Dbaibo and Hannun, 1998) (Macen *et al.*, 1996) (Macen *et al.*, 1993; Petit *et al.*, 1996; Upton *et al.*, 1990) (Dobbelstein and Shenk, 1996) |
| | Rabbitpox | SPI-2 and 2 | |
| | Myxoma | Serp1 and 2 | |

| | | | |
|---|---|---|---|
| | Vaccinia | SPI-2/B13R2 | |
| | Baculoviruses | p35 | Inhibits multiple caspases (Miller, 1997) |
| | | IAP | Inhibits multiple caspases (Deveraux and Reed, 1999) |
| | ASFV | A224L or 4-CL | IAP homolog, inhibits multiple caspases (Neilan *et al.*, 1997) |
| | Adenovirus | 14.7K | Interacts with caspase-8, FIP-1, FIP-2 and FIP-3 (Chen *et al.*, 1998; Li *et al.*, 1999; Li, Kang, andHorwitz, 1997; Li, Kang, andHorwitz, 1998) |

| | | | |
|---|---|---|---|
| Bcl-2 homologues | EBV | BHRF1 | (Henderson *et al.*, 1993) |
| | | BALF1 | (Marshall *et al.*, 1999) |
| | Adenovirus | E1B 19K also sequesters caspase-8 | (Han, Sabbatini, andWhite, 1996; Perez and White, 1998; White, 1996) |
| | HHV8 | Ksbcl-2 (ORF16) | (Cheng *et al.*, 1997) |
| | AFSV | A179L, LMW 5-HL | (Afonso *et al.*, 1996; Neilan *et al.*, 1993) |
| | HVS | ORF16 | (Nava *et al.*, 1997) |
| | MHV68 | ORFM11 | (Virgin *et al.*, 1997) |
| Regulatory | EBV | LMP-1 | Upregulates bcl-2 and other cellular survival proteins, mimics CD40/TNFR signaling (Huen *et al.*, 1995; Rowe *et al.*, 1994) |
| | | EBNA-4 (Tsai *et al.*, 1996) | Upregulate bcl-2 |
| | HIV-1 | Tat (Zauli *et al.*, 1995) | |
| | HCV | Core protein | Upregulates NF-kB (Ray, Meyer, and Ray, 1996) |
| Oxidative stress blockers | MCV | MC066L | Homologous to glutathione peroxidase, inhibits peroxide and UV induced apoptosis (Shisler *et al.*, 1998) |
| Cell cycle | HPV | E6 | Targets p53 for degradation (Scheffner *et al.*, 1990) |
| | Adenovirus | E1B 55K | Bind and inactivate p53 (Nevels *et al.*, 1997; Steegenga *et al.*, 1998) |
| | | E4orf6 | |
| | SV40 | Large T antigen | Binds and inactivate p53 and Rb protein (Mietz *et al.*, 1992) |

| | | | |
|---|---|---|---|
| Transcription factors | Marek's disease virus | MEQ | Inhibits apoptosis induced by UV, TNF-α and serum withdrawal (Liu *et al.*, 1998) |
| | HCMV | IE1, IE2 | Inhibits apoptosis induced by TNF-α but not UV (Zhu, Shen, and Shenk, 1995) |
| Unknown | HSV | Us3 | Serine/Threonine kinase (Leopardi, Van Sant, and Roizman, 1997) |
| | Aviadenovirus (CELO strain) | GAM-1 | Similar mechanism to E1B 19K (Cao, Krell, and Nagy, 1998) |
| | Myxoma | M-T4 | ER-retained |
| | | M11L | Mitochondria localized (Everett *et al.*, 2000) |
| | | M-T5 | Contains ankyrin repeats (Mossman *et al.*, 1996) |

**Figure 6. Diagram illustrating the apoptotic pathways affected by viral inhibitors of apoptosis.**

Reproduced from Tortorella et. al. (Tortorella *et al.*, 2000)

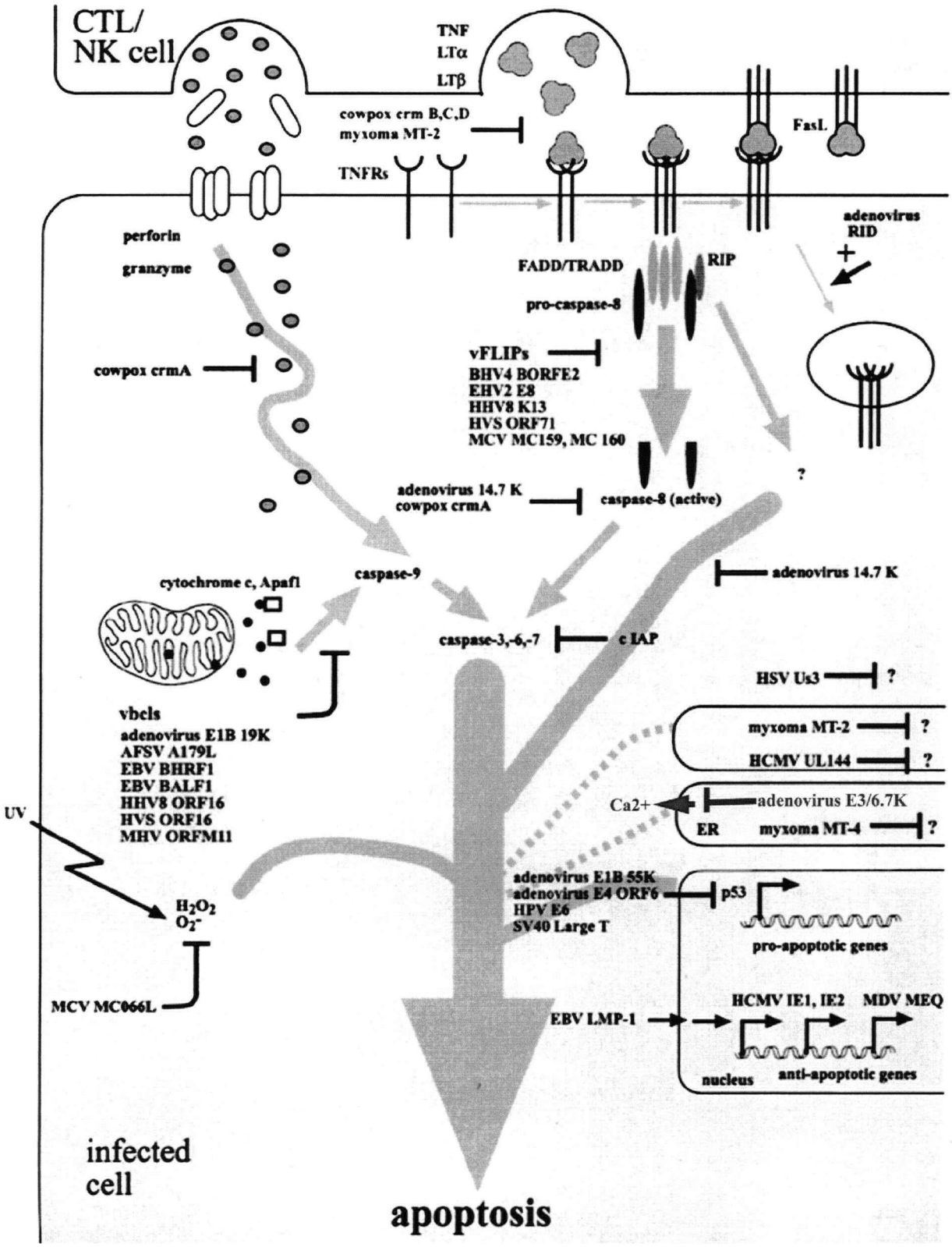

CTL/
NK cell
TNF
LTα
LTβ
cowpox crm B,C,D
myxoma MT-2
TNFRs
FasL
adenovirus
RID
perforin
granzyme
FADD/TRADD
RIP
pro-caspase-8
cowpox crmA
vFLIPs
BHV4 BORFE2
EHV2 E8
HHV8 K13
HVS ORF71
MCV MC159, MC 160
?
adenovirus 14.7 K
cowpox crmA
caspase-8 (active)
caspase-9
adenovirus 14.7 K
cytochrome c, Apaf1
caspase-3,-6,-7
c IAP
HSV Us3
?
vbcls
adenovirus E1B 19K
AFSV A179L
EBV BHRF1
EBV BALF1
HHV8 ORF16
HVS ORF16
MHV ORFM11
myxoma MT-2
?
HCMV UL144
?
UV
Ca2+
adenovirus E3/6.7K
ER
myxoma MT-4
?
adenovirus E1B 55K
adenovirus E4 ORF6
HPV E6
SV40 Large T
p53
pro-apoptotic genes
$H_2O_2$
$O_2^-$
MCV MC066L
EBV LMP-1
HCMV IE1, IE2
MDV MEQ
nucleus
anti-apoptotic genes
infected
cell
apoptosis

Figure 6

# Chapter 2. Project Rationale and General Approach

## *2.1 Investigation of the function of E3/6.7K*

The E3/6.7K protein is coded bicistronically by the same transcript that codes for the E3/19K protein. The expression of E3/6.7K protein is, however, much lower than the expression of the E3/19K protein. This appears to be due to the inefficient Kozak consensus that exists at the start site of the E3/6.7K open reading frame (Wilson-Rawls *et al.*, 1990; Wold *et al.*, 1986). The E3/6.7K sequence is not significantly homologous to any other known protein. It is, however, well conserved between Ad2 and Ad5 in group C adenovirus and between Ad3, Ad7 and Ad35 in group B adenovirus (Hawkins, Wilson-Rawls, and Wold, 1995); it is less well conserved between group C and group B.

It has been have proposed that E3/6.7K acts in concert with E3/19K as the two proteins are translated from the same mRNA. A preliminary study has also shown that E3/19K and E3/6.7K can be immunoprecipitated with one another, while there was no evidence that this interaction affects MHC class I transport (Thesis of Roger Lippe: *Modulation of the MHC Class I Antigen Processing and Presentation Pathway.* Department of Microbiology and Immunology, Faculty of Graduate Studies, University of British Columbia, June 1995). It is possible that the two proteins associate weakly as E3/19K protein binds to a variety of cellular chaperone proteins and all unfolded proteins that happen to associate with them at the time.

The first set of experiments addressed the role played by E3/6.7K in viral infection by studying the pathology of inflammation in the lungs of mice infected with viral deletion mutants that lack E3/6.7K compared to the one seen in mice infected with wild type virus. The second set of experiments attempted to explain these *in vivo* results by examining the response of cells expressing the E3/6.7K protein to TNF-$\alpha$.

Our findings indicate that the adenovirus E3/6.7K protein prevents Death Receptor-induced apoptosis in transfected cells. *In vivo* the presence of E3/6.7K resulted in a reduced inflammatory response. This *in vivo* effect can be explained by results indicating that TNF-

α, TRAIL- and Fas-induced apoptosis and TNF-α-induced release of arachidonic acid were significantly reduced in cells expressing E3/6.7K. As a result, TNF-α-induced activation of caspase-3 and cleavage of poly (ADP-ribose) polymerase were delayed in cells expressing E3/6.7K. The mechanism of action of E3/6.7K may involve the maintenance of ER calcium homeostasis. We believe this to be the case since the expression of E3/6.7K buffered the calcium ion ($Ca^{2+}$) flux generated in response to thapsigargin, an inhibitor of the ER-associated $Ca^{2+}$ ATPase. Due to its effect on $Ca^{2+}$ ion homeostasis, E3/6.7K occupies a unique place among the many viral survival factors characterized so far.

## *2.2 Investigation of the membrane topology of E3/6.7K*

The E3/6.7K protein has no sequence homology to any other known proteins. In addition, very few other anti-apoptotic factors characterized so far have been localized to the ER. The lack of sequence homology and ER subcellular localization suggest that E3/6.7K may uncover a new viral immunoevasion strategy. It is likely that the topology and mode of targeting of E3/6.7K play an important role in the mode of action of this anti-apoptotic protein. Therefore, we attempted to describe this protein's membrane structure and targeting mechanism in the hope of understanding its effect on $Ca^{2+}$ ion homeostasis.

### 2.2.1 Membrane proteins of adenovirus

E3/6.7K like all other integral membrane proteins coded by adenovirus are found in the E3 region. Figure 7 shows the current understanding of the membrane topology and post-translational modifications of the various E3 proteins as found in subgroup C adenovirus. The E3/10.4K and E3/14.5K proteins form a complex that localizes to the plasma membrane (Gooding *et al.*, 1991). The E3/19K protein is retained in the endoplasmic reticulum (ER) (Gabathuler and Kvist, 1990) as is E3/6.7K (Wilson-Rawls and Wold, 1993). The E3/11.6K protein localizes to the nuclear envelope (Scaria *et al.*, 1992). All E3 proteins have post-translational modifications. The E3/19K, E3/11.6K and E3/6.7K proteins have been shown to be

N-glycosylated (Scaria *et al.*, 1992; Wilson-Rawls and Wold, 1993; Wold *et al.*, 1985). The lumenal domain of the E3/14.5K protein was shown to be O-glycosylated not N-glycosylated (Krajcsi, Tollefson, and Wold, 1992), while its cytoplasmic domain was shown to be serine-phosporylated (Krajcsi and Wold, 1992). Intrachain and interchain disulfide bonds have been demonstrated in E3/19K and E3/10.4K, respectively (Hoffman *et al.*, 1992; Sester and Burgert, 1994). There is evidence that some cysteine residues within the cytoplasmic domain of E3/11.6K protein are modified by palmitoylation (Hausmann *et al.*, 1998). Subgroup B adenovirus (Ad-3 and Ad-7) codes for a 20.5kDa membrane protein which contains O-linked and complex N-linked oligosaccharides (Hawkins and Wold, 1995). Subgroup B also codes for the E3/16K protein which is a larger and more distant homologue of the subgroup C, E3/6.7K (Hawkins, Wilson-Rawls, andWold, 1995). The homology between Ad2 and 5 E3/6.7K and Ad3 and 7 E3/16K is depicted in Figure 8B. Our experiments focused on subgroup C (Ad-2 and Ad-5), the group most commonly associated with respiratory infections.

All E3 proteins contain a single hydrophobic stretch that could act as a transmembrane region (single-spanning). The topology of single-spanning membrane proteins is determined by the presence of a cleavable signal sequence. For type I proteins translocation is initiated by an N-terminal, cleavable signal sequence and terminated by a stop-transfer transmembrane domain. Therefore, the orientation of these proteins is N-lumenal/ C-cytoplasmic (N and C being the amino and carboxyl termini, respectively). Translocation of the E3/10.4K, E3/14.5K and E3/19K membrane proteins is signal-sequence dependent (type I). Interestingly, the signal sequence of a subset of E3/10.4K proteins is not removed, resulting in the creation of a second membrane-associated domain(Krajcsi *et al.*, 1992).

In the absence of a signal-sequence, as is the case for E3/6.7K and E3/11.6K, translocation is initiated by the same region of the polypeptide that acts as a transmembrane domain. Due to its dual functions this domain has been referred to as a signal-anchor sequence. Signal-anchor containing proteins can achieve either a type II, N-cytoplasmic/ C-lumenal or a type III, N-lumenal/ C-cytoplasmic conformation. The topology of signal-anchor proteins is determined by the distribution of charged residues in the regions of the polypeptide flanking the transmembrane

domain (von Heijne and Gavel, 1988). It has been observed that the region of a signal-anchor protein that has positively charged residues is retained in the cytoplasm. One possible explanation for this is that positive residues found in the cytoplasmic domain of these proteins interacts with the anionic phospholipids on the cytoplasmic face of the membrane (van Klompenburg *et al.*, 1997). This observation is also called "the positive inside rule". A similar theory states that the topology is determined by the net charge difference between the regions flanking the transmembrane domain (Hartmann, Rapoport, and Lodish, 1989). Consequently, the domain of the polypeptide that has a net positive charge is retained in the cytoplasm. Lastly, in the case of some signal-anchor proteins the topology was determined by the folding state of the regions flanking the transmembrane domain rather then their charge (Denzer, Nabholz, and Spiess, 1995).

The E3/11.6K and E3/6.7K have both been classified as type III proteins, because both proteins have net positive charge associated with the C-terminal region of the polypeptide. In addition the N-terminus of both proteins have been shown to have post-translational modifications that are specific to the lumen of the ER. In both proteins the only asparagine residues that are candidate acceptors for N-linked glycosylation are found in the N-terminal region of the protein. This also supports the proposed type III topology.

**Figure 7. Characteristics of the adenovirus E3 region coded membrane proteins**

Shown are the characteristics and putative membrane topology of the five membrane proteins coded by the Ad2 E3 region. The presence of a cleaved signal sequence is shown at the amino terminus of some of the proteins (in yellow). The subcellular localization is indicated as nuclear envelope-NE, endoplasmic reticulum ER and plasma membrane-PM. The known post-translational modifications are shown as disulfide bridge (S-S), N-linked carbohydrates (N-CHO), O-linked carbohydrates (S-O-CHO) and serine phosphorylation (S-O-$PO_4$) and palmitoylation S-CO-$(CH_2)_{14}$-$CH_3$.

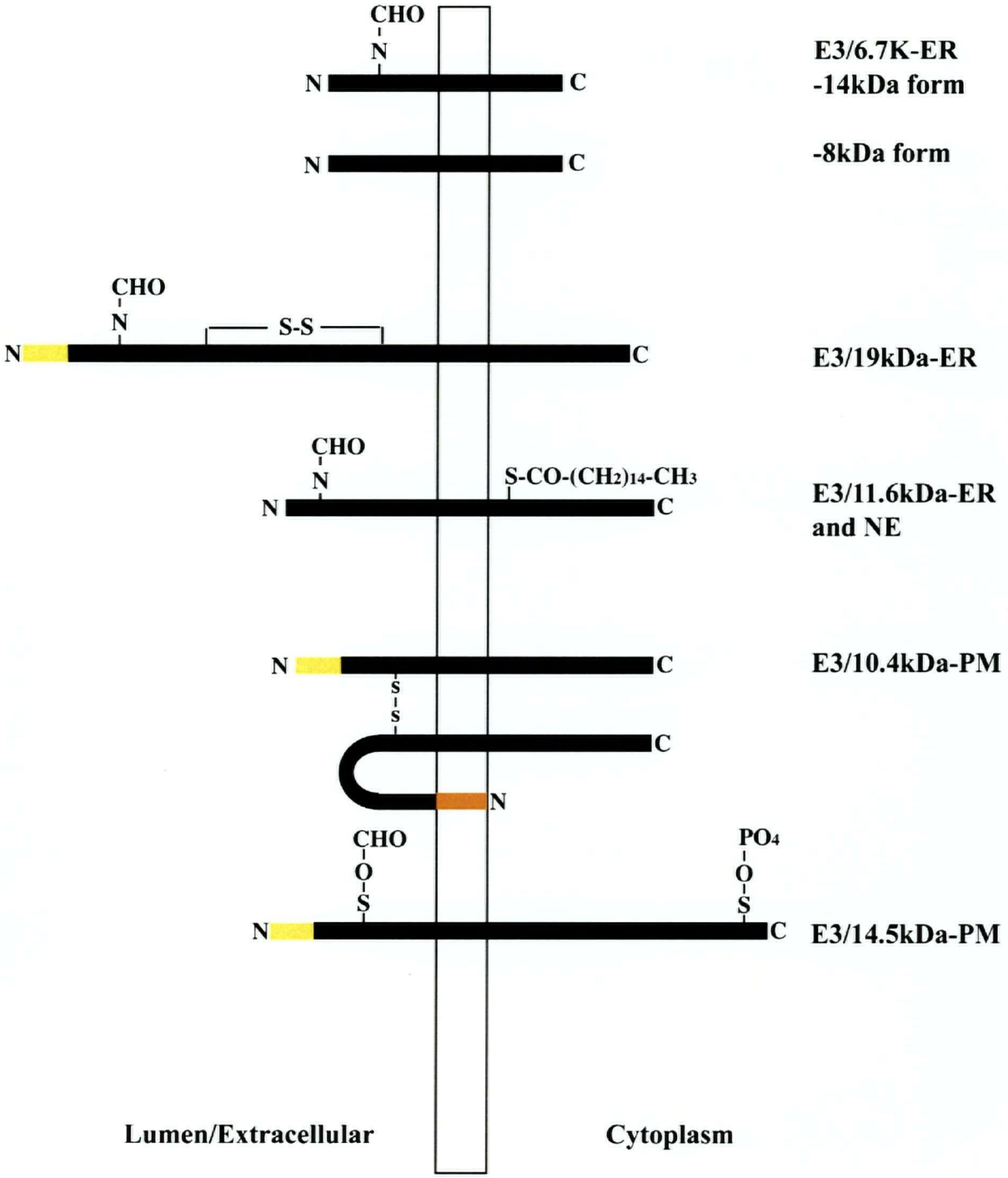

CHO
N
N
C
E3/6.7K-ER
-14kDa form
N
C
-8kDa form
CHO
N
S-S
N
C
E3/19kDa-ER
CHO
N
S-CO-(CH2)14-CH3
N
C
E3/11.6kDa-ER
and NE
N
C
E3/10.4kDa-PM
S
S
C
N
CHO
O
S
PO4
O
S
N
C
E3/14.5kDa-PM
Lumen/Extracellular
Cytoplasm

Figure 7

### 2.2.2 Previously proposed topology for the E3/6.7K protein

The E3/6.7K protein is coded bicistronically by the same mRNA that codes for E3/19K (Wold *et al.*, 1986) and co-localizes with E3/19K in the ER (Wilson-Rawls and Wold, 1993). The E3/6.7K protein is present in two forms, one unglycosylated with an apparent molecular weight of 8kDa and one glycosylated with an apparent molecular weight of 14kDa (Wilson-Rawls *et al.*, 1990). It is an integral membrane glycoprotein modified with N-linked, high mannose oligosaccharides, exclusively, indicating that it does not reach post-ER compartments (Wilson-Rawls and Wold, 1993).

Sequence analysis of Ad-2 E3/6.7K using the Kyte and Doolittle algorithm (Kyte and Doolittle, 1982) predicts an unusually long transmembrane domain of approximately 35 amino acids which also acts as the signal-anchor domain (Fig. 8A). There are three predicted Asn-linked glycosylation recognition motifs, all localized at the amino terminus. Please note that the five cysteine residues are located at the carboxyl terminus, this location will become relevant in the discussion (Fig. 8A). The charge distribution is such that all charged residues are basic and are localized at the carboxyl terminus of the protein.

Based on charged residue distribution it has been proposed that E3/6.7K is a type III protein that has a N lumenal-C cytoplasmic orientation (Wilson-Rawls and Wold, 1993). This topology is supported by the observations that the E3/6.7K is N-linked glycosylated and that the only acceptor sites for this ER lumen-specific modification are found in the N-terminal region of the polypeptide. This proposed topology implies that the signal-anchor domain of E3/6.7K also acts as a stop-transfer sequence. Therefore if one were to create a fusion protein that places E3/6.7K at the N-terminal of another protein, then the stop-transfer domain of E3/6.7K would prevent the translocation of the C-terminally located protein into the lumen of the ER. This, however does not agree with the observation that the E3/6.7K domain of an E3/6.7K-E3/19K fusion protein is fully translocated across the membrane of the ER and directs E3/19K to the membrane of the ER (Wilson-Rawls, Deutscher, and Wold, 1994), as shown in Figure 9. Consequently the E3/6.7K-E3/19K protein is N-linked glycosylated at the acceptor sites found

within the sequence of E3/19K, in addition to the ones found in the N-terminal region of E3/6.7K. In this case the transmembrane domain of E3/6.7K does not act as a stop-transfer sequence, which would be expected if E3/6.7K assumes a type III topology.

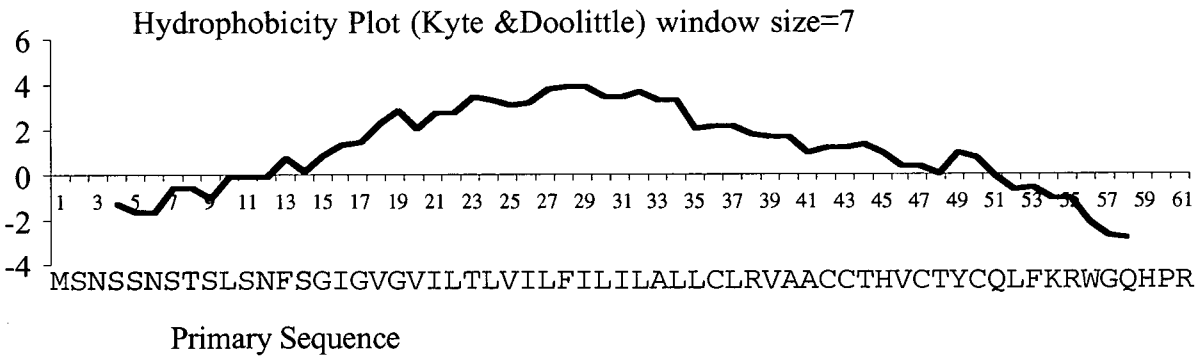
Another piece of evidence that does not support the model proposed by Wilson-Rawls et. al. (Wilson-Rawls, Deutscher, andWold, 1994; Wilson-Rawls and Wold, 1993) for the structure of E3/6.7K, comes from studies of E3/6.7K isolated from virally infected cells. Lippe et. al. pointed out that there is a mobility shift in the migration of the reduced versus non-reduced form of E3/6.7K protein by SDS-PAGE electrophoresis (see Fig. 25B) (Moise. al. manuscript submitted) . An oxidation-dependent shift in the mobility of a protein by SDS-PAGE is evidence of the existence of disulfide bonds in the native protein. A protein containing intramolecular disulfide bonds (non-reduced) is not as flexible and therefore does not move as easily through a polyacrylamide gel as a protein that lacks these bonds (reduced). Disulfide bond formation is a post-translational modification that is specific to the lumen of the ER. The existence of disulfide bonds in the native E3/6.7K protein would place the carboxyl terminus, the only domain containing disulfide bonds, in the lumen of the ER. This does not agree with the type III topology proposed by Wilson-Rawls et. al.. A figure indicating the topology proposed by Wilson-Rawls et. al. is illustrated in Figure 9.

Our findings indicate that there is another interpretation for the results obtained by Wilson-Rawls et. al.. The E3/6.7K protein is present in infected cells as two electrophoretic forms of 8 and 14kDa. *In vitro* analysis of the topology adopted by the two forms of E3/6.7K indicates that the 8kDa is unglycosylated and assumes a type II (N cytoplasmic-C lumenal) membrane topology. The 14kDa form is glycosylated and adopts a hairpin conformation with both the amino and carboxyl termini found inside the ER lumen. The unique topology of the 14kDa form is supported by the fact that post-translational modifications that are specific to the ER lumen map to both termini of the protein. The topologies of both forms of E3/6.7K constitute exceptions to "the positive inside rule".

**Figure 8. Sequence analysis of E3/6.7K.**

(A) A Kyte-Doolittle hydrophobicity plot of E3/6.7K displays the highly hydrophobic character of this protein. The three predicted Asn-linked glycosylation sites are shown in blue. (B) E3/6.7K from group C, serotypes Ad2 and Ad5, is aligned using a multi-alignment program with the highly related protein E3/16K from group B serotypes Ad3, Ad7 and Ad35. The consensus indicates that the most conserved residues are the cysteines and the basic residues located at the carboxyl terminus of all proteins. Amino acid residues conserved in all serotypes are capitalized, while the ones that are only conserved in group C serotypes are in lower case letters.

**A**



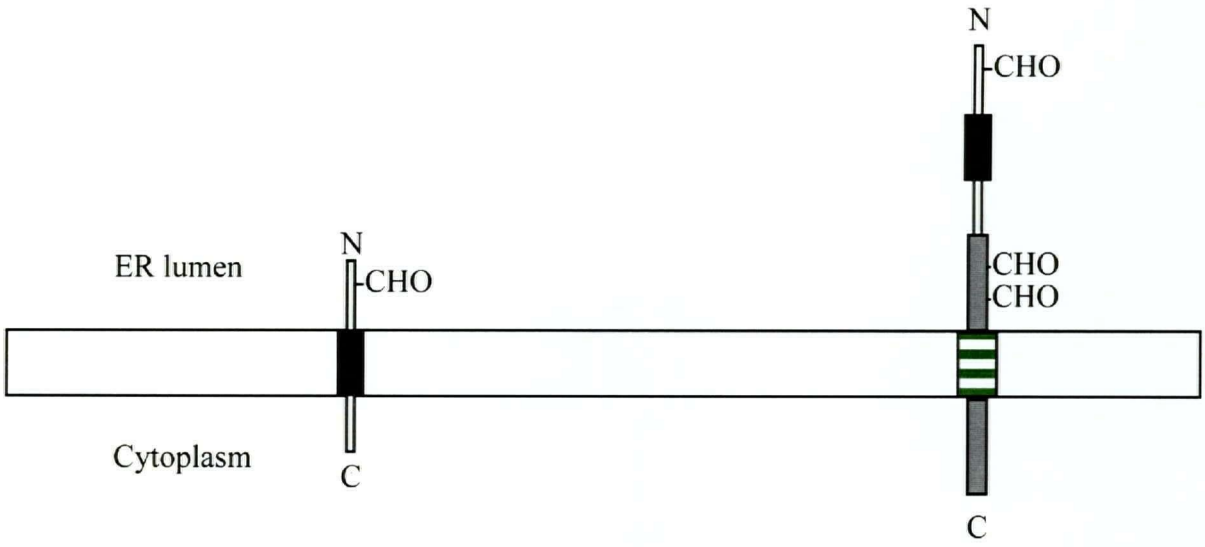

**B**

| | Conserved residues | nssnsTg sn gfs igvgVil lViLfil il llClr aaCc H ciYcqlFKrWG hpr |
|---|---|---|
| Group C | Ad2, E3/6.7K(1-61) | MSNSSNSTSLSN FSGIGVGVILTLVILFIL ILALLCLRVAACCTHVCTYCQLFKRWGQHPR |
| | Ad5, E3/7.1K(1-63) | MNNSSNSTGYSNSGFSRIGVGVILCLVILFIL IL/TLLCLRLAACCVHICIYCQLFKRWGRHPR |
| Group B | Ad3, E3/16K(83-146) | ANTITPKTGGELRGLPTDDPWVVAGFVCLGVVAGGLVLILCYLYIPCCAYLVILCCWFKKWGPY |
| | Ad7, E3/16K(83-146) | ANTITPKTGGELRGLPTDDPWVVAGFVILGVVAGGLVLILCYLYIPCCAYLVILRCWFKKWGPY |
| | Ad35, E3/15K(69-131) | TNTTF-KTGGELHGLPTENPWVEAGLVVLGILAGGLVIILCYLYTPCFTFLVVLWYWFKKWGPY |

Figure 8

**Figure 9. Previously proposed membrane topology of the Ad-2 E3/6.7K protein and the E3/6.7K-E3/19K fusion protein.**

Figure is adapted from Wilson-Rawls et. al. (Wilson-Rawls and Wold, 1993) and Wilson-Rawls et. al. (Wilson-Rawls, Deutscher, andWold, 1994).




E3/6.7K

E3/6.7K-19K fusion

E3/6.7K signal-anchor domain

E3/19K stop-transfer domain

E3/6.7K non hydrophobic region

E3/19K non hydrophobic region

C carboxyl terminus

-CHO N-linked carbohydrate

N amino terminus

Figure 9

## *2.3 Mode of translocation of E3/6.7K across the membrane of the ER*

The E3/6.7K protein is targeted to the secretory pathway through a central domain consisting of an unusually long stretch of hydrophobic amino acids (Wilson-Rawls *et al.*, 1990), while most other E3 proteins (except for E3/11.6K) have a cleavable signal sequence. The lack of an amino terminal, cleavable signal sequence and the relatively short length of E3/6.7K suggest that it may employ an unusual mode of translocation across the membrane of the ER. Even though there have been speculations that E3/6.7K is either post-translationally translocated or post-translationally glycosylated, these events have never been experimentally demonstrated. Our experiments directly addressed the question whether E3/6.7K translocates to the membrane of the ER in a co- or post-translational manner. The membrane topology and targeting of E3/6.7K are important in determining its mode of action. A brief review of the current model for protein translocation across the membrane of the ER is necessary.

### 2.3.1 Protein translocation across the membrane of the ER

Most proteins present in higher eukaryotes are targeted to the membrane of the ER by virtue of their signal sequence. Membrane targeting and synthesis of secreted proteins are closely coupled. Therefore most membrane proteins are said to be co-translationally translocated. In some rare instances the targeting event occurs after complete synthesis of the polypeptide chain, therefore resulting in post-translational translocation. A diagram depicting SRP-dependent and independent protein translocation is shown in Fig. 10.

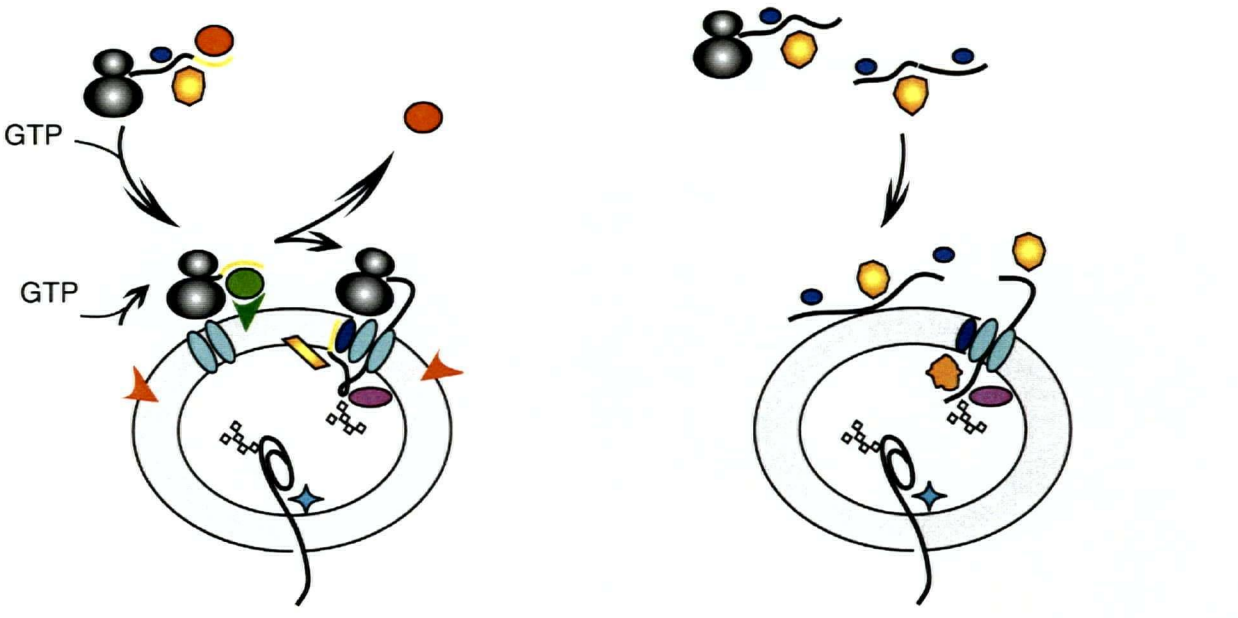
Signal peptide-dependent translocation is used mostly by proteins that have a cleavable signal sequence located at the amino terminus of the protein. The signal peptide contains a positively charged amino terminus of 1-5 amino acids, then a stretch of 10-15 hydrophobic amino acids followed by a polar domain (Kaiser *et al.*, 1987). The signal peptide binds to the signal recognition particle (SRP) as it emerges from the ribosome (Walter and Blobel, 1981). Additional proteins such as an N-ethyl maleimide (NEM)-sensitive factor (NSF) and the nascent-chain associated complex (NAC) appear to compete for the binding of the SRP to the signal peptide

(Wiedmann *et al.*, 1994). While NAC binds to nascent chains of both cytosolic and secreted proteins, the SRP prefers secreted proteins. This competition ensures the selectivity of the secretion process (Wickner, 1995).

The affinity for the signal peptide displayed by the SRP, ribonucleoprotein complex, is regulated by the existence of a GTP-GDP exchange reaction. The emerging signal peptide binds GDP-associated SRP (SRP-GDP), resulting in translation arrest. The ribosome-nascent chain-SRP complex docks at an ER membrane-associated SRP receptor (SR). Both SR and SRP are found either free (Rapiejko and Gilmore, 1997) or in their GDP-bound state prior to association and switch to a GTP-bound form following membrane anchoring of the complex (Connolly and Gilmore, 1993). The anchored complex interacts with the heterotrimeric channel complex Sec61. It is the Sec61 complex that mediates the hydrolysis of GTP, resulting in the transfer of the peptide to the channel and the dissociation of the SRP-SR anchor (Song *et al.*, 2000). After the SRP is displaced and translation resumes (Jungnickel and Rapoport, 1995), the preprotein enters the ER. A second integral membrane protein, the translocating chain-associating membrane protein (TRAM), interacts with the signal peptide and serves to orient it in the ER membrane (Mothes *et al.*, 1997). The energy required for protein translocation is generated by the polymerization of the polypeptide chain.

The mechanism of post-translational translocation is less well understood. While this mode of translocation is common in both yeast and bacteria, it is very rare in higher eukaryotes. Proteins that translocate post-translationally lack a cleavable signal sequence and in most cases have a single transmembrane, hydrophobic domain located close to the carboxyl terminus similar to E3/6.7K. Since these proteins are fully synthesized prior to translocation, they have to interact with cytosolic chaperones and be preserved unfolded in a translocation-competent state. The other main feature of this process is that the energy necessary for protein translocation is derived from the interaction between the nascent chain and the lumenal chaperone Hsc70-BiP. This mechanism operates through a chaperone-mediated trapping of protein domains (the Brownian ratchet model) as they emerge on the lumenal side of the ER (Matlack *et al.*, 1999).

**Figure 10. Simplified diagram illustrating the mechanism of co- and post-translational translocation across the membrane of the ER in higher eukaryotes**




Co-translational Translocation

Post-Translational Translocation

Ribosome

GTP-coupled Signal Recognition Particle (SRP-GTP)

GDP-coupled Signal Recognition Particle (SRP-GDP)

GTP-coupled SRP -Receptor complex (SR-GTP)

GDP-coupled SRP -Receptor complex (SR-GDP)

Sec Complex

Translocating chain-Associating Membrane Protein (TRAM)

Nascent Polypeptide-Associated Complex (NAC)

cytosolic 70kDa class Heat-Shock Proteins (Hsp70)

lumenal 70kDa class Heat-Shock Cognate Proteins (hsc70/ BiP/Kar2P)

Oligosaccharyl Transferase Complex (OST)

Core Glycosylation Moiety

Signal Peptide

Signal Peptidase

Protein Disulfide Isomerase (PDI)

Figure 10

### 2.3.2 Translocation of the E3/6.7K protein across the membrane of the ER

It is difficult to predict which proteins will employ the post-translational mode of translocation simply based on the presence of an N-terminal localized, cleavable signal sequence. There are many examples of proteins that lack a signal sequence, but which still employ a co-translational, SRP-mediated targeting mechanism. Most of these proteins have a signal-anchor domain that is recognized by the SRP. This has been observed in most type II proteins, like the invariant chain protein (Ii) (Lipp and Dobberstein, 1986) and the transferrin receptor (TfR) (Zerial *et al.*, 1986). What makes predictions of targeting mechanism for a given protein even more difficult is that, in yeast, signal sequence recognition has been implicated in post-translational translocation of some proteins into the ER (Plath *et al.*, 1998).

In spite of the alternate methods of targeting to the ER, it is important to note that there are very few confirmed examples of proteins present in higher eukaryotes that are post-translationally translocated. In fact the only higher eukaryotic proteins demonstrated to do so are the "tail-anchored" proteins. These proteins have in common the fact that their signal-anchor domain is found in close proximity to their C-terminus (tail-anchored). Tail-anchored proteins include the synaptobrevin (Whitley *et al.*, 1996), cytochrome b5 and bcl-2 (Janiak, Leber, and Andrews, 1994). These proteins insert in the ER membrane post-translationally, in the absence of the SRP and the Sec61 complex and assume a type II topology. Our results show that the E3/6.7K protein of adenovirus also translocates across the membrane of the endoplasmic reticulum (ER) in a post-translational manner to assume a type II conformation (the 8kDa form). However, we found that the protein undergoes a second translocation event that leads it to assume a hairpin topology (the 14kDa form).

### *Specific objectives*

**A)** The function of E3/6.7K may be related to the evasion of the immune response, as most other proteins coded by the E3 region. When mice are infected with viral deletion mutants that lack E3/6.7K, the infection site shows an increased inflammatory response compared to the one seen in mice infected with wild type virus. The second set of experiments will attempt to explain these *in vivo* results by looking at the response of cells transfected with E3/6.7K to inflammatory factors. Looking at the various players of the cellular signaling apparatus will then allow E3/6.7K's mechanism of action to be analyzed.

**B)** The adenovirus E3/6.7K is an unusual membrane protein due to the lack of a discernible ER-targeting mechanism as well as its highly hydrophobic nature. Wilson-Rawls et. al. have proposed that the protein forms a type III, N lumenal-C cytoplasmic, conformation (124). These experiments focus on characterizing the membrane orientation and post-translational modifications of E3/6.7K to determine whether the type III conformation hypothesis is correct. These experiments also address the mechanism of translocation of this protein.

# Chapter 3. Materials and Methods

## *3.1 Virus strains and tissue culture*

Wild Type Ad5 (Ad5wt) was obtained from the American Type Culture Collection (Rockville, Maryland, USA) and dl739, E3/6.7K-deleted viral mutant (dl739) was obtained as a gift from W.S.M Wold. These two Adenovirus group C viruses share a great degree of similarity, but differ in the expression of E3/6.7K protein, which is deleted in dl739 as described previously (Wilson-Rawls *et al.*, 1990). Both viral serotypes were propagated in monolayer culture of A549, lung carcinoma cells grown in Minimal Essential Media (Gibco BRL Life Technologies Inc., Gaithersburg, Maryland, USA) supplemented with 10% Fetal Calf Serum (FCS). Two to five days after inoculation with Ad5, cells were freeze/ thawed twice, sonicated for 30s three times and centrifuged at 500 g for 5 min. The supernatant was collected and its viral titer determined by plaque assays on A549 monolayers grown on six well plates. Titers ranged from $10^8$ to $10^9$ plaque-forming units (pfu)/ml. Control inoculum was prepared from uninfected A549 cells treated in an identical manner to the infected cells.

## *3.2 Inoculation of airway ducts and viral plaque assays*

Two groups of 24 mice were anaesthetized with Halothane. Mice from the first group were infected intranasally with $10^7$pfu of Ad5wt in 60 µl of culture media while mice from the second group were infected intranasally with $10^7$pfu of dl739 in 60 µl of culture media. In addition, six animals were infected with sterile culture media alone. Six animals from each of the two groups were sacrificed with an overdose of Halothane 2 hours, 1, 3 and 7 days post infection (p.i.). Two sham infected animals were sacrificed on days 1, 3 and 7 days p.i.. Lungs were inflated with 4% paraformaldehyde in PBS pH7.4 (0.149 M NaCl, 0.012 M $Na_2HPO_4$, 0.004M $KH_2PO_4$) and embedded in paraffin.

### *3.3 Histologic scoring*

Six lung sections were placed in a embedding ring and filled with paraffin wax (Paraplast). Four μm sections of paraffin embedded lung tissue were mounted on gelatin-subbed glass slides and stained with hematoxylin and eosin as described (Hogg *et al.*, 1989). Following an: incubation for 40 sec in hematoxylin stain the slides were rinsed twice in deionized water for 3 min. Then a rapid rinse in 0.1% ammonium hydroxide was followed by an extended wash in deionized water for 5 min then stained with eosin for 30 sec. Then the slides were rinsed ten times in 95% ethanol and ten times in 100% ethanol. Following dehydration the slides were equilibrated in xylenes, by incubating twice for 3 min. Each lung was scored in six different sections. The mean and standard deviation were calculated for each experimental group.

### *3.4 Statistical analysis*

Comparisons between the two virus were made for viral titer, inflammatory score and time using a 2-way ANOVA. The level of significance was $p<0.05$.

### *3.5 Plasmid constructs.*

To isolate the cDNA for E3/6.7K the following primers were used:
5'-ACCACCATGAGCAATTCAAGTAACTC forward primer and
5'-CCTTATCTTGGATGTTGCCCCCAG reverse primer primers and template DNA consisting of EcoRI D fragment of the E3 region of Ad2 (obtained as a kind gift from W.S.M. Wold). The reaction cocktail contained 10ng of template DNA, 0.5mM of each primer, 250mM of each nucleotide, 5U of Pfu polymerase (Canadian Life Technologies, 2270 Industrial St., Burlington , Ontario) in 1X Pfu Buffer. The reaction conditions were: melting of double stranded DNA at 95°C for 30 sec., followed by annealing at 57°C for 30 sec., followed by a 30 sec. ramp to 72°C and continued elongation for an additional 30sec.. Folllowing 30 cycles of the above PCR reaction the product was phenol chloroform extracted and examined by agarose gel electrophoresis. A band corresponding to the 200bp product was purified from the agarose plug

by centrifuging through a Wizard Miniprep Column (Promega) for 45 sec followed by phenol chloroform extraction. The newly generated cDNA for E3/6.7K contained modifications designed to enhance the translation initiation at the start site of E3/6.7. The inefficient Kozak consensus found upstream of the E3/6.7K start site was modified to an optimal context for enhanced production of the protein. The reverse primer was modified compared to the template in order to replace the TGA-Stop codon with an Ochre-Stop codon (TAA). This modification eliminated the start site of E3/19K which overlaps with the sequence of E3/6.7K, therefore eliminating the initiation of translation from the highly favorable E3/19K start site that could generate an aberrant recombinant protein.

The PCR product was cloned in the Xho1 site of the BPV based cDNA expression vector pBCMGSneo (Karasuyama and Melchers, 1988). The pIH vector was assembled by subcloning of the Spe I-Xho I fragment of pHook-1 (Invitrogen), containing the Hook gene into the Xba I-Sal I sites of pIRES (Clontech). Both strands of the inserted E3/6.7K coding region were sequenced by LoneStar Labs (Austin, Tx) to ensure accuracy.

### *3.6 Generation of U937 and Jurkat cell lines stably expressing E3/6.7K*

U937 human histiocytic lymphoma cells (Sundstrom and Nilsson, 1976) and Jurkat E6-1, T-cell lymphoma (Weiss *et al.*, 1984), obtained from ATCC (CRL 1593) were maintained in RPMI 1640, 10% FCS, 2mM L-glutamine, 10mM HEPES, 100U/ml penicillin and 100µg/ml streptomycin in an atmosphere of 5% $CO_2$ and 100% humidity.

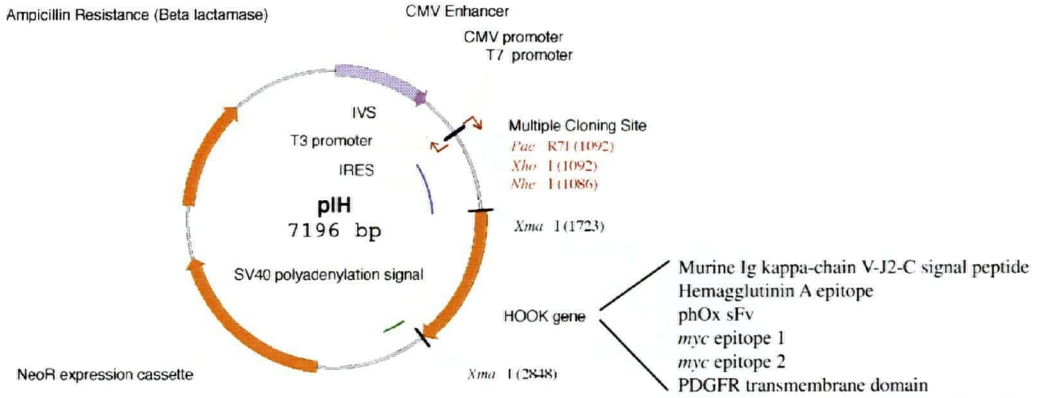
U937 cells were transfected with the either pBCMGSneo alone or pBCMGSneo carrying E3/6.7K by using the DMRIE-C cationic lipid reagent (Life Technologies) using the manufacturer's protocol. Briefly, 4µg of DNA were mixed and incubated for 30 min with 0.5 ml Opti-MEM media (Life Technologies) and a titration of 2 to 12µl of DMRIE-C in a six-well plate. The liposome-DNA mix was then mixed and incubated with 0.5ml of $5x10^5$ cells in Opti-MEM media for an additional 4 hr. Then 1ml of RPMI containing 20% FCS was added to each well. Transfected cells were selected with antibiotic 48 hr later.

Jurkat cells were transfected by electroporation. The cells were washed twice in Opti-MEM media and resuspended at a concentration of $10^8$cells/ml. Aliquots of 250µl of the cell suspension were mixed with 20µg of the appropriate plasmid and electroporated using a BioRad Electroporator at a setting of 250V/950µF. Transfected cells were selected in medium containing G-418 sulfate antibiotic at a final concentration of 1mg/ml for U937 and 2mg/ml for Jurkat cells. Media and supplements were purchased from Life Technologies. Subclones of the transfected cell lines were generated by serial dilution and examined for expression of E3/6.7K by Northern Blotting. The expression of E3/6.7K mRNA was very similar in all the clones examined. All the G-418 resistant cells that survived the selection procedure were pooled and used for the *in vitro* assays, in order to avoid clonal variations known to arise in U937 cells. The transfected Jurkat cells were also bulk transfectants.

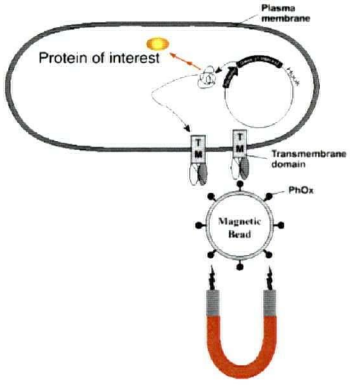
The vector pIRES (Clontech) carries the encephalomyocarditis virus (ECMV) internal ribosome entry site (IRES), which allows the co-cistronic expression of two different proteins. This vector was modified to express the single-chain antibody HOOK (Invitrogen) in the second open reading frame which allows the purification of cells using magnetic beads coated with the hapten, phenyl-oxazolone (Fig. 11) and renamed pIRES-HOOK or pIH.

Jurkat E6-1, T-cell leukemia cell line, sensitive to Fas and TRAIL, was transfected with the cDNA for E3/6.7K expressed by the eukaryotic expression vector pIH and selected with G-418. Alternatively, Jurkat E6-1 cells were transfected with vector alone and selected with G-418. The neomycin-resistant population of cells was then pooled and used in further studies to examine apoptosis. The transfected cells were examined for the surface expression of the single chain antibody HOOK in transfected cells by labeling them with an anti-Hemagglutinin Tag (HA-Tag, present in HOOK extracellular domain) antibody and analyzing them by FACS (Fig. 12). This ensured that the E3/6.7K protein was expressed.

**Figure 11. Map of pIRES-HOOK (pIH) vector indicating its main features and uses**








Description

pIH is a mammalian expression vector that allows the expression of the gene of interest at high levels in selected cells. Two MCSs are located on either side of the internal ribosome entry site (IRES) from the encephalomyocarditis virus (ECMV), which allows translation of two consecutive open reading frames from the same messenger RNA (1–3). The HOOK gene cloned into the second reading frame allows selection of cells displaying the hapten sFv. These cells specifically bind to the hapten-coated magnetic beads, and can be separated from the untransfected cells using a magnetic stand. Alternatively, transfected cells can be FACS-sorted using anti-HA or anti-cmyc antibodies.

The MCSs and IRES sequence are downstream of the immediate early promoter of cytomegalovirus(PCMV IE). The intervening sequence (IVS) between PCMV IE and the MCS is an intron that is efficiently spliced out following transcription. SV40 polyadenylation signals downstream of the MCS direct proper processing of the 3' end of the mRNA from the gene of interest. A bacteriophage T promoters is located upstream the MCS. pIH uses the neomycin resistance gene (Neo-r) to permit selection of transformed cells. Neo-r is expressed from the SV40 enhancer/promoter, and a synthetic polyadenylation signal directs proper processing of the 3' end of the Neo-r mRNA. The SV40 origin also allows for replication in mammalian cells expressing the SV40 T antigen.The vector backbone also contains the b-lactamase gene for ampicillin resistance and a ColE1 origin of replication for propagation in E. coli and an f1 origin for single-stranded DNA production.

Figure 11

**Figure 12. FACS of Jurkat cells transfected with the pIH vector (Jurkat-neo) or with pIH containing E3/6.7K (Jurkat-E3/6.7K).**

Cells were stained with an anti-HA monoclonal antibody (1:2,500 dilution) followed by an HRPO-coupled goat anti-mouse polyclonal antiserum (1:10,000 dilution). The HA tag is present in the extracellular domain of the single chain antibody HOOK which is expressed co-cistronically with E3/6.7K in cells transfected with pIH-E3/6.7K. The stained cells were examined using a FACScan (Beckton Dickinson).

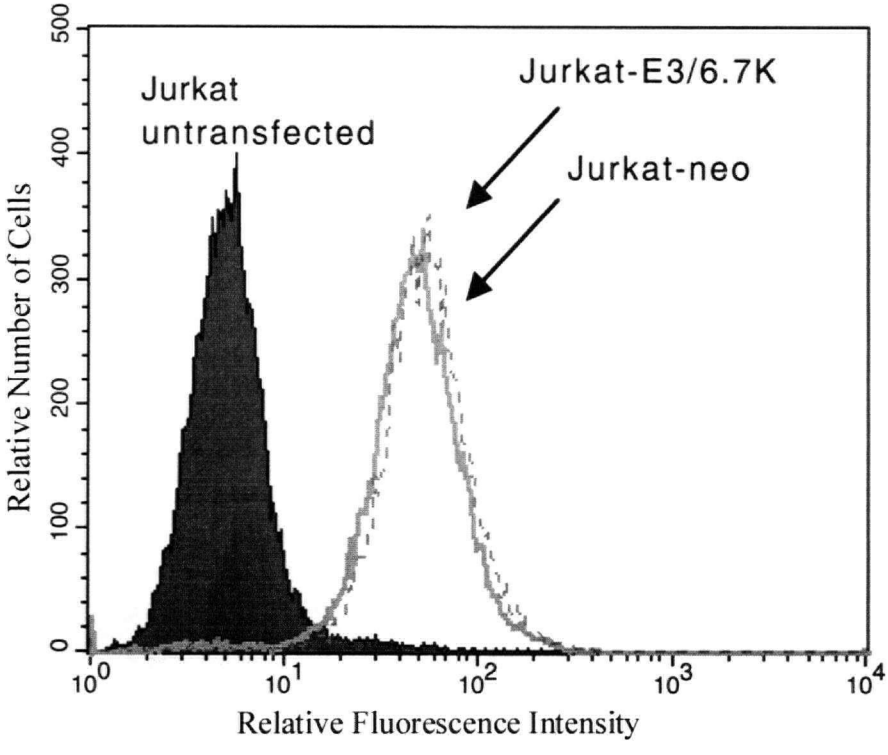

Jurkat
untransfected
Jurkat-E3/6.7K
Jurkat-neo
Relative Number of Cells
0
100
200
300
400
500
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
Relative Fluorescence Intensity

Figure 12

### *3.7 Labeling, immunoprecipitation and Western Blotting of proteins*

U937 cells transfected with vector or with vector carrying E3/6.7K were grown in suspension until they were growing exponentially, then $10^8$ cells were harvested, washed and intracellular pools of cysteine and methionine were depleted by incubation in prewarmed methionine/cysteine-free essential media without FCS for one hour at 37°C at a concentration of $5x10^6$ cells/ml. A total of $2x10^7$ cells were labeled for one hour in prewarmed methionine/cysteine-free media containing 0.5mCi/ml [$^{35}$S]-Cysteine and 0.2mCi/ml (Amersham) [$^{35}$S]-Methionine (Amersham) at a concentration of $5x10^6$ cells /ml. Cells were washed and then lysed on ice in freshly made lysis buffer containing 1% TritonX-100, 1% BSA (bovine serum albumin), 1mM iodoacetamide, 1mM phenylmethylsulfonylfluoride (PMSF), 2.5 trypsin inhibitory units (TIU)/ml aprotinin, 0.01M Tris pH8.0, 0.14M NaCl. Samples were counted by trichloroacetic acid (TCA) precipitation and approximately $10^7$cpm of each sample was precleared overnight using protein A-Sepharose CL-4B. E3/6.7K was immunoprecipitated using a polyclonal rabbit antiserum raised against the peptide CTYCQLFKRWGQHPR, corresponding to the residues 47-61 near the C-terminus of the E3/6.7K polypeptide, a generous gift from Dr. W.S.M. Wold (St. Louis University, St. Louis, MO, USA) and protein A-Sepharose (Sigma). The HLA-B27 protein was immunoprecipitated using a rabbit polyclonal antiserum generated against purified protein by Dr. Sune Kvist (Ludwig Institutet, Stockholm, Sweden).

The pellet was denatured in Tricine-SDS/sample buffer (0.1M Tris pH 6.8, 24% glycerol, 8% SDS, 0.2M DTT, 0.02% Coomassie Blue R 250) and resolved on a Tricine-SDS PAGE gel, 16.5%T (total concentration of acrylamide plus bisacrylamide) and 3%C (crosslinker) separating gel with a 10%T, 3%C spacer gel (Schagger and von Jagow, 1987).

To examine the oxidation state of E3/6.7K, the pellet was denatured in SDS/sample buffer, containing 40mM DTT final concentration (to examine the oxidation state of the protein, the protein samples were treated with or without DTT) and cooled to room temperature. Iodoacetamide, 100mM, final concentration was added and the samples were incubated for an additional 30 min in the dark at room temperature and loaded on a Tricine-SDS PAGE gel,

16.5%T, 3%C separating gel with a 10%T, 3%C spacer gel (130), unless otherwise indicated. For PNGaseF treatment, samples of immunoprecipitated protein were boiled for 2 min. in the presence of 1%SDS cooled and then incubated in 20mM phosphate buffer pH8.0, 5mM EDTA, 0.5% NP-40 and 0.1U of PNGaseF for 6 hr. at 37C following which they were denatured in SDS/sample buffer.

To analyze the cleavage of poly (ADP-ribose) polymerase (PARP), cell lysate equivalent to $10^5$ cells was denatured in SDS/sample buffer (0.1M Tris pH 6.8, 24% glycerol, 8% SDS, 0.2M DTT, 0.02% Bromophenol Blue) and resolved on a 10% glycine SDS-PAGE Laemmli gel system and blotted using the Towbin, vertical transfer, wet, system onto a 0.45μm pore size Immobilon-P PVDF membrane (Millipore) and incubated with anti-PARP mouse monoclonal antibody (Pharmingen, 1:5000 dilution). After four washes in Tris-buffered saline (TBS-50mM Tris pH 7.5, 150mM NaCl) containing 0.1% Tween-20® detergent (TBS-T), the blot was incubated with horse radish peroxidase-conjugated, goat anti-mouse antiserum (1:50,000 dilution) and visualized by chemiluminescence using the SuperSignal West Pico kit (Pierce Chemical). Alternatively, to detect caspase-3 activation, the cell lysate was resolved on a 10%T, 3%C Tricine-SDS PAGE gel without spacer, blotted using the Towbin, vertical transfer, wet, system on a 0.2μm pore size Immobilon-P PVDF membrane (Millipore) and incubated with anti-caspase-3 rabbit polyclonal antibody (Pharmingen, 1:5000 dilution). After four washes in TBS-T horse radish peroxidase-conjugated, goat anti-rabbit antiserum (1:50,000 dilution) and visualized by chemiluminescence using the SuperSignal West Pico kit (Pierce Chemical).

### *3.8 Arachidonic acid release assays*

Cells were grown at low density in 10% Hyclone FCS, RPMI 1640, 2mM L-glutamine, 10mM HEPES for several days then harvested and washed twice in PBS, 1% BSA. Approximately $5x10^6$ cells ($5x10^5$ cells/ml) were labeled for 20 hr in same media as above supplemented with 0.4 μCi/ml [$^3$H] arachidonic acid [5,6,8,9,11,12,14,15-$^3$H(N)] (0.1mCi/ml stock; New England Nuclear). Cells were washed twice in RPMI 1640, 0.2% BSA and incubated

for one hour in wash media to minimize the spontaneous release of [$^3$H] arachidonic acid. Then 400µl of cell suspension was aliquoted in each well of a 24-well plate containing 100µl of treatment media ($2x10^5$ cells/well corresponding to $1.4x10^3$ counts/well). The assay was set up in triplicate and the cells were stimulated either with media alone, 90ng/ml TNF-α (2000U/ml) (Boehringer, Mannheim), 2µg/ml cycloheximide (CHX), or with a combination of 90ng/ml TNF-α and 2µg/ml cycloheximide. After 20 hr of treatment the cells were centrifuged and 100µl of supernatant out of 500µl total was mixed with 3ml scintillation fluid and counted. For each cell line three samples were lysed in TBS containing 2% CHAPS and the lysate was used to determine the total counts of incorporated [$^3$H]arachidonic Acid. The counts per minute of released [$^3$H]arachidonic acid were expressed as percentage of the average of total incorporated [$^3$H]arachidonic acid.

### *3.9 Annexin V-FACS apoptosis assay*

Annexin V-FITC (PharMingen ) was used to determine the binding of Annexin V to externalized phosphatidyl serine. The protocol followed was based on the manufacturers Annexin V-FITC staining protocol. Cells were grown at low density in 10% Hyclone FCS, RPMI 1640, 2mM L-glutamine, 10mM HEPES for several days then $5x10^6$ cells were harvested and washed twice in PBS. Cells resuspended in above media were treated for 7 hours with media alone, 100ng/ml TNF-α, 10µg/ml cycloheximide, or a combination of 100ng/ml TNF-α and 10µg/ml cycloheximide. The cells were resuspended at $1x10^6$ cells/ml in 1xBinding Buffer (10mMHepes/NaOH, pH7.4, 140mM NaCl, 2.5mM $CaCl_2$). Then $1x10^5$ cells (100µl of above suspension) were combined with 5µl of Annexin V-FITC and analyzed with a fluorescence-activated cell sorter (FACS).

Alternatively, in the experiments examining Fas and TRAIL-induced apoptosis, Jurkat cells transfected with pIH vector or Jurkat cells transfected with pIH containing E3/6.7K were grown at low density in 10% Hyclone FCS, RPMI 1640, 2mM L-glutamine, 10mM HEPES for several days then $5x10^6$ cells were harvested and washed twice in PBS. Cells resuspended in above media were treated for 12 hours with either media alone containing potentiating reagent or

1mg/ml, DX2, anti-human Fas monoclonal antibody (Pharmingen) and a potentiator consisting of 2μg/ml goat anti-mouse polyclonal antibody or 10ng/ml of a recombinant fusion protein containing the extracellular domain of TRAIL purified from bacteria (Upstate Biotech.) and 5μg/ml of a potentiating reagent that is supplied by Upstate Biotech. consisting of a monoclonal antibody against the tag present in purified TRAIL.

### *3.10 Annexin V-Alexa-488 apoptosis assay*

This assay was based on Annexin V-Alexa 488 (Molecular Probes) labeling of cells that externalize phosphatidyl serine. A viability dye, CytoxGreen (Molecular Probes), which has much greater fluorescence than Alexa 488, was used to distinguish necrotic cells, since it is excluded by live cells but not necrotic cells.

Cells were resuspended at $1x10^6$ cells/ml in 1xBinding Buffer (10mMHepes/NaOH, pH7.4, 140mM NaCl, 2.5mM $CaCl_2$). $1x10^5$ cells (100μl of above suspension) were combined with 5μl Annexin V-Alexa 488 (Molecular Probes) and 1μl of 5mM CytoxGreen (Molecular Probes) in order to allow the discrimination between necrotic and apoptotic cells. One sample of cells was not stained and used to set up the baseline fluorescence. Cells were examined by FACS on a FACScan (Beckton Dickson).

### *3.11 YO-PRO-1 assay of membrane permeability changes*

During apoptosis, the cell membrane becomes slightly permeable to some reagents such as YO-PRO-1 but not others such as propidium iodide. This assay compared the permeability of viable and apoptotic cells to the nucleic acid stain YO-PRO-1(195), while necrotic cells were excluded using the cell-impermeant nucleic acid stain propidium iodide.

Cells were harvested, washed in PBS and resuspended at a concentration of $1x10^6$ cells/ml. For each assay 1ml of cells, 1μl of 0.1mM YO-PRO-1 in dimethyl sulfoxide and 1μl of a propidium iodide 1mg/ml solution in dimethyl sulfoxide were mixed and incubated 30 min on ice, followed by analysis of stained cells by flow cytometry using FL2~530nm and FL3~575nm

compensated emission readings on a FACScan (Beckton Dickson). After staining a cell population, apoptotic cells show green fluorescence, dead cells show red fluorescence and live cells show very little fluorescence. The percentage of viable cells undergoing apoptosis (the apoptotic index) was calculated and representative results are shown in Table IV.

### *3.12 Ratiometric intracellular [$Ca^{2+}$] determination*

Cells were washed and resuspended at a concentration of $1x10^7$ cells/ml in Opti-MEM (Life Technologies), which is reduced in phenol red and characterized in terms of its osmolality. Aliquots of 100µl of the cell suspension were incubated for 90 min at 37C with 6.0µg/ml Indo-1 AM ester, from a 0.5µg/µl stock solution in DMSO. Prior to analysis 1.9 ml of Opti-MEM were added to the cell suspension.

Intracellular $Ca^{2+}$ levels can be measured using the ratiometric $Ca^{2+}$ indicator Indo-1, since its emission maximum shifts from ~475nm in $Ca^{2+}$-free medium to ~400nm when the dye is saturated with $Ca^{2+}$. The ratio of the emission signals at 405nm and 485nm represents the ratio of $Ca^{2+}$-free to $Ca^{2+}$-bound Indo-1, yielding a very good estimate of the levels of free intracellular $Ca^{2+}$. In addition, since it measures a ratio, this method is more accurate than $Ca^{2+}$ measurements based on the increased fluorescence intensity of the bound dye, which are affected by uneven dye loading, cell thickness, photobleaching and dye leakage. Cell loading with Indo-1 is mediated by coupling it to AM esters, which can passively diffuse across cell membranes. Once inside the cell, these esters are cleaved by intracellular esterases to yield cell-impermeant fluorescent indicators. Stained cells were examined in a time dependent fashion following induction of the calcium flux using a FACS sorter equipped with an UV laser. Cells were analyzed on a FACS-Vantage sorter (Beckton Dickinson) equipped with the appropriate filters for the 405 and 485nm wavelengths.

### *3.13 In vitro transcription*

The E3 region plasmid PBR-E3 containing the EcoRI D fragment of Ad2 was obtained from Dr. W.S.M. Wold (St. Louis University, St. Louis, MO, USA). The E3/6.7K coding region was subcloned in pGEM 3Zf(+) under the control of the T7 promoter and using the E3 polyadenylation signal. Transcription of the E3/6.7K cDNA was carried out according to manufacturer's protocol using the RiboMax Large Scale RNA Production System-T7 (Promega Corp., Madison, WI, USA) or alternatively using individual components.

Prior to transcription the plasmids were linearized with a restriction enzyme that cleaves downstream of the inserted open reading frame. In some cases the enzyme used had to be an enzyme which produces a 3' protruding end. These ends have the effect of producing inhibitory antisense RNA which can affect translation efficiency. In these cases the linearized template ends were filled in with Klenow DNA polymerase prior to transcription.

Transcription was carried out at room temperature in presence of 5µl of 40U/µl RNAsin (a RNAse inhibitor available from Promega), 10µl of 5X Transcription Buffer (SP6 or T7), 2.5µl linearized plasmid DNA at 2µg/µl determined by UV spectrophotometer OD260 reading, 5µl of 0.1M DTT, 2µl of 2.5mM GpppG (methylated G cap), 7.5µl from a mix of 30µl of 3.3mM each rATP, rUTP and rCTP, 0.5µl of 10mM rGTP, 13.5µl of DEPC treated water and 1µl of 20U/µl SP6 or T7 RNA polymerase to a total volume of 47µl. All reagents were RNAse free by using only DEPC (diethyl-pyrocarbonate) treated water as well as using only baked glassware and equipment reserved for RNA work.

Following incubation at 37° C for 45min, 2µl of rGTP and an additional 1µl of SP6 or T7 polymerase were added and the sample was re-incubated at 37° for another 60min. The sample was then treated with RNAse free-DNAse RQ1 (Promega). The products of the reaction from before and after DNAse digestion were examined by agarose gel electrophoresis and spectrophotometric quantification.

The RNA was phenol-chloroform extracted and precipitated in 95% ethanol in presence of ammonium acetate. This protocol yielded usually 10-25µg of capped mRNA starting with 5µg DNA. The RNA was kept at -80C in 70% ethanol made up with DEPC water. As needed

RNA was precipitated with 1M ammonium acetate and resuspended in DEPC-water supplemented with RNAsin.

### *3.14 Preparation of microsomes*

The microsomes were prepared by using a protocol designed by R. Gabathuler and adapted from Saraste and Palade (Saraste, Palade, and Farquhar, 1986). The protocol gives a very good yield of active microsomes which can be used to study protein translocation.

Microsomes were isolated starting with 5-10 ml of packed cells (approx. $5x10^8$) after one wash with PBS. Cells were resuspended in buffer A composed of: TEA-HCl 50mM pH 7.5, sucrose 250mM, KOAc50mM, $Mg(OAc)_2$ 2.2mM, 0.1% mercaptoethanol, PMSF and Aprotinin, then homogenized with a douncer and lysed in the presence of PMSF( 2μg/ml) and aprotinin (10U/ml) as protease inhibitors. The douncer was washed once with buffer A. This was followed by centrifugation at 8000 g for 30 min. The supernatant was saved and the pellet discarded as it was composed of nuclei and cellular debris. This was followed by centrifugation at 20,000 g for 40 min to pellet all membranes, discarding the cytosolic-high speed supernatant.

The microsomal pellet was resuspended in 6 ml of buffer A, and layered on top of a 2M sucrose cushion and a 0.35M sieving fraction and centrifuged for 1 hour at 100,000g at 4°C. The microsomes were collected at the interphase of the 2M and 0.35M sucrose and diluted with 2M sucrose to reach a sucrose concentration of 50%.

To separate the submicrosomal membrane fractions, 3ml of microsomes were pipetted in SW41 ultraclear tubes. The samples were overlayed with 1ml of 45% sucrose and 0.5 ml of 40, 35, 30, 25 and 20% sucrose and centrifuged at 170,000g in a swinging bucket rotor at 4°C for 3 h. The 50-45% and 45-40% interphases contained membranes derived mostly from the rough ER. These fractions were diluted with water to ~20% sucrose (equal part of water) and centrifuged for 30 min at 100,000 g, 4°C, to remove the sucrose.

The pellets were resuspended in buffer B: TEA-HCl 50mM pH 7.5, Sucrose 250 mM, KOAc 50mM, $Mg(OAc)_2$ 2.2mM, DTT 1mM, PMSF and aprotinin. The final concentration of microsomes gave a reading of 20 Absorbance Units at 280OD. The microsomes were then

cleared of all endogenous RNA or DNA by adding: $CaCl_2$ to a final concentration of 1mM, 150 U/ml micrococcal nuclease, and 0.2mg/ml PMSF and incubating for 10, at 25°C. The reaction was stopped by addition of EGTA to a final concentration of 2mM.

The microsomes were pelleted and resuspended in buffer B to a concentration of 60 A280 units/ml. The microsomes were aliquoted, flash- frozen and stored at -80° C or in liquid nitrogen

### *3.15 In vitro translation*

The translation made use of treated reticulocyte lysate prepared as described by Pelham and Jackson (Pelham and Jackson, 1976). Because of the relatively high level of endogenous RNA (for example globin RNA) which resulted in a high background of radiolabeled protein a purchased Nuclease Treated, Rabbit Reticulocyte Lysate ® available from Promega (Promega Corp., Madison, WI, USA) was substituted in some experiments as the background translated material seemed lower in the Promega Lysate. In the later case translation was carried out according to manufacturer's protocol.

Both lysates whether made in the lab or purchased contained: phosphocreatine and phosphocreatine kinase, a mix containing all tRNA components, hemin to prevent the inhibition of the initiation of translation, 79mM potassium acetate and a 500nM-magnesium acetate buffer (optimized for our system)

The translation mix was composed of: 35µl reticulocyte lysate, 1µl of a 1mM mixture of all amino acids except methionine (or cysteine), 90µCi of ($^{35}S$) methionine (or cysteine) (>1000Ci/mmole, Amersham UK), 3µl of microsomal membranes ( the absorbance at OD280 of the stock solution of microsomes was $60A_{280}$/ml), 10 U RNAsin, 2mg/ml PMSF, 10 U aprotinin. The total reaction volume was made up to 70µl with DEPC treated water.

Translation was started by adding 50-200ng RNA and incubating at 37° C for 60-90 minutes. The reaction was terminated by adding 130µl of TNE buffer (20mM Tris-HCl, pH 8.0, 150mM NaCl and 5mM EDTA). The membranes were pelleted, washed in TNE buffer and solubilized in TNE lysis buffer containing 1% Nonindet P40 and PMSF. The non-solubilized

material consisting mainly of the phospholipids and other non-hydrolyzable material was pelleted by centrifugation. The supernatant was used for immunoprecipitation.

### *3.16 Topology assays and post-translational translocation*

To assay the membrane topology, translation was terminated with the addition of 100μg/ml cycloheximide and 1mM $CaCl_2$ final concentrations and microsomes were allowed to stabilize for 10 min. on ice. Proteinase K at a final concentration of 300μg/ml was added and samples were incubated for 30 min. on ice. PMSF was added to a final concentration of 500μg/ml to stop digestion and membranes were pelleted and solubilized. Where indicated NP-40 1% final concentration, was added concomitantly with Proteinase K. Alkaline extraction was performed by diluting the microsome suspension at the end of the translation-translocation reaction in 20 volumes of 0.1M sodium carbonate pH 11.5 followed by centrifugation and re-extraction. To assay post-translational translocation, translation was performed in the absence of microsomes for one hour. Various amounts of cycloheximide (as indicated in Fig. 26) were added and samples were incubated at room temperature for 15 min following which microsomes were added and incubated for an additional hour.

# Chapter 4. E3/6.7K prevents Death Receptor-Induced Apoptosis, Calcium Efflux from the ER and Inflammation

## *4.1 Background*

Adenovirus is a very common human pathogen that results in infections of the respiratory or gastrointestinal tract (Fox *et al.*, 1969; Fox, Hall, and Cooney, 1977), which are persistent because of the virus's elaborate evasion of the host defense mechanisms. The adenovirus genes responsible for immune evasion map to the Early 3 (E3) region of the adenovirus genome (Wold and Gooding, 1991).

Of the seven proteins expressed from the E3 region, five have been characterized while the function of the E3/6.7K and E3/12.5K proteins are not known (see map of E3 region of Ad2 genome Fig. 4 and Table II). Most E3 proteins are involved in the process of evasion of the immune response.

Since E3/6.7K gene is part of a cassette of immunomodulatory proteins it is possible that it is involved in virus-host cell interaction. Comparison of a mutant E3/6.7K-deleted virus to the wild-type virus in an *in vivo* model of adenovirus infection indicates that E3/6.7K also affects the inflammatory response *in vivo*. U937 and Jurkat cells expressing E3/6.7K by stable transfection provide a system for analyzing the effects of E3/6.7K on the cellular response to pro-inflammatory cytokines.

## *4.2 The presence of E3/6.7K reduces the inflammatory response at the site of infection*

The *in vivo* studies carried out in collaboration with Tim Vitalis and Izidor Kern indicated that the presence of E3/6.7K affected the immune response at the site of infection in the lungs of mice infected with adenovirus. The inflammation of the perivascular region of the blood vessels and the adventitia of the airways was greater in animals infected with dl739 (E3/6.7K-deleted

described by Wilson-Rawls *et. al.* (Wilson-Rawls *et al.*, 1990)) than in animals infected with wild-type virus over a seven day experimental period. As observed, the histology of the inflammatory response in mice infected with the two different virus strains indicated less lymphocytic infiltration in the alveolar, perivascular and peribronchiolar regions when E3/6.7K was present (Fig. 13).

*In vivo* the presence of E3/6.7K resulted in a reduced influx of lymphocytes into the alveoli and in the perivascular and peribronchiolar regions (Fig. 13). The presence of E3/6.7K resulted in a statistically significant reduction in the inflammatory response in the lungs of infected animals (ANOVA $p=0.025$, Fig. 14). Factors released by the epithelial cells in response to infection led to recruitment of inflammatory cells to the site of infection. The presence of E3/6.7K may inhibit the release of chemotactic factors responsible for the infiltration of inflammatory cells. Some of these factors are generated by the processing of arachidonic acid which, as observed later in our *in vitro* model, was released in significantly reduced amounts in the presence of E3/6.7K.

**Figure 13. Histopathology of lungs from mice following intranasal inoculation with wild type and E3/6.7K-deleted adenovirus.**

Mice were infected with $10^7$ pfu of wild type (C, E and G) and E3/6.7K-deleted adenovirus (D, F and H). Shown are representative hematoxylin-and-eosin stained sections from the lungs of mice infected as follows: **(A)** Ad5wt sham infected, **(C)** Ad5wt day 1 p.i., **(E)** Ad5wt day 3 p.i., **(G)** Ad5wt day 7 p.i., **(B)** dl739 sham infected, **(D)** dl739 day 1 p.i., **(F)** dl739 day 3 p.i., **(H)** dl739 day 7 p.i.. The following notations indicate (a) alveolae, (b) bronchioles small black arrows indicate lymphocyte infiltration in the peribronchiolar area, large red arrows indicate the vascular regions.

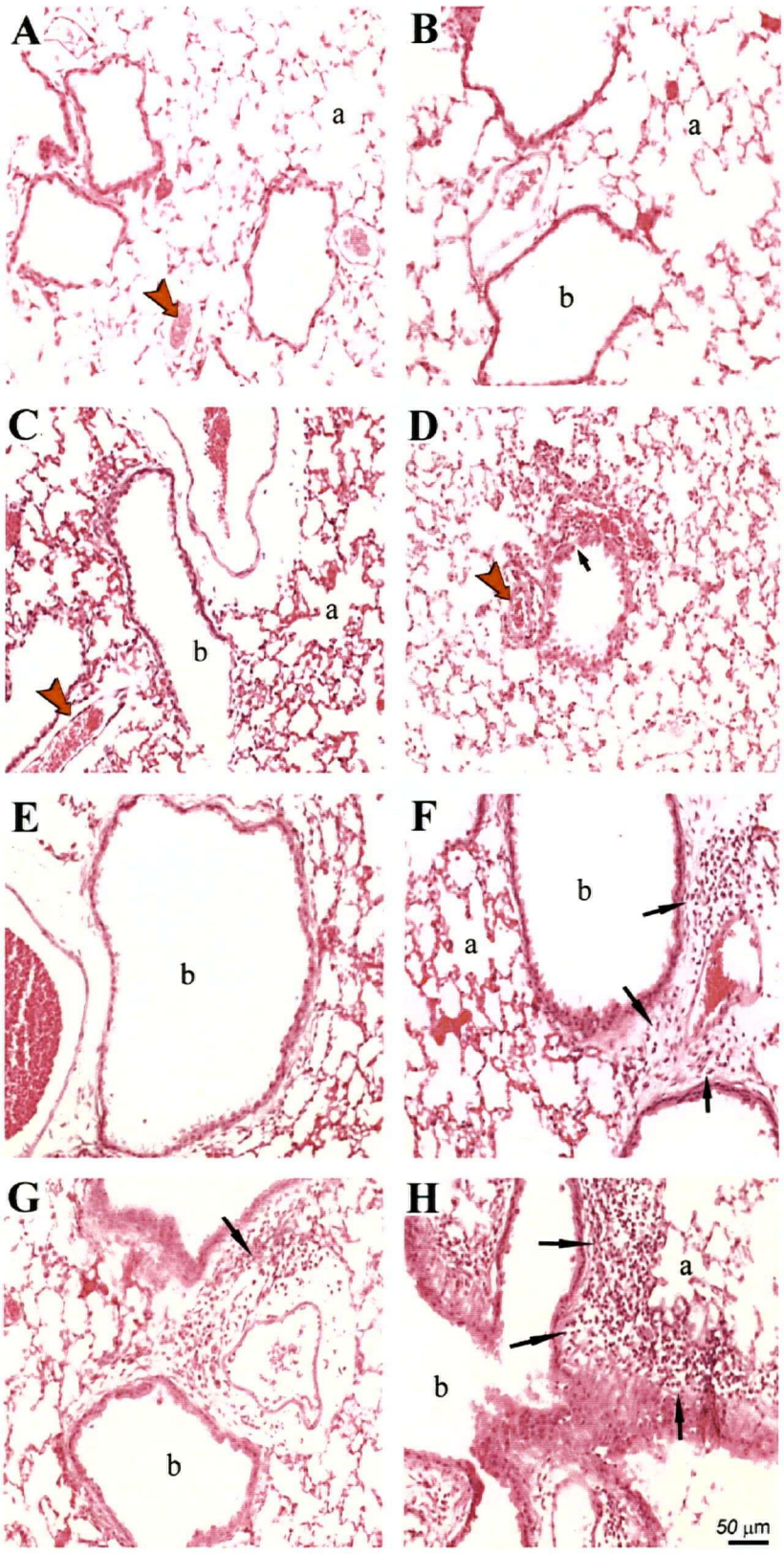
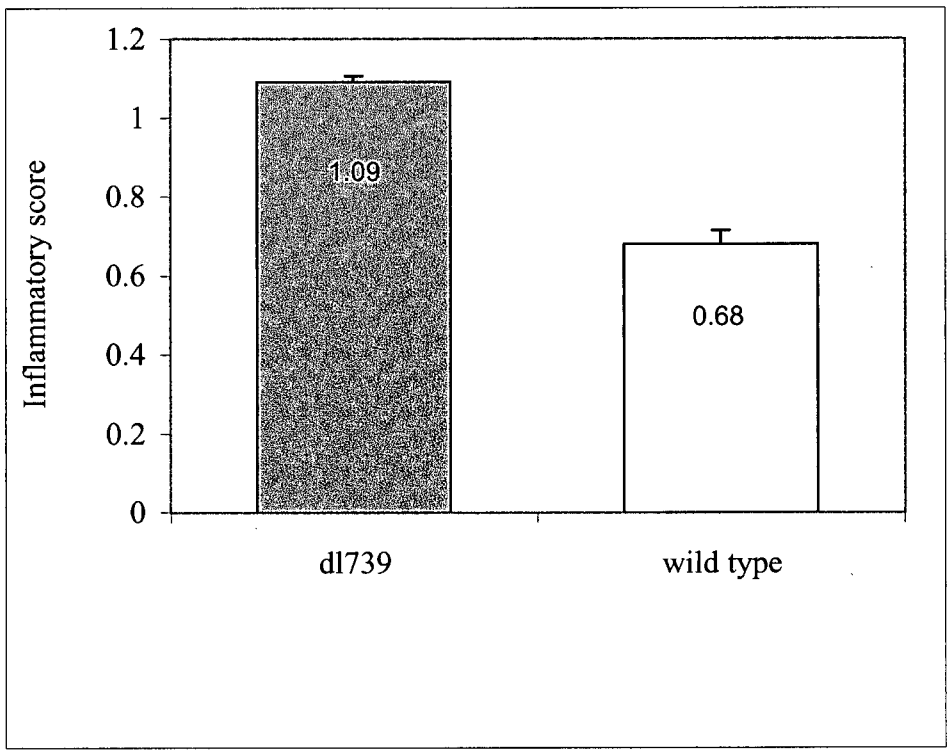



Figure 13

**Figure 14. Summary of histological scores for mice infected with virus strains lacking E3/6.7K in comparison to wild type virus.**

Mice were infected with $10^7$ pfu Ad5wt or dl739 (E3/6.7K-deleted) and evaluated for alveolar, bronchiolar and perivascular pulmonary inflammation at day 7 p.i.. Eighteen (18) mice were infected with each virus strain. An independent observer, unaware of the experimental treatment scored the airway mucosal, airway adventitia and the vascular adventitia for inflammation. The histopathologic grades were 0 - no inflammation, 1 – mild inflammation, 2 – moderate inflammation, 3 – severe inflammation for each feature. Scores for each feature were summed to give a total inflammatory score with maximum being 9 for each section. A mean inflammatory score was calculated for each lung section by dividing the total score by 3. For every mouse a total of twelve separate sections were scored and averaged. Comparisons between the two viruses were made for inflammatory score using a two-way ANOVA with a $p<0.05$ significance level.

# Figure 14



### *4.3 Death Receptor-induced apoptosis is reduced in the presence of E3/6.7K*

The first hypothesis to be tested was whether E3/6.7K dampens the immune response by preventing TNF-α-induced apoptosis, one of the key players of the immune response against adenovirus.

The U937 human histiocytic lymphoma cell line (Sundstrom and Nilsson, 1976) is sensitive to the effects of TNF-α. We transfected a batch of U937 cells with pBCMGSneo containing the cDNA for E3/6.7K another batch with the vector alone. The vector used, a Bovine Papilloma Virus (BPV) based vector, pBCMGS-neo (Karasuyama and Melchers, 1988) carries the neomycin resistance gene and is maintained by episomal transmission, does not integrate in the genome. Transfected cells were selected with G-418. All surviving, neomycin resistant, cells were pooled and used in the following experiments to avoid the clonal variation known to arise in U937 cells. The selection procedure gave rise to a pool of transfectants that express E3/6.7K (U937-E3/6.7K) and one that was not as it was transfected with the vector itself (U937neo$^r$). Expression of E3/6.7K was analyzed by Northern Blot and by immunoprecipitation. To confirm that the E3/6.7K protein was properly synthesized, the recombinant protein was immunoprecipitated with a polyclonal rabbit antiserum raised against an E3/6.7K C-terminal-derived peptide, and examined using SDS-PAGE electrophoresis (Fig. 15).

TNF-α-induced apoptosis was assayed by measuring the externalization of phosphatidyl serine by using FITC-labeled Annexin V (Martin *et al.*, 1995) and Fluorescence-Activated Cell Sorting (FACS). The presence of E3/6.7K yielded a 2.2 fold reduction in the proportion of apoptotic cells in U937-E3/6.7K (bulk transfectants) compared to U937neo$^r$ (transfected with vector alone), following stimulation with TNF-α (Fig. 16). The U937-E3/6.7K cells showed a 2.8 fold reduction in apoptosis compared to U937neo$^r$ following an augmented stimulation with a combination of TNF-α and cycloheximide (CHX). The presence of E3/6.7K decreased the apoptotic response in U937 cells upon stimulation with TNF-α or a combination of TNF-α and CHX.

**Figure 15. Tricine SDS-PAGE analysis of immunoprecipitated E3/6.7K.**

U937 cells transfected with vector alone (lane 1) or transfected with E3/6.7K cDNA (lane 2) were radiolabeled with [35S]-Cysteine and [35S]-Methionine, lysed and immunoprecipitated with a polyclonal rabbit antiserum generated against the C-terminal region of E3/6.7K, and analyzed by Tricine SDS-PAGE protocol on a 16.5%T, 3%C separating gel with a 10%T, 3%C spacer gel.

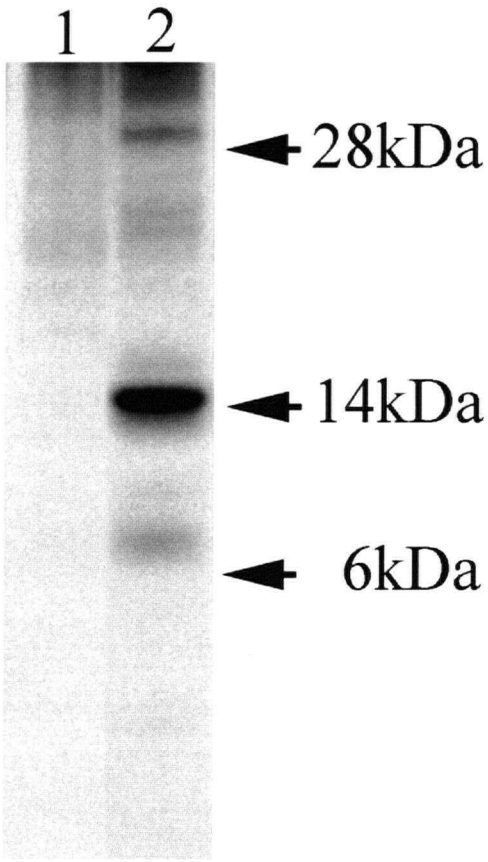
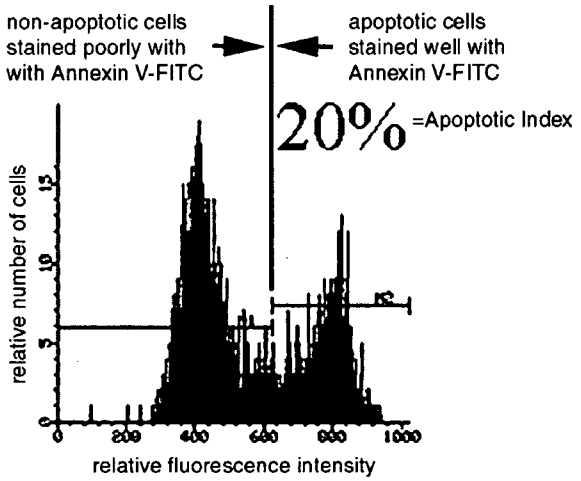



Figure 15

**Figure 16. Annexin V-FITC flow cytometry analysis of TNF-induced apoptosis.**

A population of cells transfected with vector alone (panels a, b, c, d) and a population of cells transfected with vector carrying E3/6.7K (e, f, g, h) were stimulated for 7 hours with media alone (**a** and **e**), 100ng/ml TNF-α (**b** and **f**), 10μg/ml cycloheximide (**c** and **g**), or a combination of 100ng/ml TNF-α and 10μg/ml cycloheximide (**d** and **h**). For each cell population a sample treatment was stained with Annexin V-FITC and analyzed by FACS. A second sample was analyzed by FACS in the absence of Annexin V-FITC in order to determine the background fluorescence, which corresponds to the fluorescence associated with the Annexin V-negative cell population from each sample. The percentage of Annexin V-positive, apoptotic cells is indicated for each experimental treatment and cell line. These results are representative of three repeat experiments.


non-apoptotic cells
stained poorly with
with Annexin V-FITC
apoptotic cells
stained well with
Annexin V-FITC
20%
=Apoptotic Index
relative number of cells
relative fluorescence intensity

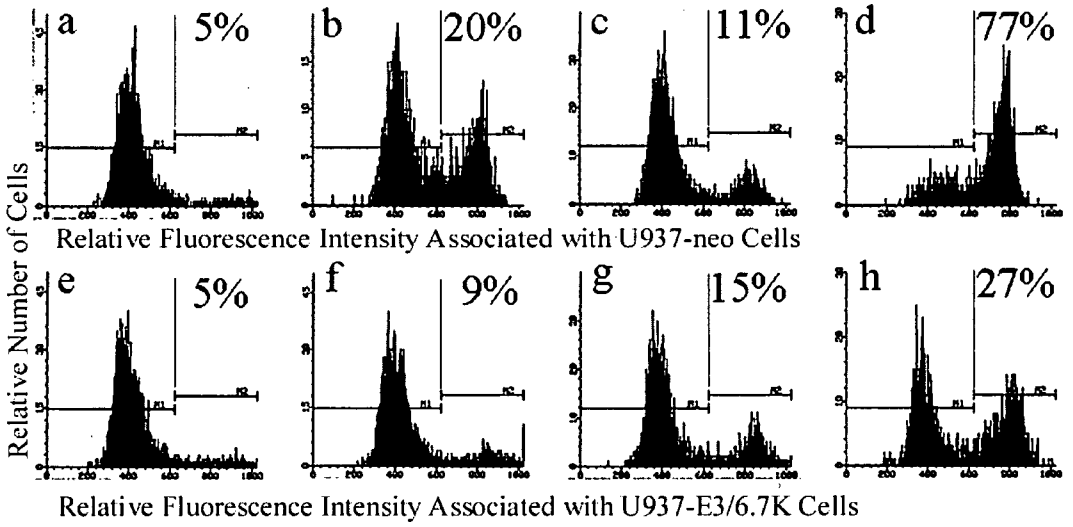

a
5%
b
20%
c
11%
d
77%
Relative Fluorescence Intensity Associated with U937-neo Cells
Relative Number of Cells
e
5%
f
9%
g
15%
h
27%
Relative Fluorescence Intensity Associated with U937-E3/6.7K Cells

Figure 16

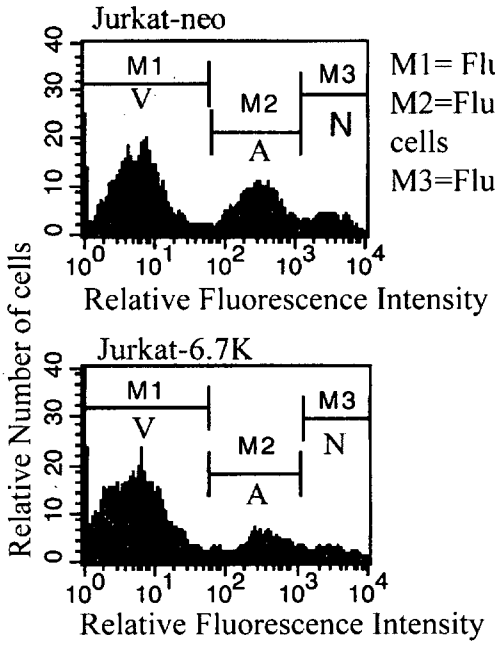
Does E3/6.7K protect against apoptosis induced through Fas or TRAIL Receptors? To find out, Jurkat E6-1, T-cell leukemia cell line (Weiss *et al.*, 1984), sensitive to Fas and TRAIL (Weis *et al.*, 1995), was transfected with the cDNA for E3/6.7K expressed by the eukaryotic expression vector pIH, and selected with G-418. The resistant population of cells was examined by FACS using an antibody directed against the HOOK protein. The expression of the bicistronically encoded HOOK protein assured that E3/6.7K expressed in the G-418 resistant population (see Methods, Fig. 12). Recombinant TRAIL and an anti-Fas, agonistic monoclonal antibody were used to induce apoptosis in both Jurkat-E3/6.7K cells and control, Jurkat-neo, which were transfected with the pIRES-HOOK vector without an insert.

The E3/6.7K protein conferred a protective effect against Death Receptor-mediated cell death due to TNF-α, Fas and TRAIL (Fig. 17, 18 and summarized in Table IV). Different methods of assaying apoptosis have different sensitivities and so they resulted in different apoptotic indices. Overall, the apoptotic indices of cells expressing E3/6.7K were consistently and significantly lower than cells transfected with vector alone. Another interesting observation was that the anti-apoptotic effect of E3/6.7K was more effective against TNF-α (Fig. 16) and TRAIL (Fig. 18) than against Fas (Fig. 17) -induced apoptosis. This may reflect the subtle differences in the signaling pathway downstream of the surface receptor.

**Figure 17. E3/6.7K protects against apoptosis induced by Fas in Jurkat cells.**

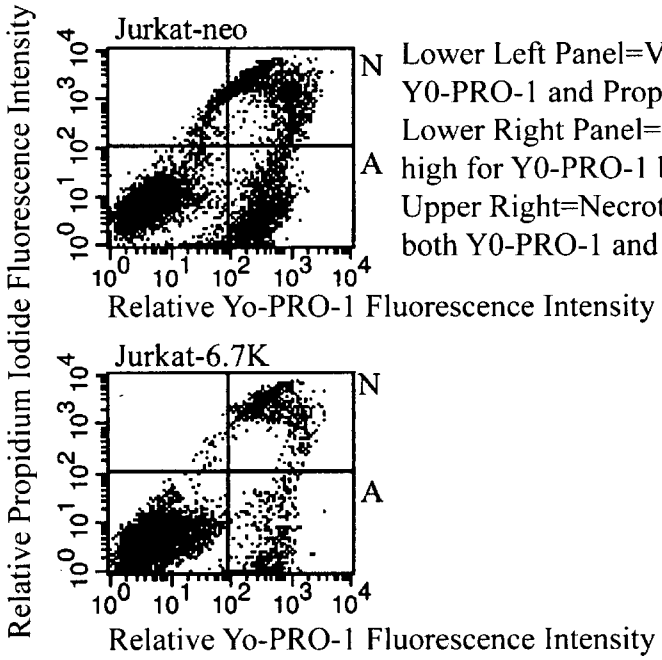
Jurkat cells transfected with vector alone (Jurkat-neo) and transfected with E3/6.7K (Jurkat-E3/6.7K). To study Fas induced apoptosis cells were stimulated for 12 hours with 1mg/ml, DX2, anti-human Fas monoclonal antibody (Pharmingen). The apoptotic cells were stained with **(A)**Annexin V-Alexa 488 indicating externalization of phosphatidyl serine or **(B)** cells were stained with YO-PRO-1 indicating an increase in membrane permeability.

A




M1= Fluorescence associated with viable (V) cells
M2=Fluorescence associated with apoptotic (A) cells
M3=Fluorescence associated with necrotic (N) cells

B



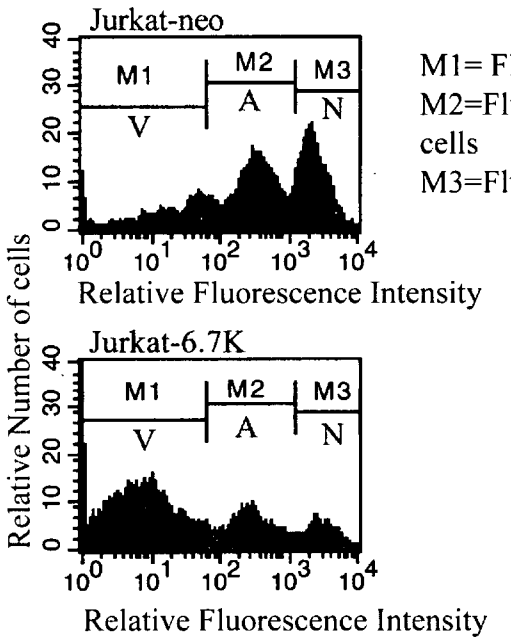

Lower Left Panel=Viable cells staining low for both Y0-PRO-1 and Propidium Iodide
Lower Right Panel=Apoptotic (A) cells staining high for Y0-PRO-1 but low for Propidium Iodide
Upper Right=Necrotic (N) cells staining high for both Y0-PRO-1 and Propidium Iodide

Figure 17

**Figure 18. E3/6.7K protects against apoptosis induced by TRAIL in Jurkat cells.**

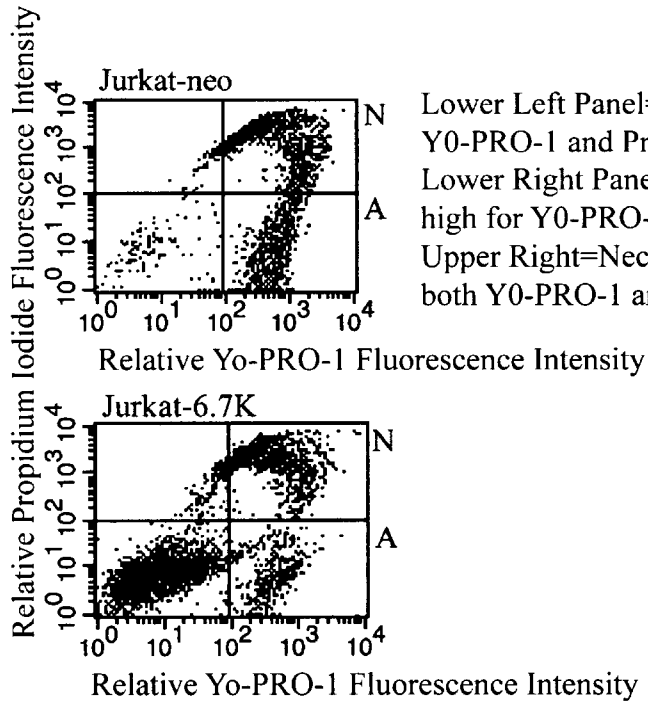
Jurkat cells transfected with vector alone (Jurkat-neo) and transfected with E3/6.7K (Jurkat-E3/6.7K). To study TRAIL induced apoptosis, cells were stimulated for 12 hours with 10ng/ml of a recombinant fusion protein containing the extracellular domain of TRAIL (Upstate Biotech.). The apoptotic cells were stained with **(A)** Annexin V-Alexa 488 indicating externalization of phosphatidyl serine or **(B)** cells were stained with YO-PRO-1 indicating an increase in membrane permeability.

A




M1= Fluorescence associated with viable (V) cells
M2=Fluorescence associated with apoptotic (A) cells
M3=Fluorescence associated with necrotic (N) cells

B




Lower Left Panel=Viable cells staining low for both Y0-PRO-1 and Propidium Iodide
Lower Right Panel=Apoptotic (A) cells staining high for Y0-PRO-1 but low for Propidium Iodide
Upper Right=Necrotic (N) cells staining high for both Y0-PRO-1 and Propidium Iodide

Figure 18

**Table IV. E3/6.7K prevents Death Receptor-induced apoptosis in bulk transfected cells.**

The presence of E3/6.7K resulted in a significant reduction in the number of apoptotic cells following Death Receptor-Induced apoptosis. It appears the protective effect was more pronounced following TRAIL and TNF-α stimulation as opposed to anti-Fas antibody stimulation of cells. The magnitude of the protective effect varied depending on the method used to assay apoptosis.

*The apoptotic index=(number of viable apoptotic cells)/(total number of viable cells)

| Cell type | Inducer | Apoptotic index* by AnnexinV Staining | Apoptotic Index* by Yo-PR0 Exclusion |
|---|---|---|---|
| U937-neo | TNF-α | 20% | -Not tested |
| U937-E3/6.7K | TNF-α | 9% reduced by 2.2 fold | -Not tested |
| U937-neo | TNF-α/CHX | 77% | - Not tested |
| U937-E3/6.7K | TNF-α/CHX | 27% reduced by 2.8 fold | - Not tested |
| Jurkat-neo | TRAIL | 75% | 84% |
| Jurkat-E3/6.7K | TRAIL | 26% reduced by 2.9 fold | 14% reduced by 6 fold |
| Jurkat-neo | anti-Fas antibody | 34% | 26% |
| Jurkat-E3/6.7K | anti-Fas antibody | 17% reduced by 2.0 fold | 12% reduced by 2.2 fold |

### *4.4 Presence of E3/6.7K delays TNF-α induced activation of caspase-3 and cleavage of PARP*

To define the pathway affected by E3/6.7K, the effect of E3/6.7K on the TNF-α induced cleavage of effector caspase-3 and of the DNA repair enzyme poly (ADP-ribose) polymerase (PARP) was tested (Fig. 19). The 17 and 11kDa proteolytic products of procaspase-3 are subunits of the heterodimeric, active form of caspase-3. The appearance of the active forms of caspase-3 was significantly delayed in U937-E3/6.7K versus U937neo$^r$ (Fig. 19A). In addition, there was a significant reduction in the amount of active caspase-3 in U937-E3/6.7K cells versus U937neo$^r$ cells. The 85kDa inactive form of PARP appeared in both U937neo$^r$ and U937-E3/6.7K cell lines at 2 hours after TNF-α stimulation (Fig. 19B). There was, however, a noticeable difference with regards to the kinetics of PARP inactivation: the amount of inactive PARP present in the U937-E3/6.7K cells was considerably reduced compared to U937neo$^r$. Therefore, the presence of E3/6.7K delayed and reduced the activation of caspase-3, and inhibits PARP inactivation during apoptosis.

**Figure 19. Effect of E3/6.7K on the induction of procaspase-3 processing and PARP cleavage during TNF-induced apoptosis in vivo.**

Cell extracts were obtained from U937neo$^r$ and U937-E3/6.7K cells that had been treated with 10ng/ml TNF-α and 0.5μg/ml CHX for various lengths of time. After electrophoresis and transfer to PVDF membranes, blots were incubated with the following: (A) anti-caspase-3 rabbit antiserum that recognizes the 17kDa and 11kDa subunits of the active, processed protein, and (B) anti-PARP mouse monoclonal that recognizes both the active 116kDa and inactive 85kDa form of the protein. The blots were developed with a secondary antibody and visualized by chemiluminescence (Pierce Chemical). Similar results were obtained in two repeat experiments.

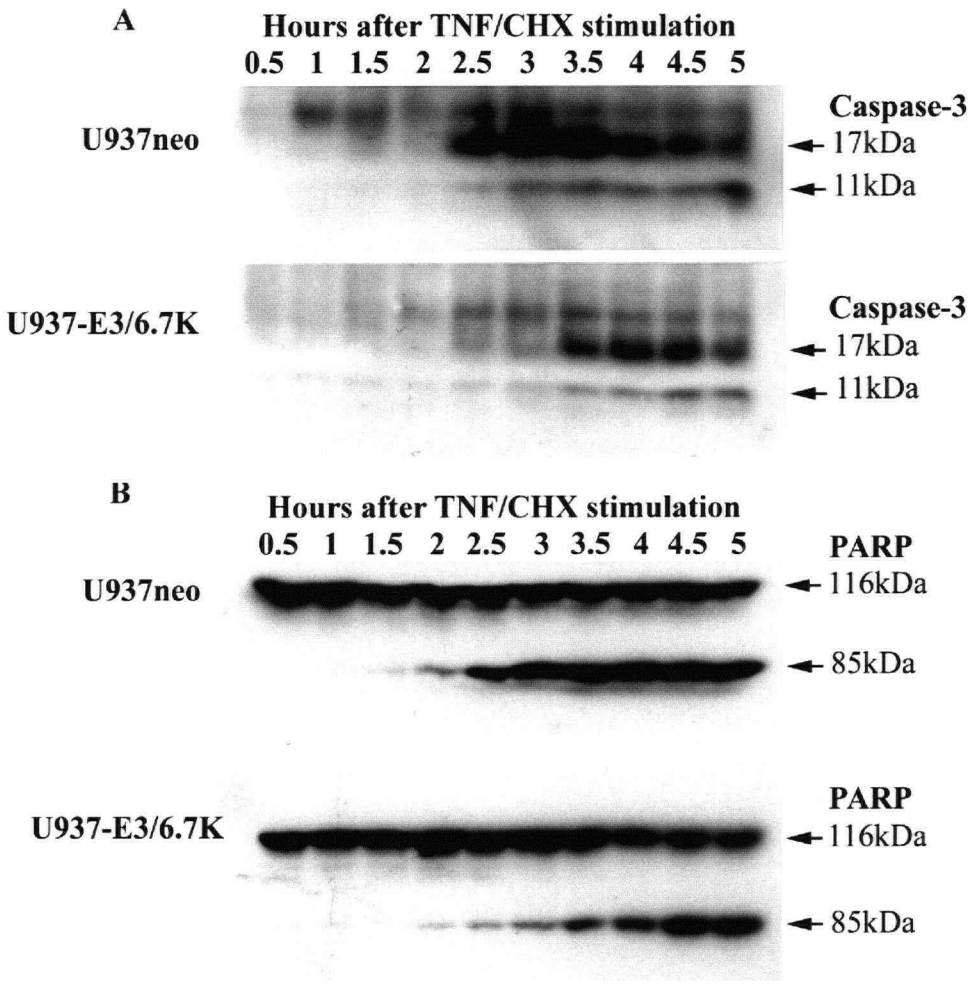

A
Hours after TNF/CHX stimulation
0.5 1 1.5 2 2.5 3 3.5 4 4.5 5
U937neo
Caspase-3
17kDa
11kDa
U937-E3/6.7K
Caspase-3
17kDa
11kDa
B
Hours after TNF/CHX stimulation
0.5 1 1.5 2 2.5 3 3.5 4 4.5 5
U937neo
PARP
116kDa
85kDa
U937-E3/6.7K
PARP
116kDa
85kDa

Figure 19

### *4.5 Efflux of $Ca^{2+}$ from the ER in response to thapsigargin is reduced in the presence of E3/6.7K*

Thapsigargin, a sesquiterpene lactone isolated from the umbelliferous plant *Thapsa garganica*, selectively inhibits the endoplasmic reticulum $Ca^{2+}$-ATPase that directs $Ca^{2+}$ uptake into the ER. In its presence, the cytosolic levels of $Ca^{2+}$ rise, mimicking the apoptotic effects of a sustained $Ca^{2+}$ flux (Jiang *et al.*, 1994). At high doses, this agent has been shown to induce apoptosis in Jurkat cells (Srivastava *et al.*, 1999b).

Thapsigargin-induced-$Ca^{2+}$ flux in Jurkat transfected cells resulted in an 80% increase in the level of intracellular calcium. In the presence of E3/6.7K, however, the observed increase is reduced to 34% (Fig. 20). Cells were loaded with the $Ca^{2+}$-sensitive fluorophore Indo-1. $Ca^{2+}$-flux was assayed using a ratiometric value that represents the amount of $Ca^{2+}$-bound Indo-1/ amount of $Ca^{2+}$-free Indo-1/cell. The graph shown in Figure 20 represents a time course of the average ratiometric values of approximately 20,000 cells/min. E3/6.7K affected the ER-calcium homeostasis, preventing the efflux of calcium from the ER. The observed decrease in calcium efflux correlates with the protective role conferred by E3/6.7K against Death Receptor-induced apoptosis.

**Figure 20. The presence of E3/6.7K results in the reduction of the thapsigargin induced-Ca2+ flux in Jurkat cells.**

Jurkat cells transfected with E3/6.7K (Jurkat-E3/6.7K) and vector alone-transfected cells (Jurkat-neo) were loaded for 90 min at 37°C with 0.5μM Indo-1 AM ester and examined on a FACS-Vantage, UV-equipped flow cytometer (Beckton Dickinson) for 5 minutes to establish the baseline fluorescence, equivalent to the resting level of intracellular calcium. At the 5 min mark, cells were treated with 5nM thapsigargin and analyzed for the next 25 min-a total number of $5x10^5$ events. The experiment was repeated three times with similar results.

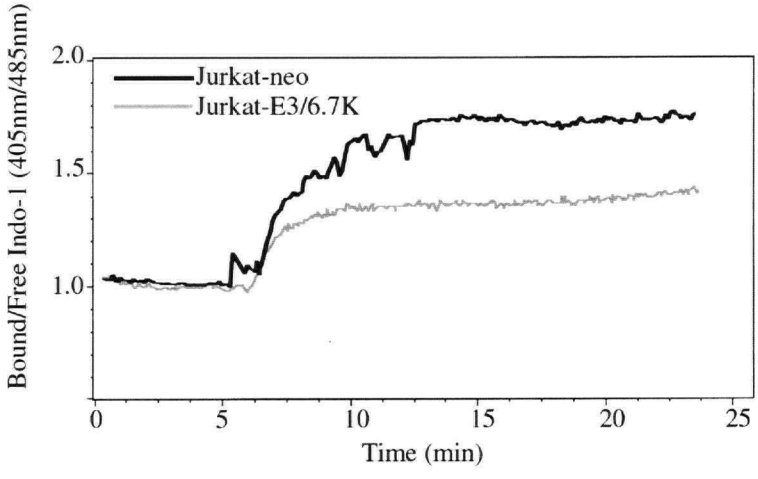

Bound/Free Indo-1 (405nm/485nm)
2.0
1.5
1.0
Jurkat-neo
Jurkat-E3/6.7K
0
5
10
15
20
25
Time (min)

Figure 20

### *4.6 TNF-mediated arachidonic acid release is reduced in the presence of E3/6.7K*

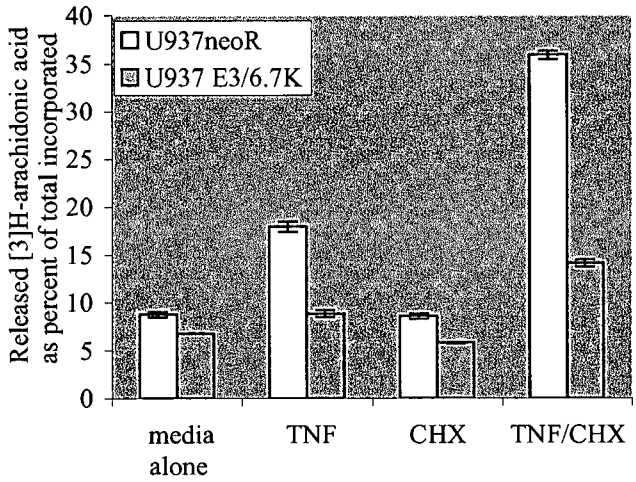
Since the ER $Ca^{2+}$efflux in transfected cells was reduced in the presence of E3/6.7K, it became imperative to test the effect of E3/6.7K on the calcium-dependent $cPLA_2$. In the presence of a sustained calcium flux $cPLA_2$ translocates preferentially to the ER and nuclear envelope where it releases arachidonic acid from the sn-2 position of various phospholipids (Peters-Golden *et al.*, 1996; Schievella *et al.*, 1995). The E3/10.4K and E3/14.5K proteins coded by adenovirus prevent the translocation of $cPLA_2$ from the cytosol to the membrane in response to TNF-α but not to A23187, a calcium ionophore.

The presence of E3/6.7K reduced the release of [$^3$H]arachidonic acid from U937-E3/6.7K by 50% compared to U937neo$^r$, following stimulation with TNF-α (Fig. 21). When the stimulus was increased by the addition of TNF-α and cycloheximide (CHX), a protein synthesis inhibitor synergistic with TNF-α, U937-E3/6.7K released 60% less [$^3$H]arachidonic acid than U937neo$^r$. The presence of E3/6.7K reduced the amount of [$^3$H]arachidonic acid released during TNF-α stimulation. Figure 21 shows that ER-localized E3/6.7K protected against TNF-α-mediated arachidonic acid release, which can be explained by the effect of E3/6.7K on $Ca^{2+}$ homeostasis. E3/6.7K's inhibition of the release of arachidonic acid also correlated well with the reduced inflammatory response observed *in vivo*.

**Figure 21. Analysis of the effect of E3/6.7K on the inducible release of radiolabeled arachidonic acid.**

The assay was set up in triplicate, and the cells were stimulated either with media alone, 90ng/ml TNF-α, 2μg/ml cycloheximide (CHX) or with a combination of 90ng/ml TNF-α and 2μg/ml CHX. The counts per minute of released [$^3$H]arachidonic acid were expressed as a percentage of the average of total incorporated [$^3$H]arachidonic acid. The experiment was repeated three times with similar results.

# Figure 21


Released [3]H-arachidonic acid as percent of total incorporated
40
35
30
25
20
15
10
5
0
U937neoR
U937 E3/6.7K
media alone
TNF
CHX
TNF/CHX

## 4.7 Discussion

Members of the TNF/Nerve Growth Factor (NGF) family of receptors include Fas (CD95 or Apo1), TNF receptor I (TNFR1, also called TNFR-55 or CD120a), TRAMP (also called DR3 or Apo3) and TNF receptor-related apoptosis-inducing ligand (TRAIL) receptors 1 (TRAIL-R1 or DR4) and 2 (TRAIL-R2, also called DR5 or Apo2). E3/6.7K prevented apoptosis induced by FasL, TNF-α and TRAIL as evidenced by three different markers of apoptosis consisting of: externalization of phosphatidyl serine, increase in membrane permeability and activation of caspases.

*In vivo*, the presence of E3/6.7K resulted in a decrease infiltration of leukocytes at the site of infection. It is possible that the *in vivo* effect of E3/6.7K was a direct result of the observed inhibition of Death Receptor induced apoptosis. This correlated well with the fact that TNF-α has been clearly implicated in the immune response to adenovirus. However, Fas-FasL interaction has not been implicated in the immune response against adenovirus (Chirmule *et al.*, 1999a) in the lungs of mice. This interaction, however, appears to be important in the liver (Chirmule *et al.*, 1999b). A recent report implicates both Fas and TNF-α in the way the cell-mediated immune system causes acute liver injury in mice infected with E1- and E3-deleted adenovirus (Liu *et al.*, 2000). Both Fas and FasL have been shown to be expressed in primary cells derived from the bronchial epithelium (Hamann *et al.*, 1998), possibly influencing the inflammatory response in the lungs. TRAIL induced apoptosis has not been examined from the point of view of an anti-viral response. Due to the similar mode of action of TRAIL and TNF-α it is probable that TRAIL also plays a role in the response against virally infected cells.

The mechanism of programmed cell death downstream of the Death Receptor is similar between the different members of the TNFR superfamily. These pathways overlap in many cases. The mitochondrial pathway and the recruitment of adapter molecules by the trimerized membrane receptor are the most interesting areas of overlap and the ones that have been studied in most detail. The ER localization of E3/6.7K, however, places it close to the events that involve the release of calcium ions $Ca^{2+}$ from the ER. Interestingly, the expression of E3/6.7K in

transfected cells prevented the sustained elevation of cytosolic $Ca^{2+}$ levels following the inhibition of the ER $Ca^{2+}$-ATPase with thapsigargin. The observed anti-apoptotic effects of E3/6.7K could be explained by the effect this protein has on ER $Ca^{2+}$ homeostasis.

$Ca^{2+}$ is involved in most biological processes as a second messenger resulting in effects as varied as membrane excitability, vesicle secretion, muscle contraction, fertilization, ciliary movement, cell death and proliferation. Very little is known about the effects of $Ca^{2+}$ efflux from the ER during apoptosis. Much of the insight on the cytotoxic effects of $Ca^{2+}$ comes from research groups directed by Sten Orrenius at the Karolinska Institutet in Sweden. The picture that begins to emerge is that $Ca^{2+}$ efflux from the ER is probably involved in most forms of apoptosis, either directly or indirectly (McConkey and Orrenius, 1997). Even though, in some forms of Death Receptor induced apoptosis such as Fas ligation, there is no detectable change in intracellular $Ca^{2+}$ levels, it appears that Fas-induced apoptosis depends on the release of inositol 1,4,5-triphosphate (IP3) (Felzen *et al.*, 1998) and a functional ER calcium channel, such as IP3 receptor-1 (IP3R-1) (Jayaraman and Marks, 1997; Jayaraman and Marks, 2000). It is therefore plausible that the currently available $Ca^{2+}$ flux analysis-techniques are inadequate to detect small $Ca^{2+}$ spikes, which may be sufficient to activate the apoptotic machinery.

Ultimately, $Ca^{2+}$-efflux is responsible for the translocation of $cPLA_2$ to the membrane of the ER. There $cPLA_2$ catalyzes the release of phospholipid-bound arachidonic acid leading to the synthesis of protaglandins and leukotrienes, which mediate inflammation. The presence of E3/6.7K effectively reduced the amount of released arachidonic acid following TNF-$\alpha$ stimulation. The results obtained in transfected cells and the results obtained *in vivo* in infected animals correlate well, reflecting the immunoevasive role of E3/6.7K, possibly through the prevention of inflammation in addition to apoptosis.

The dl739 mutant virus used in our *in vivo* studies (Fig. 13 and 14 ) was generated by the research group led by Dr. Wold. Its description, published in several papers, is that it lacks a functional E3/6.7K gene due to the deletion of the first 19 amino acid residues including the initiating methionine from the E3/6.7K open reading frame (Wilson-Rawls *et al.*, 1990). It has recently been brought to our attention by Dr. Wold that the expression of another E3 protein

may be affected in this mutant (W. S. M. Wold, personal communication). According to this unconfirmed report, the E3/10.4K or RIDα protein could not be detected by Western Blot in cells infected with dl739. E3/10.4K in concert with E3/14.5K have been shown to induce the internalization and degradation of Fas and to prevent apoptosis due to TNF-α and Fas. Consequently, this could affect the interpretation of *in vivo* results presented in this study.

It is important to note, however, that the *in vivo* effect of deletion mutants that lack the E3/10.4K or the E3/14.5K protein have been previously analyzed in a murine pneumonia model (Ginsberg *et al.*, 1991). The resulting inflammation following infection with the virus mutant dl753, which does not express the E3/10.4K protein, was not different than that following infection with wild type virus (Sparer *et al.*, 1996). At the same time, the degree of pathology due to dl739 infection observed in our study is significantly different from that of the wild type virus. Given the above evidence, it is safe to assume that the *in vivo* effects observed here are due to the deletion in the E3/6.7K gene, where the expression of E3/10.4K does not seem to play a role by itself *in vivo.*

It is regretful that this uncertainty made its way in the interpretation of our *in vivo* results. Fortunately, it does not affect the interpretation of our results from experiments using cells expressing the cDNA coding for E3/6.7K. The anti-apoptotic effect observed following transfection of cells with E3/6.7K is real and it supports the idea that E3/6.7K acts alone in the absence of any other viral factors.

# Chapter 5. Membrane topology of adenovirus E3/6.7K

## *5.1 In vitro transcription-translation system to study the topology of membrane proteins*

To determine the membrane orientation of an integral membrane protein, one of the most powerful assays is the *in vitro* transcription-translation system (Pelham and Jackson, 1976) supplemented with ER derived microsomes (Sabatini, Tashiro, and Palade, 1966). The protein synthesized in such manner is targeted to the microsomal membranes and processed and modified with appropriate post-translational modifications (Blobel and Dobberstein, 1975). Incubation of the microsomes containing the embedded protein with proteinase K (ProtK) degrades any exposed cytoplasmic domains while leaving the lumenal and transmembrane domains intact. The protected fragment is then identified by analyzing the post-translational modification present on the intact fragment, as well as by immunoprecipitating the fragment with a domain-specific polyclonal antiserum. A diagram depicting the assay system is shown in Fig. 22.

The system was assayed using human Major Histocompatibility Complex (MHC) Class I protein HLA-B27. HLA-B27 is an Asn-linked glycoprotein and liable to the action of peptide N-glycosidase F (PNGaseF) as indicated by the faster migrating unglycosylated form (Fig. 23, lane 2). HLA-B27 is a type I integral membrane protein with its carboxyl terminus found in the cytoplasm. The *in vitro* synthesized HLA-B27 was susceptible to the action of ProtK and consequently the protein lacking the carboxyl terminal migrates faster than the intact protein (Fig. 23, lane 3). These results were very consistent with previously published studies of the *in vitro* assembly of MHC Class I (Levy *et al.*, 1991).

**Figure 22. Diagram illustrating the in vitro assay used to determine the membrane topology of the embedded protein.**

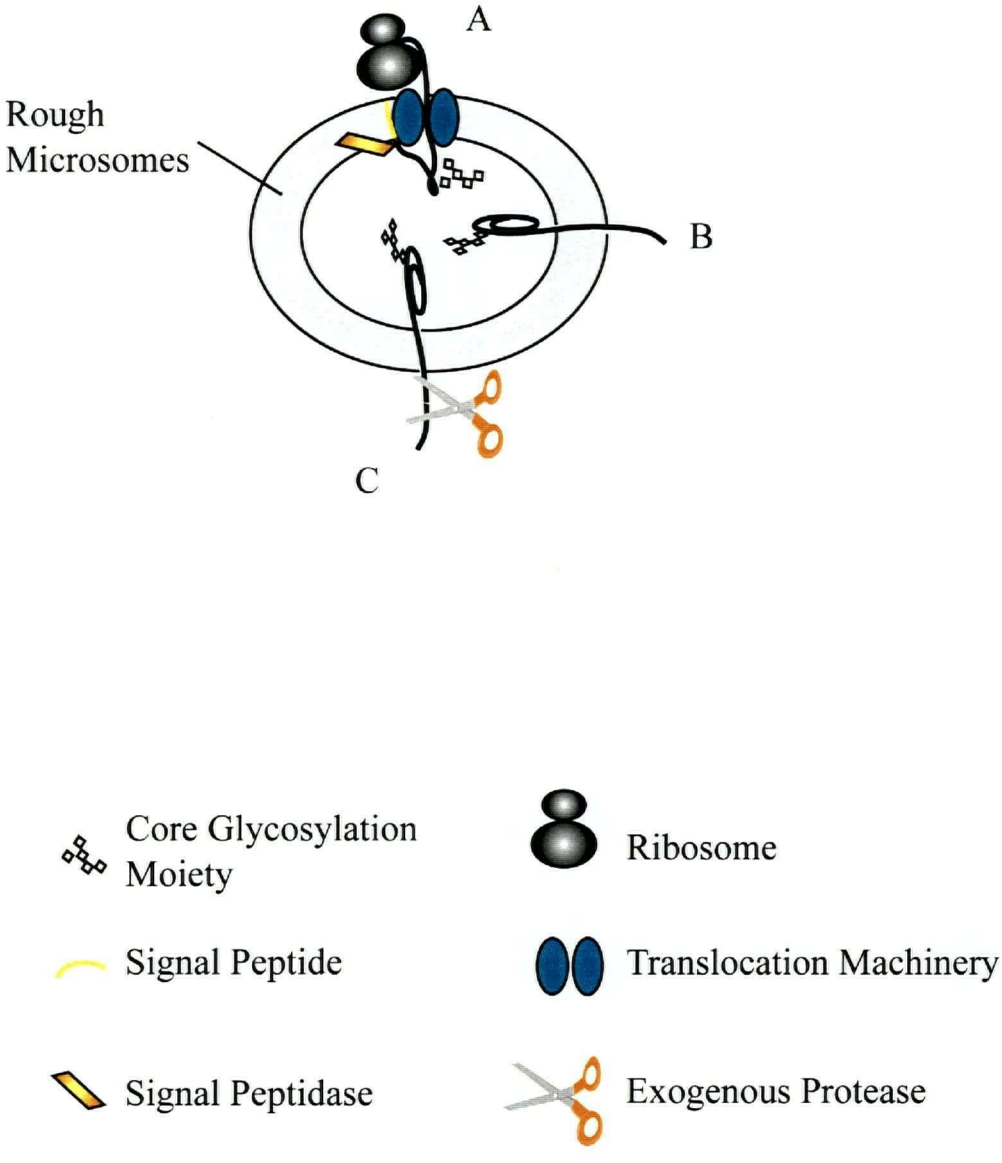

A
Rough
Microsomes
B
C
Core Glycosylation
Moiety
Ribosome
Signal Peptide
Translocation Machinery
Signal Peptidase
Exogenous Protease

Figure 22

**Figure 23. In vitro analysis of the topology adopted by HLA-B27.**

Translation of HLA-B27 mRNA in the presence of microsomes followed by the appropriate treatment and immunoprecipitation with the A425 antiserum (a gift from Dr. Sune Kvist); (+) indicates the respective treatment was carried out. **Lane 1**, HLA-B27 was translated in the presence of microsomes. **Lane 2**, HLA-B27 was translated in the presence of microsomes, immunoprecipitated and treated with PNGaseF. **Lane 3**, HLA-B27 was translated in the presence of microsomes then incubated with ProtK. **Lane 4**, HLA-B27 was translated in the presence of microsomes then incubated with ProtK and NP-40.

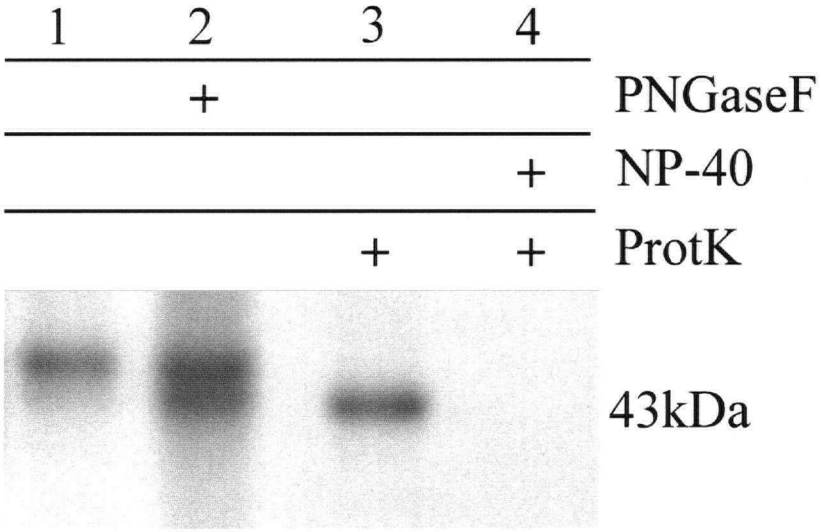



Figure 23

### *5.2 E3/6.7K assumes two different topological fates including a unique hairpin conformation*

To investigate the membrane topology of E3/6.7K the protein was translated *in vitro*, allowed to translocate into purified microsomes and then the exposed domain was digested with Proteinase K (ProtK). Analysis of the topology of E3/6.7K indicates that the protein adopts two different conformations. While embedded in the microsomal membrane the 14kDa form is completely protected from digestion by exogenous ProtK (Fig. 24A, lane 5 and Fig. 24B, lane 3). The membrane topology for the 14kDa form is that of a hairpin molecule with both termini found inside the lumen of the ER. The 14kDa form is N-linked glycosylated and can be deglycosylated to generate an 8kDa form by PNGaseF (Fig. 24A, lane 3).

The molecular form of 8kDa is not glycosylated and appears to be the primary translation product (as seen in Fig. 24A, lane 1) in the absence of microsomes. In contrast to the 14kDa form, the 8kDa form is susceptible to digestion by ProtK while embedded in the microsomal membrane. Digestion of the membrane-bound form of 8kDa with ProtK makes the protected fragment more mobile (Fig. 24A, lane 5; Fig. 24B, lane 3). The faster migrating fragment of the 8kDa form must contain the carboxyl terminus and transmembrane domains, because it can be immunoprecipitated with a polyclonal antiserum raised against a peptide corresponding to residues 47-61 of the Ad2 E3/6.7K sequence. As observed in Fig. 24A, the fragment generated by the digestion of the translocated 8kDa form with ProtK is not glycosylated, and hence is not affected by PNGaseF (Fig. 24A, lane 4).

Both forms of the protein are stably integrated in the microsomal membrane and resistant to an alkaline extraction (Fig 24B, lane 2). As a control for the level of the proteolytic digestion, in the presence of the detergent NP-40, ProtK gained access to the lumenal and transmembrane regions of the E3/6.7K protein (Fig. 24B, lane 4).

**Figure 24. In vitro analysis of the topology adopted by the two forms of E3/6.7K.**

E3/6.7K mRNA was translated to generate a radiolabeled protein product that can be immunoprecipitated with an antiserum directed against the residues 47-61 near the carboxyl terminus of E3/6.7K. **(A) Lane 1** shows the primary translation product of E3/6.7K in the absence of microsomes. **Lane 2**, E3/6.7K was translated in the presence of microsomes. **Lane 3**, E3/6.7K was translated in the presence of microsomes, immunoprecipitated and treated with PNGaseF. **Lane 4**, following translation, microsomes were incubated with ProtK and immunoprecipitated samples were treated with PNGaseF. **Lane 5**, following translation, microsomes were incubated with ProtK. **(B) Lane 1**, E3/6.7K was translated in the presence of microsomes. **Lane 2**, E3/6.7K was translated in the presence of microsomes and then subjected to alkali extraction at pH 11.5. **Lane 3**,.E3/6.7K was translated in the presence of microsomes then incubated with ProtK. **Lane 4**, E3/6.7K was translated in the presence of microsomes then incubated with ProtK and NP-40. The experiment was repeated five times with very similar results, using different batches of prepared microsomes.

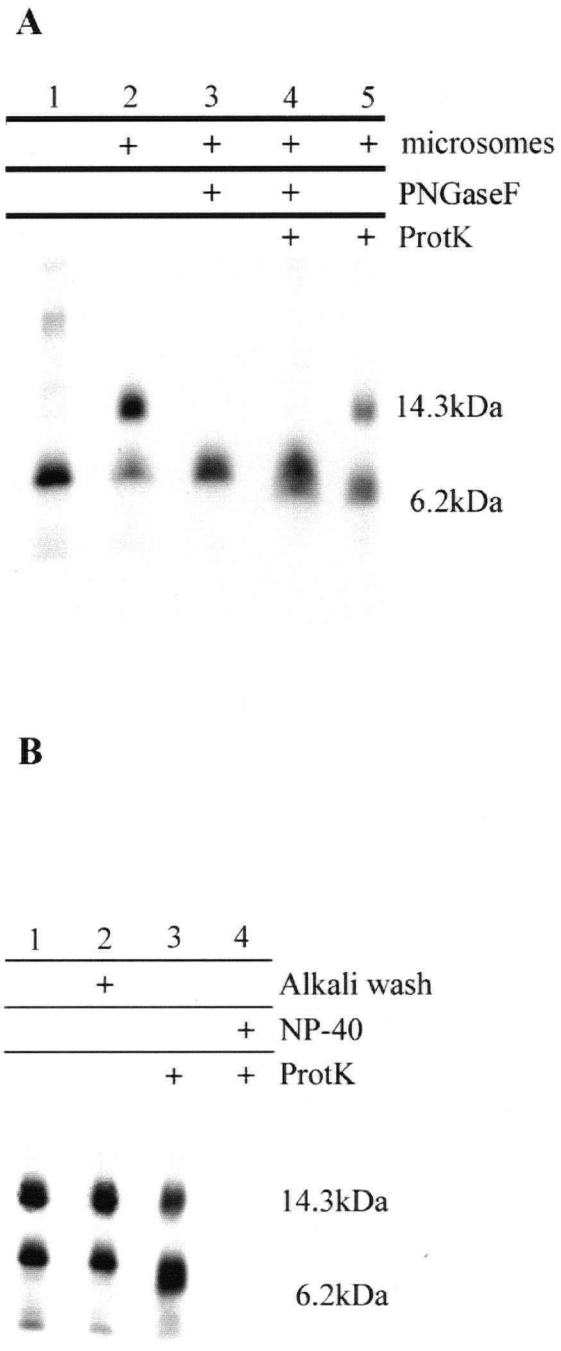

A
1 2 3 4 5
+ + + + microsomes
+ + PNGaseF
+ + ProtK
14.3kDa
6.2kDa
B
1 2 3 4
+ Alkali wash
+ NP-40
+ + ProtK
14.3kDa
6.2kDa

Figure 24

### *5.3 Evidence of intrachain disulfide bonds present in both forms of E3/6.7K*

The two proposed topologies place the cysteine-rich carboxyl termini of both forms of the protein inside the oxidizing environment of the ER lumen. This could result in the formation of disulfide bridges. As shown in Fig. 25A, *in vitro*-translated E3/6.7K was oxidized inside the lumen of the microsomes to generate disulfide bonds. Disulfide bonds were evident as a shift in electrophoretic mobility between the nonreduced (NR) protein and the reduced (R) form, which was fully linearized, and therefore slower migrating on SDS-PAGE than the NR form (Fig. 25A). The disulfide bond(s) must be intramolecular; bonds between separate molecules would have resulted in a slower form of NR-E3/6.7K due to the presence of covalently linked multimers.

The formation of disulfide bonds was also seen in cells infected with Ad2. Ad2 and dl704 produce wild-type E3/6.7K, while those mock-infected or infected with the dl739 (E3/6.7K-deleted) mutant, produced no E3/6.7K protein. The electrophoretic shift observed *in vitro* also occurs in infected cells, indicating that this modification is not an *in vitro* artifact (Fig. 25B). The presence of disulfide bonds places the carboxyl terminus in the ER lumen and provides support for the previously proposed membrane topologies for the two forms of E3/6.7K.

**Figure 25. Analysis of the oxidation state of E3/6.7K indicates the presence of intrachain disulfide bonds.**

**(A)** The *in vitro*-translated and radiolabeled E3/6.7K was immunoprecipitated with a polyclonal antiserum directed against the residues 47-61 near the carboxyl terminus of E3/6.7K. The samples were denatured in SDS-PAGE/sample buffer in the presence (R) or absence (NR) of dithiotreithol (DTT) followed by iodoacetamide (IAA)-mediated alkylation. The samples were run on a Tricine SDS-PAGE gel and analyzed by autoradiography. Arrows indicate the differential migration of the reduced and the nonreduced forms of E3/6.7K. **(B)** 293 cells were either mock infected or infected at an MOI of 50 with Ad2 and dl704, viral serotypes containing E3/6.7K, or with dl739, E3/6.7K deleted virus. From 7 to 11 hours post infection the cells were labeled with $^{35}$S-cysteine and immunoprecipitated. Samples were reduced (R) or not reduced (NR) and alkylated with IAA and analyzed by Laemmli SDS-PAGE 10-18% gradient gel. Arrows indicate the differential migration of the reduced and the nonreduced forms of E3/6.7K (Fig. 7B reproduced with permission from the Ph.D. Thesis of Roger Lippe: *Modulation of the MHC Class I Antigen Processing and Presentation Pathway.* Department of Microbiology and Immunology, Faculty of Graduate Studies, University of British Columbia, June 1995).

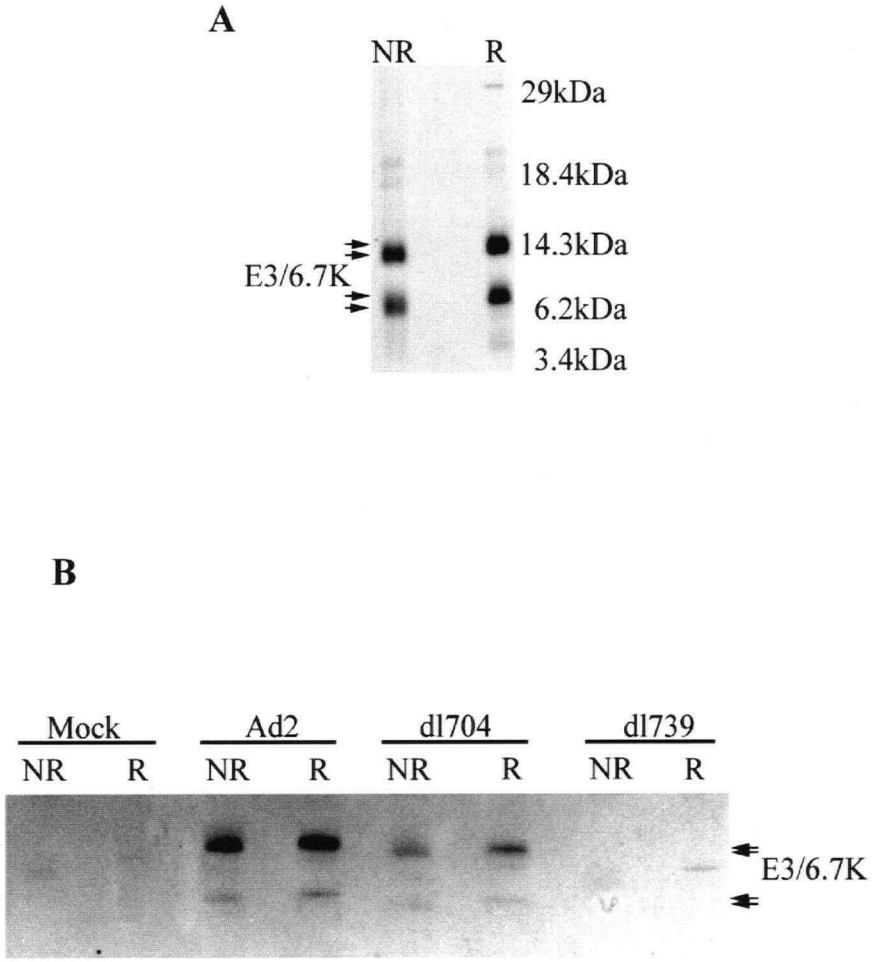

A
NR
R
29kDa
18.4kDa
14.3kDa
E3/6.7K
6.2kDa
3.4kDa
B
Mock
Ad2
dl704
dl739
NR
R
NR
R
NR
R
NR
R
E3/6.7K

Figure 25

### *5.4 Mode of translocation of E3/6.7K*

Integral membrane proteins that have a N cytoplasmic-C lumenal orientation as seen for the invariant chain protein (Ii) (Lipp and Dobberstein, 1986), the transferrin receptor (TfR) (Zerial *et al.*, 1986) and the influenza virus neuraminidase (Brown, Hogue, and Nayak, 1988) are classified as type II membrane proteins. The 8kDa form of E3/6.7K can also be classified as a type II protein. Pulse-chase experiments have shown that the 8kDa form is a precursor to the 14kDa form of E3/6.7K (Wilson-Rawls and Wold, 1993). A special group of type II proteins called tail-anchored proteins, like synaptobrevin (Whitley *et al.*, 1996), cytochrome b5 and Bcl-2 (Janiak, Leber, andAndrews, 1994), insert in the ER membrane post-translationally and in the absence of the SRP/Sec61 complex. The 8kDa form of E3/6.7K shares the orientation and possibly the mode of translocation of these tail-anchored proteins. The objective was to determine whether the precursor, 8kDa form of E3/6.7K is translocated co- or post-translationally into the ER membrane.

Cycloheximide blocks protein synthesis in eukaryotes, and the *in vitro* translation reaction was irreversibly blocked by the addition of cycloheximide. Microsomes were added once translation was stopped to assay translocation in the absence of translation. The E3/6.7K protein translocated across the membrane of the ER in the absence of protein synthesis (Fig. 26, lane 2 and 3) as efficiently as when translation was not inhibited (Fig. 26, lane 4). As shown in Figure 26 the lowest concentration of cycloheximide used was sufficient to block protein synthesis when added at the beginning of the translation reaction (Fig. 26, lane 1).

**Figure 26. Post-translational translocation of E3/6.7K.**

**Lane 1**, E3/6.7K translated in the presence of 1mM cycloheximide indicates there was no synthesized protein. **Lanes 2 and 3**, E3/6.7K translated in the absence of microsomes was first subjected to a translation block of 10mM (**lane 2**) and 1mM (**lane 3**) cycloheximide for 15 min. Then microsomes were added and incubation was continued for an additional hour. **Lane 4**, E3/6.7K was translated for one hour in the presence of microsomes. All samples were immunoprecipitated with a polyclonal antiserum directed against the residues 47-61 near the carboxyl terminus of E3/6.7K and analyzed by a Laemmli SDS-PAGE 15% gel.

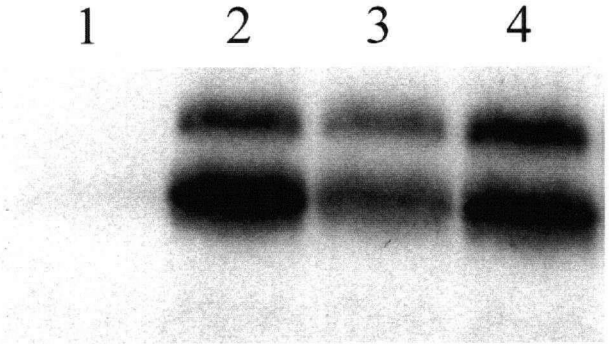



Figure 26

### 5.5 Discussion

In our studies the two forms of E3/6.7K adopted unusual topologies in the membrane of the ER (Fig. 24). The only charged residues in the sequence of E3/6.7K are positive and are found at the carboxyl terminus (Fig. 8A). The membrane topologies observed for both forms of E3/6.7K were exceptions to the "positive inside rule" since both forms of the protein have their carboxyl (positively charged) terminus in the ER lumen. The hairpin topology of the 14kDa form is the first report of such an unusual conformation. It resembles the topologies observed for caveolin and oleosin with the exception that both termini of these proteins are cytoplasmic (Abell *et al.*, 1997; Dupree *et al.*, 1993) not lumenal as in E3/6.7K. In addition, there are very few examples of proteins with dual orientations, as observed for the polytopic channel ductin (Dunlop, Jones, and Finbow, 1995), P-glycoprotein (Zhang, Duthie, and Ling, 1993) and prion protein (Yost *et al.*, 1990).

We observed that E3/6.7K was capable of translocating across the ER membrane following complete synthesis. One reason for this could be that the lack of a signal sequence and the length of the E3/6.7K protein emerging from the ribosome did not allow enough time for the nascent chain to interact with the Signal Recognition Particle (SRP). Post-translational transport in mammalian ER does not appear to require SRP-mediated targeting (Klappa *et al.*, 1991).

A possible scenario for the sequence of events could proceed as outlined in Figure 27. The nascent polypeptide chain of E3/6.7K was released from the ribosome complex in the absence of associated SRP (Fig 27, I). The unfolded E3/6.7K could be interacting with such proteins as the Nascent Polypeptide Associated Complex (NAC) (Wiedmann *et al.*, 1994), the Sec pore complex (Klappa, Zimmermann, and Zimmermann, 1994) as well as the cytosolic chaperone Heat Shock Protein (Hsp) 70 which would prevent folding and aggregation (Klappa *et al.*, 1991) (Fig. 27, II). The unfolded protein anchored in the membrane of the ER by virtue of its hydrophobic domain (Fig. 27, III and IV). In the case of some signal anchor proteins the hydrophillic domain that folds more rapidly than its counterpart on the other side of the transmembrane domain will be retained in the cytoplasm. This may be caused by secondary

structures which hinder the proteins's movement through the Sec pore (Denzer, Nabholz, andSpiess, 1995). It is likely that the highly charged carboxyl terminus of E3/6.7K remained destabilized longer than the amino terminus and therefore translocates first across the ER membrane.

Once in the oxidizing environment of the ER lumen, the protein folded, assisted by chaperones and protein disulfide isomerase, and allowed the formation of disulfide bridges which would stabilize the carboxyl terminus in a folded state (de Silva, Braakman, and Helenius, 1993; Huppa and Ploegh, 1998). The type II form of E3/6.7K representing the 8kDa form was thus generated (Fig. 27, V). A population of the type II inserted proteins underwent a second translocation event, this time of their amino termini. Once the amino terminus was translocated across the ER, the Asn-linked glycosylation sites were detected and modified with oligosaccharide moieties, generating the 14kDa form (Fig. 27, VI).

What is the role of the 8kDa form of E3/6.7K? One explanation could be that the 8kDa form is simply a topological precursor to the 14kDa form. Its presence late in infection is explained by a thermodynamically unfavourable second translocation event. In this case, only a percentage of the embedded proteins reaches the 14kDa state. It is possible that a second limiting factor interacting with the amino termini of some of the proteins is involved in transbilayer movement across the ER membrane. This trans-acting factor would then determine the rate at which E3/6.7K progresses from the 8kDa, type II form to the 14kDa, hairpin form of the protein.

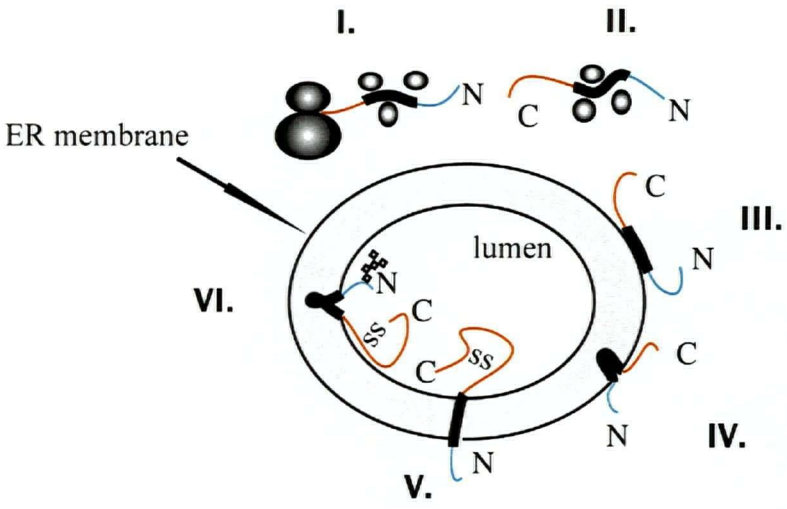
The existence of factors that can modify the topologic fate of membrane proteins has already been suggested for prion protein (PrP). Three distinct topological fates have been observed in the case of PrP, which is essential to the pathogenesis of scrapie, bovine spongiform encephalopathy, Creutzfeldt-Jakob disease (CJD) and Gerstmann-Straussler-Scheinker (GSS) disease. One form is fully translocated across the membrane of the ER and it is the precursor to the glycophosphatidyl inositol (GPI) anchored-protein found at the surface of the cell ($PrP^{C}$). It has been proposed that $PrP^{C}$ is converted into $PrP^{Sec}$, the pathogenic form, through a process whereby a portion of its α-helical and coil structure is refolded into a β-sheet (Gasset *et al.*,

1993). The two other forms of PrP are both integral membrane proteins that span the membrane of the ER once, in opposite orientations, at the same hydrophobic stretch. The $^{Ntm}$PrP has a type I topology while the $^{Ctm}$Prp has a type II topology (Hegde *et al.*, 1998). It was observed that *in vitro* translated PrP can assume different membrane conformations depending on the composition of the added microsomes. It was then proposed that the topology of PrP is controlled by a *trans*-acting factor (Hegde, Voigt, and Lingappa, 1998). Recently, it has been proposed that PrP$^{Sec}$ itself is involved in modulating *in trans* the generation or the degradation of $^{Ctm}$Prp (Hegde *et al.*, 1999).

As a protein coded by a human pathogen, E3/6.7K belongs to a very small class of mammalian proteins that are post-translationally transported into the ER. The mechanism of translocation for synaptobrevin (Kutay *et al.*, 1995), Cx26 connexin (Zhang *et al.*, 1996) and a fragment of the glucose transporter (Mueckler and Lodish, 1986) requires ATP. For the Bcl-2 and cytochrome b5 proteins, insertion only requires specific residues located at the carboxyl terminus (Janiak, Leber, andAndrews, 1994). For preprocecropin insertion is ATP and signal sequence dependent (Schlenstedt *et al.*, 1992). In addition, none of the above proteins have bitopic orientations or resemble the unusual membrane topology of the 14kDa form of E3/6.7K. Interestingly, in the case of Bcl-2 its carboxyl terminus also mediates the targeting to the mitochondrial membrane (Nguyen *et al.*, 1993) in a N-cytoplasmic/C-intermembrane space orientation.

**Figure 27. Model of the mode of translocation of E3/6.7K.**

The N and C represent the amino and carboxyl termini, respectively. The progression from nascent chain to integral membrane glycoprotein is indicated counterclockwise from I to VI. The Asn-linked high-mannose carbohydrate modification is shown at the amino terminus of the 14kDa form in VI. The disulfide bridge is shown at the carboxyl terminus of both forms of the protein in V and VI.




=cytosolic chaperones

C

=disulfide bond

=N-linked carbohydrates

=Ribosomes

=signal-anchor domain

C =carboxyl terminus (red)

N =amino terminus (blue)

Figure 27

# Chapter 6. General Discussion

## *6.1 Significance of results*

The E3/6.7K protein acts in the absence of other adenovirus proteins to prevent TNF-α–, Fas- and TRAIL-induced apoptosis in transfected cells. Its mechanism of action involves maintenance of ER $Ca^{2+}$ homeostasis. Sequence comparison of E3/6.7K to other proteins involved in apoptosis failed to indicate any sequence similarity. In addition, very few other proteins involved in apoptosis have been localized to the ER. They include the 31kDa-B cell accessory protein (BAP31) (Ng *et al.*, 1997; Ng and Shore, 1998), caspase-12 (Nakagawa *et al.*, 2000), the defender against apoptotic cell death, DAD1 which is a member of the oligosaccharyl-transferase complex (OST) (Silberstein *et al.*, 1995), the myxoma virus M-T4 protein (Hnatiuk *et al.*, 1999), and Bcl-2. Except for Bcl-2, there is very little known about the mode of action of these proteins.

It is thought that BAP31 may be a key regulator and assembly factor for apoptotic proteins such as procaspase-8 and the *Caenorhabditis elegans* CED-4 as well as the anti-apoptotic Bcl-2 and Bcl-xL (Ng *et al.*, 1997; Ng and Shore, 1998). Apaf-1 is the mammalian homologue of CED-4. Recently, CED-4 has been shown to translocate to the nucleus during apoptosis (Chen *et al.*, 2000). The details and the role of the BAP31-bridged complex during apoptosis are presently unclear. Nevertheless, the prospect of E3/6.7K interacting with BAP31 to block this process determined us to study if these two proteins associate. Using reagents generously provided by Dr. Gordon Shore, we attempted to coimmunoprecipitate BAP31 and E3/6.7K without any success (results not shown). While is still possible that these proteins interact, a different investigative approach may have to be used.

Caspase-12 is a newly characterized member of the cysteine-dependent, aspartate specific proteases known as caspases. It has been shown to be specifically involved in apoptosis resulting from inducers of ER stress, particularly, tunicamycin, brefeldin A, thapsigargin and A23187, a $Ca^{2+}$ ionophore. It does not appear to be involved in thymocyte death induced by Fas/CHX, TNF-α /CHX, staurosporine, serum deprivation or dexamethasone (Nakagawa *et al.*,

2000). This highlights the fact that there are multiple and discrete apoptotic pathways as well as apoptotic mediators exclusively dedicated to some of them. It would be interesting to test whether there is any relationship between ER localized E3/6.7K and caspase-12. As observed in Table IV, the protective mechanism of E3/6.7K also appears to have a partiality to pathways that utilize the TNFR/TRAIL receptor pathways as opposed to Fas mediated apoptosis. It is possible that $Ca^{2+}$ efflux plays a greater role in some pathways versus others.

DAD1, also known as Ost2p, is a member of the OST complex that mediates the glycosylation of nascent proteins. Even though its role in apoptosis is not known, mice deficient in DAD1 undergo very early embryonic lethality, possibly indicating the importance of protein glycosylation in organ biogenesis (Nishii *et al.*, 1999). Remarkably, it was shown that DAD1 prevents apoptosis at a stage downstream of Bcl-2 (Sugimoto *et al.*, 1995). DAD1 appears to be an integral membrane protein in close association with the ER resident OST complex.

The MT-4 appears to be a very important immunoevasive gene during myxoma virus infections (Barry *et al.*, 1997). The protein retained in the ER by virtue of its RDEL sequence is required to prevent apoptosis induction in infected lymphocytes (Hnatiuk *et al.*, 1999). According to published studies the M-T4 is the first viral ER-resident protein shown to have a role in protecting lymphocytes from apoptosis (Nash *et al.*, 1999). However, the mechanism of action of M-T4 is presently not understood.

When confronted with the complexity of apoptotic pathways and the plethora of effects generated by ER $Ca^{2+}$ efflux, we can only speculate on the anti-apoptotic mechanism employed by E3/6.7K. The mechanism's complexity is evident when we will take a closer look at the Bcl-2 cellular anti-apoptotic factor that in addition, to the mitochondria, has also been shown to localize to the ER.

There are few intriguing similarities between Bcl-2 and E3/6.7K, encompassing both their post-translational mode of targeting (described earlier) and their putative mode of action. It is clear that Bcl-2 exerts most of its anti-apoptotic effects by blocking the release of cytochrome c from the mitochondria. However, a population of Bcl-2 proteins localizes to the cytoplasmic

face of the ER membrane (Chen-Levy and Cleary, 1990), where it integrates post-translationally, similarly to E3/6.7K.

To clarify the role of the ER-localized Bcl-2 subset of proteins, researchers have employed a Bcl-2-cytochrome-b5 (cb5) fusion protein which is targeted exclusively to the ER. The anti-apoptotic effect of Bcl2-cb5 is restricted by the cell type and to the method used to induce apoptosis (Lee *et al.*, 1999; Zhu *et al.*, 1996). Recently, it has been shown that both wild type Bcl-2 and Bcl-2-cb5 block the apoptotic crosstalk between the ER and the mitochondria following induction of apoptosis using brefeldin A and CHX (Hacki *et al.*, 2000).

Like E3/6.7K, Bcl-2 has been shown to block thapsigargin-induced $Ca^{2+}$ efflux from the ER (Lam *et al.*, 1994; Magnelli *et al.*, 1994) as well as redistribution of $Ca^{2+}$ from the ER to the mitochondria (Baffy *et al.*, 1993). The role of the ER subset of this protein was then suggested to be the maintenance of ER $Ca^{2+}$ homeostasis. Later He et. al. showed that the contribution of Bcl-2 is only evident when the cells are found in physiological levels of extracellular calcium (He *et al.*, 1997). In low levels of extracellular calcium which do not allow the replenishing of intracellular stores through capacitative $Ca^{2+}$ entry, cells still undergo apoptosis in the presence of Bcl-2. This led them to suggest that it is the depletion of intracellular stores, rather than the increase in cytosolic levels of $Ca^{2+}$, following thapsigargin treatment that leads to apoptosis. In this context, the role of Bcl-2 would be to assist in the refilling of the ER stores with $Ca^{2+}$ that is, ultimately, derived from capacitative $Ca^{2+}$entry. This model was supported by evidence of the upregulation of the ER $Ca^{2+}$-ATPase that mediates $Ca^{2+}$-uptake into the ER by Bcl-2 as well as the co-immunoprecipitation of Bcl-2 and this $Ca^{2+}$ transporter.

Recently, however, the previously proposed models for the effect of Bcl-2 on $Ca^{2+}$ homeostasis have been challenged by some very elegant studies that use $Ca^{2+}$-dependent, ER-targeted fluorescent probes. Two studies using either the ER-targeted "cameleon" fluorescent protein CAM4-ER (Miyawaki *et al.*, 1999) or ER-targeted jelly-fish aequorin have demonstrated that Bcl-2 decreases the free $Ca^{2+}$ concentration within the ER. Bcl-2 achieves this by increasing the $Ca^{2+}$ permeability of the ER (Foyouzi-Youssefi *et al.*, 2000). Because of this slow depletion of the $Ca^{2+}$ reserves of the cell, there is a small pool of mobilisable $Ca^{2+}$ during agonist- or

apoptosis-induced efflux. In addition, the capacitative $Ca^{2+}$ influx is also greatly diminished in cells overexpressing Bcl-2, an adaptive consequence of low levels of ER $Ca^{2+}$ (Pinton *et al.*, 2000). These studies imply the possibility that Bcl-2 forms a slow-leak channel in the ER membrane, draining the $Ca^{2+}$ reserves. The direct involvement of Bcl-2 in ion transport is also supported by evidence that Bcl-$X_L$ can form pores in lipid bilayers that can conduct $Na^+$ but not $Ca^{2+}$, yet are regulated by the presence of $Ca^{2+}$ ions (Lam *et al.*, 1998). The specificity and the role of channels formed by Bcl-2 family members awaits further work and confirmation by independent labs. Ultimately, Bcl-2 would achieve the same goal of reducing $Ca^{2+}$ efflux from ER whether it prevented the efflux of $Ca^{2+}$ from the ER or diminished the ER $Ca^{2+}$ reserves or even if it antagonized the efflux of $Ca^{2+}$ by increasing its uptake into the ER.

Other studies indicate a role for Bcl-2 in the replenishing of mitochondrial $Ca^{2+}$ stores (Ichimiya *et al.*, 1998; Murphy *et al.*, 1996; Zhu *et al.*, 1999) and nuclear stores (Marin *et al.*, 1996) supporting the idea that Bcl-2 is involved directly or indirectly in the vectorial transport of $Ca^{2+}$ into cellular compartments.

Similar to Bcl-2, the fact that E3/6.7K prevents ER $Ca^{2+}$-depletion in response to thapsigargin does not actually tell us how this is achieved. It could be that the mechanism of E3/6.7K is related to the more current model of the mechanism of action of Bcl-2. In this case E3/6.7K should be able to induce a $Ca^{2+}$ 'leak' in the ER membrane. It could achieve this by regulating an existing channel or by forming a channel of its own. Interestingly, there is an example of a virally coded protein that enhances the membrane permeability of the ER to $Ca^{2+}$ ions. This protein is the enterovirus 2B protein of Coxsackie B virus. As a result of the diminished $Ca^{2+}$ reserves in cells transfected with the 2B protein there is a decreased flux of $Ca^{2+}$ in response to thapsigargin (van Kuppeveld *et. al.*, 1997). Analysis of the sequence of the 2B protein has revealed that it could adopt an amphipathic helix conformation, possibly forming a cationic channel leading to the disruption of $Ca^{2+}$ homeostasis. Channel-forming viral proteins that modulate the membrane permeability of cells are called viroporins. So far, viroporins have been implicated in the release of virus from the endosomal compartment following internalization

or release from the infected cell at the end of a lytic infection. Future studies will indicate whether some viroporins have anti-apoptotic effects.

Studies of Bcl-2 demonstrate that we must take into account that the initial ER $Ca^{2+}$ efflux is accompanied by the depletion of the cellular stores and the capacitative entry of $Ca^{2+}$ through SOCC channels. It is not clear at this point which of these three processes or combination thereof contributes to cell death. Consequently, any of them could be the target of apoptosis inhibitors.

In the case of ER $Ca^{2+}$ depletion, evidence suggests that it leads to the ER-stress response (Pahl and Baeuerle, 1996), which results in the shutdown of the protein synthesis machinery. This response occurs through the action of the PKR-like ER kinase, which inactivates the eukaryotic-initiator factor 2α (eIF2α) (Harding, Zhang, and Ron, 1999). Several ER-resident chaperone proteins, such as calreticulin and BiP, block repetitive $Ca^{2+}$ waves (Camacho and Lechleiter, 1995), the stress-response and consequent apoptosis induced by depletion of ER $Ca^{2+}$ stores (Liu *et al.*, 1997). Interestingly, the expression of the adenovirus E1A protein during viral infection is a very powerful inducer of the 70kDa class Heat Shock Protein (Hsp70), an indicator of ER-stress (Nevins, 1982). In addition, many viruses induce an ER-overload response through the abundant expression of secreted viral proteins, as seen for E3/19K (Pahl *et al.*, 1996). The ER-overload response can cause ER-stress (Pahl and Baeuerle, 1997) and apoptosis. Therefore, it is plausible that the ER-stress response and the disruption of ER $Ca^{2+}$ homeostasis elicited by viral infection, is prevented by E3/6.7K.

Interestingly, the activity of the adenovirus E3 promoter , which controls the expression of E3/6.7K is induced by the transcription factor NF-κB (Deryckere and Burgert, 1996; Deryckere *et al.*, 1995). NF-κB in turn is induced by TNF-α (Malinin *et al.*, 1997) and by ER-overload (Pahl and Baeuerle, 1997). It appears that the function of the E3/6.7K protein is congruous with both the requirement for TNF-α and ER-stress inhibitors during adenovirus infection as well as the control of the expression of this protein by NF-κB.

Does E3/6.7K affect the ER-stress response? How does E3/6.7K fit in the apoptotic pathways that directly involve the ER? The answers have important implications for our

understanding of how $Ca^{2+}$ efflux contributes to the induction of the effector phase of apoptosis. There is one other example of a viral protein, the African Swine Fever Virus (ASFV) A238L protein, which (though it does not affect $Ca^{2+}$ release from the ER) directly inhibits calcineurin, one of the most important downstream messengers (Miskin *et al.*, 1998). The fascinating effect of such inhibitors is that they could potentially block ER-stress leading to activation of JNK and caspase-12, activation of T-cells through the Nuclear Factor of Activated T cells (NF-AT) transcription factor and ultimately, apoptosis. There are many viral inhibitors of apoptosis, yet there are no examples of viral inhibitors of the ER-stress response. The benefits conferred to a persistent virus by the broad spectrum of such inhibitors guarantees their existence.

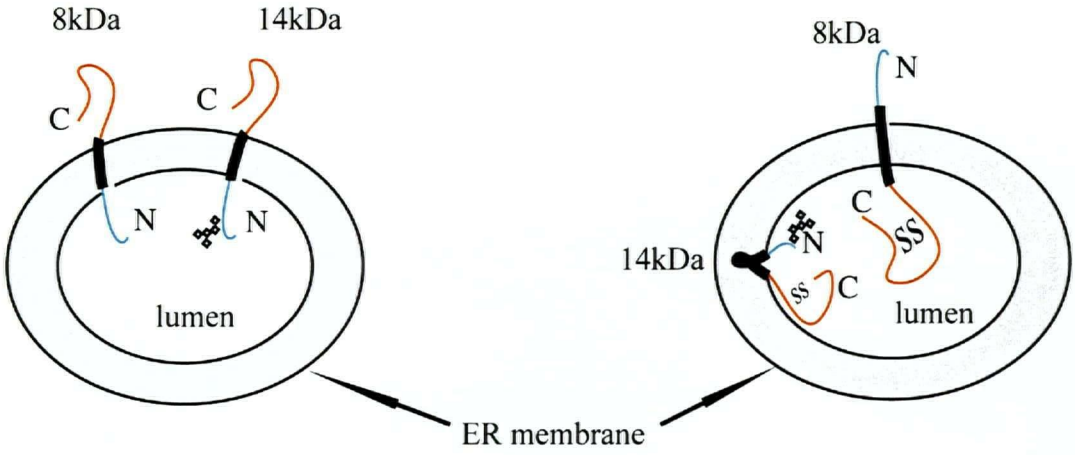
The evidence presented here demands that a new model of the topology and targeting of E3/6.7K should be considered. This evidence consists of the presence of ER specific post-translational modifications that map to both termini of the protein, as well as the protection conferred to the non-cytoplasmic domains of the protein from exogenously added protease. The currently proposed membrane structures disagree with the previously proposed topology of E3/6.7K (Fig. 28). The protein adopts two different topologies that are exceptions to 'the positive inside rule'. The occurrence of the 14kDa, hairpin topology of E3/6.7K could be dictated by accessory molecules that have been previously suggested to direct the topological fate of prion protein.

We have shown that E3/6.7K is targeted to the membrane of the ER following complete synthesis. It is likely that the translocation mechanism is very similar to the one previously described for tail-anchored proteins. In this case, the 8kDa form of E3/6.7K adopts a type II topology that contains disulfide bonds which then proceeds to the 14kDa-hairpin topology concomitant with N-glycosylation. The mechanism of targeting may involve the Sec pore, TRAM, cytosolic chaperones and probably a lumenal chaperone that drives the process. Similarly to tail-anchored proteins, E3/6.7K probably does not require SRP/SR factors.

Nevertheless, in the absence of proteoliposomes reconstituted with well-characterized components of the translocation machinery is very hard to ascertain the involvement of the various translocation factors. It is also possible that E3/6.7K is capable of translocating across

the ER membrane in the absence of protein cofactors. The acquisition of resistance to exogenously added protease in the presence of protein free liposomes would be a good approach to test this hypothesis. The only caveat to consider is that the net charge present in the naturally occurring phospholipid bilayer is hard to recreate in artificial liposomes.

**Figure 28. Previously and currently proposed models of the topology of Ad2 E3/6.7K.**




Topologies previously proposed
for the two forms of E3/6.7K protein

Currently proposed topologies
for the two forms of E3/6.7K protein

SS =disulfide bond

=N-linked carbohydrates

=signal-anchor domain

C =carboxyl terminus (red)

N =amino terminus (blue)

Figure 28

### *6.2 Future directions*

An interesting aspect about the subcellular targeting of E3/6.7K is related to its mechanism of ER retention. The sequence of E3/6.7K fails to indicate any of the previously described ER-retention signals. Therefore it is possible that its retention in the ER is mediated through its association with another ER-retained protein. In addition, due to its size, E3/6.7K is a good candidate for a mutagenesis approach to study such mechanisms as post-translational translocation or ER retention.

To investigate the mechanism employed by E3/6.7K to block apoptosis, the sequences of other proteins known to be involved in modulation of ER $Ca^{2+}$ efflux were investigated, in the hope that they would share similar motifs with the sequence of E3/6.7K. One protein that shares considerable homology over a short stretch of residues with E3/6.7K is the transmembrane activator and CAML-interactor (TACI). A member of the TNFR superfamily, TACI controls ER $Ca^{2+}$ and the activation of the nuclear factor of activated T-cells (NF-AT). The TACI protein mediates its effects through the calcium-modulating cyclophilin B ligand protein (CAML) (von Bulow and Bram, 1997), an ER-localized protein that controls $Ca^{2+}$ efflux in T-cells by interacting with cyclophilin B, the well known target of cyclosporin (Bram and Crabtree, 1994; Holloway and Bram, 1996; Holloway and Bram, 1998). The intracellular domain of TACI has also been shown to interact with TRAF, to induce apoptosis. The CAML and TRAF binding sites are separate domains within the cytoplasmic domain of TACI (Xia *et al.*, 2000).

Interestingly, the extracellular ligand of TACI is a protein previously characterized as a TNF-$\alpha$ homologue that activates apoptosis, nuclear factor-kappaB, and c-Jun NH2-terminal kinase (THANK) (Gross *et al.*, 2000; Mukhopadhyay *et al.*, 1999). THANK is also known as B cell activating factor belonging to the TNF family (BAFF) (Schneider *et al.*, 1999), B lymphocyte stimulator (BLyS) (Moore *et al.*, 1999b), TNF and ApoL related ligand (TALL-1) (Shu, Hu, and Johnson, 1999), tumor necrosis factor-like protein (zTNF4) (Gross *et al.*, 2000). THANK is a protein that can be found either membrane bound or secreted following cleavage by furin (Schneider *et al.*, 1999). THANK is synthesized by peripheral blood leukocytes and it binds

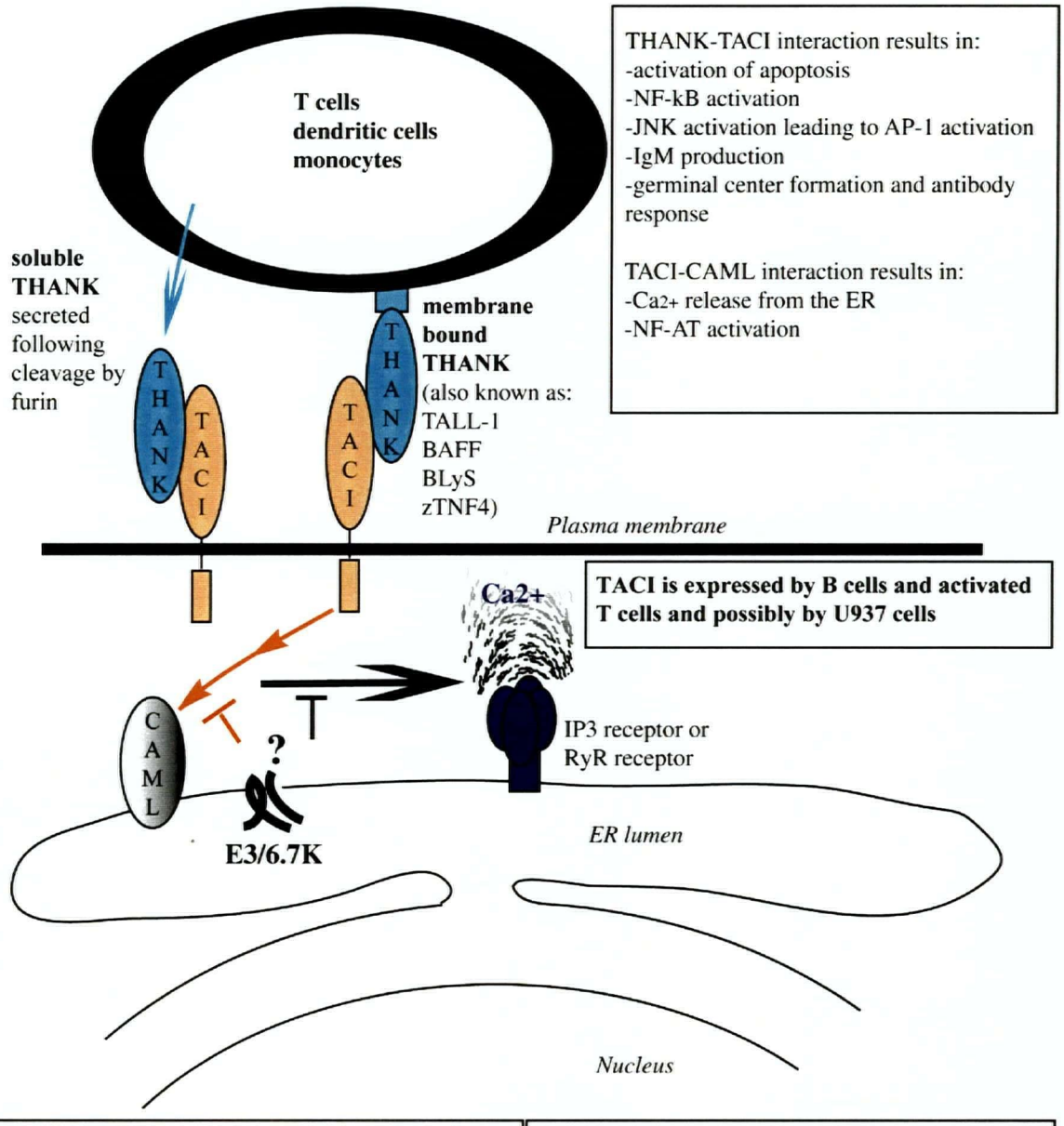
TACI as well as the B cell maturation antigen (BCMA) on activated T cells and B cells (Gross *et al.*, 2000; Thompson *et al.*, 2000; Ware, 2000). It appears that it also has an effect on U937 monocytic cells (Mukhopadhyay *et al.*, 1999), which may suggest that TACI, BCMA or an alternate ligand is found on lymphoid cells other than T or B cells.

Briefly, our working hypothesis is that E3/6.7K expression prevents $Ca^{2+}$ efflux from the ER possibly by blocking factors (such as CAML or CAML-TACI interaction) that control this process. The main arguments supporting this hypothesis and questions arising from it are illustrated in Figure 29. Our evidence is that E3/6.7K and the physiological ligand of CAML, TACI (von Bulow and Bram, 1997), share considerable sequence similarity within the well-conserved carboxyl terminus of E3/6.7K. The domain of the TACI protein that is similar to E3/6.7K overlaps with the domain that mediates TACI-CAML interaction (von Bulow and Bram, 1997). When we co-translated E3/6.7K and CAML *in vitro* in the presence of microsomes, the two proteins were shown to interact by co-immunoprecipitation. We also confirmed this result using a yeast-two-hybrid approach to study the E3/6.7K-CAML interaction. In addition, we found that E3/6.7K interacts with the same domain of CAML that was previously shown to interact with TACI (von Bulow and Bram, 1997) and to control $Ca^{2+}$ efflux (Holloway and Bram, 1996).

Establishing this hypothesis will require proof beyond the preliminary evidence presented here. Firstly, one must demonstrate that the two proteins interact *in vivo* in transfected as well as virally infected cells. In addition, the susceptibility of various cell lines to apoptosis should be assayed following expressional control of the levels of CAML through overexpression and antisense-mediated repression. These experiments are designed to implicate CAML in the ER $Ca^{2+}$ efflux-dependent apoptosis following FasL, TRAIL and TNF-$\alpha$ stimulation. Researchers who have attempted to overexpress CAML in transfected cells have already reported the high toxicity of this protein and therefore provided indirect evidence that this may be the case (Holloway and Bram, 1996). These studies, and others focused on using the yeast-two-hybrid screen to find other possible targets of E3/6.7K in a human liver cDNA library are currently underway.

**Figure 29. Current hypothesis of the mode of action of E3/6.7K.**

This model is based on the putative interaction between E3/6.7K and CAML and the possible effect of E3/6.7K on CAML induced ER $Ca^{2+}$ efflux.




THANK-TACI interaction results in:
-activation of apoptosis
-NF-kB activation
-JNK activation leading to AP-1 activation
-IgM production
-germinal center formation and antibody response

TACI-CAML interaction results in:
-$Ca^{2+}$ release from the ER
-NF-AT activation

**TACI is expressed by B cells and activated T cells and possibly by U937 cells**

Supporting arguments for above model:
-E3/6.7K shares similarity with TACI and associates with its physiological ligand, CAML
-CAML modulates calcium signaling
-E3/6.7K maintains calcium homeostasis
-THANK induces apoptosis, possibly through its ligand TACI
-E3/6.7K prevents apoptosis induced by various TNFR members (currently testing if that includes TACI)

Unresolved questions:
How does TACI ligation on the plasma membrane result in its association with ER localized CAML?
How does CAML control the efflux of $Ca^{2+}$ from the ER?
Does CAML play a role in TNF, Fas or TRAIL induced apoptosis, or in the ER-stress response?
Does E3/6.7K prevent TACI from interacting with CAML or does it prevent CAML from inducing $Ca^{2+}$ efflux?

Figure 29

## 6.3 Conclusion

The E3 region of adenovirus is rich in anti-apoptotic proteins whose main functions or modes of action are not fully characterized. Results presented here show that the expression of E3/6.7K alone is sufficient to prevent Death Receptor-induced apoptosis in transfected cells. The present results do not exclude the possibility that the effects of E3/6.7K could synergize *in vivo* with the previously characterized adenoviral inhibitors of apoptosis, in order to create a more persistent pathogen. It is possible that the redundancy of anti-apoptotic mechanisms present in adenovirus reflects the various apoptotic pathways functioning in infected cells under different conditions as well, as the need of the virus to adapt to various cellular environments.

## References


Abell, B. M., Holbrook, L. A., Abenes, M., Murphy, D. J., Hills, M. J., and Moloney, M. M. (1997). Role of the proline knot motif in oleosin endoplasmic reticulum topology and oil body targeting. *Plant Cell* **9**(8), 1481-93.

Abken, H., Butzler, C., and Willecke, K. (1987). Adenovirus type 5 persisting in human lymphocytes is unlikely to be involved in immortalization of lymphoid cells by fusion with cytoplasts or by transfection with DNA of mouse L cells. *Anticancer Res* **7**(4A), 553-8.

Adam-Klages, S., Schwandner, R., Luschen, S., Ussat, S., Kreder, D., and Kronke, M. (1998). Caspase-mediated inhibition of human cytosolic phospholipase A2 during apoptosis. *J Immunol* **161**(10), 5687-94.

Afonso, C. L., Neilan, J. G., Kutish, G. F., and Rock, D. L. (1996). An African swine fever virus Bc1-2 homolog, 5-HL, suppresses apoptotic cell death. *J Virol* **70**(7), 4858-63.

Albinsson, B., and Kidd, A. H. (1999). Adenovirus type 41 lacks an RGD alpha(v)-integrin binding motif on the penton base and undergoes delayed uptake in A549 cells. *Virus Res* **64**(2), 125-36.

Ankarcrona, M., Dypbukt, J. M., Orrenius, S., and Nicotera, P. (1996). Calcineurin and mitochondrial function in glutamate-induced neuronal cell death. *FEBS Lett* **394**(3), 321-4.

Antonsson, B., Conti, F., Ciavatta, A., Montessuit, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J. J., Mazzei, G., Maundrell, K., Gambale, F., Sadoul, R., and Martinou, J. C. (1997). Inhibition of Bax channel-forming activity by Bcl-2. *Science* **277**(5324), 370-2.

Antonsson, B., Montessuit, S., Lauper, S., Eskes, R., and Martinou, J. C. (2000). Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria. *Biochem J* **345 Pt 2**, 271-8.

Atsumi, G., Tajima, M., Hadano, A., Nakatani, Y., Murakami, M., and Kudo, I. (1998). Fas-induced arachidonic acid release is mediated by Ca2+-independent phospholipase A2 but not cytosolic phospholipase A2, which undergoes proteolytic inactivation. *J Biol Chem* **273**(22), 13870-7.

Babiss, L. E. (1989). The cellular transcription factor E2f requires viral E1A and E4 gene products for increased DNA-binding activity and functions to stimulate adenovirus E2A gene expression. *J Virol* **63**(6), 2709-17.

Babiss, L. E., Ginsberg, H. S., and Darnell, J. E., Jr. (1985). Adenovirus E1B proteins are required for accumulation of late viral mRNA and for effects on cellular mRNA translation and transport. *Mol Cell Biol* **5**(10), 2552-8.

Baffy, G., Miyashita, T., Williamson, J. R., and Reed, J. C. (1993). Apoptosis induced by withdrawal of interleukin-3 (IL-3) from an IL-3- dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 oncoprotein production. *J Biol Chem* **268**(9), 6511-9.

Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science* **281**(5383), 1674-7.

Barnes, P. J., and Karin, M. (1997). Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* **336**(15), 1066-71.

Barry, M., Hnatiuk, S., Mossman, K., Lee, S. F., Boshkov, L., and McFadden, G. (1997). The myxoma virus M-T4 gene encodes a novel RDEL-containing protein that is retained within

the endoplasmic reticulum and is important for the productive infection of lymphocytes. *Virology* **239**(2), 360-77.

Baud, V., Liu, Z. G., Bennett, B., Suzuki, N., Xia, Y., and Karin, M. (1999). Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino-terminal effector domain. *Genes Dev* **13**(10), 1297-308.

Beijerinck, M. W., Ivanovskii, D., Mayer, A., Baur, E., and American Phytopathological, S. (1942). "Concerning the mosaic disease of tobacco. Concerning the mosaic disease of the tobacco plant. Concerning a contagium vivum fluidum as a cause of the spot-disease of tobacco leaves. On the etiology of infectious variegation.". Phytopathological classics ; no.7 American Phytopathological Society, St. Paul, Minn.

Bennett, E. M., Bennink, J. R., Yewdell, J. W., and Brodsky, F. M. (1999). Cutting edge: adenovirus E19 has two mechanisms for affecting class I MHC expression. *J Immunol* **162**(9), 5049-52.

Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L., and Finberg, R. W. (1997). Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* **275**(5304), 1320-3.

Bhattacharya, S., Eckner, R., Grossman, S., Oldread, E., Arany, Z., D'Andrea, A., and Livingston, D. M. (1996). Cooperation of Stat2 and p300/CBP in signalling induced by interferon- alpha. *Nature* **383**(6598), 344-7.

Blobel, G., and Dobberstein, B. (1975). Transfer to proteins across membranes. II. Reconstitution of functional rough microsomes from heterologous components. *J Cell Biol* **67**(3), 852-62.

Bodmer, J. L., Holler, N., Reynard, S., Vinciguerra, P., Schneider, P., Juo, P., Blenis, J., and Tschopp, J. (2000). TRAIL receptor-2 signals apoptosis through FADD and caspase-8. *Nat Cell Biol* **2**(4), 241-3.

Boldin, M. P., Goncharov, T. M., Goltsev, Y. V., and Wallach, D. (1996). Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell* **85**(6), 803-15.

Boyd, J. M., Malstrom, S., Subramanian, T., Venkatesh, L. K., Schaeper, U., Elangovan, B., D'Sa-Eipper, C., and Chinnadurai, G. (1994). Adenovirus E1B 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins [see comments] [published erratum appears in Cell 1994 Dec 16;79(6):following 1120]. *Cell* **79**(2), 341-51.

Boyer, G. S., Denny, F. W. J., and Ginsberg, H. S. (1959). Sequential cellular changes produced by types 5 and 7 adenoviruses in HeLa cells and in human amnion cells: cytological changes aided by fluorescein-labelled antibody. *J. Exp. Med.* **110**, 827-844.

Bram, R. J., and Crabtree, G. R. (1994). Calcium signalling in T cells stimulated by a cyclophilin B-binding protein. *Nature* **371**(6495), 355-8.

Brandt, C. D., Kim, H. W., Vargosko, A. J., Jeffries, B. C., Arrobio, J. O., Rindge, B., Parrott, R. H., and Chanock, R. M. (1969). Infections in 18,000 infants and children in a controlled study of respiratory tract disease. I. Adenovirus pathogenicity in relation to serologic type and illness syndrome. *Am J Epidemiol* **90**(6), 484-500.

Bridge, E., Hemstrom, C., and Pettersson, U. (1991). Differential regulation of adenovirus late transcriptional units by the products of early region. *Virology* **183**(1), 260-6.

Brown, D. J., Hogue, B. G., and Nayak, D. P. (1988). Redundancy of signal and anchor functions in the NH2-terminal uncharged region of influenza virus neuraminidase, a class II membrane glycoprotein. *J Virol* **62**(10), 3824-31.

Burgert, H. G., and Kvist, S. (1985). An adenovirus type 2 glycoprotein blocks cell surface expression of human histocompatibility class I antigens. *Cell* **41**(3), 987-97.

Burgert, H. G., Maryanski, J. L., and Kvist, S. (1987). "E3/19K" protein of adenovirus type 2 inhibits lysis of cytolytic T lymphocytes by blocking cell-surface expression of histocompatibility class I antigens. *Proc Natl Acad Sci U S A* **84**(5), 1356-60.

Camacho, P., and Lechleiter, J. D. (1995). Calreticulin inhibits repetitive intracellular Ca2+ waves. *Cell* **82**(5), 765-71.

Cao, J. X., Krell, P. J., and Nagy, E. (1998). Sequence and transcriptional analysis of terminal regions of the fowl adenovirus type 8 genome [In Process Citation]. *J Gen Virol* **79**(Pt 10), 2507-16.

Carlin, C. R., Tollefson, A. E., Brady, H. A., Hoffman, B. L., and Wold, W. S. (1989). Epidermal growth factor receptor is down-regulated by a 10,400 MW protein encoded by the E3 region of adenovirus. *Cell* **57**(1), 135-44.

Chaudhary, P. M., Eby, M., Jasmin, A., Bookwalter, A., Murray, J., and Hood, L. (1997). Death receptor 5, a new member of the TNFR family, and DR4 induce FADD- dependent apoptosis and activate the NF-kappaB pathway. *Immunity* 7(6), 821-30.

Chen, F., Hersh, B. M., Conradt, B., Zhou, Z., Riemer, D., Gruenbaum, Y., and Horvitz, H. R. (2000). Translocation of C. elegans CED-4 to nuclear membranes during programmed cell death. *Science* **287**(5457), 1485-9.

Chen, G., Ray, R., Dubik, D., Shi, L., Cizeau, J., Bleackley, R. C., Saxena, S., Gietz, R. D., and Greenberg, A. H. (1997). The E1B 19K/Bcl-2-binding protein Nip3 is a dimeric mitochondrial protein that activates apoptosis. *J Exp Med* **186**(12), 1975-83.

Chen, P., Tian, J., Kovesdi, I., and Bruder, J. T. (1998). Interaction of the adenovirus 14.7-kDa protein with FLICE inhibits Fas ligand-induced apoptosis. *J Biol Chem* **273**(10), 5815-20.

Chen, P. H., Ornelles, D. A., and Shenk, T. (1993). The adenovirus L3 23-kilodalton proteinase cleaves the amino-terminal head domain from cytokeratin 18 and disrupts the cytokeratin network of HeLa cells. *J Virol* **67**(6), 3507-14.

Chen-Levy, Z., and Cleary, M. L. (1990). Membrane topology of the Bcl-2 proto-oncogenic protein demonstrated in vitro. *J Biol Chem* **265**(9), 4929-33.

Cheng, E. H., Nicholas, J., Bellows, D. S., Hayward, G. S., Guo, H. G., Reitz, M. S., and Hardwick, J. M. (1997). A Bcl-2 homolog encoded by Kaposi sarcoma-associated virus, human herpesvirus 8, inhibits apoptosis but does not heterodimerize with Bax or Bak. *Proc Natl Acad Sci U S A* **94**(2), 690-4.

Chinnadurai, G., Rho, H. M., Horton, R. B., and Green, M. (1976). mRNA from the transforming segment of the adenovirus 2 genome in productively infected and transformed cells. *J Virol* **20**(1), 255-63.

Chirmule, N., Moffett, J., Dhagat, P., Tazelaar, J., and Wilson, J. M. (1999a). Adenoviral vector-mediated gene therapy in the mouse lung: no role of Fas-Fas ligand interactions for elimination of transgene expression in bronchioepithelial cells. *Hum Gene Ther* **10**(17), 2839-46.

Chirmule, N., Moscioni, A. D., Qian, Y., Qian, R., Chen, Y., and Wilson, J. M. (1999b). Fas-Fas ligand interactions play a major role in effector functions of cytotoxic T lymphocytes after adenovirus vector-mediated gene transfer. *Hum Gene Ther* **10**(2), 259-69.

Clark, J. D., Schievella, A. R., Nalefski, E. A., and Lin, L. L. (1995). Cytosolic phospholipase A2. *J Lipid Mediat Cell Signal* **12**(2-3), 83-117.

Clem, R. J., Fechheimer, M., and Miller, L. K. (1991). Prevention of apoptosis by a baculovirus gene during infection of insect cells. *Science* **254**(5036), 1388-90.

Cohen, O., Inbal, B., Kissil, J. L., Raveh, T., Berissi, H., Spivak-Kroizaman, T., Feinstein, E., and Kimchi, A. (1999). DAP-kinase participates in TNF-alpha- and Fas-induced apoptosis and its function requires the death domain. *J Cell Biol* **146**(1), 141-8.

Connolly, T., and Gilmore, R. (1993). GTP hydrolysis by complexes of the signal recognition particle and the signal recognition particle receptor. *J Cell Biol* **123**(4), 799-807.

Cook, J. L., Walker, T. A., Lewis, A. M., Jr., Ruley, H. E., Graham, F. L., and Pilder, S. H. (1986). Expression of the adenovirus E1A oncogene during cell transformation is sufficient to induce susceptibility to lysis by host inflammatory cells. *Proc Natl Acad Sci U S A* **83**(18), 6965-9.

Daugas, E., Susin, S. A., Zamzami, N., Ferri, K. F., Irinopoulou, T., Larochette, N., Prevost, M. C., Leber, B., Andrews, D., Penninger, J., and Kroemer, G. (2000). Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. *Faseb J* **14**(5), 729-39.

Dbaibo, G. S., and Hannun, Y. A. (1998). Cytokine response modifier A (CrmA): a strategically deployed viral weapon. *Clin Immunol Immunopathol* **86**(2), 134-40.

de Jong, P. J., Valderrama, G., Spigland, I., and Horwitz, M. S. (1983). Adenovirus isolates from urine of patients with acquired immunodeficiency syndrome. *Lancet* **1**(8337), 1293-6.

de Silva, A., Braakman, I., and Helenius, A. (1993). Posttranslational folding of vesicular stomatitis virus G protein in the ER: involvement of noncovalent and covalent complexes. *J Cell Biol* **120**(3), 647-55.

Debbas, M., and White, E. (1993). Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes Dev* **7**(4), 546-54.

Dekker, J., Kanellopoulos, P. N., Loonstra, A. K., van Oosterhout, J. A., Leonard, K., Tucker, P. A., and van der Vliet, P. C. (1997). Multimerization of the adenovirus DNA-binding protein is the driving force for ATP-independent DNA unwinding during strand displacement synthesis. *Embo J* **16**(6), 1455-63.

Denzer, A. J., Nabholz, C. E., and Spiess, M. (1995). Transmembrane orientation of signal-anchor proteins is affected by the folding state but not the size of the N-terminal domain. *Embo J* **14**(24), 6311-7.

Deryckere, F., and Burgert, H. G. (1996). Tumor necrosis factor alpha induces the adenovirus early 3 promoter by activation of NF-kappaB. *J Biol Chem* **271**(47), 30249-55.

Deryckere, F., Ebenau-Jehle, C., Wold, W. S., and Burgert, H. G. (1995). Tumor necrosis factor alpha increases expression of adenovirus E3 proteins. *Immunobiology* **193**(2-4), 186-92.

Deveraux, Q. L., and Reed, J. C. (1999). IAP family proteins--suppressors of apoptosis. *Genes Dev* **13**(3), 239-52.

Diaz, J. L., Oltersdorf, T., Horne, W., McConnell, M., Wilson, G., Weeks, S., Garcia, T., and Fritz, L. C. (1997). A common binding site mediates heterodimerization and homodimerization of Bcl-2 family members. *J Biol Chem* **272**(17), 11350-5.

Dobbelstein, M., Roth, J., Kimberly, W. T., Levine, A. J., and Shenk, T. (1997). Nuclear export of the E1B 55-kDa and E4 34-kDa adenoviral oncoproteins mediated by a rev-like signal sequence. *Embo J* **16**(14), 4276-84.

Dobbelstein, M., and Shenk, T. (1996). Protection against apoptosis by the vaccinia virus SPI-2 (B13R) gene product. *J Virol* **70**(9), 6479-85.

Dobner, T., Horikoshi, N., Rubenwolf, S., and Shenk, T. (1996). Blockage by adenovirus E4orf6 of transcriptional activation by the p53 tumor suppressor. *Science* **272**(5267), 1470-3.

Duan, H., and Dixit, V. M. (1997). RAIDD is a new 'death' adaptor molecule. *Nature* **385**(6611), 86-9.

Dunlop, J., Jones, P. C., and Finbow, M. E. (1995). Membrane insertion and assembly of ductin: a polytopic channel with dual orientations. *Embo J* **14**(15), 3609-16.

Dupree, P., Parton, R. G., Raposo, G., Kurzchalia, T. V., and Simons, K. (1993). Caveolae and sorting in the trans-Golgi network of epithelial cells. *Embo J* **12**(4), 1597-605.

Elkon, K. B., Liu, C. C., Gall, J. G., Trevejo, J., Marino, M. W., Abrahamsen, K. A., Song, X., Zhou, J. L., Old, L. J., Crystal, R. G., and Falck-Pedersen, E. (1997). Tumor necrosis factor alpha plays a central role in immune-mediated clearance of adenoviral vectors. *Proc Natl Acad Sci U S A* **94**(18), 9814-9.

Elsing, A., and Burgert, H. G. (1998). The adenovirus E3/10.4K-14.5K proteins down-modulate the apoptosis receptor Fas/Apo-1 by inducing its internalization. *Proc Natl Acad Sci U S A* **95**(17), 10072-7.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998). A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD [see comments] [published erratum appears in Nature 1998 May 28;393(6683):396]. *Nature* **391**(6662), 43-50.

Everett, H., Barry, M., Lee, S. F., Sun, X., Graham, K., Stone, J., Bleackley, R. C., and McFadden, G. (2000). M11L: a novel mitochondria-localized protein of myxoma virus that blocks apoptosis of infected leukocytes. *J Exp Med* **191**(9), 1487-98.

Felzen, B., Shilkrut, M., Less, H., Sarapov, I., Maor, G., Coleman, R., Robinson, R. B., Berke, G., and Binah, O. (1998). Fas (CD95/Apo-1)-mediated damage to ventricular myocytes induced by cytotoxic T lymphocytes from perforin-deficient mice: a major role for inositol 1,4,5-trisphosphate. *Circ Res* **82**(4), 438-50.

Ferrari, F. K., Samulski, T., Shenk, T., and Samulski, R. J. (1996). Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. *J Virol* **70**(5), 3227-34.

Fields, B. N. (1985). "Virology." Raven Press, New York.

Fox, J. P., Brandt, C. D., Wassermann, F. E., Hall, C. E., Spigland, I., Kogon, A., and Elveback, L. R. (1969). The virus watch program: a continuing surveillance of viral infections in metropolitan New York families. VI. Observations of adenovirus infections: virus excretion patterns, antibody response, efficiency of surveillance, patterns of infections, and relation to illness. *Am J Epidemiol* **89**(1), 25-50.

Fox, J. P., Hall, C. E., and Cooney, M. K. (1977). The Seattle Virus Watch. VII. Observations of adenovirus infections. *Am J Epidemiol* **105**(4), 362-86.

Foyouzi-Youssefi, R., Arnaudeau, S., Borner, C., Kelley, W. L., Tschopp, J., Lew, D. P., Demaurex, N., and Krause, K. H. (2000). Bcl-2 decreases the free Ca2+ concentration within the endoplasmic reticulum. *Proc Natl Acad Sci U S A* **97**(11), 5723-8.

Gabathuler, R., and Kvist, S. (1990). The endoplasmic reticulum retention signal of the E3/19K protein of adenovirus type 2 consists of three separate amino acid segments at the carboxy terminus. *J Cell Biol* **111**(5 Pt 1), 1803-10.

Gabler, S., Schutt, H., Groitl, P., Wolf, H., Shenk, T., and Dobner, T. (1998). E1B 55-kilodalton-associated protein: a cellular protein with RNA- binding activity implicated in nucleocytoplasmic transport of adenovirus and cellular mRNAs. *J Virol* **72**(10), 7960-71.

Gasset, M., Baldwin, M. A., Fletterick, R. J., and Prusiner, S. B. (1993). Perturbation of the secondary structure of the scrapie prion protein under conditions that alter infectivity. *Proc Natl Acad Sci U S A* **90**(1), 1-5.

Germain, M., Affar, E. B., D'Amours, D., Dixit, V. M., Salvesen, G. S., and Poirier, G. G. (1999). Cleavage of Automodified Poly(ADP-ribose) Polymerase during Apoptosis. Evidence for involvement of caspase-7. *J Biol Chem* **274**(40), 28379-28384.

Ginsberg, H. S. (1999). The life and times of adenoviruses. *Adv Virus Res* **54**, 1-13.

Ginsberg, H. S., Moldawer, L. L., Sehgal, P. B., Redington, M., Kilian, P. L., Chanock, R. M., and Prince, G. A. (1991). A mouse model for investigating the molecular pathogenesis of adenovirus pneumonia. *Proc Natl Acad Sci U S A* **88**(5), 1651-5.

Gooding, L. R., Ranheim, T. S., Tollefson, A. E., Aquino, L., Duerksen-Hughes, P., Horton, T. M., and Wold, W. S. (1991). The 10,400- and 14,500-dalton proteins encoded by region E3 of adenovirus function together to protect many but not all mouse cell lines against lysis by tumor necrosis factor. *J Virol* **65**(8), 4114-23.

Goodrum, F. D., Shenk, T., and Ornelles, D. A. (1996). Adenovirus early region 4 34-kilodalton protein directs the nuclear localization of the early region 1B 55-kilodalton protein in primate cells. *J Virol* **70**(9), 6323-35.

Grable, M., and Hearing, P. (1992). cis and trans requirements for the selective packaging of adenovirus type 5 DNA. *J Virol* **66**(2), 723-31.

Greber, U. F., Suomalainen, M., Stidwill, R. P., Boucke, K., Ebersold, M. W., and Helenius, A. (1997). The role of the nuclear pore complex in adenovirus DNA entry. *Embo J* **16**(19), 5998-6007.

Greber, U. F., Webster, P., Weber, J., and Helenius, A. (1996). The role of the adenovirus protease on virus entry into cells. *Embo J* **15**(8), 1766-77.

Greber, U. F., Willetts, M., Webster, P., and Helenius, A. (1993). Stepwise dismantling of adenovirus 2 during entry into cells. *Cell* **75**(3), 477-86.

Gross, J. A., Johnston, J., Mudri, S., Enselman, R., Dillon, S. R., Madden, K., Xu, W., Parrish-Novak, J., Foster, D., Lofton-Day, C., Moore, M., Littau, A., Grossman, A., Haugen, H., Foley, K., Blumberg, H., Harrison, K., Kindsvogel, W., and Clegg, C. H. (2000). TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease [see comments]. *Nature* **404**(6781), 995-9.

Hacki, J., Egger, L., Monney, L., Conus, S., Rosse, T., Fellay, I., and Borner, C. (2000). Apoptotic crosstalk between the endoplasmic reticulum and mitochondria controlled by Bcl-2. *Oncogene* **19**(19), 2286-95.

Hamann, K. J., Dorscheid, D. R., Ko, F. D., Conforti, A. E., Sperling, A. I., Rabe, K. F., and White, S. R. (1998). Expression of Fas (CD95) and FasL (CD95L) in human airway epithelium. *Am J Respir Cell Mol Biol* **19**(4), 537-42.

Han, J., Modha, D., and White, E. (1998). Interaction of E1B 19K with Bax is required to block Bax-induced loss of mitochondrial membrane potential and apoptosis. *Oncogene* **17**(23), 2993-3005.

Han, J., Sabbatini, P., Perez, D., Rao, L., Modha, D., and White, E. (1996). The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein. *Genes Dev* **10**(4), 461-77.

Han, J., Sabbatini, P., and White, E. (1996). Induction of apoptosis by human Nbk/Bik, a BH3-containing protein that interacts with E1B 19K. *Mol Cell Biol* **16**(10), 5857-64.

Hanson, P. I., Meyer, T., Stryer, L., and Schulman, H. (1994). Dual role of calmodulin in autophosphorylation of multifunctional CaM kinase may underlie decoding of calcium signals. *Neuron* **12**(5), 943-56.

Harding, H. P., Zhang, Y., and Ron, D. (1999). Protein translation and folding are coupled by an endoplasmic-reticulum- resident kinase [published erratum appears in Nature 1999 Mar 4;398(6722):90] [see comments]. *Nature* **397**(6716), 271-4.

Hartmann, E., Rapoport, T. A., and Lodish, H. F. (1989). Predicting the orientation of eukaryotic membrane-spanning proteins. *Proc Natl Acad Sci U S A* **86**(15), 5786-90.

Hausmann, J., Ortmann, D., Witt, E., Veit, M., and Seidel, W. (1998). Adenovirus death protein, a transmembrane protein encoded in the E3 region, is palmitoylated at the cytoplasmic tail. *Virology* **244**(2), 343-51.

Hawkins, L. K., Wilson-Rawls, J., and Wold, W. S. (1995). Region E3 of subgroup B human adenoviruses encodes a 16-kilodalton membrane protein that may be a distant analog of the E3-6.7K protein of subgroup C adenoviruses. *J Virol* **69**(7), 4292-8.

Hawkins, L. K., and Wold, W. S. (1995). The E3-20.5K membrane protein of subgroup B human adenoviruses contains O-linked and complex N-linked oligosaccharides. *Virology* **210**(2), 335-44.

He, H., Lam, M., McCormick, T. S., and Distelhorst, C. W. (1997). Maintenance of calcium homeostasis in the endoplasmic reticulum by Bcl- 2. *J Cell Biol* **138**(6), 1219-28.

Hegde, R. S., Mastrianni, J. A., Scott, M. R., DeFea, K. A., Tremblay, P., Torchia, M., DeArmond, S. J., Prusiner, S. B., and Lingappa, V. R. (1998). A transmembrane form of the prion protein in neurodegenerative disease. *Science* **279**(5352), 827-34.

Hegde, R. S., Tremblay, P., Groth, D., DeArmond, S. J., Prusiner, S. B., and Lingappa, V. R. (1999). Transmissible and genetic prion diseases share a common pathway of neurodegeneration [see comments]. *Nature* **402**(6763), 822-6.

Hegde, R. S., Voigt, S., and Lingappa, V. R. (1998). Regulation of protein topology by trans-acting factors at the endoplasmic reticulum. *Mol Cell* **2**(1), 85-91.

Henderson, S., Huen, D., Rowe, M., Dawson, C., Johnson, G., and Rickinson, A. (1993). Epstein-Barr virus-coded BHRF1 protein, a viral homologue of Bcl-2, protects human B cells from programmed cell death. *Proc Natl Acad Sci U S A* **90**(18), 8479-83.

Herelle, F. d. (1922). "The bacteriophage, its rôle in immunity; authorized translation by George H. Smith." Williams & Wilkins, Baltimore.

Hiebert, S. W., Blake, M., Azizkhan, J., and Nevins, J. R. (1991). Role of E2F transcription factor in E1A-mediated trans activation of cellular genes. *J Virol* **65**(7), 3547-52.

Higashino, F., Pipas, J. M., and Shenk, T. (1998). Adenovirus E4orf6 oncoprotein modulates the function of the p53-related protein, p73. *Proc Natl Acad Sci U S A* **95**(26), 15683-7.

Hilleman, M. R., and Werner, J. R. (1954). Recovery of new agent in patients with acute respiratory illness. *Proc Soc Exp Biol Med* **85**, 183-188.

Hnatiuk, S., Barry, M., Zeng, W., Liu, L., Lucas, A., Percy, D., and McFadden, G. (1999). Role of the C-terminal RDEL motif of the myxoma virus M-T4 protein in terms of apoptosis regulation and viral pathogenesis. *Virology* **263**(2), 290-306.

Hofer, A. M., Fasolato, C., and Pozzan, T. (1998). Capacitative Ca2+ entry is closely linked to the filling state of internal Ca2+ stores: a study using simultaneous measurements of ICRAC and intraluminal [Ca2+]. *J Cell Biol* **140**(2), 325-34.

Hoffman, P., Yaffe, M. B., Hoffman, B. L., Yei, S., Wold, W. S., and Carlin, C. (1992). Characterization of the adenovirus E3 protein that down-regulates the epidermal growth factor receptor. Evidence for intermolecular disulfide bonding and plasma membrane localization. *J Biol Chem* **267**(19), 13480-7.

Hogan, M. J., and Crawford, J. W. (1942). Epidemic keratoconjunctivitus. *Am J Ophtalmol* **25**, 1059-1078.

Hogg, J. C., Irving, W. L., Porter, H., Evans, M., Dunnill, M. S., and Fleming, K. (1989). *In situ* hybridization studies of adenoviral infections of the lung and their relationship to follicular bronchiectasis. *Am Rev Respir Dis* **139**, 1531-5.

Holloway, M. P., and Bram, R. J. (1996). A hydrophobic domain of Ca2+-modulating cyclophilin ligand modulates calcium influx signaling in T lymphocytes. *J Biol Chem* **271**(15), 8549-52.

Holloway, M. P., and Bram, R. J. (1998). Co-localization of calcium-modulating cyclophilin ligand with intracellular calcium pools. *J Biol Chem* **273**(26), 16346-50.

Honda, R., Tanaka, H., and Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett* **420**(1), 25-7.

Howe, J. A., Mymryk, J. S., Egan, C., Branton, P. E., and Bayley, S. T. (1990). Retinoblastoma growth suppressor and a 300-kDa protein appear to regulate cellular DNA synthesis. *Proc Natl Acad Sci U S A* **87**(15), 5883-7.

Hsu, H., Shu, H. B., Pan, M. G., and Goeddel, D. V. (1996). TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* **84**(2), 299-308.

Hu, F. Q., Smith, C. A., and Pickup, D. J. (1994). Cowpox virus contains two copies of an early gene encoding a soluble secreted form of the type II TNF receptor. *Virology* **204**(1), 343-56.

Huang, M. M., and Hearing, P. (1989). The adenovirus early region 4 open reading frame 6/7 protein regulates the DNA binding activity of the cellular transcription factor, E2F, through a direct complex. *Genes Dev* **3**(11), 1699-710.

Huen, D. S., Henderson, S. A., Croom-Carter, D., and Rowe, M. (1995). The Epstein-Barr virus latent membrane protein-1 (LMP1) mediates activation of NF-kappa B and cell surface phenotype via two effector regions in its carboxy-terminal cytoplasmic domain. *Oncogene* **10**(3), 549-60.

Huppa, J. B., and Ploegh, H. L. (1998). The eS-Sence of -SH in the ER. *Cell* **92**(2), 145-8.

Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. (1997). Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* **275**(5296), 90-4.

Ichimiya, M., Chang, S. H., Liu, H., Berezesky, I. K., Trump, B. F., and Amstad, P. A. (1998). Effect of Bcl-2 on oxidant-induced cell death and intracellular Ca2+ mobilization. *Am J Physiol* **275**(3 Pt 1), C832-9.

Ilan, Y., Droguett, G., Chowdhury, N. R., Li, Y., Sengupta, K., Thummala, N. R., Davidson, A., Chowdhury, J. R., and Horwitz, M. S. (1997). Insertion of the adenoviral E3 region into a recombinant viral vector prevents antiviral humoral and cellular immune responses and permits long-term gene expression. *Proc Natl Acad Sci U S A* **94**(6), 2587-92.

Imai, Y., Kimura, T., Murakami, A., Yajima, N., Sakamaki, K., and Yonehara, S. (1999). The CED-4-homologous protein FLASH is involved in Fas-mediated activation of caspase-8 during apoptosis [see comments] [published erratum appears in Nature 1999 Jul 1;400(6739):89]. *Nature* **398**(6730), 777-85.

Irmler, M., Steiner, V., Ruegg, C., Wajant, H., and Tschopp, J. (2000). Caspase-induced inactivation of the anti-apoptotic TRAF1 during Fas ligand-mediated apoptosis. *FEBS Lett* **468**(2-3), 129-33.

Janiak, F., Leber, B., and Andrews, D. W. (1994). Assembly of Bcl-2 into microsomal and outer mitochondrial membranes. *J Biol Chem* **269**(13), 9842-9.

Jayaraman, T., and Marks, A. R. (1997). T cells deficient in inositol 1,4,5-trisphosphate receptor are resistant to apoptosis. *Mol Cell Biol* **17**(6), 3005-12.

Jayaraman, T., and Marks, A. R. (2000). Calcineurin Is Downstream of the Inositol 1,4,5-Trisphosphate Receptor in the Apoptotic and Cell Growth Pathways. *J Biol Chem* **275**(9), 6417-6420.

Jenkins, J. R., Rudge, K., and Currie, G. A. (1984). Cellular immortalization by a cDNA clone encoding the transformation- associated phosphoprotein p53. *Nature* **312**(5995), 651-4.

Jenner, E. (1923). "An inquiry into the causes and effects of the Variolae vaccinae : a disease discovered in some of the western counties of England, particularly Gloucestershire, and known by the name of the cow pox." [R. Lier & Co.], [Milan].

Jiang, S., Chow, S. C., Nicotera, P., and Orrenius, S. (1994). Intracellular Ca2+ signals activate apoptosis in thymocytes: studies using the Ca(2+)-ATPase inhibitor thapsigargin. *Exp Cell Res* **212**(1), 84-92.

Jungnickel, B., and Rapoport, T. A. (1995). A posttargeting signal sequence recognition event in the endoplasmic reticulum membrane [see comments]. *Cell* **82**(2), 261-70.

Jurgensmeier, J. M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D., and Reed, J. C. (1998). Bax directly induces release of cytochrome c from isolated mitochondria. *Proc Natl Acad Sci U S A* **95**(9), 4997-5002.

Kafri, T., Morgan, D., Krahl, T., Sarvetnick, N., Sherman, L., and Verma, I. (1998). Cellular immune response to adenoviral vector infected cells does not require de novo viral gene expression: implications for gene therapy. *Proc Natl Acad Sci U S A* **95**(19), 11377-82.

Kaiser, C. A., Preuss, D., Grisafi, P., and Botstein, D. (1987). Many random sequences functionally replace the secretion signal sequence of yeast invertase. *Science* **235**(4786), 312-7.

Karasuyama, H., and Melchers, F. (1988). Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5, using modified cDNA expression vectors. *Eur J Immunol* **18**(1), 97-104.

Kass, G. E., Juedes, M. J., and Orrenius, S. (1992). Cyclosporin A protects hepatocytes against prooxidant-induced cell killing. A study on the role of mitochondrial Ca2+ cycling in cytotoxicity. *Biochem Pharmacol* **44**(10), 1995-2003.

Kastan, M. B., Zhan, Q., el-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., and Fornace, A. J., Jr. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* **71**(4), 587-97.

Kelly, H. A. (1923). "Walter Reed and yellow fever." 3d , rev. ed. The Norman Remington company, Baltimore, Md.,.

King, A. J., and van der Vliet, P. C. (1994). A precursor terminal protein-trinucleotide intermediate during initiation of adenovirus DNA replication: regeneration of molecular ends in vitro by a jumping back mechanism. *Embo J* **13**(23), 5786-92.

Kinnaird, L. (1866). "Rinderpest; or the treatment of cattle." [s.n.], London.

Klappa, P., Mayinger, P., Pipkorn, R., Zimmermann, M., and Zimmermann, R. (1991). A microsomal protein is involved in ATP-dependent transport of presecretory proteins into mammalian microsomes. *Embo J* **10**(10), 2795-803.

Klappa, P., Zimmermann, M., and Zimmermann, R. (1994). The membrane proteins TRAMp and sec61 alpha p may be involved in post- translational transport of presecretory proteins into mammalian microsomes. *FEBS Lett* **341**(2-3), 281-7.

Konig, C., Roth, J., and Dobbelstein, M. (1999). Adenovirus type 5 E4orf3 protein relieves p53 inhibition by E1B-55- kilodalton protein. *J Virol* **73**(3), 2253-62.

Kornblihtt, A. R. (1993). [The unexpected complexity of eukaryotic genes. Nobel Prize of Medicine or Physiology 1993, Philip Sharp and Richard Roberts (editorial)]. *Medicina* **53**(6), 563-5.

Korppi, M., Leinonen, M., Makela, P. H., and Launiala, K. (1991). Mixed infection is common in children with respiratory adenovirus infection. *Acta Paediatr Scand* **80**(4), 413-7.

Krajcsi, P., Dimitrov, T., Hermiston, T. W., Tollefson, A. E., Ranheim, T. S., Vande Pol, S. B., Stephenson, A. H., and Wold, W. S. (1996). The adenovirus E3-14.7K protein and the E3-10.4K/14.5K complex of proteins, which independently inhibit tumor necrosis factor (TNF)-

induced apoptosis, also independently inhibit TNF-induced release of arachidonic acid. *J Virol* **70**(8), 4904-13.

Krajcsi, P., Tollefson, A. E., Anderson, C. W., Stewart, A. R., Carlin, C. R., and Wold, W. S. (1992). The E3-10.4K protein of adenovirus is an integral membrane protein that is partially cleaved between Ala22 and Ala23 and has a Ccyt orientation. *Virology* **187**(1), 131-44.

Krajcsi, P., Tollefson, A. E., and Wold, W. S. (1992). The E3-14.5K integral membrane protein of adenovirus that is required for down-regulation of the EGF receptor and for prevention of TNF cytolysis is O-glycosylated but not N-glycosylated. *Virology* **188**(2), 570-9.

Krajcsi, P., and Wold, W. S. (1992). The adenovirus E3-14.5K protein which is required for prevention of TNF cytolysis and for down-regulation of the EGF receptor contains phosphoserine. *Virology* **187**(2), 492-8.

Kratzer, F., Rosorius, O., Heger, P., Hirschmann, N., Dobner, T., Hauber, J., and Stauber, R. H. (2000). The adenovirus type 5 E1B-55K oncoprotein is a highly active shuttle protein and shuttling is independent of e4orf6, p53 and mdm2 [In Process Citation]. *Oncogene* **19**(7), 850-7.

Krilov, L. R., Rubin, L. G., Frogel, M., Gloster, E., Ni, K., Kaplan, M., and Lipson, S. M. (1990). Disseminated adenovirus infection with hepatic necrosis in patients with human immunodeficiency virus infection and other immunodeficiency states. *Rev Infect Dis* **12**(2), 303-7.

Kumar, A., Haque, J., Lacoste, J., Hiscott, J., and Williams, B. R. (1994). Double-stranded RNA-dependent protein kinase activates transcription factor NF-kappa B by phosphorylating I kappa B. *Proc Natl Acad Sci U S A* **91**(14), 6288-92.

Kutay, U., Ahnert-Hilger, G., Hartmann, E., Wiedenmann, B., and Rapoport, T. A. (1995). Transport route for synaptobrevin via a novel pathway of insertion into the endoplasmic reticulum membrane. *Embo J* **14**(2), 217-23.

Kyte, J., and Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157**(1), 105-32.

Laidlaw, P. P. (1938). "Virus diseases and viruses." The University press, Cambridge [Eng.].

Lam, M., Bhat, M. B., Nunez, G., Ma, J., and Distelhorst, C. W. (1998). Regulation of Bcl-xl channel activity by calcium. *J Biol Chem* **273**(28), 17307-10.

Lam, M., Dubyak, G., Chen, L., Nunez, G., Miesfeld, R. L., and Distelhorst, C. W. (1994). Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca2+ fluxes. *Proc Natl Acad Sci U S A* **91**(14), 6569-73.

Lasky, L. A., Nakamura, G., Smith, D. H., Fennie, C., Shimasaki, C., Patzer, E., Berman, P., Gregory, T., and Capon, D. J. (1987). Delineation of a region of the human immunodeficiency virus type 1 gp120 glycoprotein critical for interaction with the CD4 receptor. *Cell* **50**(6), 975-85.

Latinis, K. M., Carr, L. L., Peterson, E. J., Norian, L. A., Eliason, S. L., and Koretzky, G. A. (1997). Regulation of CD95 (Fas) ligand expression by TCR-mediated signaling events. *J Immunol* **158**(10), 4602-11.

Lavoie, J. N., Nguyen, M., Marcellus, R. C., Branton, P. E., and Shore, G. C. (1998). E4orf4, a novel adenovirus death factor that induces p53-independent apoptosis by a pathway that is not inhibited by zVAD-fmk. *J Cell Biol* **140**(3), 637-45.

Lechevalier, H. (1972). Dmitri Iosifovich Ivanovski (1864-1920). *Bacteriol Rev* **36**(2), 135-45.

Lee, S. T., Hoeflich, K. P., Wasfy, G. W., Woodgett, J. R., Leber, B., Andrews, D. W., Hedley, D. W., and Penn, L. Z. (1999). Bcl-2 targeted to the endoplasmic reticulum can inhibit apoptosis induced by Myc but not etoposide in Rat-1 fibroblasts. *Oncogene* **18**(23), 3520-8.

Lees-Miller, S. P., Chen, Y. R., and Anderson, C. W. (1990). Human cells contain a DNA-activated protein kinase that phosphorylates simian virus 40 T antigen, mouse p53, and the human Ku autoantigen. *Mol Cell Biol* **10**(12), 6472-81.

Leonard, G. T., and Sen, G. C. (1996). Effects of adenovirus E1A protein on interferon-signaling. *Virology* **224**(1), 25-33.

Leopardi, R., Van Sant, C., and Roizman, B. (1997). The herpes simplex virus 1 protein kinase US3 is required for protection from apoptosis induced by the virus. *Proc Natl Acad Sci U S A* **94**(15), 7891-6.

Leppa, S., and Bohmann, D. (1999). Diverse functions of JNK signaling and c-Jun in stress response and apoptosis. *Oncogene* **18**(45), 6158-62.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. *Cell* **88**(3), 323-31.

Levy, F., Gabathuler, R., Larsson, R., and Kvist, S. (1991). ATP is required for in vitro assembly of MHC class I antigens but not for transfer of peptides across the ER membrane. *Cell* **67**(2), 265-74.

Li, E., Stupack, D., Bokoch, G. M., and Nemerow, G. R. (1998a). Adenovirus endocytosis requires actin cytoskeleton reorganization mediated by Rho family GTPases. *J Virol* **72**(11), 8806-12.

Li, E., Stupack, D., Klemke, R., Cheresh, D. A., and Nemerow, G. R. (1998b). Adenovirus endocytosis via alpha(v) integrins requires phosphoinositide- 3-OH kinase. *J Virol* **72**(3), 2055-61.

Li, H., Zhu, H., Xu, C. J., and Yuan, J. (1998c). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94**(4), 491-501.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**(4), 479-89.

Li, Y., Kang, J., Friedman, J., Tarassishin, L., Ye, J., Kovalenko, A., Wallach, D., and Horwitz, M. S. (1999). Identification of a cell protein (FIP-3) as a modulator of NF-kappaB activity and as a target of an adenovirus inhibitor of tumor necrosis factor alpha-induced apoptosis. *Proc Natl Acad Sci U S A* **96**(3), 1042-7.

Li, Y., Kang, J., and Horwitz, M. S. (1997). Interaction of an adenovirus 14.7-kilodalton protein inhibitor of tumor necrosis factor alpha cytolysis with a new member of the GTPase superfamily of signal transducers. *J Virol* **71**(2), 1576-82.

Li, Y., Kang, J., and Horwitz, M. S. (1998). Interaction of an adenovirus E3 14.7-kilodalton protein with a novel tumor necrosis factor alpha-inducible cellular protein containing leucine zipper domains. *Mol Cell Biol* **18**(3), 1601-10.

Lill, N. L., Grossman, S. R., Ginsberg, D., DeCaprio, J., and Livingston, D. M. (1997). Binding and modulation of p53 by p300/CBP coactivators. *Nature* **387**(6635), 823-7.

Lipp, J., and Dobberstein, B. (1986). Signal recognition particle-dependent membrane insertion of mouse invariant chain: a membrane-spanning protein with a cytoplasmically exposed amino terminus. *J Cell Biol* **102**(6), 2169-75.

Liston, P., Roy, N., Tamai, K., Lefebvre, C., Baird, S., Cherton-Horvat, G., Farahani, R., McLean, M., Ikeda, J. E., MacKenzie, A., and Korneluk, R. G. (1996). Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature* **379**(6563), 349-53.

Liu, H., Bowes, R. C., 3rd, van de Water, B., Sillence, C., Nagelkerke, J. F., and Stevens, J. L. (1997). Endoplasmic reticulum chaperones GRP78 and calreticulin prevent oxidative stress, Ca2+ disturbances, and cell death in renal epithelial cells. *J Biol Chem* **272**(35), 21751-9.

Liu, J. L., Ye, Y., Lee, L. F., and Kung, H. J. (1998). Transforming potential of the herpesvirus oncoprotein MEQ: morphological transformation, serum-independent growth, and inhibition of apoptosis. *J Virol* **72**(1), 388-95.

Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* **86**(1), 147-57.

Liu, Z. G., Smith, S. W., McLaughlin, K. A., Schwartz, L. M., and Osborne, B. A. (1994). Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene nur77. *Nature* **367**(6460), 281-4.

Liu, Z. X., Govindarajan, S., Okamoto, S., and Dennert, G. (2000). Fas- and Tumor Necrosis Factor Receptor 1-Dependent But Not Perforin- Dependent Pathways Cause Injury in Livers Infected With an Adenovirus Construct in Mice. *Hepatology* **31**(3), 665-673.

Loparev, V. N., Parsons, J. M., Knight, J. C., Panus, J. F., Ray, C. A., Buller, R. M., Pickup, D. J., and Esposito, J. J. (1998). A third distinct tumor necrosis factor receptor of orthopoxviruses. *Proc Natl Acad Sci U S A* **95**(7), 3786-91.

Lowe, S. W., and Ruley, H. E. (1993). Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. *Genes Dev* **7**(4), 535-45.

Lundblad, J. R., Kwok, R. P., Laurance, M. E., Harter, M. L., and Goodman, R. H. (1995). Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP. *Nature* **374**(6517), 85-8.

Luschen, S., Ussat, S., Kronke, M., and Adam-Klages, S. (1998). Cleavage of human cytosolic phospholipase A2 by caspase-1 (ICE) and caspase-8 (FLICE). *Biochem Biophys Res Commun* **253**(1), 92-8.

Macen, J. L., Garner, R. S., Musy, P. Y., Brooks, M. A., Turner, P. C., Moyer, R. W., McFadden, G., and Bleackley, R. C. (1996). Differential inhibition of the Fas- and granule-mediated cytolysis pathways by the orthopoxvirus cytokine response modifier A/SPI-2 and SPI-1 protein. *Proc Natl Acad Sci U S A* **93**(17), 9108-13.

Macen, J. L., Upton, C., Nation, N., and McFadden, G. (1993). SERP1, a serine proteinase inhibitor encoded by myxoma virus, is a secreted glycoprotein that interferes with inflammation. *Virology* **195**(2), 348-63.

Magnelli, L., Cinelli, M., Turchetti, A., and Chiarugi, V. P. (1994). Bcl-2 overexpression abolishes early calcium waving preceding apoptosis in NIH-3T3 murine fibroblasts. *Biochem Biophys Res Commun* **204**(1), 84-90.

Malinin, N. L., Boldin, M. P., Kovalenko, A. V., and Wallach, D. (1997). MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. *Nature* **385**(6616), 540-4.

Marcellus, R. C., Lavoie, J. N., Boivin, D., Shore, G. C., Ketner, G., and Branton, P. E. (1998). The early region 4 orf4 protein of human adenovirus type 5 induces p53- independent cell death by apoptosis. *J Virol* **72**(9), 7144-53.

Marin, M. C., Fernandez, A., Bick, R. J., Brisbay, S., Buja, L. M., Snuggs, M., McConkey, D. J., von Eschenbach, A. C., Keating, M. J., and McDonnell, T. J. (1996). Apoptosis suppression by bcl-2 is correlated with the regulation of nuclear and cytosolic Ca2+. *Oncogene* **12**(11), 2259-66.

Marshall, W. L., Yim, C., Gustafson, E., Graf, T., Sage, D. R., Hanify, K., Williams, L., Fingeroth, J., and Finberg, R. W. (1999). Epstein-Barr virus encodes a novel homolog of the bcl-2 oncogene that inhibits apoptosis and associates with Bax and Bak. *J Virol* **73**(6), 5181-5.

Martin, M. E., and Berk, A. J. (1998). Adenovirus E1B 55K represses p53 activation in vitro. *J Virol* **72**(4), 3146-54.

Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFace, D. M., and Green, D. R. (1995). Early redistribution of plasma membrane phosphatidylserine is

a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* **182**(5), 1545-56.

Martinon, F., Holler, N., Richard, C., and Tschopp, J. (2000). Activation of a pro-apoptotic amplification loop through inhibition of NF-kappaB-dependent survival signals by caspase-mediated inactivation of RIP. *FEBS Lett* **468**(2-3), 134-6.

Matlack, K. E., Misselwitz, B., Plath, K., and Rapoport, T. A. (1999). BiP acts as a molecular ratchet during posttranslational transport of prepro-alpha factor across the ER membrane. *Cell* **97**(5), 553-64.

McConkey, D. J., and Orrenius, S. (1997). The role of calcium in the regulation of apoptosis. *Biochem Biophys Res Commun* **239**(2), 357-66.

Mietz, J. A., Unger, T., Huibregtse, J. M., and Howley, P. M. (1992). The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. *Embo J* **11**(13), 5013-20.

Miller, L. K. (1997). Baculovirus interaction with host apoptotic pathways. *J Cell Physiol* **173**(2), 178-82.

Miskin, J. E., Abrams, C. C., Goatley, L. C., and Dixon, L. K. (1998). A viral mechanism for inhibition of the cellular phosphatase calcineurin. *Science* **281**(5376), 562-5.

Miyamoto, S., Teramoto, H., Coso, O. A., Gutkind, J. S., Burbelo, P. D., Akiyama, S. K., and Yamada, K. M. (1995). Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. *J Cell Biol* **131**(3), 791-805.

Miyawaki, A., Griesbeck, O., Heim, R., and Tsien, R. Y. (1999). Dynamic and quantitative Ca2+ measurements using improved cameleons. *Proc Natl Acad Sci U S A* **96**(5), 2135-40.

Montero, M., Brini, M., Marsault, R., Alvarez, J., Sitia, R., Pozzan, T., and Rizzuto, R. (1995). Monitoring dynamic changes in free Ca2+ concentration in the endoplasmic reticulum of intact cells. *Embo J* **14**(22), 5467-75.

Moore, A. N., Kampfl, A. W., Zhao, X., Hayes, R. L., and Dash, P. K. (1999a). Sphingosine-1-phosphate induces apoptosis of cultured hippocampal neurons that requires protein phosphatases and activator protein-1 complexes. *Neuroscience* **94**(2), 405-15.

Moore, P. A., Belvedere, O., Orr, A., Pieri, K., LaFleur, D. W., Feng, P., Soppet, D., Charters, M., Gentz, R., Parmelee, D., Li, Y., Galperina, O., Giri, J., Roschke, V., Nardelli, B., Carrell, J., Sosnovtseva, S., Greenfield, W., Ruben, S. M., Olsen, H. S., Fikes, J., and Hilbert, D. M. (1999b). BLyS: member of the tumor necrosis factor family and B lymphocyte stimulator. *Science* **285**(5425), 260-3.

Mossman, K., Lee, S. F., Barry, M., Boshkov, L., and McFadden, G. (1996). Disruption of M-T5, a novel myxoma virus gene member of poxvirus host range superfamily, results in dramatic attenuation of myxomatosis in infected European rabbits. *J Virol* **70**(7), 4394-410.

Mothes, W., Heinrich, S. U., Graf, R., Nilsson, I., von Heijne, G., Brunner, J., and Rapoport, T. A. (1997). Molecular mechanism of membrane protein integration into the endoplasmic reticulum. *Cell* **89**(4), 523-33.

Muckelbauer, J. K., Kremer, M., Minor, I., Diana, G., Dutko, F. J., Groarke, J., Pevear, D. C., and Rossmann, M. G. (1995). The structure of coxsackievirus B3 at 3.5 A resolution. *Structure* **3**(7), 653-67.

Mueckler, M., and Lodish, H. F. (1986). Post-translational insertion of a fragment of the glucose transporter into microsomes requires phosphoanhydride bond cleavage. *Nature* **322**(6079), 549-52.

Mukhopadhyay, A., Ni, J., Zhai, Y., Yu, G. L., and Aggarwal, B. B. (1999). Identification and characterization of a novel cytokine, THANK, a TNF homologue that activates apoptosis, nuclear factor-kappaB, and c-Jun NH2-terminal kinase. *J Biol Chem* **274**(23), 15978-81.

Muller, U., Steinhoff, U., Reis, L. F., Hemmi, S., Pavlovic, J., Zinkernagel, R. M., and Aguet, M. (1994). Functional role of type I and type II interferons in antiviral defense. *Science* **264**(5167), 1918-21.

Murphy, A. N., Bredesen, D. E., Cortopassi, G., Wang, E., and Fiskum, G. (1996). Bcl-2 potentiates the maximal calcium uptake capacity of neural cell mitochondria. *Proc Natl Acad Sci U S A* **93**(18), 9893-8.

Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998). An induced proximity model for caspase-8 activation. *J Biol Chem* **273**(5), 2926-30.

Nagata, K., Guggenheimer, R. A., Enomoto, T., Lichy, J. H., and Hurwitz, J. (1982). Adenovirus DNA replication in vitro: identification of a host factor that stimulates synthesis of the preterminal protein-dCMP complex. *Proc Natl Acad Sci U S A* **79**(21), 6438-42.

Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B. A., and Yuan, J. (2000). Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* **403**(6765), 98-103.

Nakajima, T., Morita, K., Tsunoda, H., Imajoh-Ohmi, S., Tanaka, H., Yasuda, H., and Oda, K. (1998). Stabilization of p53 by adenovirus E1A occurs through its amino- terminal region by modification of the ubiquitin-proteasome pathway. *J Biol Chem* **273**(32), 20036-45.

Nalefski, E. A., Sultzman, L. A., Martin, D. M., Kriz, R. W., Towler, P. S., Knopf, J. L., and Clark, J. D. (1994). Delineation of two functionally distinct domains of cytosolic phospholipase A2, a regulatory Ca(2+)-dependent lipid-binding domain and a Ca(2+)-independent catalytic domain. *J Biol Chem* **269**(27), 18239-49.

Nash, P., Barrett, J., Cao, J. X., Hota-Mitchell, S., Lalani, A. S., Everett, H., Xu, X. M., Robichaud, J., Hnatiuk, S., Ainslie, C., Seet, B. T., and McFadden, G. (1999). Immunomodulation by viruses: the myxoma virus story. *Immunol Rev* **168**, 103-20.

Nava, V. E., Cheng, E. H., Veliuona, M., Zou, S., Clem, R. J., Mayer, M. L., and Hardwick, J. M. (1997). Herpesvirus saimiri encodes a functional homolog of the human bcl-2 oncogene. *J Virol* **71**(5), 4118-22.

Neilan, J. G., Lu, Z., Afonso, C. L., Kutish, G. F., Sussman, M. D., and Rock, D. L. (1993). An African swine fever virus gene with similarity to the proto-oncogene bcl-2 and the Epstein-Barr virus gene BHRF1. *J Virol* **67**(7), 4391-4.

Neilan, J. G., Lu, Z., Kutish, G. F., Zsak, L., Burrage, T. G., Borca, M. V., Carrillo, C., and Rock, D. L. (1997). A BIR motif containing gene of African swine fever virus, 4CL, is nonessential for growth in vitro and viral virulence. *Virology* **230**(2), 252-64.

Nelson, K. E., Gavitt, F., Batt, M. D., Kallick, C. A., Reddi, K. T., and Levin, S. (1975). The role of adenoviruses in the pertussis syndrome. *J Pediatr* **86**(3), 335-41.

Neumann, R., Genersch, E., and Eggers, H. J. (1987). Detection of adenovirus nucleic acid sequences in human tonsils in the absence of infectious virus. *Virus Res* **7**(1), 93-7.

Nevels, M., Rubenwolf, S., Spruss, T., Wolf, H., and Dobner, T. (1997). The adenovirus E4orf6 protein can promote E1A/E1B-induced focus formation by interfering with p53 tumor suppressor function. *Proc Natl Acad Sci U S A* **94**(4), 1206-11.

Nevins, J. R. (1981). Mechanism of activation of early viral transcription by the adenovirus E1A gene product. *Cell* **26**(2 Pt 2), 213-20.

Nevins, J. R. (1982). Induction of the synthesis of a 70,000 dalton mammalian heat shock protein by the adenovirus E1A gene product. *Cell* **29**(3), 913-9.

Ng, F. W., Nguyen, M., Kwan, T., Branton, P. E., Nicholson, D. W., Cromlish, J. A., and Shore, G. C. (1997). p28 Bap31, a Bcl-2/Bcl-XL- and procaspase-8-associated protein in the endoplasmic reticulum. *J Cell Biol* **139**(2), 327-38.

Ng, F. W., and Shore, G. C. (1998). Bcl-XL cooperatively associates with the Bap31 complex in the endoplasmic reticulum, dependent on procaspase-8 and Ced-4 adaptor. *J Biol Chem* **273**(6), 3140-3.

Nguyen, M., Millar, D. G., Yong, V. W., Korsmeyer, S. J., and Shore, G. C. (1993). Targeting of Bcl-2 to the mitochondrial outer membrane by a COOH- terminal signal anchor sequence. *J Biol Chem* **268**(34), 25265-8.

Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., and et al. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis [see comments]. *Nature* **376**(6535), 37-43.

Nishii, K., Tsuzuki, T., Kumai, M., Takeda, N., Koga, H., Aizawa, S., Nishimoto, T., and Shibata, Y. (1999). Abnormalities of developmental cell death in Dad1-deficient mice [In Process Citation]. *Genes Cells* **4**(4), 243-52.

Nordqvist, K., Ohman, K., and Akusjarvi, G. (1994). Human adenovirus encodes two proteins which have opposite effects on accumulation of alternatively spliced mRNAs. *Mol Cell Biol* **14**(1), 437-45.

Ohman, K., Nordqvist, K., and Akusjarvi, G. (1993). Two adenovirus proteins with redundant activities in virus growth facilitates tripartite leader mRNA accumulation. *Virology* **194**(1), 50-8.

Okano, M., Thiele, G. M., and Purtilo, D. T. (1990). Severe chronic active Epstein-Barr virus infection syndrome and adenovirus type-2 infection. *Am J Pediatr Hematol Oncol* **12**(2), 168-73.

Ornelles, D. A., and Shenk, T. (1991). Localization of the adenovirus early region 1B 55-kilodalton protein during lytic infection: association with nuclear viral inclusions requires the early region 4 34-kilodalton protein. *J Virol* **65**(1), 424-9.

Pahl, H. L., and Baeuerle, P. A. (1996). Activation of NF-kappa B by ER stress requires both Ca2+ and reactive oxygen intermediates as messengers. *FEBS Lett* **392**(2), 129-36.

Pahl, H. L., and Baeuerle, P. A. (1997). The ER-overload response: activation of NF-kappa B. *Trends Biochem Sci* **22**(2), 63-7.

Pahl, H. L., Sester, M., Burgert, H. G., and Baeuerle, P. A. (1996). Activation of transcription factor NF-kappaB by the adenovirus E3/19K protein requires its ER retention. *J Cell Biol* **132**(4), 511-22.

Pelham, H. R., and Jackson, R. J. (1976). An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur J Biochem* **67**(1), 247-56.

Perez, D., and White, E. (1998). E1B 19K inhibits Fas-mediated apoptosis through FADD-dependent sequestration of FLICE. *J Cell Biol* **141**(5), 1255-66.

Persson, H., Kvist, S., Ostberg, L., Peterson, P. A., and Philipson, L. (1980). Adenoviral early glycoprotein E3-19K and its association with transplantation antigens. *Cold Spring Harb Symp Quant Biol* **44**(Pt 1,), 509-17.

Peters-Golden, M., Song, K., Marshall, T., and Brock, T. (1996). Translocation of cytosolic phospholipase A2 to the nuclear envelope elicits topographically localized phospholipid hydrolysis. *Biochem J* **318**(Pt 3), 797-803.

Petit, F., Bertagnoli, S., Gelfi, J., Fassy, F., Boucraut-Baralon, C., and Milon, A. (1996). Characterization of a myxoma virus-encoded serpin-like protein with activity against interleukin-1 beta-converting enzyme. *J Virol* **70**(9), 5860-6.

Pinton, P., Ferrari, D., Magalhaes, P., Schulze-Osthoff, K., Di Virgilio, F., Pozzan, T., and Rizzuto, R. (2000). Reduced loading of intracellular Ca(2+) stores and downregulation of capacitative Ca(2+) influx in Bcl-2-overexpressing cells. *J Cell Biol* **148**(5), 857-62.

Plath, K., Mothes, W., Wilkinson, B. M., Stirling, C. J., and Rapoport, T. A. (1998). Signal sequence recognition in posttranslational protein transport across the yeast ER membrane. *Cell* **94**(6), 795-807.

Pombo, A., Ferreira, J., Bridge, E., and Carmo-Fonseca, M. (1994). Adenovirus replication and transcription sites are spatially separated in the nucleus of infected cells. *Embo J* **13**(21), 5075-85.

Pruijn, G. J., van Driel, W., and van der Vliet, P. C. (1986). Nuclear factor III, a novel sequence-specific DNA-binding protein from HeLa cells stimulating adenovirus DNA replication. *Nature* **322**(6080), 656-9.

Querido, E., Marcellus, R. C., Lai, A., Charbonneau, R., Teodoro, J. G., Ketner, G., and Branton, P. E. (1997). Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells. *J Virol* **71**(5), 3788-98.

Querido, E., Teodoro, J. G., and Branton, P. E. (1997). Accumulation of p53 induced by the adenovirus E1A protein requires regions involved in the stimulation of DNA synthesis. *J Virol* **71**(5), 3526-33.

Rapiejko, P. J., and Gilmore, R. (1997). Empty site forms of the SRP54 and SR alpha GTPases mediate targeting of ribosome-nascent chain complexes to the endoplasmic reticulum [see comments]. *Cell* **89**(5), 703-13.

Ray, R. B., Meyer, K., and Ray, R. (1996). Suppression of apoptotic cell death by hepatitis C virus core protein. *Virology* **226**(2), 176-82.

Ridgley, E. L., Xiong, Z. H., and Ruben, L. (1999). Reactive oxygen species activate a Ca2+-dependent cell death pathway in the unicellular organism Trypanosoma brucei brucei. *Biochem J* **340**(Pt 1), 33-40.

Rivers, T. M., and Amos, H. L. (1928). "Filterable viruses." Williams & Wilkins, Baltimore.

Rizzuto, R., Pinton, P., Carrington, W., Fay, F. S., Fogarty, K. E., Lifshitz, L. M., Tuft, R. A., and Pozzan, T. (1998). Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca2+ responses. *Science* **280**(5370), 1763-6.

Roth, J., Konig, C., Wienzek, S., Weigel, S., Ristea, S., and Dobbelstein, M. (1998). Inactivation of p53 but not p73 by adenovirus type 5 E1B 55-kilodalton and E4 34-kilodalton oncoproteins. *J Virol* **72**(11), 8510-6.

Rott, R. (1999). The post-Loeffler-Frosch era: contribution of German virologists. *Arch Virol Suppl* **15**, 43-61.

Roulston, A., Marcellus, R. C., and Branton, P. E. (1999). Viruses and apoptosis. *Annu Rev Microbiol* **53**, 577-628.

Rowe, M., Peng-Pilon, M., Huen, D. S., Hardy, R., Croom-Carter, D., Lundgren, E., and Rickinson, A. B. (1994). Upregulation of bcl-2 by the Epstein-Barr virus latent membrane protein LMP1: a B-cell-specific response that is delayed relative to NF-kappa B activation and to induction of cell surface markers. *J Virol* **68**(9), 5602-12.

Rowe, W. P., Huebner, A. J., Gilmore, L. K., Parrott, R. N., and Ward, T. G. (1952). Isolation of a cytopthogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc Soc Exp Biol Med* **84**, 570-573.

Ruska, H. (1950). "Virus; eine kurze Zusammenfassung der Kenntnisse über das Virusproblem." Akademische Verlagsgesellschaft Athenaion, Potsdam,.

Sabatini, D. D., Tashiro, Y., and Palade, G. E. (1966). On the attachment of ribosomes to microsomal membranes. *J Mol Biol* **19**(2), 503-24.

Sakahira, H., Enari, M., and Nagata, S. (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis [see comments]. *Nature* **391**(6662), 96-9.

Samulski, R. J., and Shenk, T. (1988). Adenovirus E1B 55-Mr polypeptide facilitates timely cytoplasmic accumulation of adeno-associated virus mRNAs. *J Virol* **62**(1), 206-10.

Sanna, M. G., Duckett, C. S., Richter, B. W., Thompson, C. B., and Ulevitch, R. J. (1998). Selective activation of JNK1 is necessary for the anti-apoptotic activity of hILP. *Proc Natl Acad Sci U S A* **95**(11), 6015-20.

Saraste, J., Palade, G. E., and Farquhar, M. G. (1986). Temperature-sensitive steps in the transport of secretory proteins through the Golgi complex in exocrine pancreatic cells. *Proc Natl Acad Sci U S A* **83**(17), 6425-9.

Sarnow, P., Hearing, P., Anderson, C. W., Halbert, D. N., Shenk, T., and Levine, A. J. (1984). Adenovirus early region 1B 58,000-dalton tumor antigen is physically associated with an early region 4 25,000-dalton protein in productively infected cells. *J Virol* **49**(3), 692-700.

Scaria, A., Tollefson, A. E., Saha, S. K., and Wold, W. S. (1992). The E3-11.6K protein of adenovirus is an Asn-glycosylated integral membrane protein that localizes to the nuclear membrane. *Virology* **191**(2), 743-53.

Schagger, H., and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* **166**(2), 368-79.

Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. J., and Howley, P. M. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* **63**(6), 1129-36.

Schievella, A. R., Regier, M. K., Smith, W. L., and Lin, L. L. (1995). Calcium-mediated translocation of cytosolic phospholipase A2 to the nuclear envelope and endoplasmic reticulum. *J Biol Chem* **270**(51), 30749-54.

Schlenstedt, G., Gudmundsson, G. H., Boman, H. G., and Zimmermann, R. (1992). Structural requirements for transport of preprocecropinA and related presecretory proteins into mammalian microsomes. *J Biol Chem* **267**(34), 24328-32.

Schmedt, C., Green, S. R., Manche, L., Taylor, D. R., Ma, Y., and Mathews, M. B. (1995). Functional characterization of the RNA-binding domain and motif of the double-stranded RNA-dependent protein kinase DAI (PKR). *J Mol Biol* **249**(1), 29-44.

Schneider, P., MacKay, F., Steiner, V., Hofmann, K., Bodmer, J. L., Holler, N., Ambrose, C., Lawton, P., Bixler, S., Acha-Orbea, H., Valmori, D., Romero, P., Werner-Favre, C., Zubler, R. H., Browning, J. L., and Tschopp, J. (1999). BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J Exp Med* **189**(11), 1747-56.

Schreiber, M., Rajarathnam, K., and McFadden, G. (1996). Myxoma virus T2 protein, a tumor necrosis factor (TNF) receptor homolog, is secreted as a monomer and dimer that each bind rabbit TNFalpha, but the dimer is a more potent TNF inhibitor. *J Biol Chem* **271**(23), 13333-41.

Schreiber, M., Sedger, L., and McFadden, G. (1997). Distinct domains of M-T2, the myxoma virus tumor necrosis factor (TNF) receptor homolog, mediate extracellular TNF binding and intracellular apoptosis inhibition. *J Virol* **71**(3), 2171-81.

Sedger, L., and McFadden, G. (1996). M-T2: a poxvirus TNF receptor homologue with dual activities. *Immunol Cell Biol* **74**(6), 538-45.

Sester, M., and Burgert, H. G. (1994). Conserved cysteine residues within the E3/19K protein of adenovirus type 2 are essential for binding to major histocompatibility complex antigens. *J Virol* **68**(9), 5423-32.

Shalloway, D., Johnson, P. J., Freed, E. O., Coulter, D., and Flood, W. A., Jr. (1987). Transformation of NIH 3T3 cells by cotransfection with c-src and nuclear oncogenes. *Mol Cell Biol* **7**(10), 3582-90.

Shao, R., Hu, M. C., Zhou, B. P., Lin, S. Y., Chiao, P. J., von Lindern, R. H., Spohn, B., and Hung, M. C. (1999). E1A sensitizes cells to tumor necrosis factor-induced apoptosis through inhibition of IkappaB kinases and nuclear factor kappaB activities. *J Biol Chem* **274**(31), 21495-8.

Shisler, J., Yang, C., Walter, B., Ware, C. F., and Gooding, L. R. (1997). The adenovirus E3-10.4K/14.5K complex mediates loss of cell surface Fas (CD95) and resistance to Fas-induced apoptosis. *J Virol* **71**(11), 8299-306.

Shisler, J. L., Senkevich, T. G., Berry, M. J., and Moss, B. (1998). Ultraviolet-induced cell death blocked by a selenoprotein from a human dermatotropic poxvirus [see comments]. *Science* **279**(5347), 102-5.

Shtrichman, R., Sharf, R., Barr, H., Dobner, T., and Kleinberger, T. (1999). Induction of apoptosis by adenovirus E4orf4 protein is specific to transformed cells and requires an interaction with protein phosphatase 2A. *Proc Natl Acad Sci U S A* **96**(18), 10080-5.

Shu, H. B., Hu, W. H., and Johnson, H. (1999). TALL-1 is a novel member of the TNF family that is down-regulated by mitogens. *J Leukoc Biol* **65**(5), 680-3.

Silberstein, S., Collins, P. G., Kelleher, D. J., and Gilmore, R. (1995). The essential OST2 gene encodes the 16-kD subunit of the yeast oligosaccharyltransferase, a highly conserved protein expressed in diverse eukaryotic organisms. *J Cell Biol* **131**(2), 371-83.

Slee, E. A., Harte, M. T., Kluck, R. M., Wolf, B. B., Casiano, C. A., Newmeyer, D. D., Wang, H. G., Reed, J. C., Nicholson, D. W., Alnemri, E. S., Green, D. R., and Martin, S. J. (1999). Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9- dependent manner. *J Cell Biol* **144**(2), 281-92.

Smith, C. A., Davis, T., Wignall, J. M., Din, W. S., Farrah, T., Upton, C., McFadden, G., and Goodwin, R. G. (1991). T2 open reading frame from the Shope fibroma virus encodes a soluble form of the TNF receptor. *Biochem Biophys Res Commun* **176**(1), 335-42.

Smith, C. A., Hu, F. Q., Smith, T. D., Richards, C. L., Smolak, P., Goodwin, R. G., and Pickup, D. J. (1996). Cowpox virus genome encodes a second soluble homologue of cellular TNF receptors, distinct from CrmB, that binds TNF but not LT alpha. *Virology* **223**(1), 132-47.

Song, W., Raden, D., Mandon, E. C., and Gilmore, R. (2000). Role of Sec61alpha in the regulated transfer of the ribosome-nascent chain complex from the signal recognition particle to the translocation channel. *Cell* **100**(3), 333-43.

Sparer, T. E., Tripp, R. A., Dillehay, D. L., Hermiston, T. W., Wold, W. S., and Gooding, L. R. (1996). The role of human adenovirus early region 3 proteins (gp19K, 10.4K, 14.5K, and 14.7K) in a murine pneumonia model. *J Virol* **70**(4), 2431-9.

Srivastava, R. K., Sasaki, C. Y., Hardwick, J. M., and Longo, D. L. (1999a). Bcl-2-mediated drug resistance: inhibition of apoptosis by blocking nuclear factor of activated T lymphocytes (NFAT)-induced Fas ligand transcription. *J Exp Med* **190**(2), 253-65.

Srivastava, R. K., Sollott, S. J., Khan, L., Hansford, R., Lakatta, E. G., and Longo, D. L. (1999b). Bcl-2 and Bcl-X(L) block thapsigargin-induced nitric oxide generation, c-Jun NH(2)-terminal kinase activity, and apoptosis. *Mol Cell Biol* **19**(8), 5659-74.

Stanley, W. M., and Valens, E. G. (1961). "Viruses and the nature of life." [1st ] ed. Dutton, New York,.

Steegenga, W. T., Riteco, N., Jochemsen, A. G., Fallaux, F. J., and Bos, J. L. (1998). The large E1B protein together with the E4orf6 protein target p53 for active degradation in adenovirus infected cells. *Oncogene* **16**(3), 349-57.

Steegenga, W. T., Shvarts, A., Riteco, N., Bos, J. L., and Jochemsen, A. G. (1999). Distinct regulation of p53 and p73 activity by adenovirus E1A, E1B, and E4orf6 proteins. *Mol Cell Biol* **19**(5), 3885-94.

Stewart, A. R., Tollefson, A. E., Krajcsi, P., Yei, S. P., and Wold, W. S. (1995). The adenovirus E3 10.4K and 14.5K proteins, which function to prevent cytolysis by tumor necrosis factor and to down-regulate the epidermal growth factor receptor, are localized in the plasma membrane. *J Virol* **69**(1), 172-81.

Sugimoto, A., Hozak, R. R., Nakashima, T., Nishimoto, T., and Rothman, J. H. (1995). dad-1, an endogenous programmed cell death suppressor in Caenorhabditis elegans and vertebrates. *Embo J* **14**(18), 4434-41.

Sundstrom, C., and Nilsson, K. (1976). Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int J Cancer* **17**(5), 565-77.

Suomalainen, M., Nakano, M. Y., Keller, S., Boucke, K., Stidwill, R. P., and Greber, U. F. (1999). Microtubule-dependent plus- and minus end-directed motilities are competing processes for nuclear targeting of adenovirus. *J Cell Biol* **144**(4), 657-72.

Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D. R., Aebersold, R., Siderovski, D. P., Penninger, J. M., and Kroemer, G. (1999). Molecular characterization of mitochondrial apoptosis-inducing factor [see comments]. *Nature* **397**(6718), 441-6.

Susin, S. A., Zamzami, N., Castedo, M., Daugas, E., Wang, H. G., Geley, S., Fassy, F., Reed, J. C., and Kroemer, G. (1997). The central executioner of apoptosis: multiple connections between protease activation and mitochondria in Fas/APO-1/CD95- and ceramide- induced apoptosis. *J Exp Med* **186**(1), 25-37.

Szalai, G., Krishnamurthy, R., and Hajnoczky, G. (1999). Apoptosis driven by IP(3)-linked mitochondrial calcium signals. *Embo J* **18**(22), 6349-61.

Teodoro, J. G., Shore, G. C., and Branton, P. E. (1995). Adenovirus E1A proteins induce apoptosis by both p53-dependent and p53- independent mechanisms. *Oncogene* **11**(3), 467-74.

Tewari, M., and Dixit, V. M. (1995). Fas- and tumor necrosis factor-induced apoptosis is inhibited by the poxvirus crmA gene product. *J Biol Chem* **270**(7), 3255-60.

Thome, M., Schneider, P., Hofmann, K., Fickenscher, H., Meinl, E., Neipel, F., Mattmann, C., Burns, K., Bodmer, J. L., Schroter, M., Scaffidi, C., Krammer, P. H., Peter, M. E., and Tschopp, J. (1997). Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* **386**(6624), 517-21.

Thompson, J. S., Schneider, P., Kalled, S. L., Wang, L., Lefevre, E. A., Cachero, T. G., MacKay, F., Bixler, S. A., Zafari, M., Liu, Z. Y., Woodcock, S. A., Qian, F., Batten, M., Madry, C., Richard, Y., Benjamin, C. D., Browning, J. L., Tsapis, A., Tschopp, J., and Ambrose, C. (2000). BAFF binds to the tumor necrosis factor receptor-like molecule B cell maturation antigen and is important for maintaining the peripheral B cell population [In Process Citation]. *J Exp Med* **192**(1), 129-36.

Thorne, T. E., Voelkel-Johnson, C., Casey, W. M., Parks, L. W., and Laster, S. M. (1996). The activity of cytosolic phospholipase A2 is required for the lysis of adenovirus-infected cells by tumor necrosis factor. *J Virol* **70**(12), 8502-7.

Ting, A. T., Pimentel-Muinos, F. X., and Seed, B. (1996). RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. *Embo J* **15**(22), 6189-96.

Tollefson, A. E., Hermiston, T. W., Lichtenstein, D. L., Colle, C. F., Tripp, R. A., Dimitrov, T., Toth, K., Wells, C. E., Doherty, P. C., and Wold, W. S. (1998). Forced degradation of Fas inhibits apoptosis in adenovirus-infected cells. *Nature* **392**(6677), 726-30.

Tollefson, A. E., Ryerse, J. S., Scaria, A., Hermiston, T. W., and Wold, W. S. (1996a). The E3-11.6-kDa adenovirus death protein (ADP) is required for efficient cell death: characterization of cells infected with adp mutants. *Virology* **220**(1), 152-62.

Tollefson, A. E., Scaria, A., Hermiston, T. W., Ryerse, J. S., Wold, L. J., and Wold, W. S. (1996b). The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. *J Virol* **70**(4), 2296-306.

Tollefson, A. E., Scaria, A., Saha, S. K., and Wold, W. S. (1992). The 11,600-MW protein encoded by region E3 of adenovirus is expressed early but is greatly amplified at late stages of infection. *J Virol* **66**(6), 3633-42.

Tollefson, A. E., Stewart, A. R., Yei, S. P., Saha, S. K., and Wold, W. S. (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**(6), 3095-105.

Tortorella, D., Gewurz, B. E., Furman, M. H., Schust, D. J., and Ploegh, H. L. (2000). Viral subversion of the immune system. *Annu Rev Immunol* **18**, 861-926.

Townsend, A. R., Rothbard, J., Gotch, F. M., Bahadur, G., Wraith, D., and McMichael, A. J. (1986). The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* **44**(6), 959-68.

Tsai, H. L., Kou, G. H., Chen, S. C., Wu, C. W., and Lin, Y. S. (1996). Human cytomegalovirus immediate-early protein IE2 tethers a transcriptional repression domain to p53. *J Biol Chem* **271**(7), 3534-40.

Tschopp, J., Irmler, M., and Thome, M. (1998). Inhibition of fas death signals by FLIPs. *Curr Opin Immunol* **10**(5), 552-8.

Universite de, L. (1923). "Les Travaux de Pasteur : discours et conferences faits a l'Universite de Lille en 1922-1923 en commemoration du centenaire de Louis Pasteur." Marquant, Lille.

Upton, C., Macen, J. L., Schreiber, M., and McFadden, G. (1991). Myxoma virus expresses a secreted protein with homology to the tumor necrosis factor receptor gene family that contributes to viral virulence. *Virology* **184**(1), 370-82.

Upton, C., Macen, J. L., Wishart, D. S., and McFadden, G. (1990). Myxoma virus and malignant rabbit fibroma virus encode a serpin-like protein important for virus virulence. *Virology* **179**(2), 618-31.

van Klompenburg, W., Nilsson, I., von Heijne, G., and de Kruijff, B. (1997). Anionic phospholipids are determinants of membrane protein topology. *Embo J* **16**(14), 4261-6.

van Kuppeveld, F. J., Hoenderop, J. G., Smeets, R. L., Willems, P. H., Dijkman, H. B., Galama, J. M., and Melchers, W. J. (1997). Coxsackievirus protein 2B modifies endoplasmic reticulum membrane and plasma membrane permeability and facilitates virus release. *Embo J* **16**(12), 2519-32

Vanags, D. M., Porn-Ares, M. I., Coppola, S., Burgess, D. H., and Orrenius, S. (1996). Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis. *J Biol Chem* **271**(49), 31075-85.

Villalba, M., Kasibhatla, S., Genestier, L., Mahboubi, A., Green, D. R., and Altman, A. (1999). Protein kinase ctheta cooperates with calcineurin to induce Fas ligand expression during activation-induced T cell death. *J Immunol* **163**(11), 5813-9.

Vincenz, C., and Dixit, V. M. (1997). Fas-associated death domain protein interleukin-1beta-converting enzyme 2 (FLICE2), an ICE/Ced-3 homologue, is proximally involved in CD95- and p55-mediated death signaling. *J Biol Chem* **272**(10), 6578-83.

Virgin, H. W. t., Latreille, P., Wamsley, P., Hallsworth, K., Weck, K. E., Dal Canto, A. J., and Speck, S. H. (1997). Complete sequence and genomic analysis of murine gammaherpesvirus 68. *J Virol* **71**(8), 5894-904.

Voelkel-Johnson, C., Thorne, T. E., and Laster, S. M. (1996). Susceptibility to TNF in the presence of inhibitors of transcription or translation is dependent on the activity of cytosolic phospholipase A2 in human melanoma tumor cells. *J Immunol* **156**(1), 201-7.

von Bulow, G. U., and Bram, R. J. (1997). NF-AT activation induced by a CAML-interacting member of the tumor necrosis factor receptor superfamily. *Science* **278**(5335), 138-41.

von Heijne, G., and Gavel, Y. (1988). Topogenic signals in integral membrane proteins. *Eur J Biochem* **174**(4), 671-8.

Walter, P., and Blobel, G. (1981). Translocation of proteins across the endoplasmic reticulum III. Signal recognition protein (SRP) causes signal sequence-dependent and site- specific arrest of chain elongation that is released by microsomal membranes. *J Cell Biol* **91**(2 Pt 1), 557-61.

Wang, H. G., Pathan, N., Ethell, I. M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T. F., and Reed, J. C. (1999). Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD. *Science* **284**(5412), 339-43.

Wang, K., Guan, T., Cheresh, D. A., and Nemerow, G. R. (2000). Regulation of adenovirus membrane penetration by the cytoplasmic tail of integrin beta5 [In Process Citation]. *J Virol* **74**(6), 2731-9.

Wang, K., Huang, S., Kapoor-Munshi, A., and Nemerow, G. (1998). Adenovirus internalization and infection require dynamin. *J Virol* **72**(4), 3455-8.

Ware, C. F. (2000). Decoy receptors thwart B cells [news; comment]. *Nature* **404**(6781), 949-50.

Weeks, D. L., and Jones, N. C. (1983). E1A control of gene expression is mediated by sequences 5' to the transcriptional starts of the early viral genes. *Mol Cell Biol* **3**(7), 1222-34.

Weis, M., Schlegel, J., Kass, G. E., Holmstrom, T. H., Peters, I., Eriksson, J., Orrenius, S., and Chow, S. C. (1995). Cellular events in Fas/APO-1-mediated apoptosis in JURKAT T lymphocytes. *Exp Cell Res* **219**(2), 699-708.

Weiss, A., Imboden, J., Shoback, D., and Stobo, J. (1984). Role of T3 surface molecules in human T-cell activation: T3-dependent activation results in an increase in cytoplasmic free calcium. *Proc Natl Acad Sci U S A* **81**(13), 4169-73.

White, E. (1996). Life, death, and the pursuit of apoptosis. *Genes Dev* **10**(1), 1-15.

White, E., Cipriani, R., Sabbatini, P., and Denton, A. (1991). Adenovirus E1B 19-kilodalton protein overcomes the cytotoxicity of E1A proteins. *J Virol* **65**(6), 2968-78.

White, E., Sabbatini, P., Debbas, M., Wold, W. S., Kusher, D. I., and Gooding, L. R. (1992). The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor alpha. *Mol Cell Biol* **12**(6), 2570-80.

Whitley, P., Grahn, E., Kutay, U., Rapoport, T. A., and von Heijne, G. (1996). A 12-residue-long polyleucine tail is sufficient to anchor synaptobrevin to the endoplasmic reticulum membrane. *J Biol Chem* **271**(13), 7583-6.

Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A., and Harlow, E. (1988). Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature* **334**(6178), 124-9.

Whyte, P., Williamson, N. M., and Harlow, E. (1989). Cellular targets for transformation by the adenovirus E1A proteins. *Cell* **56**(1), 67-75.

Wickham, T. J., Filardo, E. J., Cheresh, D. A., and Nemerow, G. R. (1994). Integrin alpha v beta 5 selectively promotes adenovirus mediated cell membrane permeabilization. *J Cell Biol* **127**(1), 257-64.

Wickham, T. J., Mathias, P., Cheresh, D. A., and Nemerow, G. R. (1993). Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell* **73**(2), 309-19.

Wickner, W. (1995). The nascent-polypeptide-associated complex: having a "NAC" for fidelity in translocation [comment]. *Proc Natl Acad Sci U S A* **92**(21), 9433-4.

Wiedmann, B., Sakai, H., Davis, T. A., and Wiedmann, M. (1994). A protein complex required for signal-sequence-specific sorting and translocation. *Nature* **370**(6489), 434-40.

Wienzek, S., Roth, J., and Dobbelstein, M. (2000). E1B 55-kilodalton oncoproteins of adenovirus types 5 and 12 inactivate and relocalize p53, but not p51 or p73, and cooperate with E4orf6 proteins to destabilize p53. *J Virol* **74**(1), 193-202.

Williams, B. R. (1999). PKR; a sentinel kinase for cellular stress. *Oncogene* **18**(45), 6112-20.

Wilson-Rawls, J., Deutscher, S. L., and Wold, W. S. (1994). The signal-anchor domain of adenovirus E3-6.7K, a type III integral membrane protein, can direct adenovirus E3-gp19K, a type I integral membrane protein, into the membrane of the endoplasmic reticulum. *Virology* **201**(1), 66-76.

Wilson-Rawls, J., Saha, S. K., Krajcsi, P., Tollefson, A. E., Gooding, L. R., and Wold, W. S. (1990). A 6700 MW membrane protein is encoded by region E3 of adenovirus type 2. *Virology* **178**(1), 204-12.

Wilson-Rawls, J., and Wold, W. S. (1993). The E3-6.7K protein of adenovirus is an Asn-linked integral membrane glycoprotein localized in the endoplasmic reticulum. *Virology* **195**(1), 6-15.

Wold, W. S., Cladaras, C., Deutscher, S. L., and Kapoor, Q. S. (1985). The 19-kDa glycoprotein coded by region E3 of adenovirus. Purification, characterization, and structural analysis. *J Biol Chem* **260**(4), 2424-31.

Wold, W. S., Deutscher, S. L., Takemori, N., Bhat, B. M., and Magie, S. C. (1986). Evidence that AGUAUAUGA and CCAAGAUGA initiate translation in the same mRNA region E3 of adenovirus. *Virology* **148**(1), 168-80.

Wold, W. S., and Gooding, L. R. (1991). Region E3 of adenovirus: a cassette of genes involved in host immunosurveillance and virus-cell interactions. *Virology* **184**(1), 1-8.

Wondrak, E. (1991). [Dr. Stanislav Prowazek (1875-1915) at the closing of his life]. *Cas Lek Cesk* **130**(2), 57-9.

Woronicz, J. D., Calnan, B., Ngo, V., and Winoto, A. (1994). Requirement for the orphan steroid receptor Nur77 in apoptosis of T- cell hybridomas. *Nature* **367**(6460), 277-81.

Wu, Y. L., Jiang, X. R., Newland, A. C., and Kelsey, S. M. (1998). Failure to activate cytosolic phospholipase A2 causes TNF resistance in human leukemic cells. *J Immunol* **160**(12), 5929-35.

Xia, X. Z., Treanor, J., Senaldi, G., Khare, S. D., Boone, T., Kelley, M., Theill, L. E., Colombero, A., Solovyev, I., Lee, F., McCabe, S., Elliott, R., Miner, K., Hawkins, N., Guo, J., Stolina, M., Yu, G., Wang, J., Delaney, J., Meng, S. Y., Boyle, W. J., and Hsu, H. (2000). TACI is a TRAF-interacting receptor for TALL-1, a tumor necrosis factor family member involved in B cell regulation [In Process Citation]. *J Exp Med* **192**(1), 137-44.

Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases [see comments]. *Nature* **366**(6456), 701-4.

Xue, D., and Horvitz, H. R. (1995). Inhibition of the Caenorhabditis elegans cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein. *Nature* **377**(6546), 248-51.

Yang, Y., Li, Q., Ertl, H. C., and Wilson, J. M. (1995). Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J Virol* **69**(4), 2004-15.

Yang, Y., Trinchieri, G., and Wilson, J. M. (1995). Recombinant IL-12 prevents formation of blocking IgA antibodies to recombinant adenovirus and allows repeated gene therapy to mouse lung [see comments]. *Nat Med* **1**(9), 890-3.

Yoshida, H., Kong, Y. Y., Yoshida, R., Elia, A. J., Hakem, A., Hakem, R., Penninger, J. M., and Mak, T. W. (1998). Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell* **94**(6), 739-50.

Yost, C. S., Lopez, C. D., Prusiner, S. B., Myers, R. M., and Lingappa, V. R. (1990). Non-hydrophobic extracytoplasmic determinant of stop transfer in the prion protein. *Nature* **343**(6259), 669-72.

Youn, H. D., Sun, L., Prywes, R., and Liu, J. O. (1999). Apoptosis of T cells mediated by Ca2+-induced release of the transcription factor MEF2. *Science* **286**(5440), 790-3.

Yujiri, T., Sather, S., Fanger, G. R., and Johnson, G. L. (1998). Role of MEKK1 in cell survival and activation of JNK and ERK pathways defined by targeted gene disruption. *Science* **282**(5395), 1911-4.

Zamzami, N., Susin, S. A., Marchetti, P., Hirsch, T., Gomez-Monterrey, I., Castedo, M., and Kroemer, G. (1996). Mitochondrial control of nuclear apoptosis [see comments]. *J Exp Med* **183**(4), 1533-44.

Zauli, G., Gibellini, D., Caputo, A., Bassini, A., Negrini, M., Monne, M., Mazzoni, M., and Capitani, S. (1995). The human immunodeficiency virus type-1 Tat protein upregulates Bcl-2 gene expression in Jurkat T-cell lines and primary peripheral blood mononuclear cells. *Blood* **86**(10), 3823-34.

Zerial, M., Melancon, P., Schneider, C., and Garoff, H. (1986). The transmembrane segment of the human transferrin receptor functions as a signal peptide. *Embo J* **5**(7), 1543-50.

Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996). Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L) [see comments]. *Cell* **87**(4), 619-28.

Zhang, J. T., Chen, M., Foote, C. I., and Nicholson, B. J. (1996). Membrane integration of in vitro-translated gap junctional proteins: co- and post-translational mechanisms. *Mol Biol Cell* **7**(3), 471-82.

Zhang, J. T., Duthie, M., and Ling, V. (1993). Membrane topology of the N-terminal half of the hamster P-glycoprotein molecule. *J Biol Chem* **268**(20), 15101-10.

Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V. M., and Salvesen, G. S. (1997). Target protease specificity of the viral serpin CrmA. Analysis of five caspases. *J Biol Chem* **272**(12), 7797-800.

Zhu, H., Shen, Y., and Shenk, T. (1995). Human cytomegalovirus IE1 and IE2 proteins block apoptosis. *J Virol* **69**(12), 7960-70.

Zhu, L., Ling, S., Yu, X. D., Venkatesh, L. K., Subramanian, T., Chinnadurai, G., and Kuo, T. H. (1999). Modulation of mitochondrial Ca(2+) homeostasis by Bcl-2. *J Biol Chem* **274**(47), 33267-73.

Zhu, W., Cowie, A., Wasfy, G. W., Penn, L. Z., Leber, B., and Andrews, D. W. (1996). Bcl-2 mutants with restricted subcellular location reveal spatially distinct pathways for apoptosis in different cell types. *Embo J* **15**(16), 4130-41.

Zilli, D., Voelkel-Johnson, C., Skinner, T., and Laster, S. M. (1992). The adenovirus E3 region 14.7 kDa protein, heat and sodium arsenite inhibit the TNF-induced release of arachidonic acid. *Biochem Biophys Res Commun* **188**(1), 177-83.