EFFECT OF ADENOVIRUS E3/19K PROTEIN
ON CELLULAR PROCESSES IN THE ENDOPLASMIC RETICULUM

by

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Abstract

The human adenovirus has developed several methods to evade the immune system. One mechanism by which it accomplishes this involves the endoplasmic reticulum (ER) retained adenovirus E3/19K protein. This protein interferes with antigen presentation by binding and retaining Major Histocompatibility Complex Class I (MHC Cl I) proteins in the ER.

E3/19K binds all human and all but one mouse MHC Cl I molecules tested to date. Differences in mouse MHC Cl I sequences were exploited to determine the structures involved in binding. Human 293 cells transfected with the mouse H-2 alleles K^d, K^b, K^k, D^d, D^b, L^d were infected with adenovirus 2. It was found that MHC Cl I alleles could be grouped into three categories. The H-2 allelic proteins K^d and K^b were found to be binders; K^k and D^d non binders and D^b and L^d slow binders.

Examination of a cell line transfected with the slow-binding H-2D^b protein revealed that D^b is expressed at a reduced level at the cell surface. To determine the cause of this, cells were subjected to conditions previously used to restore defective cell surface expression of MHC Cl I including culture at reduced temperature, addition of excess β_2m and exposure to gamma interferon. All these methods were unsuccessful in increasing cell surface expression of D^b. Rather than being due to a missing co-factor or unstable conformation, the accumulation of the allelic proteins in the ER in this transfectant was due to an undetermined mechanism.

Because E3/19K binding quickly stabilised a mature MHC Cl I conformation in the presence of tunicamycin it was suggested that it bound MHC Cl I like a chaperone. It was found that

E3/19K binding to MHC Cl I did not block the association of endogenous ER resident chaperones calnexin and TAP. Peptide binding to the MHC Cl I-E3/19K complex could also occur. These experiments showed that E3/19K did not associate with MHC Cl I through the peptide binding groove and did not disrupt the interaction between the MHC Cl I and the processing machinery found in the ER, namely calnexin and TAP, and therefore does not retain MHC molecules by making them conformationally immature.

This study shows E3/19K works independently of chaperones. E3/19K may be a tool to trap MHC Cl I - chaperone complexes at a specific point in their maturation.

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Nomenclature

293	human cell line derived from embryonic kidney carcinoma
293.12	293 cell line transfected with E3/19K
621	293 cell line transfected with truncated E3/19K
Ad	adenovirus
$\beta_2 m$	beta-2-microglobulin
BiP	heat shock protein in the ER
BSA	bovine serum albumin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSP	dithiobis (succinimidylpropionate)
DTT	dithiothreitol
E3/19K	19 kilodalton adenovirus protein coded in early region 3
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
Endo H	endoglycosidase H
ER	endoplasmic reticulum
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorter
Gal	galactose
γIFN	gamma interferon
H-2	mouse MHC Cl I region The mouse versions are K, D and L
HEPES	2-(2-hydroxyyethyl)-1-piperadineethanesulfonic acid
HLA	human MHC Cl I region HLA A, B, and C
HRPO	horse radish peroxidase
Ig	immunoglobulin
IR	immune response
K^d, K^b, etc	Alleles d and b of the mouse H-2K gene
kDa	kilodalton
mAb	monoclonal antibody
MHC	Major Histocompatibility Complex
MW	molecular weight
NRS	normal rabbit sera
PMSF	phenylmethylsufonylfluoride
R425, R426	rabbit anti MHC Cl antisera
SA-HRPO	streptavidin horse radish peroxidase
SDS	sodium dodecyl sulfate
TAP	transporters associated with antigen processing
TCA	trichloroacetic acid
TcR	T cell receptor
UPR	unfolded protein response
W6/32	monoclonal that detects mature Cl I HLA

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1. Introduction

The human body with its immune system is like a heavily fortified medieval donjon. While a great number of attacks on the integrity of the body are thwarted on a continual basis, breaches of the immune system by pathogens can result in discomfort, disease and worse.

Persistent viral infections result when viruses escape detection by the immune system. Many viruses have evolved a set of mechanisms that effectively target susceptible components of the immune system (1). These viruses can propagate undetected and unchallenged by the host immune system. Persistent viral infections offer a good model to examine the immune system. Investigation of the strategies used by viruses to escape the immune system provides a useful method for understanding the components involved in antigen presentation and the interactions between them.

This study investigates the role of the endoplasmic reticulum (ER) resident E3/19K protein of human adenovirus in the immune system, protein processing and maturation through the ER and Golgi, antigen presentation and viral clearing.

1.1. Immunity

An immune system is present in all vertebrates and many invertebrates. It is a mechanism employed by organisms to protect themselves from pathogens and other foreign materials. This system also serves as a method to detect self from non-self. While in many lower organisms this differentiation of self versus non-self is a simple mechanical differentiation between 'in and out', in higher vertebrates the immune system is more sophisticated with multi-faceted methods for preserving the integrity of the living body (2).

The immune system in humans and mice has both specific and non-specific methods for eliminating foreign substances. Non-specific mechanisms include physical barriers, temperature levels and pH to make living conditions hostile to potential pathogens.

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Additionally, lysozymes, interferons, complement and natural killer (NK) cells are present and non-specifically respond to foreign objects.

A second, more sophisticated method available to the body to defend itself are the specific responses to foreign material. These are known as the acquired immune response. Some features of the acquired immune response include immunological memory, the ability to respond to a high number and diverse set of antigens and the ability to recognize foreign antigens in the context of self antigens (3).

1.2. Pluripotent Stem Cells

The acquired immune response is driven by the action of lymphocytes. Pluripotent stem cells are the progenitors of two lines of cells, the lymphoid and the myeloid. The lymphoid line can be further divided into several cell types including B and T cells. B Cells have characterizing immunoglobulins present on their surface; T Cells are distinguished by the presence of a T Cell Receptor and Cluster Differentiation marker CD3.

1.3. B Cells

B Cells are specialized for immunoglobulin (antibody) production. Antibodies can be raised against almost any type of foreign antigen. Antibodies are very specific and one antibody recognizes one epitope. Immunoglobulins consist of a heavy (H) and light (L) chain. Different combinations of H and L chains can be assembled to generate a large diversity of antibodies specific for different antigens. The diversity of antigens against which antibodies can be raised is considerable.

1.4. T Cells

T Cells recognize fragments of proteins bound to self MHC molecules (4). Viral (and other) proteins are degraded into peptides. These peptides are bound by major

histocompatibility complex (MHC) molecules and recognized by T cell receptors (TCR) at the surface of infected cells (5). Both MHC Cl I and Cl II molecules present foreign (and endogenous) antigens to the TCR of circulating T cells (6). The T Cell population can be further divided in $CD4^+$ and $CD8^+$ populations. $CD4^+$ cells are involved in the recognition of MHC Cl II with bound peptide; $CD8^+$ cells are involved in the recognition of MHC Cl I with bound peptide.

Immunoglobulins and the TCR are structurally related to one another and are part of the immunoglobulin gene superfamily. Additional members of this family also include MHC Cl I, Cl II, CD4, CD8, β_2m, MRC-OX2, ICAM-1, ICAM-2 and many others. These proteins have a characteristic 110 residue domain and a cysteine bridge (7). It is likely that these proteins evolved from a limited number of original genes.

The great diversity present in both immunoglobulins and the TCR is the result of gene rearrangements. This diversity is produced by rearrangements between V, D and J segments. RAG-1 and RAG-2 genes are responsible for this genetic rearrangement (8).

As the primary methods for clearing viral infections include removing infected cells from the host before the viral cycle has completed, detection of infected cells is very important. T Cells effect the killing, recognizing the processed foreign proteins presented at the cell surface of an infected cell by a MHC Cl I protein (9). In cases where the immune system is unable to clear the viral infection, persistent infection occurs. The effects of the persistent infection will not become apparent until the general health of the victim declines, often as the patient succumbs to old age or another illness (such as HIV). Some of the viruses that cause persistent infections include Herpes Simplex Virus (HSV), Cytomegalovirus (CMV), Adenovirus (Ad), and Human Immunodeficiency Virus (HIV). Each has particular and differing strategies to achieve a persistent infection due to 'stealth' (10).

1.5. MHC

The major histocopatibility complex (MHC) is one target of persistent viruses. The MHC region is found in all vertebrates and many invertebrates (11). Precursors to the MHC are also found in some invertebrates. The region codes for more than one thousand proteins including many that play key roles in the immune system. These proteins include MHC Cl I , Cl II, complement, TAP 1 and 2.

The MHC region is on chromosome 17 in humans and chromosome 6 in mice. There are three MHC Cl I loci in both humans (HLA-A, B and C) and mice (H-2 K, D and L) (12). Polymorphism at the MHC Cl I locus is extensive. With as many as one hundred alleles at each loci, the diversity found within the population is very high. MHC Cl I molecules have been studied extensively, with Cl I regions studied in vertebrates ranging from xenopus to human (figure 1).

Figure 1. The MHC Cl I regions for different species

MHC regions for many different species have been identified based on similarities between species (12). MHC Cl I genes are represented by white boxes; MHC Cl II genes by black boxes.
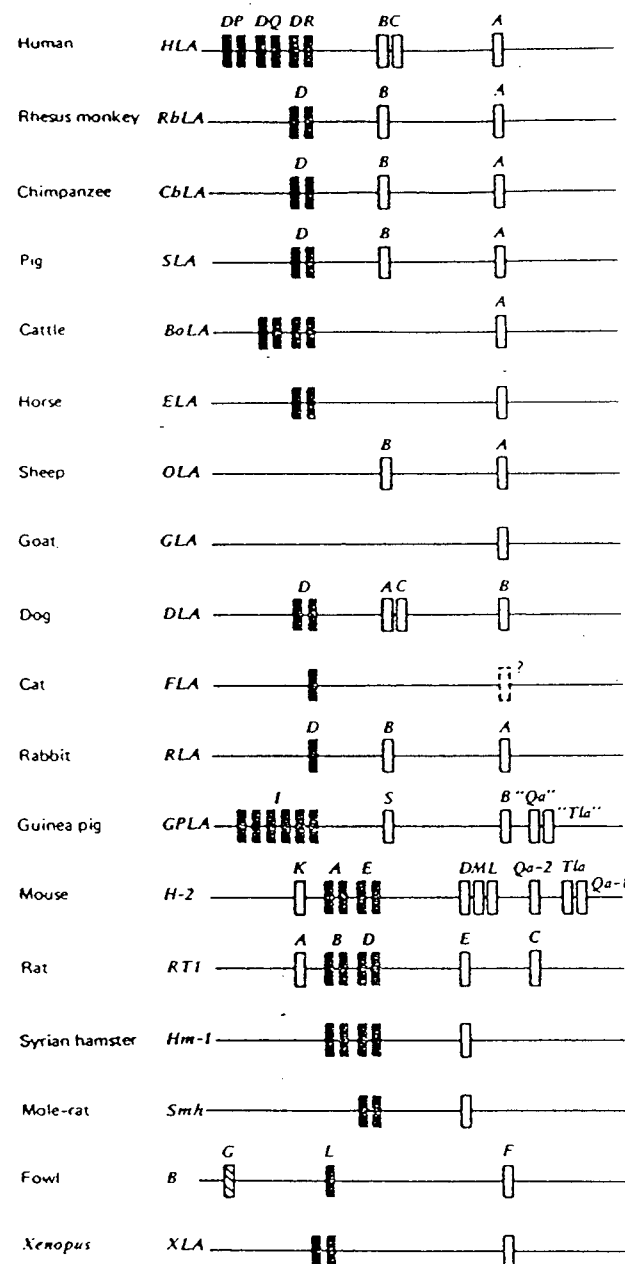
MHC Cl I is found noncovalently associated with beta-2-microglobulin (β_2m) at the cell surface of most nucleated cells in the body. MHC Cl I molecules consist of three extracellular domains (alpha 1-3), a transmembrane region and a cytoplasmic tail. Each extracellular domain consists of about 90 amino acids and is also a member of the Ig superfamily. The MHC Cl I molecule is glycosylated at asparagine 86 in humans with a second (and sometimes third) glycosylation site in mice. The mature MHC Cl I molecule is about 46-55 kilodaltons (kDa). The polymorphic regions of the MHC Cl I molecule are found mainly in the alpha 1 and alpha 2 regions. The genetic organization of the MHC Cl I region reveals many exons and introns. Each of the three alpha regions and the transmembrane region are encoded by a separate exon and the cytoplasmic tail is comprised of three exons (figure 2).

Figure 2. The MHC Cl I gene

The MHC Cl I gene is made up of large exon and intron regions (12). Similarities between the exons (black boxes) and introns of mouse (K^d and L^d, non classical Q6), human HLA 12.4 and $\beta_2 m$ are depicted below.
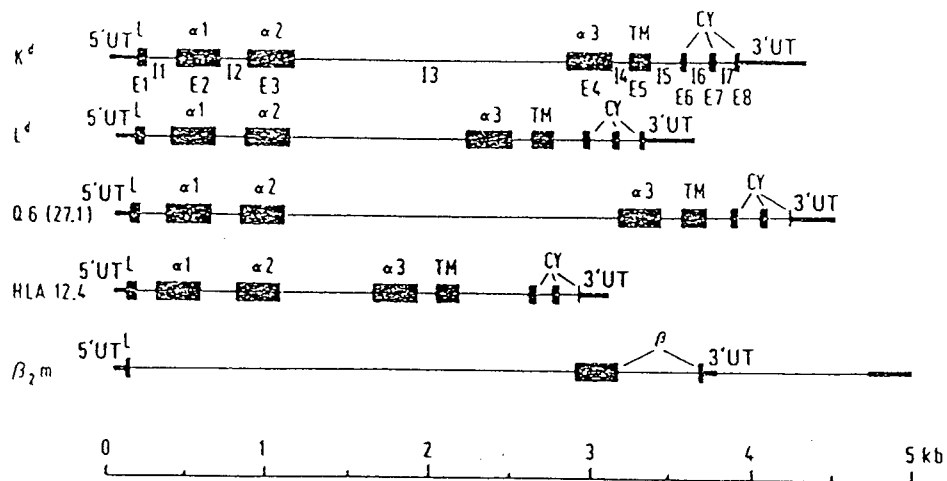
Since antigens are presented by MHC molecules, extensive polymorphism allows for great variability in the number and types of antigens that can be presented within a population. Some allelelic forms of MHC Cl I proteins present specific antigens far more efficiently than others. Individuals with certain alleles are more susceptible to some disease because their MHC Cl I is simply unable to present the antigen to their immune system. For example, certain autoimmune diseases have been linked to specific HLA alleles.

Structurally, the MHC Cl I molecule consists of two antiparallel alpha helices (alpha 1 and 2) resting on top of two antiparallel beta pleated sheets (alpha 3) (13). The helices form a 25 Å long by 10 Å wide groove. The highly conserved 12 kDa protein β_2m is non-covalently associated with the alpha 3 domain.

The crystal structure for MHC Cl I has been determined for many allelic proteins. The ribbon structure is demonstrated in figure 3. Peptide is bound in the groove between the alpha 1 and alpha 2 regions (fig 3a). Peptide hypervariable regions of the MHC Cl I molecule are found near the peptide binding groove. The resolved crystal structures have demonstrated the existence of pockets inside the groove into which the R-groups of bound peptides fit tightly. Peptide sequence constrains which peptides fit into the groove. Different allelic forms of MHC Cl I bind sets of peptides limited in sequence by key 'anchor' positions. Peptides derived from viral (and endogenous) proteins are tightly bound in the peptide binding groove. Figure 3b illustrates the peptide binding groove from the top. This association is a requisite step in the assembly of a stable MHC Cl I molecule (14).

Figure 3. Crystal structure of MHC Cl I

The crystal structure of MHC Cl I reveals the presence a large peptide binding groove at the top. In a) the peptide binding groove is demonstrated lying between $\alpha 1$ and $\alpha 2$ regions. Also shown is the close association of $\beta_2 m$. In b) and c) the peptide binding groove is seen from above. The physical constraints of the groove permit limited sets of peptides to bind. The constraints on the peptide include pockets within the groove for amino acid R group side chains and well conserved anchor positions (13).

a α_1 α_2

$\beta_2 m$ α_3

b

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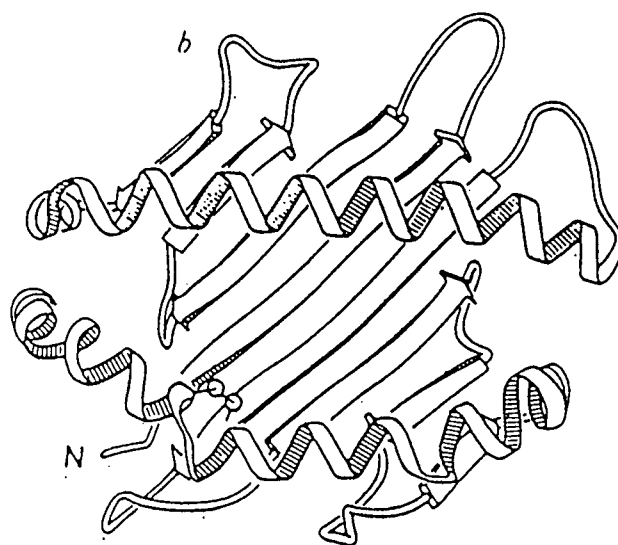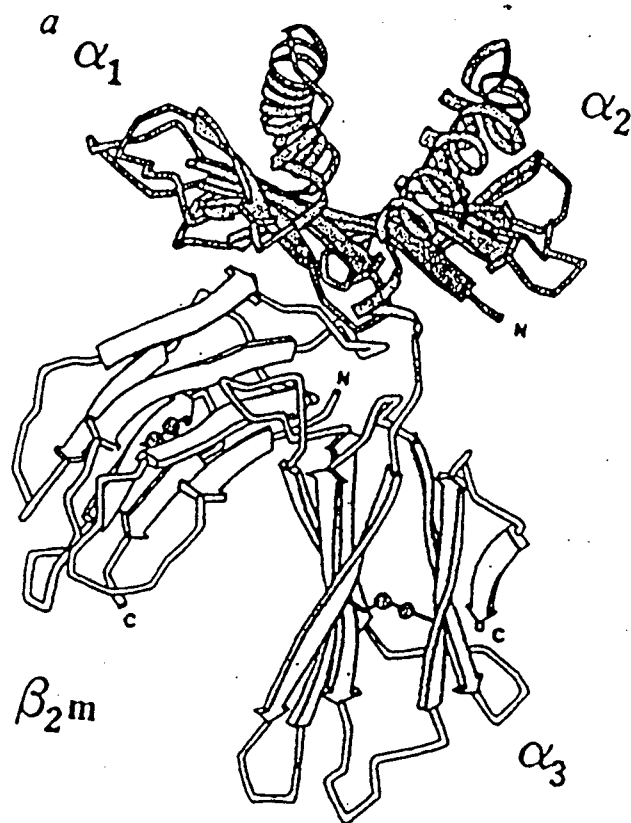
1.6. Cellular Mechanics

1.6.1. Peptides

The ability of the immune system to respond to millions of potential pathogens is due to the large number of different peptides that can be bound by the MHC Cl I molecule. Some investigators have suggested that potentially millions of different peptides can occupy the peptide binding groove of MHC Cl I. The TCR found on the surface of T cells recognises both the polymorphic region of the MHC Cl I molecule and the peptide contained within the groove. Recent studies demonstrate direct contact between the TCR and the peptide occurs (15).

A study in which self peptides eluted from MHC Cl I were sequenced identified many motifs of peptides associated with a single allelic protein (16). Other studies that examined the stability of MHC Cl I molecules associated with peptides showed that there is a hierarchy of peptide sequences that confer stability (17). The peptide specificity of different MHC Cl I allelic proteins is in part determined by the side chains of the amino acids lining the polymorphic peptide groove of the MHC Cl I molecule. Different allelic proteins of MHC Cl I have preferences for peptides ranging from 8 to 11 amino acids in length (18). Amino acid side chains of bound peptides extending into the groove dictate that certain peptide residue positions are regarded as anchor residues (19, 20). For example, the H-2 protein K^b preferentially binds octapeptides with a tyrosine or phenylalanine at position 5 and a Met or Ile at position 8 (18).

1.6.2. Proteasome

Peptides that bind to MHC Cl I molecules are generated within the cytoplasm by the proteasome. The proteasome is a barrel shaped structure made up of many distinct subunits.

Viral and endogenous proteins are tagged with ubiquitin and targeted for degradation by the proteasome.

The proteasome is well-conserved throughout many species ranging from Archaebacterium (21) through to humans and serves as a general cellular method to remove abnormal, short lived and otherwise redundant proteins. The proteolytic process starts with the tagging of the doomed protein with ubiquitin to mark the protein for hydrolysis by the 26S proteasome (22). The proteasome is also able to function without ubiquitin. The proteasome consists of a 20S Multi Catalytic Protease complex (MCP) catalytic core with additional subunits. The 20S core is approximately 650 kDa (23), composed of 13-15 subunits of similar size (24) stacked in a barrel shape and is found in the cytosol and the nucleus of all eukaryotic cells. It has multiple catalytic sites and comprises up to 1% of the total protein of a mammalian cell. Association of an ATPase complex results in a 26S proteasome which degrades ubiquitinated proteins in the presence of ATP (25).

In another configuration of the proteasome, the two subunits, LMP 2 and LMP 7, are also found. They are encoded in the MHC region, are upregulated by γ-IFN and have been shown to participate in a proteasome involved in the generation of peptides bound by MHC Cl I molecules (26).

Proteasome-generated peptides are presented to immature MHC Cl I molecules present in the ER by the MHC-encoded transporter associated with antigen presentation (TAP) proteins (27). Encoded in the MHC Cl II region, TAP 1 and 2 form a heterodimer that pumps peptides across the ER membrane in an ATP-dependent fashion. These two proteins are non-covalently associated in the ER. The TAP proteins are members of the ATP binding cassette (ABC) transporter protein family and show homology across species. The binding of TAP proteins with empty MHC Cl I heavy (H) chains and β_2m in the ER (28-30) is required for MHC loading with peptides (31). Cells with deficiency in the TAP proteins show reduced peptide

loading and antigen presentation (32-35).

Studies have demonstrated that stringency of these transporters is not high for peptides is not high. Peptides must be longer than 7 residues and peptides longer than 12 residues are transported with lower efficiency (36, 37). Some specificity in peptides transported by TAP has been shown. In rats, different alleles of TAP transporters favour different sets of peptides (38).

Like most plasma membrane and secretory proteins, MHC Cl I molecules are synthesized on ER bound ribosomes and transported across the ER membrane to the lumen of the ER. Processing occurs in the lumen of the ER and continues with transport from the ER to the cis, medial and trans sections of the Golgi to the cell surface. In general, proteins are transported out of the ER when they have achieved a correct conformation for exit. Treating cells with reducing agents or otherwise altering the conditions found in the ER can delay or even block transport of proteins out of the ER (39, 40). Several studies have shown that in the case of MHC Cl I molecules, exiting the ER can be the rate limiting step for cell surface expression.

1.7. Antigen Presentation

The maturation of the MHC Cl I into a trimolecular complex made up of heavy (H) chain, β_2m and bound peptide involves several specific proteins in the ER. Peptide is supplied by TAP 1 and TAP 2. Recent findings suggest that a 48 kDa glycoprotein called tapasin is also associated with the MHC Cl I-β_2m-TAP complex (41). Calnexin functions in a chaperone like manner for glycoproteins such as transferrin (42) and is required for association of MHC Cl I with TAP (43). The calcium-binding calreticulin protein also associates with nascent proteins following the association of calnexin. Both calnexin and calreticulin improve the chances of a mature conformation by preventing aggregation and premature disulphide bond formation (44).

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The interaction of many different intermediates results in antigen presentation by MHC Cl I at the cell surface. The cytokine gamma Interferon (γ-IFN) can upregulate many aspects of antigen presentation. In cell lines with low MHC Cl I expression, treatment with γ-IFN results in increased cell surface expression (45). Studies in MHC Cl I deficient cells demonstrate that the addition of γ-IFN upregulates the association of β_2m with MHC Cl I H chains and the subsequent cell surface expression (46). In addition, γ-IFN upregulates the proteasome subunits encoded in the MHC region and MHC Cl I H chain production (47).

1.7.1. Nature of the ER

Like other membrane and cell surface proteins, the initial assembly of the MHC Cl I complex occurs in the lumen of the ER. The endoplasmic reticulum is an interconnected network of membranes forming a tubular system within the cell that is continuous with the nuclear membrane. Proteins destined for secretion or cell surface expression are processed in the ER soon after translation. These proteins contain a N terminal signal sequence consisting of about 20 residues that mediates transport across the ER membrane. Once inside, the signal is quickly cleaved and the nascent protein associates with ER resident proteins that bind to and further process the protein. For membrane bound proteins, a second signal sequence found on the protein stops translocation across the membrane and causes this region to become embedded in the ER, serving as the transmembrane anchor (48).

Processing in the ER is not yet completely understood. There are many types of processing enzymes that act in concert on nascent proteins. These include signal peptidase, protein disulphide isomerase (PDI), protein prolyl isomerase (PPI), ERGIC 53 (49), oligosaccharyl transferase, alpha glucosidase I, alpha glucosidase II, ER alpha 1,2-mannosidase, thiol dependent reductase ERp57, calnexin, calreticulin and BiP. Different sets

of molecules associate together and modify the newly formed ER protein. Modifications include the addition of oligosaccharides, oligomerization and the formation of disulphide bridges. When complete, a protein with a mature and correct conformation can exit the ER and proceed to further processing through the cis Golgi.

1.7.2. Chaperones

Proper assembly of ER proteins involves ER chaperone proteins. Chaperones can be defined as proteins that interact with immature or misfolded proteins and either help them to achieve a proper conformation or direct them to be degraded. Some well known chaperones include the stress proteins (50) including BiP (51, 52) and calnexin. BiP is involved in both translocation of nascent proteins into the ER and in the binding and retaining of misfolded proteins in the ER, whereas calnexin binds and retains nascent glycoproteins in the ER until they achieve a mature conformation (53, 54).

The chaperone calnexin (55) associates with nascent glycoproteins in the ER in a lectin-like manner by binding to the intermediate $Glc_1Man_9GlcNAc_2$ (56, 57). Calnexin association stabilises intermediate structures preventing their rapid degradation (58) and enhances efficient assembly.

1.7.3. Other chaperones

In addition to interacting with calnexin, it is clear that membrane proteins bound for the cell surface (and other glycoproteins) must also interact with other chaperones. These include chaperone molecules such as protein disulphide isomerase, glucosylases and cis-prolyl isomerase. Also involved directly with MHC Cl I molecules and antigen presentation are the TAP proteins and tapasin and BiP.

Calnexin associates with immature MHC Cl I (and other membrane glycoproteins) in

the ER. Calnexin binds immature glycoproteins through a lectin-like domain (56). The lectin action (and subsequent association) of calnexin requires a mono-glucosylated oligosaccharide. The glycosylation/de-glycosylation cycle is an effective means of generating and re-generating substrate for calnexin binding and re-binding, causing incorrectly folded or immature proteins to be retained by calnexin (59).

The binding step will apply steric constraints to the bound protein, and during this step inter- and intra-chain disulphide bonds can be formed. Glucosylation of glycoproteins regulates transport of glycoproteins out of the ER (60). Once the calnexin-bound protein achieves correct conformation, the final glucose residue is removed and association with calnexin terminates (57). With MHC Cl I , the correct conformation is achieved with the addition of the peptide and β_2m forming a tri-molecular complex (61).

Studies with reagents that inhibit some of the above mentioned chaperones demonstrate that disruption of regular maturation results in incorrectly folded or immature proteins. These proteins are either retained in the ER or they are tagged for degradation. They are not further modified in the ER and are not further processed through the Golgi. Recent studies using lactacystin to inhibit the proteasome have suggested that such tagged proteins are exported back to the cytoplasm for degradation (62). Studies with daudi cells that do not produce correctly folded MHC Cl I because they are missing β_2m protein showed the accumulation of heavy (H) chains in the ER when treated with a proteasome inhibitor (63).

Use of the reducing agent dithiothreitol (DTT) and permeabilised cells showed that disulphide bridge formation in nascent proteins is a fairly early reaction and is mediated by protein disulphide isomerase. Preventing disulphide bridge formation prevents proper folding of MHC Cl I , but is reversible upon removal of DTT (64). While formation of the disulphide bridges occurs during protein folding, it is likely that this step does not influence folding, but rather promotes rapid reshuffling of incorrect disulphide pairings and stabilising of a correct

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one (65, 66)

Excess unfolded protein in the ER can trigger the unfolded protein response (UPR). The UPR is an upregulation of ER lumenal proteins including BiP and PDI in response to malfolded proteins in the ER. Stresses that can cause the UPR include inhibition of glycosylation by starvation or the addition of drugs, addition of reducing agents, expression of folding mutants, or addition of calcium ionophores that reduce calcium stores in the ER. In yeast, a transmembrane serine/threonine kinase has been identified that is involved in the UPR, possibly transmitting the UPR signal to the nucleus (67). It has also been found that protein overload of the ER can trigger NF-kB in a manner independent of the UPR, possibly by causing the release of Ca^{2+} from the ER (68). This may be a generalised cellular antiviral response.

1.7.4. Glycosylation

Nascent proteins are glycosylated in the ER. Amongst the early acting ER chaperone proteins are the glycosylation mediators. An oligosaccharide assembled on the lipid carrier dolichol phosphate is transferred to a nascent glycoprotein. This oligosaccharide consists of two GlcNAc, 9 mannose and three glucose residues ($Glc_3Man_9GlcNAc_2$) and is able to efficiently associate cotranslationally at most Asn-X-Ser/Thr sites. Steric hindrance and accessibility of the site seem to influence transfer of the oligosaccharide to the glycoprotein; different glycosylation sites on the same protein exhibit different but consistent glycosylation patterns (69, 70). Once a protein has folded, the sites of glycoprotein attachment may no longer be accessible to the cellular glycosylation machinery.

Upon transfer, this oligosaccharide is almost immediately set upon by ER resident proteins. Glucosidase I acts almost immediately on the outermost glucose of the N-linked core glycan, removing it. The second glucose is then sequentially removed by glucosidase II.

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Glucosidase II also subsequently removes the third glucose, leaving a high mannose form of the sugar. Replacement of the third glucose residue is mediated by UDP-glucose:glycoprotein glucosyltransferase (60) resulting in a mono-glucosylated glycan. A glucosylation/de-glucosylation cycle (71) seems to be one method employed to effect quality control within the ER (57, 72).

Studies with tunicamycin, which blocks the synthesis of the dolichol linked oligosaccharide, show that glycosylation is an essential step in the processing of a glycoprotein. Failure to become glycosylated results in immature proteins that collect in the ER, associated with BiP (73).

The above studies show that each step in the maturation of a glycoprotein is essential for any subsequent steps. Studies with castanospermine reveal that inhibiting the glucosidases has a similar effect, resulting in rapid degradation of unassembled MHC Cl I (74). Indeed, newly formed proteins are subject to quality control with those that are misfolded being retained and degraded (75). There is some evidence that degraded proteins exit the ER and are degraded in the cytosol by the proteasome machinery (76).

1.8. The Golgi complex and membrane traffic

Proteins are further modified as they travel from the ER into the cis Golgi. They continue through to the trans face of the Golgi and are packaged in vesicles or make their way to the cell surface. While the 100 year anniversary of the discovery of the Golgi apparatus was recently celebrated (77), much of the structure, function and organization of the Golgi have yet to be determined. The Golgi is the location of hundreds of enzymes.

Much debate has centered on the nature of glycoprotein maturation. What is agreed upon is that proteins with signal sequences are subject to the 'export' mechanism of the cell (78). Proteins are exported from the cytosol to the lumen of the ER where they have access to the exterior of the cell through the Golgi. Proteins make their way through the medial and trans

Golgi before being packaged in vesicles that can transport them to the cell surface where they are either released outside the cell or they form part of the outer layer of the plasma membrane.

What is less clear are the rules governing the transport of material between the various compartments of the ER-Golgi system. The bulk flow theory suggests that there is a bulk flow from the ER through the Golgi to the cell surface (79, 80). Any resident in the ER that is not tagged with a retention signal will eventually make its way through the ER, the Golgi complex and through to the cell surface. Recently, investigators elucidating the nature of vesicular budding in yeast have challenged the 'bulk flow model', postualting that proteins (known as cargo) associated with membrane bound vesicles are transported through to the Golgi from the ER in response to some positive signal (81-83). A sugar may mediate this positive signal (84) and ERGIC-53 (that has some lectin-like properties) is the protein mediating this (49, 85).

1.9. T Cell Receptor/Ligand Interactions

Once an MHC Cl I molecule has matured through the ER-Golgi processing machinery, it presents bound peptide at the cell surface for recognition by T cells. CD 8^+ cytotoxic T lymphocytes (CTL) are involved in viral clearance. Recognition and subsequent destruction of virally infected cells is effected by T Cell Receptors (TCR) that must recognize foreign antigens in the context of a host or 'self' protein (86). The antigen that stimulates a response is the bound peptide fragment derived from foreign protein. Studies have shown that CTL responses may be raised against a single immunodominant epitope (87, 88). The crystal structure of the TCR interacting with the MHC Cl I molecule has allowed insight into the interaction. The MHC Cl I peptide groove allows binding of the peptide in a single orientation; much of the peptide is hidden from the TCR. Studies with mutants suggest that the TCR binds diagonally across the alpha helices (89), avoiding the protruding N-terminals of each of the a1 and a2 alpha helices and maximising contact with the peptide-MHC Cl I complex (13, 90).

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Recent elucidation of the crystal structure of a TCR interacting with the polymorphic region of MHC Cl I and peptide shows the intimacy (and specificity) of the contact between the two proteins. Figure 4 reveals the tight association of the α and β chains of the TCR with the $\alpha 1$ and $\alpha 2$ regions of MHC Cl I and the bound peptide.

Figure 4. The interaction between MHC Cl I and the TCR

This figure demonstrates the tight association between the α and β chains of the TCR (shown in pink and blue respectively) and the MHC Cl I protein (depicted in light green) with bound peptide (yellow). The peptide in the peptide groove is sandwiched between the TCR and the $\alpha1$ and $\alpha2$ sections of the MHC Cl molecule. Close association of β_2m (dark green) with the $\alpha3$ regionis also observed (15).
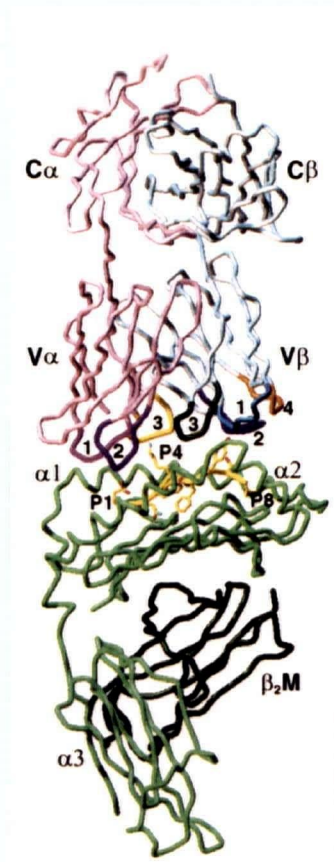
1.10. Viruses

Most viral infections are cleared in the procedures mentioned above. In general, the viral life cycle starts with infection, followed by transcription of early viral genes that hijack the cellular machinery. The host machinery is then used to replicate the viral genome and synthesize viral proteins. Proteins and the genetic material are packaged to produce more viruses which are released into the surrounding area. In short, a virus turns its host cell into a virus-making factory.

Different cell types are more or less susceptible to infection by particular viruses. Non-permissive cell types do not readily support infection and propagation of virus whereas permissive cell types readily succumb to viral infection and contribute to the viral life cycle.

The immune system attempts to destroy virally infected cells prior to the release of more virus. CTLs target infected cells and cause their imminent destruction. T Cells recognize antigens within the context of allelic forms of MHC molecules. This is known as allelic restriction (4).

Figure 5. The antigen presentation pathway

The steps occurring in antigen processing are detailed here. a) Proteins are synthesised within the cell. Proteins tagged with ubiquitin are degraded by the multicatalytic protealytic complex or proteasome. The proteasome is a large (650 kD), well conserved found within the cytoplams. Peptides of 7 to 12 residues are transported into the ER through the action of the ATP dependant TAP proteins.

b) MHC Cl I proteins are co-translationally translocated into the ER where the associate with the chaperone calnexin. The H chain also associates with L chain (β_2m) and other chaperones such as Erp57 and calreticulin to name a few. Tapasin mediates the association of the immature MHC Cl I H chain-(β_2m) complex with TAP. Peptide loading by TAP proteins results in a mature conformation accompanied with release by calnexin. The tri-molecular complex is subsequently expressed on the cell surface where it can be recognised by the TCR of passing T Cells. (91)

Cell surface

(a) Protein synthesis

Antigen

+/− Ubiquitin

Proteasome

Peptides

(b) HLA class I synthesis

Calnexin

β2m
ERp57
Calreticulin

Tapasin

TAP

(c)

Endoplasmic reticulum

Golgi

(d) Exocytosis

Complex of HLA class I and peptide

HLA
heavy
chain

β2m

Calreticulin

ERp57

Tapasin

24
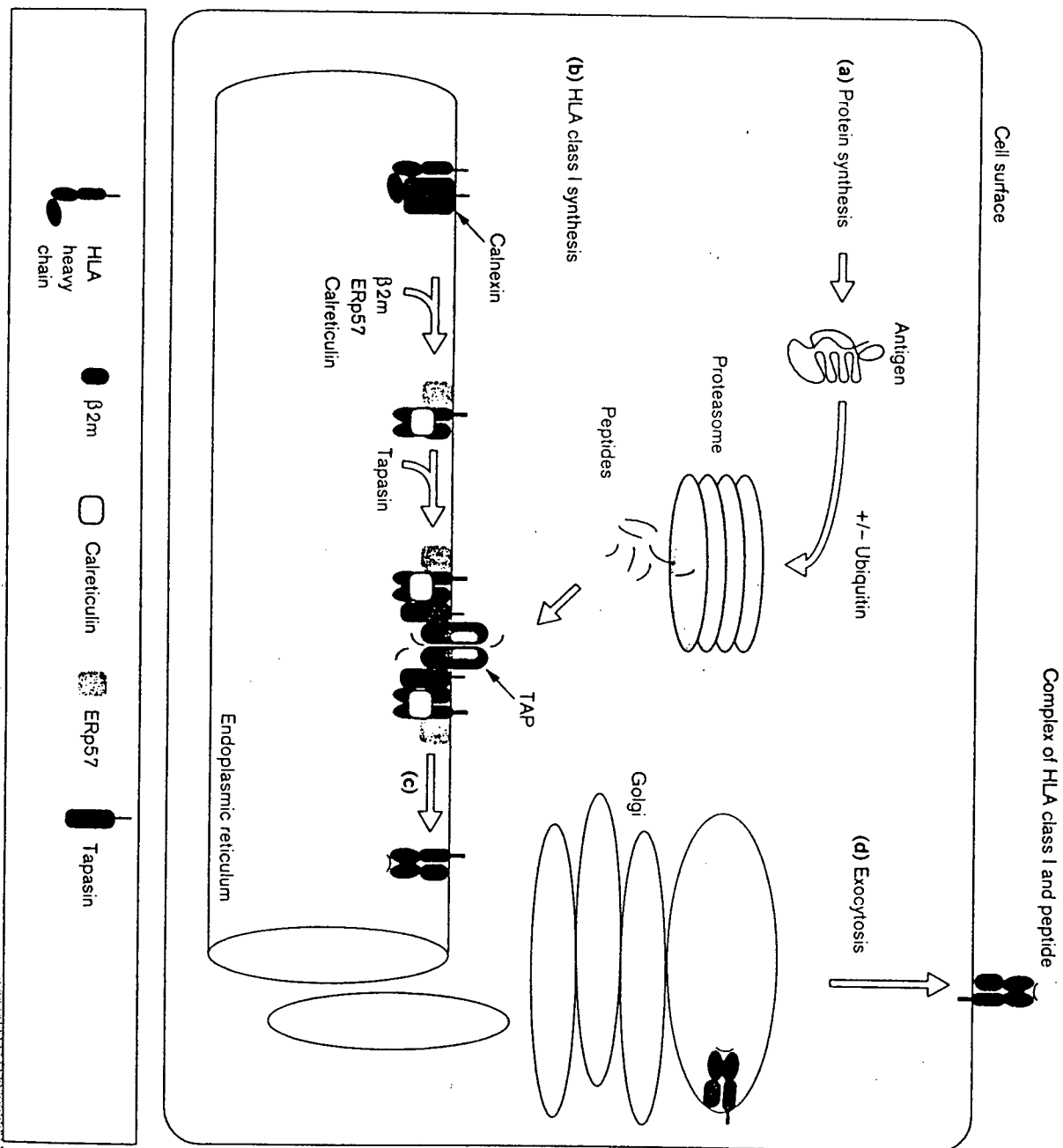
1.11. Adenovirus

There exist at least 70 different serotypes of human adenovirus which are divided into 6 groups termed A through F. The pathological effects of adenovirus are quite variable, but in general, they can cause acute disease of the eye, respiratory tract and gastrointestinal tract (92). While adenovirus rarely causes a chronic medical condition in healthy individuals, the virus has the ability to evade the host immune system and cause a persistent infection. In several studies, infection by viruses in general and adenovirus in particular tended to result in a sub clinical effect (93). In addition, adenoviruses may become persistent and shed in feces for years post infection in otherwise healthy individuals (94). Adenoviruses can exist in a latent state and cause disseminated disease in immunocompromised patients due to reactivation (95). For example, it has been demonstrated that over 10% of patients with acquired immunodeficiency syndrome (AIDS) shed adenoviruses in their urine (94). It has been suggested that infection and persistence of adenoviruses in early childhood may result in respiratory illness in adulthood (96).

The adenovirus is a non-enveloped icosahedral structure made up of an outer protein capsid and an inner core composed of a tightly packed DNA-protein core (97). The protein capsid is composed of 240 hexons and 12 penton bases that are noncovalently attached to fibre proteins. Each penton is surrounded by five hexons and is found at each of the twelve 'corners' of the virion with an attached protruding fiber protein. The core consists of virus DNA and core proteins. The virion is approximately 600-700 Å in diameter (97).

The capsid enters host cells by association of the fiber proteins with specific receptors on the cell surface (98) followed by import of the virion into the cell. The virion is transported to the nucleus where the capsid is stripped away and the DNA is transcribed either in an 'early' or a 'late' phase. The early phase precedes viral replication; the late phase follows viral replication. Early genes code proteins that are able to disrupt normal cellular processes,

25

including those that involve presenting peptides derived from foreign proteins at the cell surface. Late phase proteins have functions that aid packaging the large amounts of viral DNA and viral capsids into new virions.

The genome of adenovirus consists of about 36,000 base pairs of linear, double stranded DNA. It contains multiple overlapping spliced mRNAs generated from a single mRNA precursor (Figure 6). The early genes are divided into 6 major transcriptional units, consisting of the E1A, E1B, E2, E3, E4 and L1. The immediate early region consists of E1A gene products including a 289 amino acid (aa) protein and a 243aa protein that act as trans-acting regulators of other early region genes (99) and as suppressors of certain enhancer-dependent genes. E1B gene products are poorly understood. E2 region gene products participate in viral DNA replication. The E3 region codes for several gene products that enhance the ability of a virally infected cell to evade the immune system. The E4 region also codes for a trans-activating protein and little is known about the functions of the L1 gene products.

Figure 6. The adenovirus genome

The adenovirus genome is made up of early and late transcribing units.   In this illustrration, the double line represents the dsDNA.   Arrows represent transcription products and numbers refer to proteins. Rightward transcription products are depicted by the r; leftward by the l. (100).
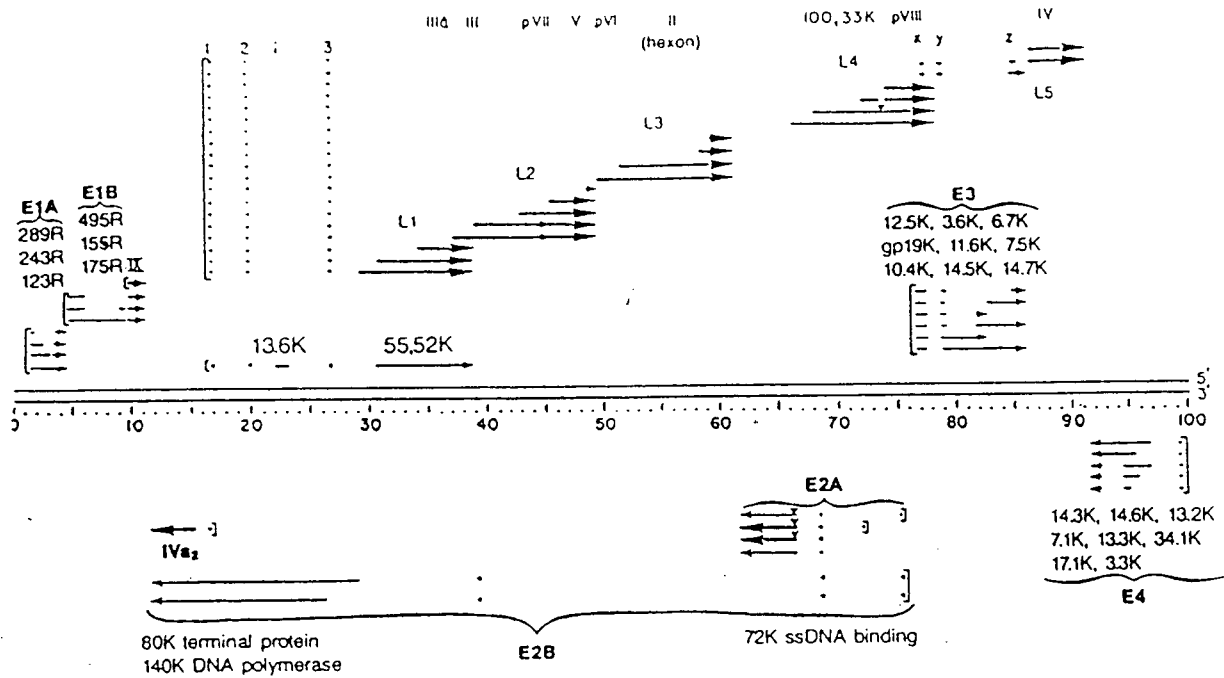
The adenovirus has developed several mechanisms to evade the immune system. Perhaps the best characterised is its ability to downregulate the cell surface expression of MHC Cl I molecules and therefore evade the immune surveillance mechanism. Viral sub-groups achieve this result by different means. Ad 12 blocks transcription of MHC Cl I mRNA, causing a repression of cell surface expression (101). Ad2 and 5 have a 25 kDa protein (E3/19K) that binds the MHC Cl I molecule and causes its retention inside the infected cell. Other mechanisms utilised by adenovirus include downregulation of the EGF-Receptors at the cell surface and protection from TNF-induced cell destruction.

1.11.1. E3 region

While the E3 region has been shown to be non-essential to adenovirus replication in cultured cells (102), it is the region that codes for several proteins that may confer an ability to evade the immune system of the host (100). While the differences in sequence between the E3 region of adenovirus of different serotypes is significant, there is consensus in the open reading frames (ORFs) generating some of the proteins. In Ad2 and Ad 5, variable splicing of a common mRNA precursor gives rise to at least 6 different ORFs.

Figure 7: The E3 region of adenovirus

The E3 region encodes several proteins that aid in evasion of the immune system. Solid arrows show mRNA; dotted lines show introns. Hatched bars show identified peptides and grey bars are those proposed to exist. (100).
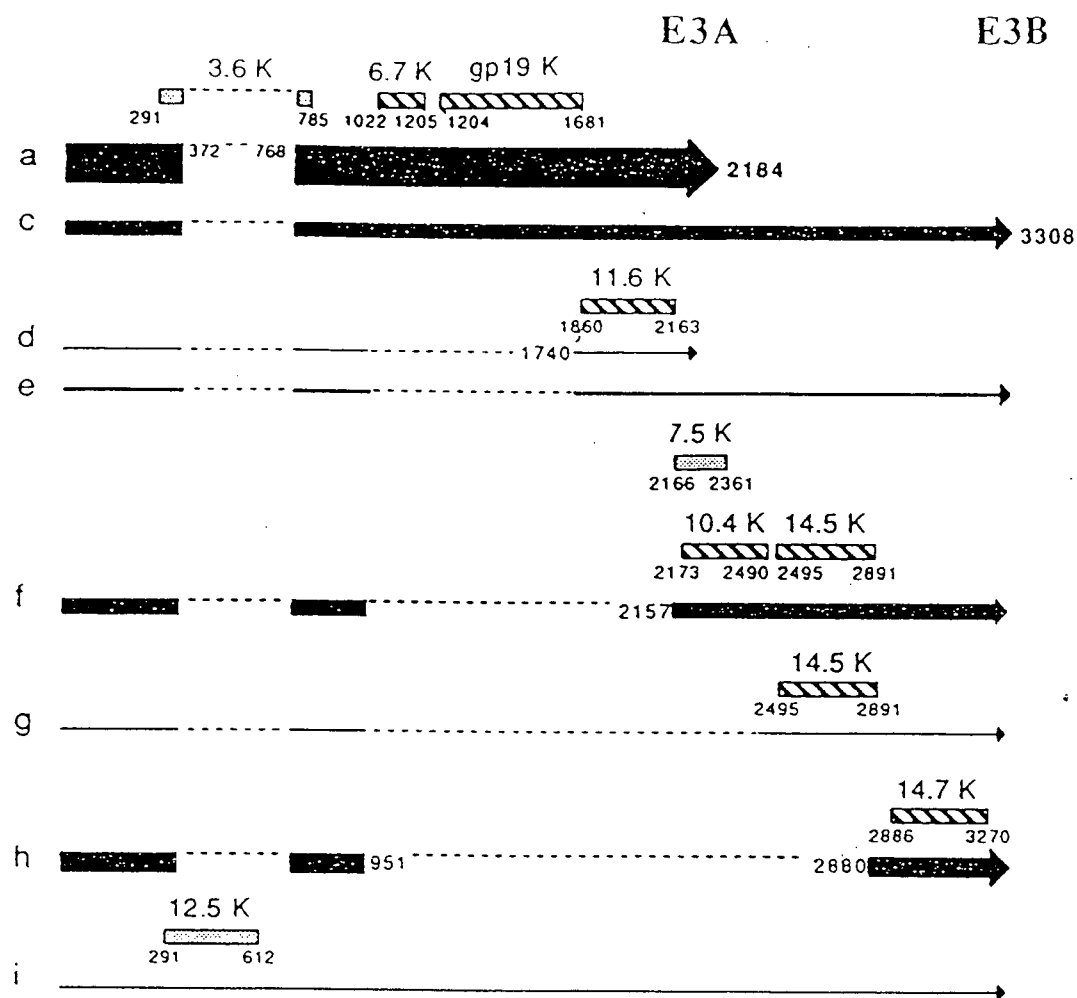
1.11.2. Overview of proteins and effects of the E3 region

Several gene products of the E3 region have been studied. E3/6.7 has been shown not to participate in downregulating the cell surface level of MHC Cl I molecules in concert with E3/19K. E3/19K is the focus of this study and the features shall be discussed in detail below. The protein E3/14.7K has been shown to prevent cytolysis of adenovirus-infected cultured cells by tumour necrosis factor (103, 104). E3/10.4 downregulates the EGF receptor (105).

Table 1.   Summary of the effects of E3 region proteins

E3/19K	Blocks surface expression of MHC Cl I (106)
E3/14.7K	Prevents lysis of infected cells by Tumour Necrosis Factor (TNF) (103)
E3/10.4K	Downregulation of Epidermal Growth Factor (EGF) receptor (107) -also works in conjunction with 14.5 to prevent TNF cytolysis (108)
E3/14.5K	Downregulation of Epidermal Growth Factor (EGF) receptor (107) -also works in conjunction with 10.4 to prevent TNF cytolysis (108)
E3/11.6K	Adenovirus Death Protein-induces apoptosis (109)
E3/6.7K	No published function

The E3/19K protein is conserved across several serotypes. The E3/19K protein of Ad2 is a 142 amino acid Type 1 ER resident transmembrane glycoprotein. The protein consists of a 15 amino acid (aa) cytoplasmic tail, a transmembrane region, a conserved 20 amino acid spacer region (110), and then a variable region. This lumenal section of the protein is glycosylated at positions 12 and 61 (111). At positions 11 and 28 are the first pair of cysteines that participate

in a disulphide bond; the second spans from Cys 22 to Cys 83. These disulphide bonds are conserved across several serotypes (112) and confer stability and contribute to the structure of E3/19K. The conserved spacer and cysteine residues have been shown to be important for binding to MHC Cl I molecules (110, 113). Other studies have shown that truncated E3/19K molecules lacking the cytoplasmic tail and some of the transmembrane regions retain the ability to bind MHC Cl I molecules (114). Site-directed mutagenesis of this protein has revealed that almost every stretch of the lumenal domain is required for association of this protein with MHC Cl I (110).

Figure 8 The E3/19K protein

The adenovirus E319K protein (114).
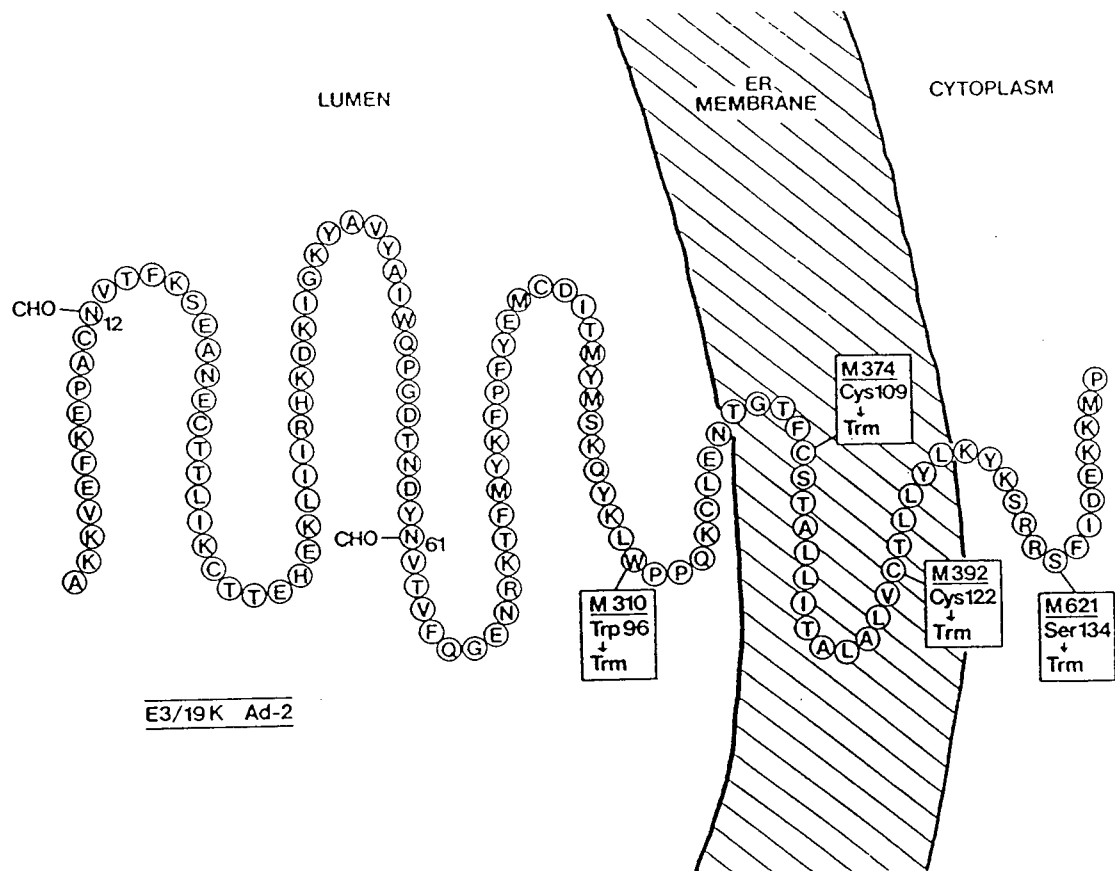
The E3/19K protein is cotranslationally translocated into the ER. Upon transcription, its signal sequence is cleaved. Some processing occurs and it migrates as far as the cis Golgi before returning to the RER. ER retention is mediated by a short stretch on its cytoplasmic tail; truncation of the tail results in E3/19K at the cell surface (114). E3/19K associates cotranslationally with nascent MHC Cl I molecules. By virtue of the ER retention site, it retains MHC Cl I molecules in the ER preventing travel through the Golgi to the cell surface. The E3/19K molecule has been shown to associate with rodent, human, and simian MHC Cl I molecules (115, 116). E3/19K shows varying affinities for different MHC Cl I molecules (117, 118). While it binds to every human MHC Cl I molecule tested to date, it is unable to bind to at least one mouse H-2 protein. During viral infections, the E3/19K protein of adenovirus type-2 (Ad2) binds to major histocompatibility complex (MHC) Class I proteins in the endoplasmic reticulum (ER) where these molecules are assembled. This prevents MHC Cl I transport to the cell surface where they can present adenovirus peptides to cytotoxic T lymphocytes (CTL) (100, 119).

Despite studies showing the E3 region not to be essential for virus growth in cultured cells (102) , data supporting the role of the E3/19K protein in persistent infections is provided by experiments in which this protein is deleted from an otherwise normal virus and shown to result in the early clearance of the virus in Ad2 infections in mice (120). It is likely that E3/19K is involved in permitting adenovirus infection to spread and become persistent in humans (96). Lack of a good animal model has hampered study in this area.

Objectives

While adenovirus may have many strategies to evade the immune system, the best studied mechanism is the retention of MHC Cl I proteins within the ER away from detection by roving T cells. This study exploited the properties of the adenovirus E3/19K protein to perturb the antigen processing component of the immune system. As E3/19K is an ER retained protein, study of this protein allowed detailed analysis of many functions of the ER including antigen presentation and protein maturation. Additionally, the role of chaperones in quality control and large functional complexes was studied.

The objectives of this study were to use the effects of the E3/19K protein to study antigen processing occurring within the cell. Blocking of antigen presentation revealed details of protein processing and maturation in the ER. The identification of and the interactions between several ER resident proteins was revealed.

The exact nature of the blocking of antigen presentation was explored by examining the role of E3/19K in the ER. E3/19K was bound to many different MHC Cl I proteins in order to establish a minimum conformation or sequence of residues for binding. Instead the nature of binding was shown to be influenced by other factors including the inherent ability of a MHC Cl I molecule to be processed by the endogenous ER maturation pathway. Additionally, it was possible to group MHC Cl I allelic proteins into three categories: binders (those that bound E3/19K), non-binders and a new category called slow-binders. Slow-binders bind MHC Cl I quite strongly but require a longer period of association to achieve this binding.

Investigation of one slowbinder revealed the retention of large amounts of protein in the ER with comparatively small amounts at the cell surface. Attempts to reverse the retention led to the examination of many factors which influence protein maturation in the ER and showed this process was more complicated than originally suspected. This in turn suggested that E3/19K retention of MHC Cl I may involve more interactions than simply MHC Cl I-E3/19K association.

Regular maturation of MHC Cl I with and without E319K and the association of this complex with ER resident chaperones was examined. E3/19K rapidly promoted a stable conformation under adverse conditions acting like a chaperone. E3/19K association with MHC Cl I did not block the interaction of endogenous chaperones suggesting that the size of complexes in the ER were quite large. Other mechanisms such as the unfolded protein response may be involved in the E3/19K effect on infected cells.

Peptide binding by MHC Cl I is a step in antigen presentation. The effect of E3/19K binding to MHC Cl I on peptide binding was examined. Explicit peptide binding to MHC Cl I in the presence of E3/19K was demonstrated. This showed that both peptide and E3/19K bound MHC Cl I concurrently, reducing the likelihood that the association was through the peptide binding groove.

Demonstration of multiple proteins binding to MHC Cl I suggested that large complexes of proteins were formed in the ER. E3/19K with these complexes also occurred. Steric hindrance between members of these large complexes seems likely. To examine if E3/19K and other molecules occurred as multimers, complexes were precipitated and separated on a sucrose density gradient. These findings lend further credence to the idea that other effects of E3/19K remain to be discovered.

2. Materials and Methods

2.1 Cellular Methods

2.1.1 Tissue Culture

The cell lines used in these studies are listed in table 2. Most experiments used either 293 cells or transfected 293 cells. 293 cells are adherent human embryonic kidney cells transformed with Ad 5 (121). Adherent cells were grown to 85-95% confluence in DMEM supplemented with L-Glu (2 mM), 10 mM HEPES (pH 7.2), and 10% Fetal Calf Serum. Antibiotics were omitted from culture media. Cells were passaged using 0.05% trypsin (wt/vol) in phosphate buffered saline (PBS) with 1 mM EDTA at 37°C, diluting cells 1:10 in fresh culture media. Non-adherent cells were grown in flasks with the media supplemented as above. Living cell concentration was monitored by trypan blue exclusion and cultures were grown to a concentration of $1x10^6$ to $5x10^6$ cells/ml before passaging at a 1:10 dilution. With the exception noted below, all cells were incubated at 37°C in a humidified 5% CO_2/95% air environment.

Large scale cultures were grown in sealed Belco spinner flasks at 37°C in incubator not supplemented with CO_2. Cell spinners were set at 60-70 rpm. Media was supplemented with Ca^{2+}. Cell concentration was monitored as above with trypan blue and was maintained between $1 x 10^6$ and $5 x10^7$ cells/ml.

Table 2. Reagents used

Cell Lines	
293	human embryonic kidney (ATCC CRL 1573)
293.12	293 transfected with E3/19K
A549	human lung carcinoma (ATCC CCL 185)
293p39	Low passage 293
Monoclonal Antibodies (TCS)	
W6/32	mouse anti HLA A, B, C (ATCC HB 95)
PA 2.1	mouse anti HLA A2, Aw69 (ATCC HB 117)
OKT6	mouse anti transferrin receptor
34.5.8s + (34-1-2s)	mouse anti D^d (ATCC HB 102)
15.5.5s + (H100.27.55)	mouse anti D^k (ATCC HB 24)
34.1.2s	mouse anti K^d (ATCC HB 79)
16.3.1N	mouse anti K^k (ATCC HB 25)
28.14.8s	mouse anti D^b, L^d (ATCC HB 27)
Antisera	
R418	Rabbit anti E3/19K (122)
R426 R425	Rabbit anti MHC Cl I H chains (123)
anti calnexin	Rabbit anti calnexin(124)
anti transporter	Rabbit anti TAP (125)
Anti human β_2m	Rabbit anti human β_2m (126)
Lysis Buffers	
0.6% Chaps	0.6% Chaps, PBS
1% NP40	1% NP40, 120 mM NaCl, 4mM $MgCl_2$, 20 m M Tris
1% N-Octyl Glucoside	1% N-Octyl Glucoside, 50mM $NaPO_4$ pH 7.0
PBS + 1% SDS	1% SDS, PBS w/o Mg^{2+} or Ca^+
Media	
Hams F12	
DMEM	
alpha MEM	

2.1.2. Transfection

293 cells were transfected using Lipofectin (GibcoBRL). Genomic DNA in PBR322 was purified using the Promega MaxiPrep kit. 10 μg DNA was combined in 10 fold excess with a neomycin resistance marker and transfected following the manufacturers instructions. Cells were allowed to grow 36-48 hours before the selection agent, G418 (neomycin) (GibcoBRL) was added at a final concentration of 1 mg/ml. Dead cells were removed with frequent media changes. Surviving colonies were isolated with cloning rings. Individual clones were grown up and tested for gene expression.

2.1.3. Viruses

Adenovirus 2 (Ad2) stock was obtained from Dr. Frank Graham through Roger Lippe. Stocks were grown in bulk on low passage 293 cells (121) in complete alpha MEM, harvested and stored at -80°C in 10% glycerol/PBS^{++}at $2x10^9$ plaque forming units per ml (PFU). PFU were calculated using viral stock serially diluted onto A549 cells and overlaid with 2x F11 media and 1% Agarose. Plaques were scored 7 days after infection. Prior to use, virus stock was gently thawed at 4°C. Vaccinia virus containing the human β_2m gene was generously provided by Dr. J. Yewdell (National Institutes of Health, Bethesda, MD).

Prior to infection cells were washed in PBS^{++}. Cells were infected at a multiplicity of infection (MOI) of 5. Virus was added to the cells and allowed to attach for one hour. Media was added back after the first hour and infection was allowed to proceed for approximately 18 hours.

2.1.4. Peptides

Synthetic peptides were purchased from the Alberta Peptide Institute. Peptides were biotinylated at the N terminal using NHS-Ester biotin (Sigma). Peptides dissolved at 1 mM in NaPhosphate buffer (pH 7.5) were mixed with 100x excess NHS-Biotin Ester for two hours. Unreacted NHS-ester was neutralised with Tris-HCl (pH 7.4) to a final concentration of 10 mM. Peptide (biotinylated and non-biotinylated) was added to growing cell cultures 24 hours prior to labelling at concentrations ranging from 5nM to 5μM.

2.1.5. Fluorescence activated cell sorting (FACS)

Adherent cells were pre-treated with versene or 0.05% trypsin and washed in FACS buffer to remove them from the plate. Cells were suspended at 1×10^6 cells/ml in DMEM supplemented with 200 mM L-Glutamine, 20 mM Hepes (pH 7.2) and 20 mM NaAzide (hereafter referred to as FACS buffer). Cell suspensions and all manipulations were performed at 4°C for the duration of the antibody labelling. Suspensions were washed twice with FACS buffer and incubated with primary antibody (200 μl of monoclonal TCS or 3 μl of ascites) for 45 minutes. Unbound primary antibody was washed out with two more FACS buffer washes and the samples were incubated with 100 μl of 10 mg/ml stock fluorescein isothiocyanate (FITC) labelled secondary antibody (Jackson) for 45 minutes. Excess secondary antibody was washed out with two more FACS buffer washes followed by a wash with PBS with Ca^{2+} or Mg^+ (PBS⁻). The cells were fixed in a 1.5% paraformaldehyde/PBS and stored in the dark at 4°C until analyzed. Analysis was performed on a Becton Dickson (BD) FACScan. 5000 gated events were collected and subsequently analyzed using the Lysis II software from BD.

An alternate protocol involved substituting FITC coupled secondary antibody with biotinylated secondary antibody. A third incubation using FITC coupled streptavidin was also added.

2.1.6. Animals

Rabbits used for antisera generation were housed in the University of British Columbia south campus animal facility. They were kept under the care of Willem Schoorl according to the guidelines of the Canadian Council on Animal Care. Rabbits were initially injected with antigen in the lymph nodes and subsequently bled to raise antisera. When necessary they were sacrificed by CO_2 asphyxiation.

2.2. Protein Techniques

2.2.1. Antibodies

Monoclonal antibodies (mAbs) were used as Tissue Culture Supernatant (TCS) but in some cases ascites fluid was available. Rabbit antisera to E3/19K (R418) and to MHC Cl I (R425 and R426) were donated by Dr. Sune Kvist. Anti calnexin antisera (124) was a gift from Dr. Bergeron, McGill University, Montreal, Canada and mouse TAP 1 and TAP 2 antisera were obtained from Dr. G. Butcher, AFRC, Cambridge, UK. Antisera to E3/19K was raised in rabbits using synthetic peptide coupled to KLH.

2.2.2. Metabolic Labelling

An 85-95% confluent 65 mm plate with approximately $1x10^6$-$2x10^6$ cells received 300 μCi ^{35}S in 2 mls during labelling. Cell cultures were washed in methionine and cysteine free DMEM (Gibco) one hour prior to labelling and subsequently labelled with 150 μCi/ml Pro-Mix

(^{35}S-Met-Cys) (Amersham) for periods ranging from 5 min to one hour. In pulse chase

experiments, the labelling media was replaced with standard media for the chase. All labelling

was performed under standard cell culture conditions. Cells were lysed with a variety of lysis

buffers (see table 2) supplemented with PMSF (40 µg/ml) on ice. .   Efficiency of labelling

was determined by counting small aliquots (3 µl) of lysate following TCA precipitation of

labelled proteins.

2.2.3.   Immunoprecipitation

Lysates were spun at 10,500 g for 30 minutes. Volumes of lysates were normalised to

account for differences in labelling and aliquots ranging from 200 µl to 1ml were used for

immunoprecipitation. All immunoprecipitation steps were performed at 4°C. Samples were pre-

cleared by incubation with 3 µl Normal Rabbit Sera (NRS) for 45 minutes, followed by a 45

minute incubation with 45 µl of a 1:1 Lysis Buffer:Prot-A-Sepharose (Pharmacia) slurry. Specific

antibodies were added to pre-cleared samples. In general, 100 µl of TCS from monoclonal cell

lines or 1 µl of ascites fluid or sera was incubated with the samples for 45 minutes. Following

specific incubation, 45 µl of a Prot-A-Sepharose slurry was added and allowed to incubate for a

further 45 minutes. Beads were washed several times with buffers of varying salt concentration

(three times with buffer B: 0.2% NP40, 150 mM NaCl, 2mM EDTA, 10 mM Tris, two times with

buffer C: 0.2% NP40, 500 mM NaCl, 2 mM EDTA, 10 mM Tris, once with Buffer D: 10mM

Tris) and finally sucked dry. Beads were then either stored at -80°C or boiled at 95°C for 5

minutes with bromomix (60% sucrose, bromophenolblue 0.1%, Tris-HCl pH 8.8 with freshly

made 0.5M dithiothreitol)-20% SDS mixture. Samples were then blocked with 0.5mM

iodoacetimide, and separated with SDS polyacrylamide gels electrophoresis (SDS-PAGE).

2.2.4. SDS-PAGE

Immunoprecipitated samples were separated on SDS-PAGE. Large gels were prepared as gradient gels with 10-15% polyacrylamide topped with a 5% stacking gel. 25 µl samples were loaded and run O/N at 18 mAmps (350 V). Gels of 10, 12 and 15% were also used. Upon complete migration of the dye front across the gel, gels were fixed (10% Acetic Acid, 30% v/v Methanol) for 30 minutes and then enhanced in 20mM NaSalicylate (known as enhance). Gels were dried and placed on Kodak X-AR radiographic film at -80°C for periods ranging from one day to several months. Alternatively, samples were separated on the BioRad Minigel (10 x 7 cm) system. Gels were made as above and samples separated according to manufacturers instructions.

2.2.5. Western Blotting

As an alternative to fixing after PAGE, western blotting was performed. In addition to ^{14}C labelled molecular weight (MW) standards, biotinylated standards were also present on the gels. Following complete migration of the dye front in the electric field, the gel equilibrated in western transfer buffer () for 30 - 90 minutes. The gel was then sandwiched against a sheet of Immobilon P polyvinylidene difluoride (PVDF) membrane, submerged in western transfer buffer and blotted in an electric field for time periods between one and twelve hours. Alternatively, the BioRad mini western apparatus was used according to manufacturers instructions. After transfer, membranes were washed 2 times in western wash buffer (0.1% BSA, 0.05% Tween 20, 0.01M thimerasol in PBS), and blocked for one hour in BSA (2.5%) fortified western wash buffer. Membranes were washed 3 more times and exposed to antisera.

Antisera was added at dilutions of 1:10 to 1:5000 in 45 mls total volume. Alternatively, the glass plate techniquewas used to conserve antisera. Briefly, the membrane was placed on a

glass plate with 100 µl of diluted antisera. Antisera was distributed over the membrane with a glass spreader every 30 minutes for two hours creating a thin film of antisera between the plate and the membrane. Following the two hour incubation, the primary antibody was washed away and the secondary goat anti rabbit coupled with biotin (Jackson) was added at a dilution of 1:10,000 for 45 minutes. Membranes were then washed several times and incubated with HRPO coupled to streptavidin. Subsequent detection was performed using Enhanced Chemiluminescence (Amersham ECL) system. Membranes were exposed to Kodak X-AR film for periods ranging from 5 seconds to overnight. Subsequently they were stored damp at 4°C for future use.

2.2.6. Tunicamycin

Tunicamycin was used to block N-linked glycosylation prior to pulse-chase experiments. Cells were incubated with varying concentrations of tunicamycin in regular media for one hour. Media was replaced with labelling media (prior to addition of ^{35}S-Met-Cys) supplemented with tunicamycin for one hour. Cells were then labelled in the presence of tunicamycin. Subsequent treatment of the cells is described below in section 2.2.7.

2.2.7. Cross linking

Cross linking was performed during lysis with the addition of DSP [dithiobis(succinimidylpropionate)] (Pierce) to the lysis buffer. Cells were prepared, labelled and lysed as above with the exception that the lysis buffer contained DSP (200 µg/ml). The lysis buffer used with DSP was PBS with 1% SDS. Crosslinking was stopped with 100 µl of 1M Tris-HCl pH 7.5 prior to spinning the lysate down.

2.2.8. Endoglycosidase H (Endo H) digestions

Endo H (Boehringer Mannheim) was prepared according to manufacturers instructions with the addition of PMSF (800 μl NaCitrate, 200 μl Endo H, 1 μl PMSF). Precipitates treated with Endo H were washed as above (2.2.7) except that in the final wash with Buffer D, beads were resuspended and divided into two aliquots prior to final drying. The first aliquot was incubated with 12.5 μl Endo H for 12 hours at 37°C. A second 12.5 μl was added for an additional 12 hours. The second aliquot was incubated in the same mixture without Endo H. Following a 24 hour incubation, the Endo H was washed out with Buffer D and the samples were prepared for SDS-PAGE as above.

2.2.9. Sucrose density centrifugation

Sucrose density centrifugation was performed as described in the literature (127). Continuous 5% to 20% sucrose gradients were created using stock sucrose solutions. The detergents CHAPs, digitonin or N-Octyl glucoside were utilised in the lysis buffer and sucrose solutions. Gradients were made using the Pharmacia gradient maker. 300 μl of sample was placed on top of a 12 ml cushion in a Beckman SW 41 UltraClear tube and centrifuged at 4°C and 148,000g (36,800 rpm) for 28 hours in an SW41 rotor. Fractions were manually collected in 1 ml aliquots from the top of the tube. Samples were then diluted and run on SDS-PAGE.

2.2.10. Densitometry

Densitometry was performed using the Molecular Dynamics scanner. Gels were prepared without enhance solution and exposed using the PhosphorImaging (Molecular Dynamics) cassette. Computer analysis including densitometric traces were performed on the

resulting data.

2.2.11. Data Imaging

Data were scanned and manipulated using Adobe Photoshop and MacDraw and printed on a Codonix printer.

3. Comparison of Differential Binding of E3/19K to MHC Class I allelic proteins

3.1 Introduction

This study relates the transport rates of MHC Cl I molecules to their ability to interact with the E3/19K molecule. E3/19K binds to every human allelic protein tested so far and all but one H-2 protein. Previous studies involved many groups of researchers using cell lines from different species (128-132). However, since the original E3/19K studies were performed, elucidation of the steps in antigen presentation has occurred. Many different proteins, some of which are allele specific, may be involved. Therefore the rate of maturation for different MHC Cl I proteins is likely to vary when studied in different cell lines. Alleles of mouse MHC H-2 proteins were transfected into human cells to establish a model system with a consistent cellular background.

3.2 Rationale and Goals

The goals of this study were to elucidate the binding requirements of E3/19K for MHC Cl I. By comparing differences between those allelic forms of MHC Cl I proteins that did and did not bind, it was hoped that a set of factors influencing binding would be elucidated. These data would also shed further light on the nature of adenovirus evasion of the immune system and further understanding of MHC function.

3.3   Results

Previous studies had assessed the ability of E3/19K to bind to different MHC Cl I allelic proteins in different cell lines from different species. Comparison of differences of E3/19K binding MHC Cl I alleles in these experiments reflected many variables, some of

which related to differences between species. By transfecting many different MHC alleles into the same cell line, some of the variation due to species differences could be removed. At the same time, because of the allele specific nature of these effects, this also represents a limitation of the study; effects seen in 293 cells may be completely different from those seen in other cell lines.

3.3.1. Transfected H-2 allelic proteins are expressed at different levels at the surface of 293 cells

In order to establish the relative level of surface expression of the H-2 allelic proteins in 293 cells, FACS analysis was performed on the transfectants. Several clones from each transfection were analyzed, and the clone with the highest expression for each transfected allele of mouse MHC Cl I genes was used in all subsequent experiments. The decision to use the clone with the highest level of expression was based on the assumptions that E3/19K had an innate ability to bind particular alleles of MHC Cl I and that large amounts of mouse MHC Cl I proteins expressed in 293 cells would provide more ligand for E3/19K. Limiting amounts of E3/19K seemed unlikely since in adenovirus infected cells, E3/19K is expressed in excess.

Data in Figure 9a shows that these transfectants express the H-2 proteins at the surface in varying levels. The level of endogenous HLA expression in untransfected 293 cells is shown on the left (labelled HLA). While some of the allelic proteins show low levels of expression, all show a detectable level of expression. As a control for transport to the cell surface, in Fig 10c the cell surface levels of the transferrin receptor show that transport to the cell surface is not disrupted.

To determine whether low surface expression of some MHC Cl I proteins was due to competition for factors with endogenous HLA, the level of HLA-A, B and C expression was assessed using W6/32. A comparison of the W6/32 cell surface expression in the transfected and untransfected 293 cells is shown in Figure 9b. These data showed that in every

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transfectant except the K^d transfectant, the level of cell surface expression of the W6/32 epitope was reduced. The level of reduction of W6/32 expression indirectly allowed the monitoring of the effect of the foreign MHC Cl I molecule on the endogenous MHC Cl I. In both the D^b and K^b transfectants the level of W6/32 expression was reduced to a level of 58% and 5%, respectively, of untransfected 293 cells. The K^d transfectant alone showed an increase in the level of W6/32 expression; this was due to cross reactivity of W6/32 with K^d (133). Comparison with levels of K^d expression assessed with the K^d specific monoclonal 34.1.2s and W6/32 expression in the non-transfected 293 cells suggests that the K^d and endogenous expression were additive.

Reduction in cell surface expression of endogenous MHC Cl I proteins could reflect a competition for limited resources such as β_2m or peptides. Mouse MHC Cl I proteins have a higher affinity for human β_2m than endogenous MHC Cl I (133). Excess mouse MHC Cl I may leave little β_2m available for endogenous HLA alleles, resulting in lowered W6/32 expression. The phenomenon of internal competition modulating HLA expression is previously unreported.

Figure 9. FACS analysis of 293 cells and 293 transfectants

   Confluent plates of 293 cells and 293 transfectants were analyzed by FACS. Cells were harvested as described in Materials and Methods. In all cases the untransfected 293 cell data is in the first column and labelled HLA. a) Levels of cell surface expression of transfected MHC Cl I was determined by incubation with allele specific antibodies as listed in table 2. Calculation of arbitrary fluorescence units (AFU) involved taking the difference between mock treated and specifically labelled FITC labelled samples. .. b) Levels of endogenous HLA-A2 expression was assessed using the monoclonal antibody W6/32. c) Transferrin receptor levels were determined using the OKT9 monoclonal antibody.

Specific Cl I Expression
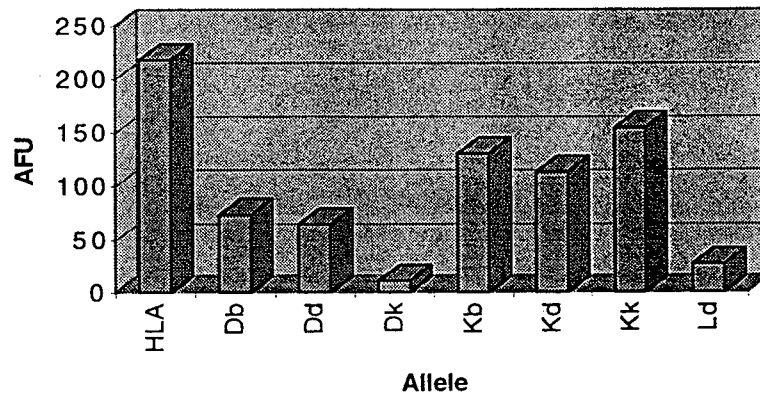
W6/32 Expression
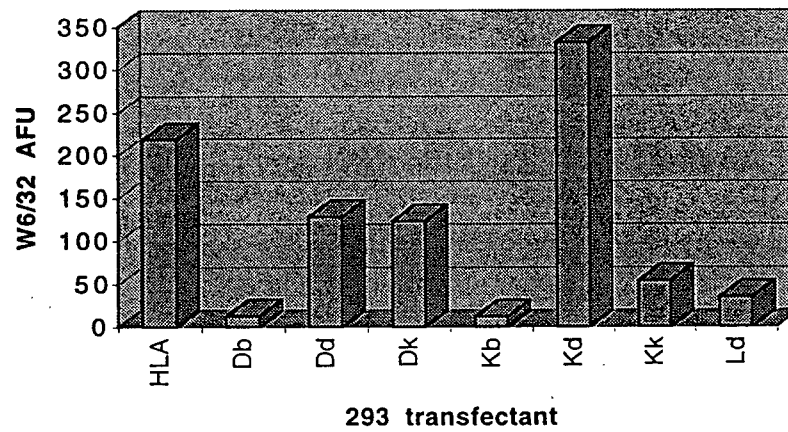
OKT9 Expression
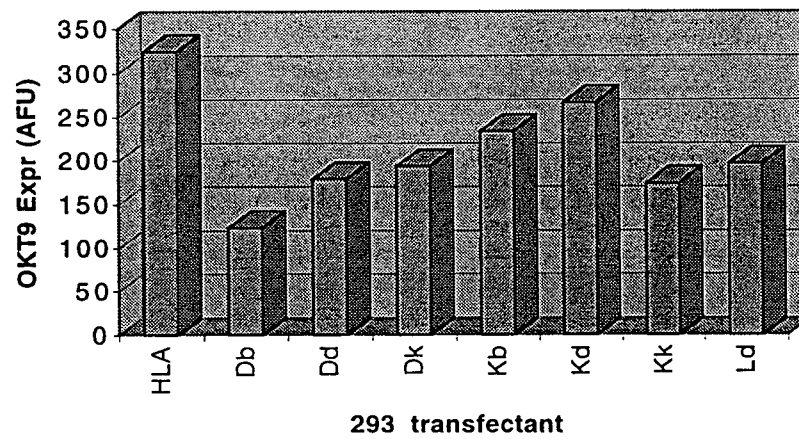
3.3.2. Transport of H-2 allelic proteins to the cell surface of 293 cells

To determine levels of expression of transfected MHC Cl I proteins, monoclonal antibodies were used to precipitate MHC Cl I from cells pulsed for 15 minutes and chased for 0, 30, 120, 240 and 480 minutes. During the course of the chase, the mobility of the precipitated MHC Cl I decreases, reflecting modification of the proteins. MHC Cl I proteins undergo processing from a 43 kDa protein at two hours to a 46 kD MW form present at four hours of the chase. The rate limiting step for transport to the cell surface is escape from the ER. To assess whether the MHC Cl I proteins had left the ER and migrated to the cis-Golgi, Endo H digestion was performed on half of the MHC Cl I precipitates. Glycans of glycoproteins are modified from a high mannose form to a more complex Endo H resistant form in the cis-Golgi.

The analysis shows that a portion of the population of D^b molecules exit the ER (as demonstrated by their insensitivity to Endo H) after two hours. However, a large proportion of the D^b molecules remains Endo H sensitive after four hours, suggesting an extremely slow transport rate. The half life $(t_{1/2})$ for this protein to leave the ER is greater than four hours. In the D^d transfectant, the D^d molecules achieve a mature conformation after four hours. D^d is present in an immature lower molecular weight form for most of the four hour chase. The higher molecular weight form appears after four hours of chase. In the samples treated with Endo H, most of the sample has disappeared from all but the 480 minute timepoint. This likely reflects an unstable D^d MHC Cl I protein, with most of it falling apart during the 24 hour, 37°C Endo H incubation. The Endo H resistant form visible at the final four hour chase point suggests that the mature protein is more stable. The L^d molecule is processed marginally over the four hour chase. It appears that the epitope recognized by the monoclonal used may not be stable under the conditions of the 24 hour incubation with the Endo H buffer.

The K^b molecule is partially Endo H resistant after about one hour and is therefore rapidly transported. Analysis of the K^d precipitation demonstrates that an epitope recognised by the conformation specific mAb for the K^d protein does not appear until 30 minutes post pulse. Endo H digestion reveals that the forms observed at 30 minutes and one hour have not exited the ER; by two hours, much of this molecule is Endo H insensitive and at the end of four hours, almost no Endo H sensitive K^d molecules remain. A similar pattern is observed for K^k. Endo H digestion reveals that the K^k protein has left the ER by two hours post pulse, suggesting that it is even more rapidly transported than K^d.

Figure 10.   Levels of expression and rates of transport of H-2 MHC Cl I proteins transfected into 293 cells

    Lysates from confluent 293 cells transfected with different mouse MHC Cl I (H-2) proteins were precipitated with allele specific monoclonal antibodies as listed in table 2.   Cells were pulsed with Pro-Mix (^{35}S Cys-Met) for 30 minutes and chased for 0, 30, 60 120 and 240 minutes (lanes 1 to 5 respectively).   Aliquots which have been incubated with Endo H are in lanes 6-10.   Levels of expression for each allele were determined.   MHC Cl I proteins with different mobilities reflecting differently processed forms are labelled with arrows.   Precipitates were split and alternately mock treated (lanes 1-5) or treated with Endo H (lanes 6 -10) for 24 hours and then separated by SDS-PAGE.   Endo H resistant proteins have reduced mobility and are labelled by the upper arrow.

Kb
Db — 43 kD

Kd
Dd

Kk
Ld

54
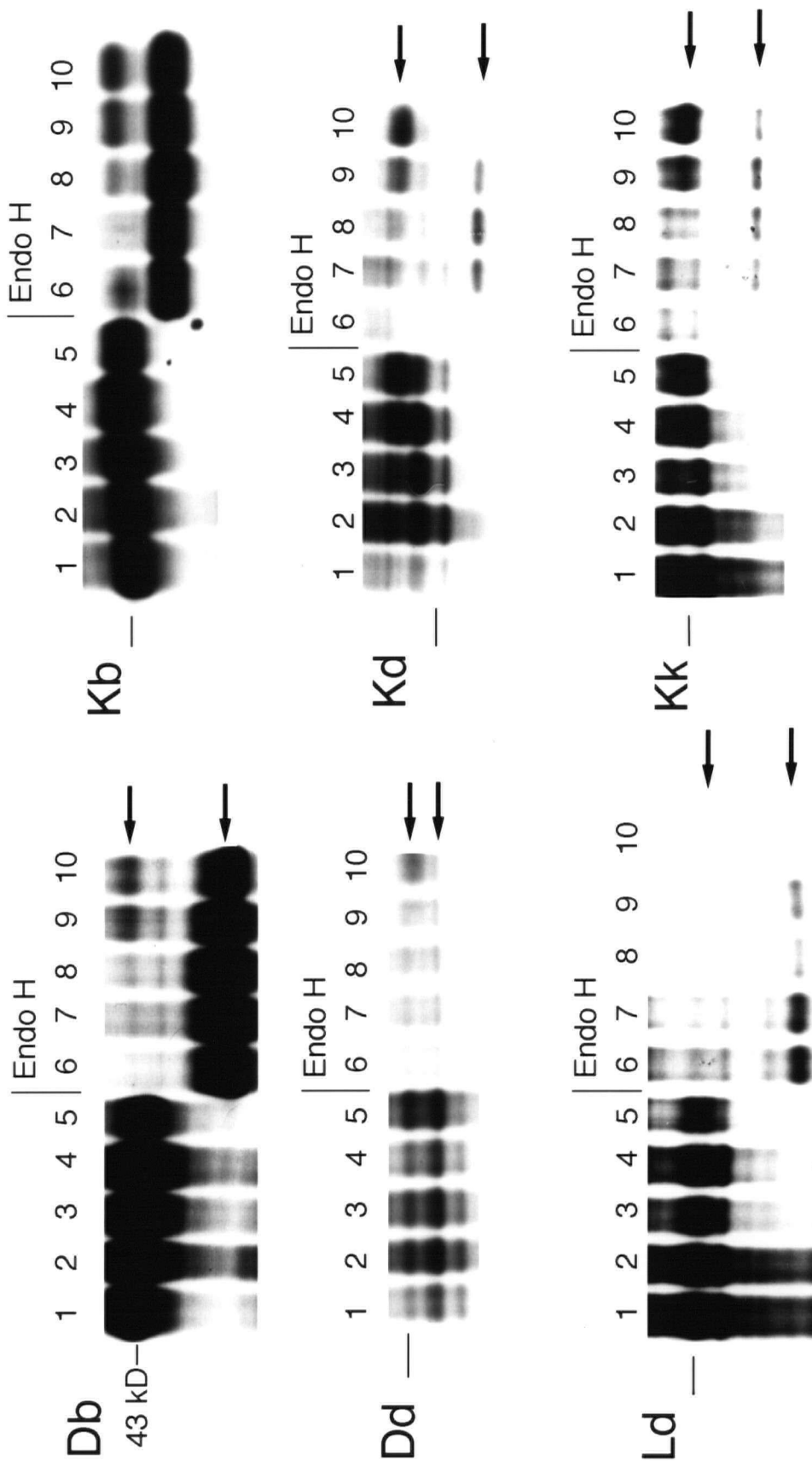
Once expression of H-2 proteins in human 293 cells was established, the effect of the E3/19K on the different proteins was examined. Initially, it was hoped that expression of the transfected mouse H-2 alleles would be uniformly strong and that this level of expression would provide a clear picture about whether E3/19K bound or not. In transfectants with MHC Cl I allelic proteins that bound E3/19K, reduced cell surface expression was expected. In transfectants with proteins that did not bind, no reduction in cell surface expression was expected. For proteins that bound less effectively, it was postulated that temporary association with E3/19K may result in slower transport to the cell surface.

It should be noted that the level of expression for each allele was quite variable in the 293 cells. This variable expression probably reflects the accessory proteins and chaperones available for the transfected allele. Additionally, as shown in Fig 11b, the expression of the transfected allele did affect the cell surface expression of the endogenous MHC Cl I alleles. Highly expressed transfectants such as K^b cause a large reduction in cell surface expression of HLA A, B and C detectable by the conformation specific monoclonal antibody W6/32. This could reflect limited β_2m or peptide.

3.3.3. Co-precipitation of the adenovirus E3/19K protein with MHC Cl I in 293 transfectants

To address the binding capacity of H-2 proteins to E3/19K, the transfectants were infected with Ad2 at a MOI of 5 for 18 hours and metabolically labelled with a pulse of ^{35}S methionine and then chased for 0-4 hours. After lysis, the H-2 allele expressed in each transfectant was immunoprecipitated and examined by SDS-PAGE and autoradiography. Controls were carried out to confirm that the infected transfectants expressed equal amounts of the E3/19K protein (appendix 1). Half of the precipitated samples were digested for 24 hours

at 37°C with Endo H to determine whether the MHC Cl I molecules had acquired mature N-linked glycans. Results are shown in figure 11.

E3/19K is detected by co-precipitation with MHC Cl I in Ad2 infected cells. While confirmation of the identity of E3/19K by subsequent western blotting was not performed in the mouse MHC Cl I transfectants, comparison with parallel immunoprecipitations using the 293.12 cell line helped to establish the identity of E3/19K. 293.12 cells have transfected with the EcoRI d fragment containing the E3/19K protein from Ad2 and exhibit reduced cell surface expression of MHC Cl I in FACS experiments. Comparison of immunoprecipitation of MHC Cl I from 293, Ad2 infected 293 cells and 293.12 cells reveals a protein corresponding to E3/19K (131).

After a chase of 2 and 4 hours, the D^b molecule co-precipitates a protein at about 25 kDa that corresponds to E3/19K. A corresponding band also appears at the 2 and 4 hour time points in the Endo H digested samples. In the Endo H digested samples, the band corresponding to E3/19K has the lower MW consistent with digestion of the N-linked sugars on this molecule. The three bands in the Endo H digested samples correspond to differences in sensitivity in the two carbohydrates present on E3/19K. Co-precipitating with D^b is the $\beta_2 m$ molecule, suggesting that transfected D^b proteins are being assembled with human $\beta_2 m$. The appearance of $\beta_2 m$ in the samples that have been exposed to 37°C for 24 hours in the Endo H treatment suggests that the D^b molecule has adopted a stable conformation.

The D^d molecule does not co-precipitate with E3/19K. The Endo H digestion of D^d reveals it exits the ER at the same rate as in the uninfected cells. An Endo H resistant protein is observed at 4 hours. The L^d molecule also does not co-precipitate E3/19K during the four hour chase. However, in both infected and uninfected cells, the L^d molecule remains Endo H

sensitive throughout the four hour chase.

The K^b protein co-precipitates E3/19K. E3/19K appears to co-precipitate instantaneously with the K^b molecule appearing at all time points. At the 2 and 4 hour time points, the molecular weight of E3/19K has decreased marginally, suggesting carbohydrate processing. The Endo H digestion shows that two more forms of E3/19K appear after two hours; they differ in carbohydrate attachments. After two hours, a second protein at about 22 kDa appears. At four hours post pulse an additional band at the same molecular weight as the untreated precipitates appears. This band likely represents an Endo H resistant form of E3/19K.

The K^d molecule co-precipitates E3/19K strongly at all timepoints. K^d is assembled by about 30 minutes post chase. At this point, the heavy chain and β_2m is observed. The K^d molecule remains Endo H sensitive throughout the entire four hour chase. Once again, two forms of partially Endo H resistant E3/19K are observed co-precipitating with Endo H sensitive K^d molecules. These data suggest that E3/19K is cycled back and forth through more mature compartments such as the cis Golgi and is able to bind MHC Cl I molecules in an earlier compartment and retain them there. Differences in the maturity of the carbohydrates on E3/19K bound by H-2 MHC Cl I proteins suggests differing affinities for E3/19K based on its processing. The K^k molecule does not co-precipitate with E3/19K. The K^k is entirely Endo H resistant after about four hours.

To summarize results from figure 11, the E3/19K protein clearly co-precipitates with K^b, K^d, and D^b proteins. L^d does not co-precipitate E3/19K during the chase times performed in this experiment. The Endo H sensitivity of K^d, D^b, and K^b during the four hour chase confirms that they do not exit the ER during this period and thus the E3/19K binding of these

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molecules. The K^k, and D^d molecule are Endo H insensitive after four hours. Comparison with Figure 10 shows differences between MHC Cl I transport in adenovirus infected and non infected cells. E3/19K had no effect in retarding the egress of these allelic forms of MHC Cl I proteins from the ER. The D^d protein is able to achieve Endo H sensitivity at the same rate that it did in non-infected cells (Figure 10), becoming Endo H resistant after 4 hours. Interaction between D^d and E3/19K is likely to be weak or nonexistent.

Figure 11. The effect of Ad2 infection on the rate of transport of transfected MHC Cl I proteins in 293 cells

     293 cells and 293 transfectants were infected with Ad2. Infection was at MOI of 5 eighteen hours prior to labelling with Pro Mix (^{35}S-Met/Cys). The cells were pulsed for 30 minutes and chased for 0, 30, 60 120 and 240 minutes (lanes 1 to 5) respectively. Aliquots which have been incubated with Endo H are in lanes 6-10. MHC Cl I was precipitated with allele specific antibodies as listed in table 1. Samples were divided and alternately mock treated or treated with Endo H for 24 and then separated by SDS-PAGE. E3/19K migrates at ~25 kDa and is denoted by arrows. β_2m also co-precipitates and is seen on the gel at 12kDa.
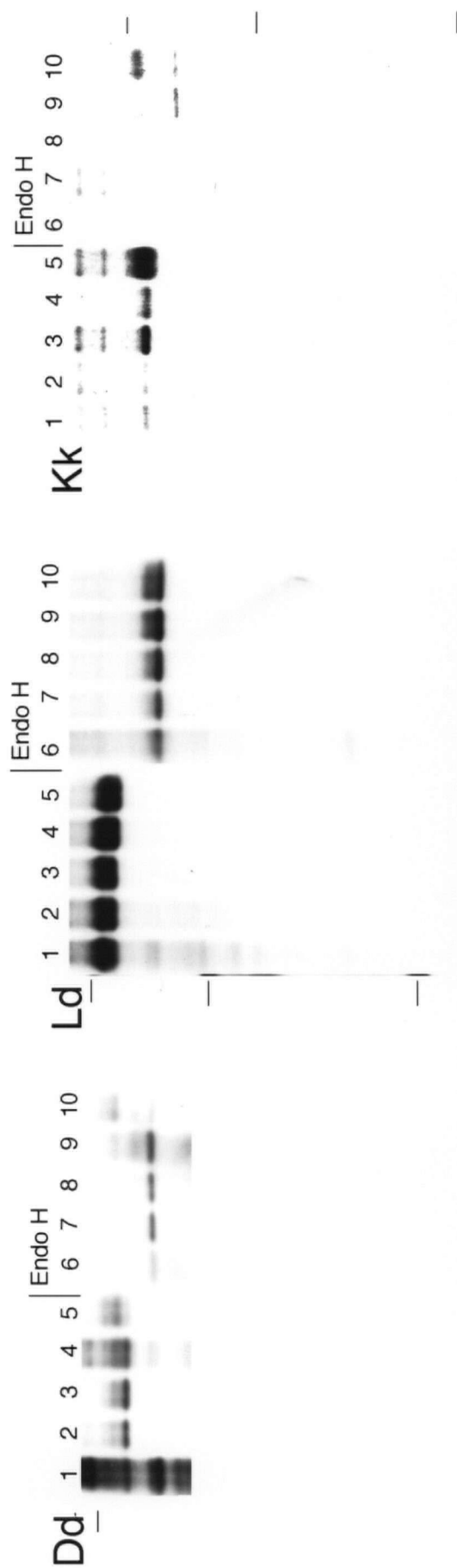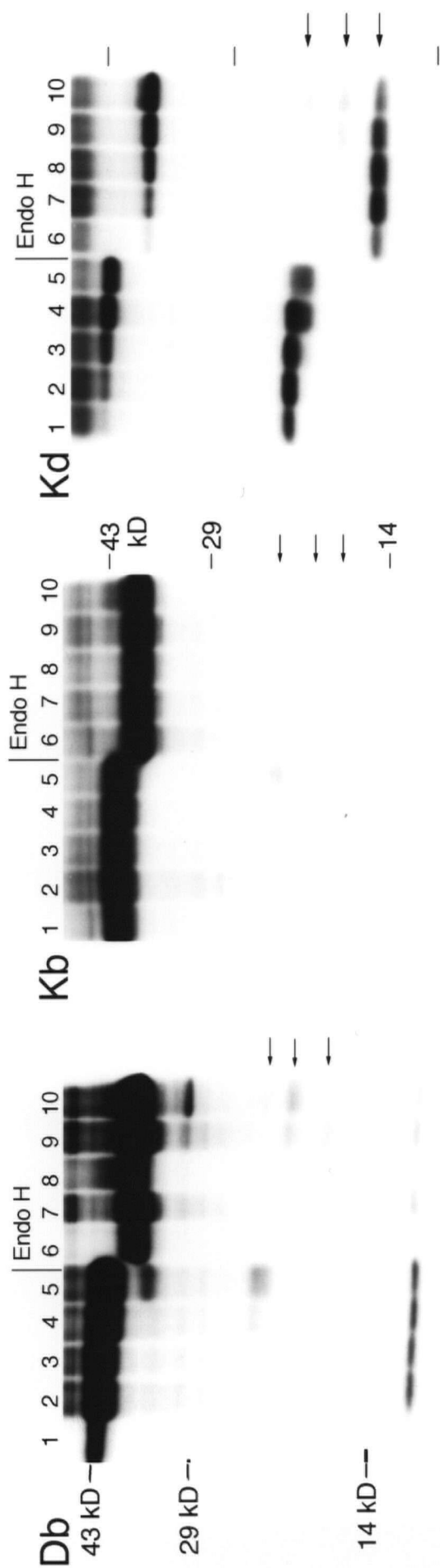
3.3.4. Adenovirus infection of 293 transfectants reduces cell surface expression of MHC Cl I proteins

To assess the effect of E3/19K on cell surface expression, FACS analysis of Ad2 infected transfectants was performed (figure 12). While the cell surface expression of transfected H-2 proteins is variable, when infected with Ad2, cell surface expression of many of these MHC Cl I proteins is reduced. The FACS data show that in the untransfected 293 cells, infection with Ad2 causes reduction of the cell surface HLA (W6/32 epitope) to 62% of uninfected expression. The difference in cell surface expression of the transferrin receptor in infected and mock infected cells is shown in figure 12b demonstrating that the effect of adenovirus infection is not blocking all transport to the cell surface. In the transfected 293 cells, Ad2 infection causes the cell surface expression of the H-2 transfectants to be reduced as well. A comparison of levels of cell surface expression of H-2 proteins in infected and uninfected cells shows which alleles are susceptible to E3/19K binding. Figure 12 shows the greatest level of reduction of cell surface expression was highest for D^b which expressed 43% of the D^b that the uninfected transfectant expressed. L^d also showed a large reduction at 44%. At the other end of the spectrum, the proteins that were affected the least were K^k and D^k, which showed 93% and 95% of uninfected expression respectively. The cell surface expression of K^d (62%), K^b (73%) and D^d (81%) were moderately inhibited. These data support the hypothesis that amongst the H-2 molecules K^d, D^b and L^d bind to the E3/19K molecule with the greatest affinity.

However, it should be noted that while a FACS experiment represents 5000 individual events, the results are subject to some presorting. Sampled cells are gated by size and cells are selected to represent a healthy population. While size selection is performed prior to observing

results, the outcome of the experiment could reflect arbitrary sampling of a larger, more diverse population.

Figure 12. The effect of Ad2 infection on MHC Cl I expression in 293 cells and 293 transfectants

a) 293 cells and transfectants were alternately infected or mock infected with Ad2 at MOI of 5. Infection was started eighteen hours prior to harvest for FACS analysis. Cell suspensions were specifically labelled with MHC Cl I allelic protein specific antibodies as listed in table 1. Suspensions were analyzed using flow cytometry. Arbitrary fluorescence units (AFU) are the difference between mock treated and specifically labelled FITC labelled samples for each transfectant. The first bar of each pair shows the uninfected cell surface expression; the second the level of cell surface expression in infected transfectants.
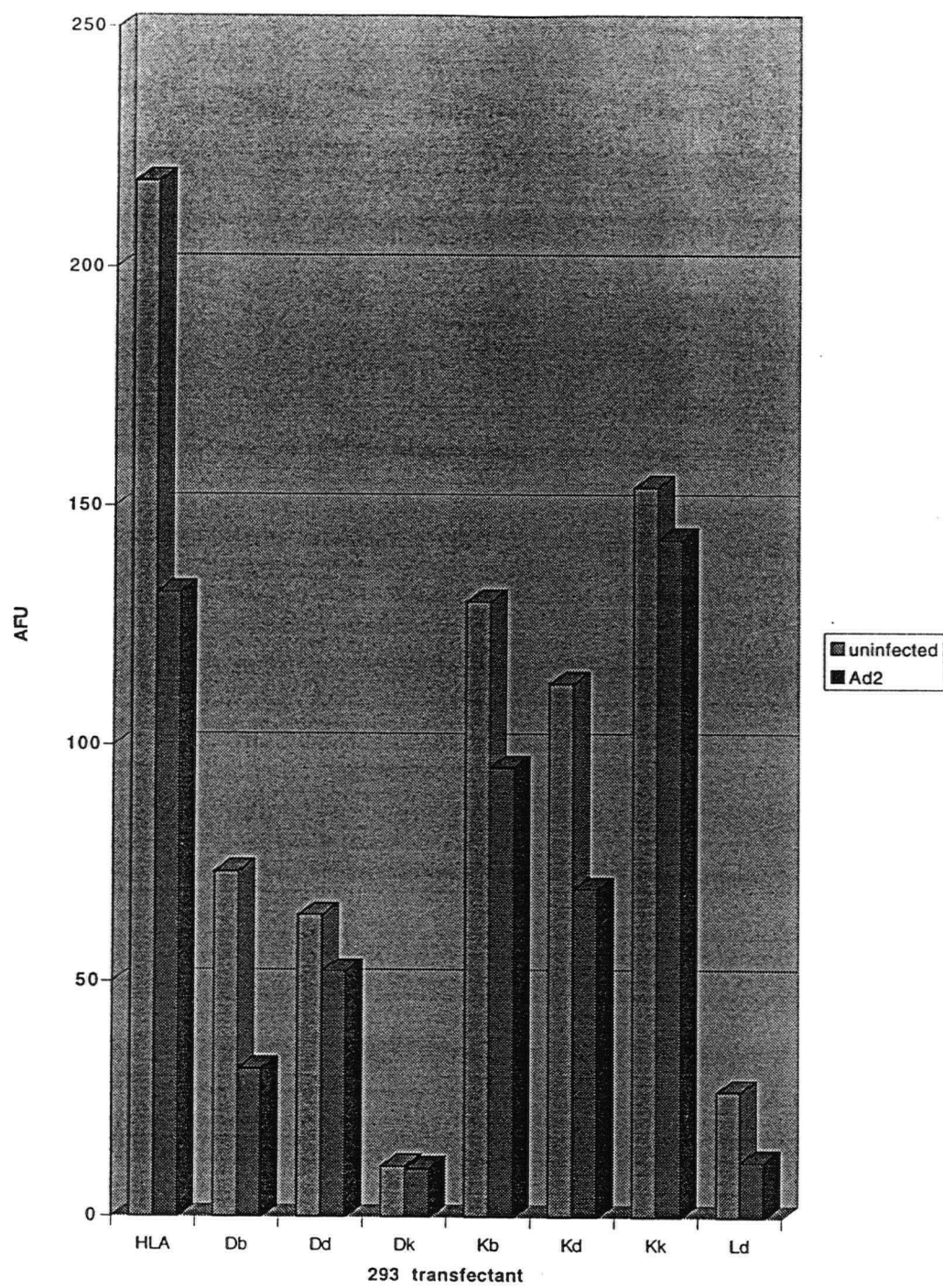
b) The levels of the transferrin receptor at the cell surface are compared in infected and uninfected transfectants.

Transferrin Receptor Expression
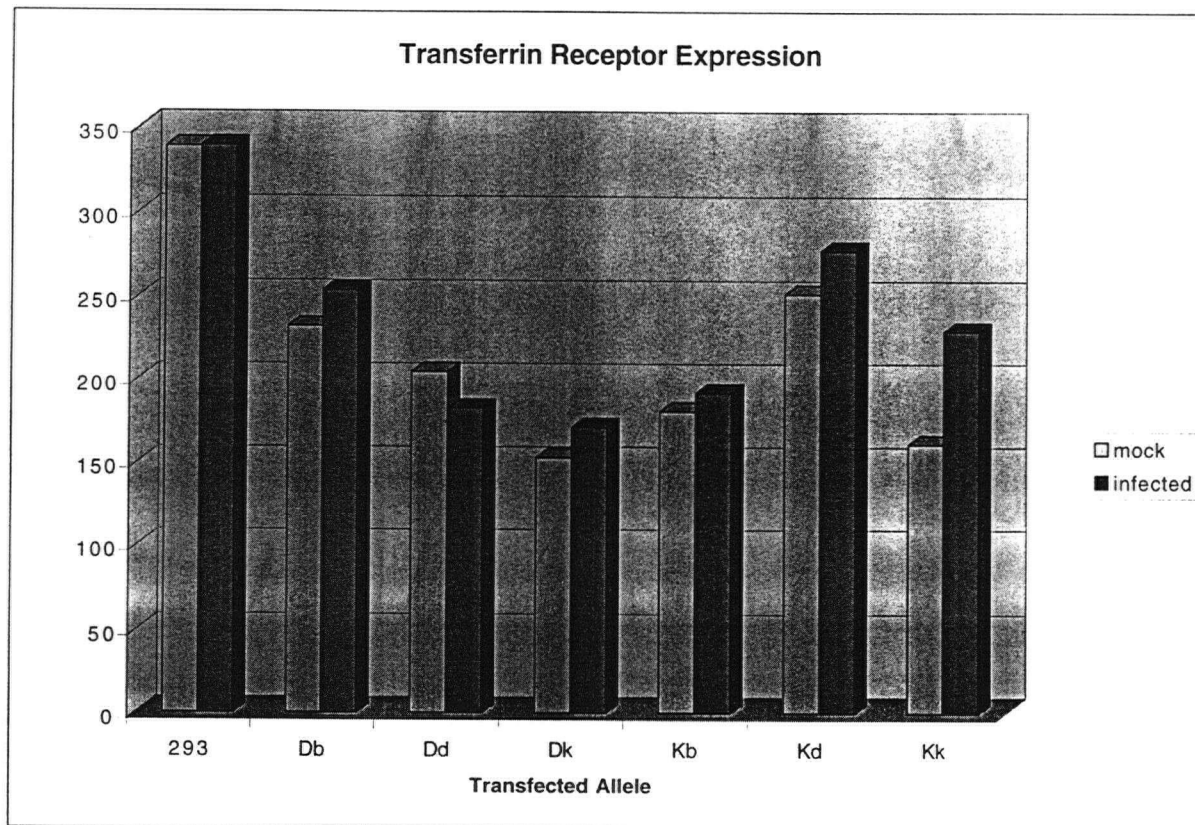
Results representative of these experiments are summarized in table 3. As determined by immunoprecipitation, K^d, K^b and D^b all co-immunoprecipitate with E3/19K, but the products of alleles K^k and D^d do not. The data for L^d is less conclusive. Column two shows the reduction of cell surface expression of these proteins as detected in FACS analyses. For the proteins that co-immunoprecipitate E3/19K, a reduction in cell surface expression is noted. Reduction in cell surface expression results range from 43% of the level of uninfected

expression to 73%. For the L^d allelic protein, cell surface expression of 44% that of uninfected is observed in the presence of E3/19K (Ad infection). This is suggestive of down regulation due to association with E3/19K. Surface expression of D^k is unaffected by Ad infection. Finally, in the third column, the rate of exit from the ER assessed by the Endo H sensitivity (or the difference in Endo H sensitivity) of MHC Cl I proteins from infected and non-infected cells is summarized. In this column, it is observed that there are no differences in Endo H sensitivity in the K^k and D^d proteins in infected and non-infected cells.

Table 3. Summary of E3/19K association with H-2 allelic proteins

Mouse Allelic proteins		Co-IP (a)	FACS data (b)	Endo H* sensitivity
Binders	K^d	+	62%	+
	K^b	+	73%	+
Slow-binders	D^b	+/-	43%	+/-
	L^d	-	44%	-
Non-binders	K^k	-	93%	-
	D^d	-	81%	-
	D^k	-	95%	?

*After 4 hours

An overview of all the techniques employed gives an indication of the ability of each H-2 transfectant to associate with E3/19K. The highest expressing clone for each transfectant was selected and tested above. Based on each technique, the H-2 proteins tested are grouped into three categories. The binders show co-immunoprecipitation (a). The slow-binders are not observed to co-immunoprecipitate, but do result in reduced cell surface expression as seen in the FACS experiment demonstrated here (b). Finally, the non-binders have not been shown to co-precipitate and also do not reduce the cell surface expression of MHC Cl I.

3.4 Discussion

The strong point of this study is that it is the first in which the binding of the H-2[b],[d], and [k] proteins to the Ad2 E3/19K protein in the context of a common cellular background has been examined. This set of experiments made the initial assumption that variable cell surface expression of transfected mouse genes was likely due in part to varying transport rates through the cell. These transport rates likely reflect incompatibilities between human antigen presentation proteins and mouse MHC Cl I alleles. Additionally, it is possible that accessory proteins required for mature, stable MHC Cl I molecules may also be missing or of an inadequate fit.

The initial assumption made in this set of experiments was that overexpression of MHC Cl I alleles would provide more ligand for potential E3/19K binding limits the conclusions that may be drawn from the data. While overexpression of transfected proteins in these experiments results in clear demonstrations of E3/19K binding and non-binding for some allelic proteins, in other cases it results in unstable, short lived MHC Cl I proteins. The instability observed in Endo H digestions suggest that some of the transfected alleles may be very short lived in 293 cells. The ability of E3/19K to bind these alleles is likely low and reflects limits of the expression system rather than the ability to bind these alleles. These instability seen in these MHC Cl I proteins may be due to our detection system. Perhaps the monoclonals selected do not recognize or act to destabilize transfected MHC Cl I proteins. Alternately, some MHC Cl I-β_2m-peptide complexes may be more susceptible to disruption by the detergents used in these experiments.

This set of experiments revealed many problems associated with protein trafficking and re-establishment of antigen presentation by mouse MHC proteins in human cells. Further experiments which would more finely control the many variables could include a comparison

of unfolded or improperly folded proteins to those detected by conformation specific monoclonal antibodies. This would allow some comparison of total levels of expression to transport of mature proteins out of the transfected cell. A wider sampling of the clones of transfectants to include those which expressed mouse MHC Cl I at lower levels would allow a better assessment innate affinity between MHC Cl I proteins and E3/19K. Finally, to broaden the scope of the conclusions, this experiment could be repeated in other cell lines.

Within the constraints outlined above, this study has identified those allelic proteins that interact with E3/19K in our model system. This study also extends those studies done earlier with K^d, K^k and the hybrid MHC Cl I molecules containing regions of K^k spliced to regions of K^d molecules (131). Furthermore, this study has identified a new sub-group of slow binding molecules. The identification of those allelic MHC Cl I proteins that bind E3/19K has been determined in three ways. Co-immunoprecipitation of E3/19K with MHC Cl clearly demonstrates binding. In addition to co-immunoprecipitation, the effect of E3/19K is assessed by comparing the rate of exit of MHC Cl I proteins from the ER in infected and mock infected transfectants. The third method to determine binding is by detecting reduction of MHC Cl I expression at the cell surface. Taken together, these methods identified which allelic proteins were binders, non-binders and slowbinders.

These data are summarised in table 3. The first method shows E3/19K clearly binds to D^b, K^d and K^b but not K^k, D^d and L^d. The second method confirms that E3/19K does not slow the exit from the ER of D^d and K^k. Differences in Endo H sensitivity between infected and mock infected transfectants are observed for D^b, K^d and K^b. L^d is poorly transported to the cell surface, but is more stable (i.e. does not fall apart during Endo H treatment) in Ad2 infected cells.

FACS analysis shows a reduction in cell surface expression for all transfected allelic proteins in the presence of E3/19K. While overall expression is of transfected alleles is quite variable, the percentage difference between infected and uninfected expression was compared. This comparison shows the level of reduction of MHC Cl I cell surface expression is not as dramatic for the K^k and D^d alleles as for K^d, K^b, D^b, and L^d. While the data used here was from a single experiment, repetitions of the FACS experiments demonstrated that while overall cell surface expression varied greatly between experiments, the differences in expression due to E3/19K (between infected and non-infected) remained fairly constant, varying no more than 10%.

The results above suggest that the nature of the L^d association with E3/19K is different from that of other allelic proteins. The first method employed shows that over the course of a four hour chase, L^d does not co-immunoprecipitate E3/19K. This could be due to the fact that the monoclonal antibody used interferes with E3/19K binding. It could also be because that over the course of the four hour chase, L^d does not fold into a conformation that promotes binding to E3/19K. Figure 10 shows that during a four hour experiment, only a limited amount of this protein is detected and Endo H digestion and exposure to 37°C for 24 hours suggests that what little is there is quite unstable. FACS analysis confirms the level of cell surface expression of the L^d protein is lower than that of other allelic proteins, but is still detectable. FACS analyses, which assay a steady state level of expression, demonstrate that L^d does fold into a mature conformation and can be expressed on the cell surface (Figure 9a), and that this level of expression is reduced by E3/19K (figure 12).

This finding allows the proposal of a new subgroup of allelic proteins that associate with E3/19K. L^d (and D^b) illustrate the subgroup of allelic proteins that is slowly transported out of the ER and will bind E3/19K, but only once having achieved a transport competent

conformation. These proteins can be called "slow-binders" because the rate at which they fold this mature conformation and are able to bind E3/19K is greatly reduced. Slow-binders are characterised by their slow maturation rate and are transported out of the ER quite slowly. It is likely the length of time that they are present in the same compartment with E3/19K that enhances the opportunity to associate and be retained.

The D^b protein is another member of this "slow binding" group. One hour after the beginning of the pulse, only trace amounts of E3/19K are co-immunoprecipitated; it is only after four hours that significant amounts of E3/19K are seen. In contrast, K^d binding strongly to E3/19K occurs almost immediately, or shortly after translation. The E3/19K molecule co-precipitates even at time zero suggesting that E3/19K made during the pulse can bind already pre-existing K^d molecules. In addition, at the 30 minute timepoint, K^d is still available for the E3/19K to bind. This contrasts sharply with the D^b and L^d molecules that appear to mature (acquire $\beta_2 m$, peptide, dissociate calnexin) more slowly than K^d. However, once a mature conformation has been achieved, E3/19K binds the "slow-binders" with great efficiency. The conformation of D^b that E3/19K binds is initially not present in the D^b transfectant, but appears with subsequent maturation. This suggests that E3/19K binding is dependant on overall MHC Cl I conformation rather than specific residues. This view is in contrast with previous studies that have attempted to map specific residues crucial for E3/19K binding (128, 131).

In cells transfected with the MHC Cl I alleles classified as slow-binders, the level of endogenous MHC Cl I (or W6/32) cell surface expression is quite low. The cellular components that participate in the maturation of MHC Cl I molecules (peptide, peptide transporters, calnexin, and other chaperones) may be occupied with or competed for by the slow binding H-2 MHC Cl I and therefore have reduced availability for the endogenous HLA. The reduction of endogenous MHC Cl I indicates that there is some limit to the number of

MHC Cl I proteins that can be correctly folded and/or expressed by a cell.

The K^k protein is one of the allelic proteins which does not bind E3/19K. It is also exits the ER very quickly. The $t_{1/2}$ for Endo H resistance is approximately 15 minutes; the average for endogenous MHC Cl I is 45 minutes to 1 hour. Rapid exit from the ER may reflect a lack of competition for cofactors such as β_2m or peptide resulting in very rapid folding. Alternately, it could reflect a reduced ability for the human chaperones present in 293 cells to recognize and retain the K^k protein and ensure quality control. However, K^k has limited exposure to E3/19K (reduced period of time) and the opportunity to associate may simply not occur. The inability of K^k to bind E3/19K may not due to lack of affinity but rather lack of exposure.

If E3/19K binding to MHC Cl I is partly due to being in the right place for the appropriate period of time, then the contribution of affinity between these proteins becomes less clear. In studies where E3/19K is precipitated directly, the ratio of E3/19K to MHC Cl I is always much higher than when MHC Cl I is precipitated. Clearly there exists a large pool of E3/19K that is not binding to MHC Cl I. Conversely, the level of E3/19K expression within a cell correlates inversely to the level of cell surface MHC Cl I expression (134). While affinity between MHC Cl I proteins and E3/19K has been noted, an estimation of the relative strength of this affinity has never been made.

Secondly, the period of time required for maturation, and hence the length of exposure to E3/19K is influenced by many other factors including but not limited to availability of correct peptide and the ability of chaperones such as calnexin, calreticulin, tapasin, TAP, glucosyltransferases, protein disulphide isomerase and others to bind to mouse MHC Cl I proteins. Our evidence shows that for the small sample of alleles expressed in 293 cells, great

variations in stability were observed. Our findings are limited by the sources of variability inherent in our expression system. This variability reflects the complexity of antigen presentation and protein folding in general.

Some specific limitations include the level of expression of the transfected allele. As mentioned above, endogenous HLA cell surface expression was reduced when mouse allelic proteins were expressed suggesting that there is a limit to the total MHC Cl I that can be processed by a particular cell. Overexpression of transfected genes may perturb regulation of this limit. Secondly, species differences in proteins involved in antigen presentation may affect the expression of stable conformations of mouse MHC Cl I proteins in human cells. However, it is likely that such limitations will be present in any study of E3/19K binding MHC Cl I proteins and that a better picture of MHC Cl I binding by E3/19K could be obtained by using multiple expression systems.

The binding of E3/19K by MHC Cl I allelic proteins was examined against a common cellular background. 293 cells were chosen as they are amongst the best characterised cell lines for Ad 2 infection and are easily infected. The highest expressing clones were chosen to be representative for each allelic protein, with differences in levels of gene expression observed for some alleles. However, when infected with Ad 2, levels of E3/19K expression are well in excess (appendix 1); therefore limited access to E3/19K is unlikely a factor in this study. Differences in allele expression may influence proper folding and exit from the ER.

Based on the this set of experiments, a speculation about E3/19K binding can be made. E3/19K binds to those MHC Cl I molecules that it is exposed to for. MHC Cl I alleles which are normally processed through the ER remain there for the period of time necessary for peptide loading and proper folding. Chaperones such as calnexin serve effect the quality control. MHC Cl I molecules that fold quickly probably escape the attention of E3/19K.

Rapid folding may reflect bypassing the quality control mechanism altogether. Reduced affinity between chaperones and MHC Cl I proteins and could result in empty or non-functional MHC Cl I at the cell surface which in adenovirus infected cells, are not bound by E3/19K.

Figure 13 outlines the different fates for MHC Cl I. The different paths that can be taken are represented by arrows (labelled 1, 2 or 3). The length of the arrow gives an indication of the amount of time the process takes. The dashed arrow indicates an expedited path. The binders are assumed to follow the first path. They are efficiently and rapidly transported through the ER and Golgi to the cell surface. The slow-binders take the second path. These allelic proteins are less efficiently transported and spend more time in the ER. Finally, the non-binders are very rapidly transported to the cell surface and may possibly bypass the processing machinery altogether. This results in low chance of contact with E3/19K.

Figure 13. Overview of maturation paths for the MHC Cl I complex

The maturation path through the ER and Golgi is illustrated. Different potential paths are shown for each of the types of MHC Cl I proteins based on their ability to bind E3/19K. Binders, non-binders and slow-binders are depicted. 1) Binders travel through the ER and Golgi in the most straightforward manner. Interaction with chaperones in the ER and Golgi ensures proper processing and maturation. 2) Slow-binders interact with the same chaperones, but the rate of transport through the ER is slower. This is depicted as a circuitious route through different ER compartements. 3) Non-binders travel quickly through the entire ER-Golgi maturation pathway, arriving at the cell surface very rapidly. This is depicted with a dashed line.

Complex of HLA class I and peptide

Cell surface

(a) Protein synthesis ⟹ Antigen

+/− Ubiquitin

(d) Exocytosis

Proteasome

Golgi

Peptides

(b) HLA class I synthesis

TAP

Calnexin

β2m
ERp57
Calreticulin

Tapasin

(c)

1 2 3

Endoplasmic reticulum

HLA heavy chain   β2m   Calreticulin   ERp57   Tapasin

75
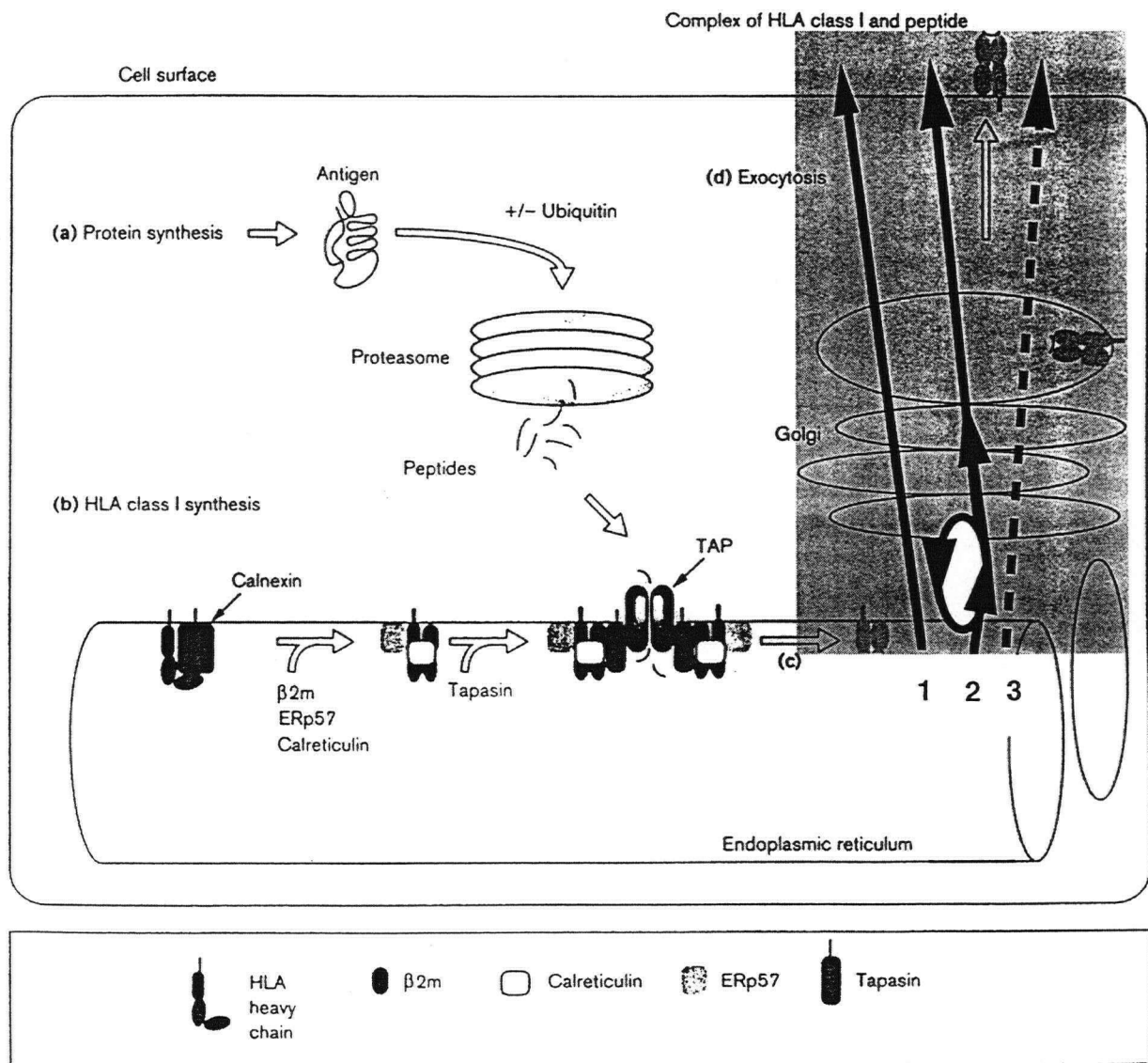
4. Efforts to increase the cell surface expression of H-2 D^b

4.1. Introduction

In the previous chapter experiments with different H-2 transfected 293 cells were discussed. Of the transfectants studied, the 293D^b transfectant was the most intriguing. The D^b protein was unusual because while the cell surface expression was quite high, a much larger proportion of the D^b molecules remained inside the cell and was Endo H sensitive. This protein was slowly transported through the ER with the majority remaining intracellular. Other proteins seemed to mature through the ER properly, indicating that mouse allelic proteins can be successfully transfected and expressed in the human 293 cells. Much of the D^b that is made is still within the cell at four hours post pulse. In the last chapter it was shown that in transfected cell lines, surface expression for endogenous MHC Cl I was reduced. This suggested that the H-2 proteins were competing for factors required for MHC Cl I maturation. The limit for levels of cell surface expression in the 293D^b transfectant could be a shortage of an essential co-factor required for proper conformation. Improperly folded H-2 Db proteins could be prevented from escaping from the ER.

Previous studies in cell lines exhibiting low cell surface expression revealed many strategies to restore expression to regular levels. These studies were instrumental in determining which components of a cell were crucial for regular expression of the MHC Cl I molecule. In general, the deficiencies in these cells could be broken down into two or three categories. In order to determine the nature of the incomplete expression of the D^b protein, strategies previously applied to other surface expression mutants were utilised.

Some of the earliest MHC Cl I cell surface negative mutants characterised were those that lacked β_2m. The Daudi cell line, a mouse cell line lacking β_2m and consequently MHC Cl

I cell surface expression, was restored to a normal phenotype when fused with a cell line expressing β_2m (135). Other experiments also demonstrated the dependence of the H chain on the β_2m for cell surface expression (136).

Another effective treatment for cells with reduced cell surface expression is the incubation with γ-IFN (137). γ-IFN is part of a normal cellular antiviral response; many elements of the antigen presentation pathway have interferon regulatory elements in their promoters. The antiviral effects of γ-IFN have been widely documented (138). The effects of γ-IFN include widespread upregulation of antigen presentation elements. γ-IFN reactive elements upregulate transcription of many of the factors necessary for MHC Cl I gene upregulation. These factors include β_2m, MHC Cl I, TAPs and LMPs. Upregulation of these elements enhances the ability of infected cells to present antigens at the cell surface. In cells that present little MHC Cl I at the cell surface, γ-IFN increases expression. The mutant mouse cell line CMT-64 has reduced MHC Cl I expression at the cell surface due to missing transporters (139). Similar results have been reported with other cell lines (140). Restoration of MHC Cl I expression has been achieved by transfecting the cell line with TAP 1, TAP 2 and both TAP 1 and TAP 2 (47). In the absence of transfected TAPs, cell surface expression can be stimulated by treating these cells with γ-IFN. Treatment with γ-IFN upregulates the transporters, as well as β_2m and MHC Cl I, resulting in cell surface expression (139).

Studies with the TAP 2 defective mouse cell line RMA-S and the parental line RMA demonstrated that a population of MHC Cl I molecules devoid of peptide exists. These 'empty' MHC Cl I molecules are expressed at the cell surface (141). Indeed, some of the

earliest studies with peptides and MHC Cl I were done showing RMA-S cells becoming sensitized to CTL killing by the addition of extracellular peptide (142). These cells have a mutation in the TAP 2 peptide transporter and are unable to form a TAP 1/ TAP 2 heterodimer required to transport a full complement of peptides into the ER. Many of the D^b and K^b molecules in this cell line are never loaded with peptides. They are quite unstable and consequently expressed at a low levels on the cell surface. Growing cells at 26°C increases the thermodynamic stability of characteristically unstable MHC Cl I molecules devoid of peptide and permits them to survive longer at the cell surface. The phenomenon of empty MHC Cl I molecules on the cell surface was shown to be normally occurring when empty MHC Cl I molecules were not only found on mutant cells growing at low temperature but also found on the surface of the non-mutated parental cell line RMA (35).

One of the mechanisms above might be responsible for the lack of cell surface expression of the D^b protein in the transfected $293D^b$ cell line.

4.2 Rationale and Goals

The goals of this study were to examine a MHC Cl I protein that remained intracellular. Several different strategies were employed to determine if intracellular stores of the D^b protein could be coerced to travel to the cell surface. These studies would shed light on the nature of antigen presentation and also protein maturation and transport. The analysis of regular transport of cell surface bound glycoproteins may allow elucidation of several of the mechanisms present for quality control in protein transport through the ER and Golgi.

4.3   Results

4.3.1.   The D^b protein remains intracellular in the $293D^b$ transfectant

Endo H analysis of the $293D^b$ transfectant reveals that the D^b protein is well expressed. Figure 14 shows large amounts of D^b expressed imunoprecipitated from lysates of these cells. However, Endo H digestion reveals that only a small fraction of the transfected H-2 D^b protein is able to exit the ER. This figure shows a pulse-chase experiment. At the right side of the figure the lysates have been incubated with Endo H. Most of the lysates are Endo H sensitive; at 2 and 4 hours post chase Endo H resistant D^b is observed (upper arrow).

Figure 14. Intracellular accumulation of H-2D^b in 293 cells

Confluent 293 cells transfected with MHC Cl I H-2 D^b (293D^b) were pulsed for 30 minutes and chased for 0, 30, 60 120 and 240 minutes (lanes 1 to 5) respectively. MHC Cl I was precipitated with protein specific antibodies as listed in table 1. Samples were divided and alternately mock treated or treated with Endo H for 24 and then separated by SDS-PAGE. Endo H sensitive proteins are identified by the lower arrow; Endo H resistant proteins by the upper arrow.
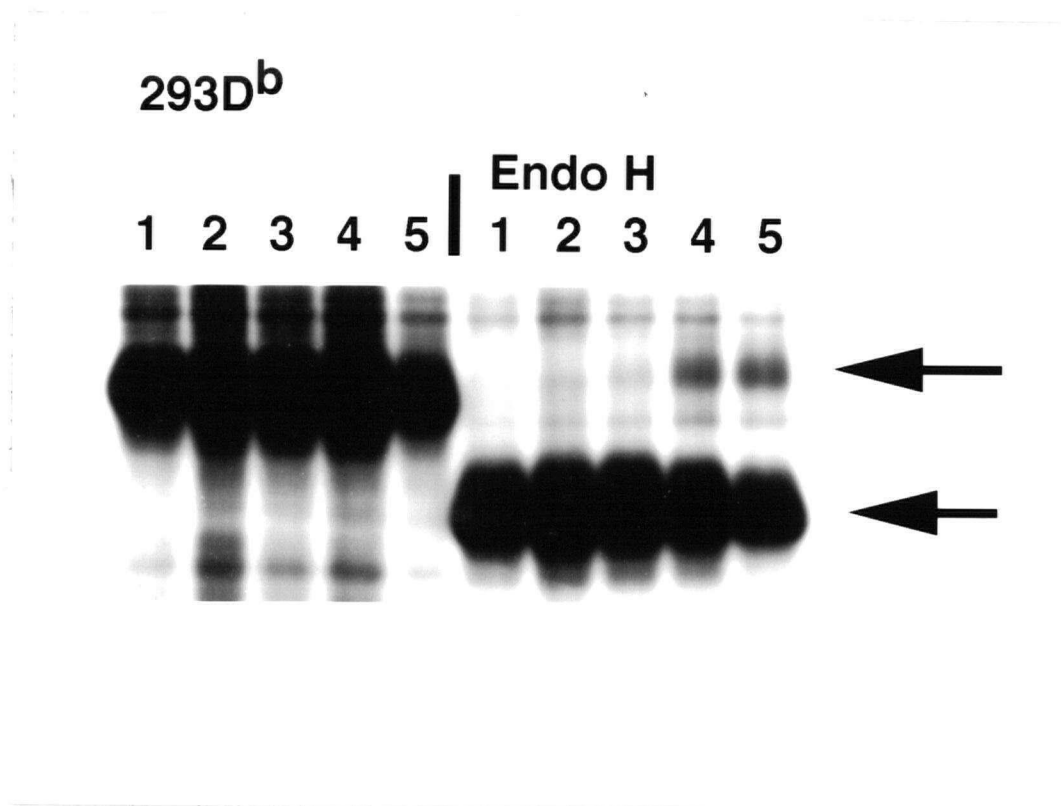
4.3.2. Growth at 26°C does not result in the bulk of the intracellular stores of the D^b protein escaping the ER

In figure 15 the effects of growth at 26° are shown. The MHC Cl I allele D^b was precipitated and half of the lysate was incubated with Endo H.Culture at 26°C results in many contaminating proteins co-immunoprecipitating with the mouse MHC Cl I protein. These contaminants are characteristic of precipitates from temperature shocked cells; incubation at 37°C for 24 greatly reduces the levels observed suggesting that they are quite unstable. While growth at 26°C results in more Endo H resistant D^b proteins, most of the population remains Endo H sensitive. It was reasoned that 293 cells may lack the peptide repertoire bound by the D^b protein. Only the earliest studies examining peptide transporters had suggested there existed allele specificity to the peptide transporters (38, 143). However, if the D^b molecules in the 293 cell were unable to obtain the correct peptide to form a trimolecular complex, they would be unable to achieve the correct conformation to exit the ER. This would result in large amounts of the D^b population remaining in the ER. Alternately, D^b complexes devoid of peptide may be formed, but be thermodynamically unstable and hence very short lived. These short lived species would likely be too unstable to be detected outside of the ER. It had previously been demonstrated that unstable MHC Cl I molecules lacking peptide could be stabilised and detected at the cell surface by culturing cells at 26°C (144). Table 4 shows the FACS results from growing D^b cells at 26°C and 37°C. The level of D^b at the cell surface grown at 26°C is comparable to the cell surface expression grown at 37°C.

Figure 15. The effect of growth at 26°C on the intracellular accumulation of H-2D^b in 293D^b cells

293 D^b transfectants were alternately cultured at 26°C or 37°C for 24 hours prior to labelling. Cells were pulsed for 30 minutes and chased for two hours at 26°C or 37°C respectively. Cells were lysed on ice and lysates precipitated with a D^b specific monoclonal antibody (as listed in table 2). Prior to SDS-PAGE, precipitates and molecular weight (MW) standards were either treated or mock treated with Endo H for 24 hours.
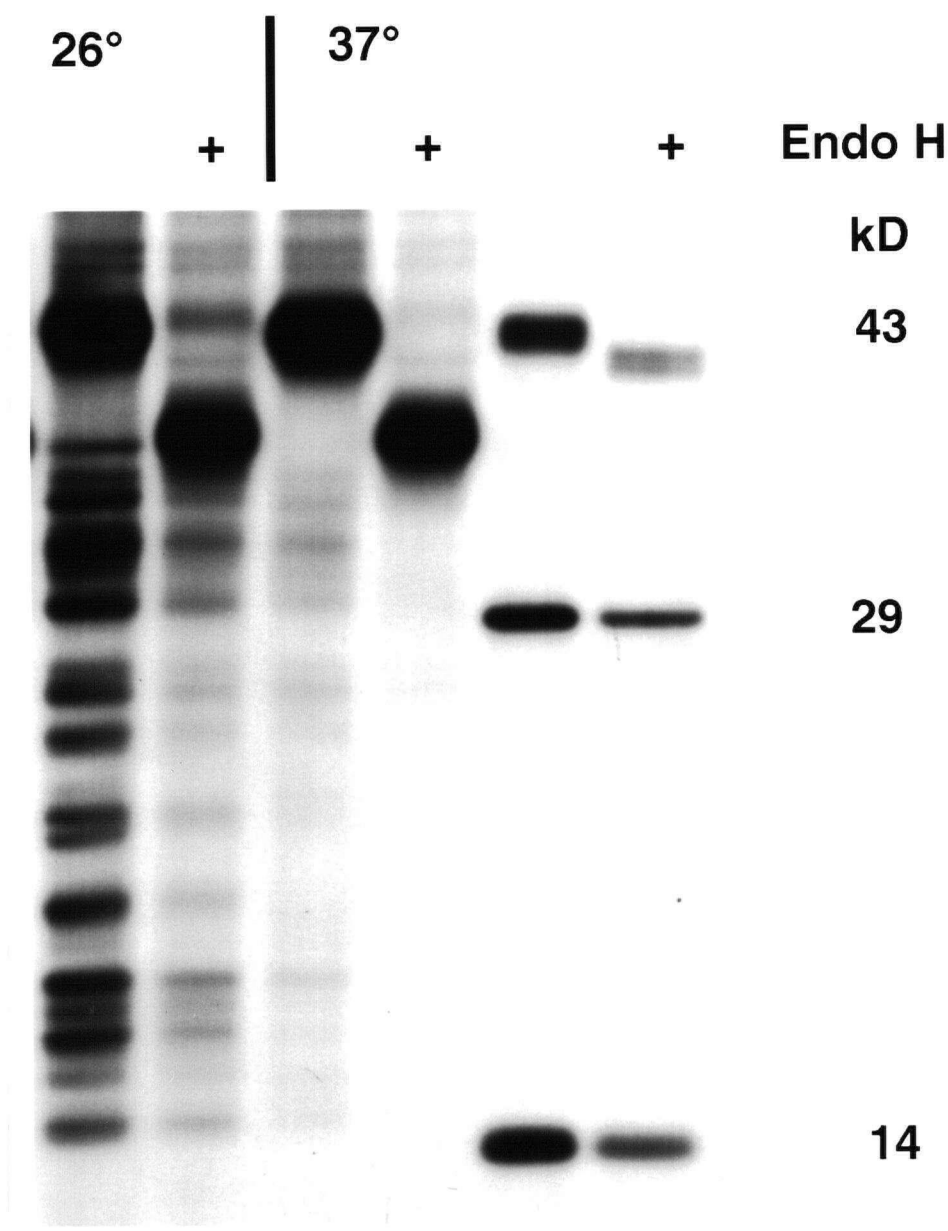
4.3.3. Excess β_2m does not promote proper folding of the the bulk of the intracellular store of the D^b protein

While 293 cells show no shortage of β_2m normally, the extra burden of large amounts of transfected D^b proteins could result in insufficient quantities of β_2m to supply both the transfected and the endogenous MHC Cl I molecules. A comparison of the levels of endogenous HLA (W6/32) expression in both 293 and the transfectants suggested that in almost every case, the presence of an H-2 transfectant reduced the level of endogenous MHC Cl I expressed at the cell surface (Figure 9). One explanation for this observation is that levels of β_2m in the transfectants were limiting the total number of MHC Cl I molecules that could achieve a mature conformation and make it to the cell surface. By adding more β_2m to these cells, it would be possible to promote more H chain-β_2m interactions and thereby upregulate the surface expression of D^b. $293D^b$ cells were infected with the β_2m-Vaccinia construct. Infected and mock infected cells were FACS analyzed (table 4). While the level of β_2m was increased when this method was used, the cell surface expression was unchanged.

In figure 16 we demonstrate that infection with the human-β_2m-Vaccinia construct markedly increases the amount of β_2m precipitated with D^b proteins. $293D^b$ cells infected or mock infected with a human β_2m vaccinia construct were precipitated with anti β_2m antisera. The precipitated β_2m from the infected cells co-immunoprecipitates many other proteins

characteristic of vaccinia infections.

Figure 16. The effect of infection with a vaccinia virus human-β_2m construct on β_2m expression

293D[b] cells were alternately infected (+) or mock infected (-) with a human-β_2m construct expressed in vaccinia virus (hu-β_2m-Vac). Cells were infected 18 hours prior to labelling with Pro Mix (^{35}S-Met/Cys). The cells were pulsed for 30 minutes and chased for 120 minutes. β_2m was precipitated with rabbit anti human-β_2m antisera (as listed in table 2). MHC Cl I and β_2m are denoted by the arrows.
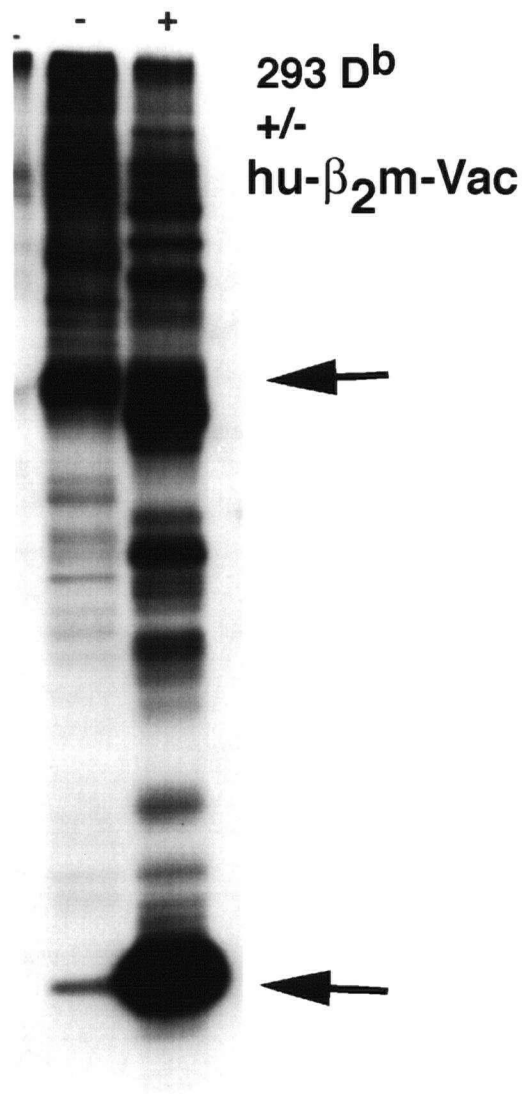
Table 4. A comparison of the cell surface expression of the H-2 D^b protein at 26°C and 37°C with and without excess β_2m

	non-infected (AFU)	infected (AFU)	percentage
26°C	44.8	64.4	144
37°C	58.9	68.1	116
percentage	132	106	

The 293D^b transfectant was alternately cultured at 26°C and 37°C. Additionally, cells were either mock infected or infected with the hu-β_2m-Vaccinia as described in Materials and Methods. At 18 hours post infection, the cells were harvested and FACS analyzed as described in Materials and Methods. Levels of cell surface expression are given in arbitrary fluorescence units (AFU). Calculation of AFU involved taking the difference between mock and specifically labelled FITC labelled samples. Percentage of cell surface expression is determined by comparing changes in AFU along rows or down columns. e.g Uninfected 293D^b transfectants at 37°C express 132% the level of D^b expressed at 26°C at the cell surface.

The result of adding excess human β_2m in cells cultured at both the standard 37°C and 26°C is shown in table 4. Addition of β_2m results in an increase in cell surface expression of 15%. When cells cultured at 26°C are exposed to excess β_2m, the level of cell surface expression increases by 43%. Figure 17 shows the analyses of the Endo H resistance of these transfectants. While the percentage of cell surface expression of D^b increases, the overall amount that is Endo H resistant remains much less than the Endo H sensitive population.

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Figure 17. **The effect of growth at 26°C and infection with a vaccinia virus human-β$_2$m on escape of intracellular stores of H-2D^b protein from the ER**

293D^b cells cultured at 26°C and 37°C were alternately infected or mock infected with a human-β$_2$m construct expressed in vaccinia virus (hu-β$_2$m-Vac). Cells were infected 18 hours prior to labelling with Pro Mix (^{35}S-Met/Cys). The cells were pulsed for 30 minutes and chased for 120 minutes. MHC Cl I H-2D^b was precipitated with a D^b specific antibody (as listed in table 1). Prior to SDS-PAGE, precipitates and MW standards were alternately mock treated or treated with Endo H for 24 hours.
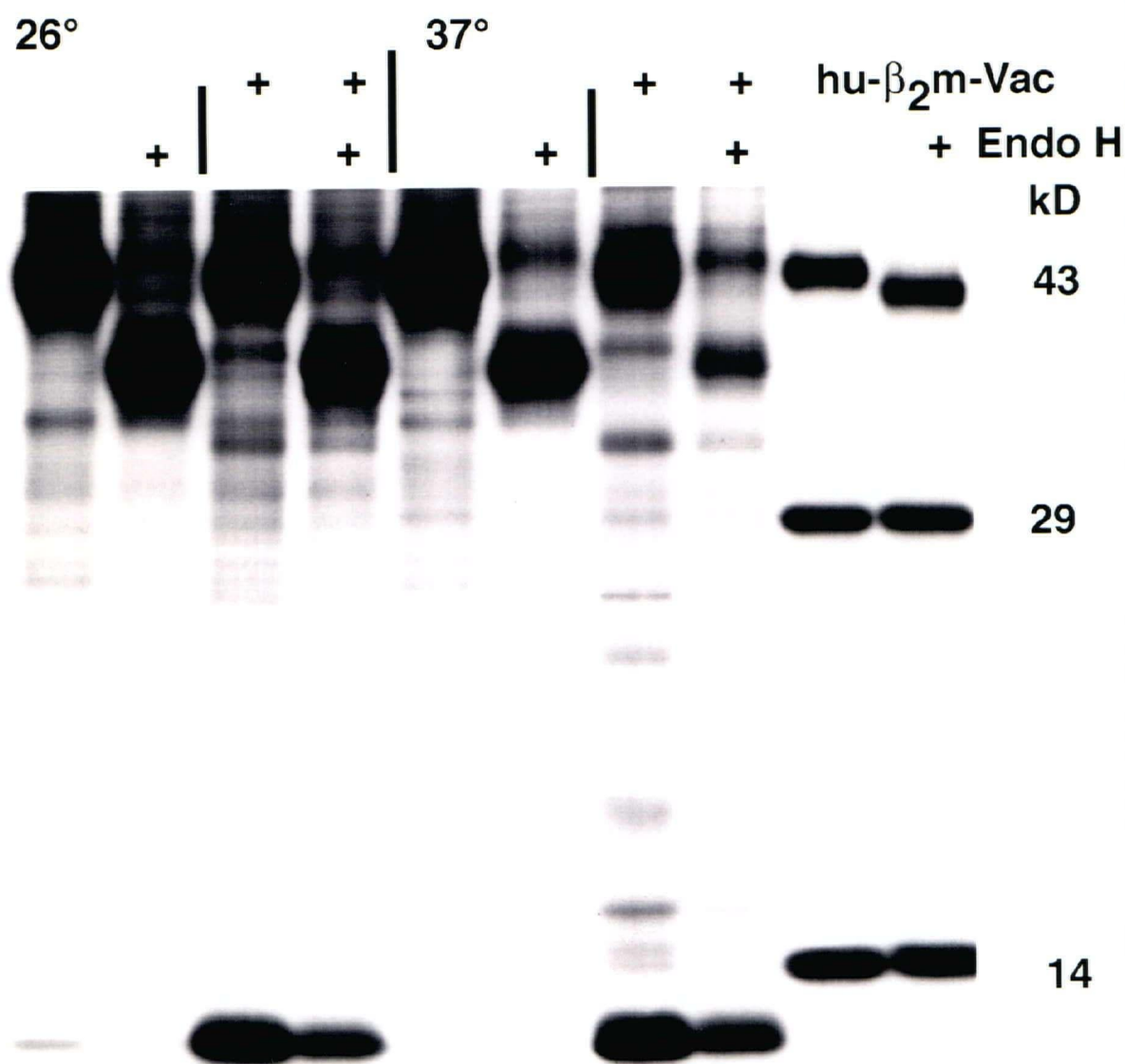
4.3.5. γ-IFN has no effect in upregulating cell surface expression of D^b

Previous studies had demonstrated that γ-IFN had been used to upregulate cell surface expression of MHC Cl I molecules in cell lines that normally had low levels. In these studies, the non-specific effects of γ-IFN included upregulating many components involved in antigen presentation, with the obvious final effect of increasing cell surface expression of MHC Cl I. This treatment was applied to 293 D^b cells to determine if some other element required for the efficient transport of D^b was regulated with the other antigen processing elements.

Table 5 shows the results of this treatment. 293 cells tranfected with E3/19K (293.12 cells) and the 293 D^b transfectant cell lines were pretreated with γ-IFN. While the effect of γ-IFN can be observed on MHC Cl I expression in 293 cells, treatment with γ-IFN has absolutely no effect in upregulating the cell surface expression of D^b in the transfectants. In 293.12 cells, which normally exhibit reduced cell surface expression, γ-IFN is able to upregulate cell surface expression to levels close to that observed in 293 cells exposed to γ-IFN.

Table 5. The effect of γ-IFN on MHC Cl I cell surface expression

Cell Line	normal (AFU)	γ-IFN (AFU)	percentage
293	12.8	20.4	160
293.12	3.91	19.5	498
293D^b	6.43	5.89	91.6

Transfectants were alternately treated or mock treated with γ-IFN. Cells were cultured in the presence of γ-IFN for 24 hours. FACS analysis was performed to assess the effect of γ-IFN on cell surface expression. Levels of cell surface expression were detected with W6/32 in 293 and 293.12 cells and 28.14.8s in 293Db cells and are given in arbitrary fluorescence units (AFU). Calculation of AFU involved taking the difference between mock and specifically labelled FITC labelled samples. Percentage of cell surface expression is determined by comparing changes in AFU along rows. e.g γ-IFN treated 293D^b transfectants express 91.6% the level of D^b at the cell surface expressed in untreated cells.

4.4 Discussion

Techniques documented in the literature were attempted to increase cell surface expression in the 293D^b transfectant to levels closer to the overall level of expression of D^b. In figure 15 upregulation of cell surface expression of H-2 D^b does not occur when cells are cultured at 26°C. Previously, it was thought that the regulation of cell surface expression of MHC Cl I relied on factors such as rate of transport to the cell surface and turnover of surface molecules. The bulk flow theory predicts that proteins deposited in the ER will eventually pass through the ER, cis and trans Golgi and either out to the cell surface or into endosomes unless they are retained by an organelle specific retention or retrieval signal. The rate of transport of MHC Cl I molecules was believed to be directly related to the ability of the MHC Cl I molecule to achieve a stable and mature conformation and escape the ER. In many mutants, the factors that affect this are often the cofactors such as β_2m (Daudi) and peptide (RMA-S, CMT-64). Peptide is controlled by TAP 1 and TAP 2. The experiments in this chapter suggest that in this case, some other factor is responsible.

Results reported in the previous chapter demonstrated that D^b is a slow binder to E3/19K. The length of time that this molecule spends in the same compartment as E3/19K enhances its ability to be bound by E3/19K. FACS experiments show that this protein is expressed at the cell surface in uninfected cells at a level similar to that of endogenous MHC Cl I. These experiments also showed that when this (or other allelic proteins) are expressed at the cell surface, the level of the endogenous MHC Cl I expression goes down correspondingly. The reduction in cell surface expression likely is related to a shortage of cofactors. Results reported in this chapter suggest that the addition of an excess of co-factors still has no effect on upregulating cell surface expression.

It is possible that there is feedback control of total MHC Cl I molecules at the cell surface. When a certain number of MHC Cl I molecules is expressed on the cell surface, a signal is generated that reduces the number to follow. Rather than each allelic protein being regulated differently and by separate mechanisms, each protein expressed may have a 'set' contribution or ratio of the final cell surface population. In 293D^b, the level of the endogenous MHC Cl I does not drop to zero; the block in the maturation and transport of one allelic protein (in this case the D^b) is clearly not blocking the maturation and transport of the endogenous allelic proteins.

It has been shown that the retention of E3/19K in the ER triggers a UPR (68). The level of activation of NF-κB has been shown to be dependant on the amount of E3/19K present in the ER. It is not the binding of E3/19K with MHC Cl I that is the trigger of NF-κB; it is its appearance in the ER. An excess of D^b molecules in the ER may trigger the UPR. Others show that the release of Ca^{2+} from the ER into the cytoplasm seems to trigger this response (68). Further studies in yeast suggest that activation of a membrane serine/threonine kinase triggers the alternate splicing of a transcription factor, HAC1, in the nucleus (145, 146). HAC1 has an affinity for the unfolded protein response element (UPRE) and upregulates expression of genes with this element. Some known products include heat shock proteins such as BiP. In a recent review (145) it is speculated that free BiP plays a role in the regulation of this process. Excess protein in the ER may mop up all free BiP in the ER. In yeast, free BiP may associate with a membrane protein called Ire1p. Dissociation of BiP allows Ire1p to dimerise and autophosphorylate, sending a signal through the UPR to upregulate transcription of more BiP. Presumably, the increased levels of Bip should establish a new equilibrium of unfolded proteins to unfolded protein binding capacity in the ER. In these studies, the investigators suggest that the UPR is instrumental in preventing apoptosis.

The regulation of the UPR may influence the turnover of molecules at the cell surface. The UPR upregulates expression of heat shock protein in response to proteins that remain in the ER. Cell surface regulation is probably influenced by a combination of concentration, regular turnover at a specific rate and a positive signal to either keep proteins at the cell surface or to tag them for turnover.

5. E3/19K binding to MHC Cl I does not exclude association with calnexin

5.1 Introduction

The observation that E3/19K very rapidly bound and stabilised a mature conformation of MHC Cl I proteins leads to the speculation that E3/19K behaves like a chaperone. Examination of E3/19K binding to MHC Cl I in the ER involving the ER resident chaperones leads to the proposal that E3/19K binding in the ER will disrupt or involve the chaperones involved in the MHC Cl I processing pathway.

Chaperones are molecules that bind to immature peptide sequences. They bind to proteins before they fully mature and form either a transient or permanent association with them. Chaperones are usually associated with a protein that has not yet been transported to its final destination. The role of a chaperone protein is to bind to immature and misfolded proteins and to cooperatively act with them to allow them either to achieve their correct conformation (e.g. calnexin) or to shuttle for degradation (BiP).

Many chaperones act with protein intermediates in the ER. Ribosomes transcribing nascent peptides are directed to the ER by way of the signal sequence;the peptide is directed into the lumen of the ER. The newly made protein is modified by many enzymes that act in concert to further process it. The signal sequence is removed by signal peptidase. Transport through the ER membrane may be enhanced by association with BiP, which acts as a ratchet pulling the peptide into the ER. Glycans are added and modified by a series of glycosylation enzymes. Disulphide bridges are established between cysteine residues; proline isomerisation is mediated by protein prolyl isomerase (PPI). Calnexin and calreticulin act in concert as a type of quality control mechanism; once proteins have achieved a correct conformation, they are released by calnexin and permitted to progress further through the ER-Golgi maturation pathway. More recently, other participants such as ERp57 have been shown to have a role in

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the glycoprotein quality control mechanisms in the ER. BiP/Kar2p has been implicated in playing a regulatory role in the UPR of cells (147).

These proteins are involved with most, if not all, glycoproteins that travel through the ER. In addition to the concerted action of these ER resident proteins, the MHC Cl I molecules that are involved in antigen presentation are acted on by a further set of specialised chaperones. This list has grown lately as the interactions among all the participants are better defined. Some chaperones involved in the antigen presentation path include calnexin and calreticulin with specialized roles. Additionally, since MHC Cl I molecules are composed of a trimolecular complex that includes a peptide fragment, the TAP proteins also have been demonstrated to act as chaperones. More recently, essential roles for tapasin (148) have been elucidated.

5.1.1. Maturation of a Cell Surface Membrane Protein

A cell surface membrane protein comes in contact with this progression of chaperones, mentioned above, on its way to the cell surface. If it is properly folded, the interaction with the chaperone proteins will be temporary. If it is not properly folded, there exist many chaperones that will bind the improperly folded protein and feed it to the degradation machinery of the cell. Almost any path that a peptide will take through the cell will involve the participation of chaperones. It is in this manner that E3/19K also functions as a chaperone. E3/19K binds to immature peptides and seals their fate in a manner not unlike BiP. It stabilises a mature epitope and halts its progression through the cell.

In the pulse chase studies with HLA proteins, the W6/32 antibody was used to follow the rate of appearance of mature MHC Cl I molecules. Acquisition of the W6/32 conformation has been used as an indication of maturity (133, 149). In contrast to the Rabbit antiserum R426, W6/32 recognises only the MHC Cl I molecules that have escaped the ER. While the epitope recognised by the W6/32 epitope is coincident with a mature epitope, it should be noted that the converse is not necessarily true; recognition by W6/32 does not guarantee that a MHC

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Cl I protein is mature.

Calnexin is one effector of quality control in the ER, indiscriminately binding glycoproteins in the ER until they achieve a mature conformation and are fit to be transported to the cell surface. Calnexin binds MHC Cl I molecules at some point in their maturation.

5.2. Rationale and Goals

The goal of this study was to determine whether the association of E3/19K with MHC Cl I complex in the ER would disrupt the association of any other chaperones in the ER. This set of experiments set out to determine if an E3/19K bound protein would still associate with calnexin. This data would give information on the physical association of participants in complexes. It would also shed light on the timing of different events occurring in protein maturation and quality control.

5.3. Results

5.3.1. E3/19K preserves W6/32 binding of MHC Cl I HLA in the presence of tunicamycin

Many studies have demonstrated that proper glycosylation of MHC Cl I molecules is required for maturation of MHC Cl I molecules (150). Growth in the presence of a glycosylation inhibitor such as tunicamycin results in increased levels of improperly folded proteins in the presence of BiP in the ER and can trigger the UPR. When assessed with W6/32 the mature epitope is lost when 293 cells are grown in the presence of tunicamycin (figure 18). Unglycosylated MHC Cl I glycoprotein is unable to exit the ER. An unglycosylated MHC Cl I molecule is likely unable to associate with calnexin (56, 151) and therefore can proceed no further down the maturation pathway. When the 293.12 cells are treated with tunicamycin the MHC Cl I loses its carbohydrates but the W6/32 epitope is still detected and MHC Cl I co-precipitates with E3/19K. This finding suggested that E3/19K can bind to MHC Cl I in the

absence of carbohydrates and is able to force it into a mature conformation.

In figure 18b the effect of a tunicamycin titration on 293 and 293.12 cells is examined. In this experiment, the non-specific epitope recognized by the antisera R426 (and the identical R425) can be precipitated at all concentrations of tunicamycin. A similar result is observed in 293.12 cells. As the tunicamycin concentration increases, the mobility of the MHC Cl I increases coincident with the loss of carbohydrates.

Figure 18a illustrates the results of the same experiment using W6/32. When the mature conformation specific monoclonal W6/32 is used, MHC Cl I ceases to be precipitated from 293 cells at ~7µg/ml Tunicamycin. In 293.12 cells, MHC Cl I can be detected at all tunicamycin concentrations. E3/19K is also observed co-immunoprecipitating at all points. This result demonstrates that E3/19K stabilises a mature (W6/32) epitope under adverse conditions. In this case, E3/19K is able to cause conformation specific mAb to bind even in the absence of carbohydrates.

Figure 18. The effect of increasing levels of tunicamycin on MHC Cl I in 293 and 293.12 cells

       293 and the E3/19K transfected 293.12 cell lines were cultured in the presence of increasing concentrations of tunicamycin. Cells were grown in media supplemented with 0, 3.5, 7, 14 and 21 µg/ml (lanes 1-5) respectively for 18 hours prior to labelling. Both labelling and chase media were also supplemented with tunicamycin. Cells were labelled for 30 minutes and chased for two hours. Immunoprecipitation was performed with a) the conformation specific monoclonal W6/32 or b) non conformation-specific sera R426 followed by SDS-PAGE.
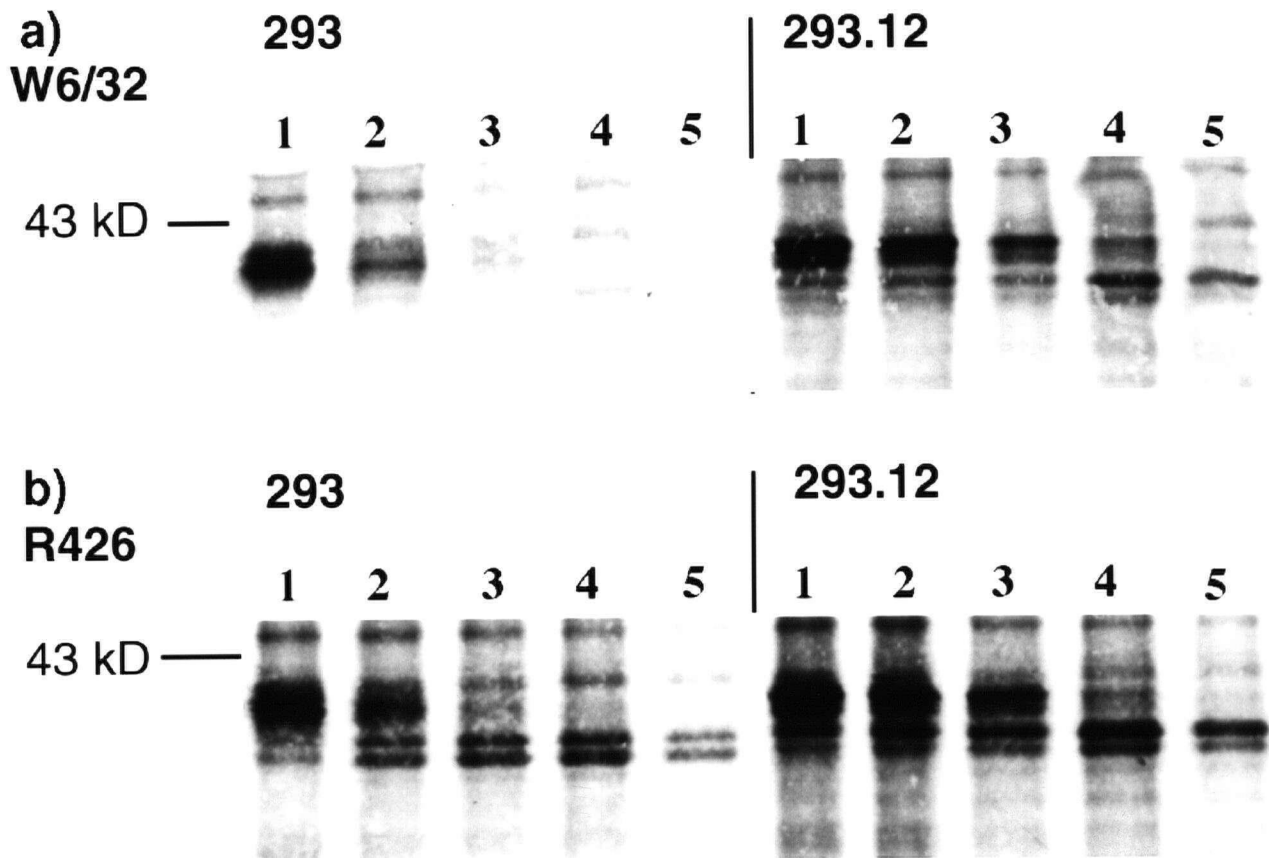
5.3.2. E3/19K rapidly stabilises a mature epitope

The rapidity of the formation of a mature epitope was investigated in figure 19. This figure illustrates R426 and W6/32 precipitated products at various time points post chase. The R426 population appears immediately in both 293 and 293.12 cells. The increased mobility of E3/19K with time reflecting modification of sugars.

In figure 19b an immunoprecipitation with W6/32 is shown. In 293 cells it is apparent that the rate of transport through the ER and coincident mature conformation is 2-4 hours. The first appearance of the mature W6/32 epitope starts at about one hour post chase.

In the 293.12 cells a W6/32 epitope can be observed almost immediately, with some precipitate visible at 0 minutes post pulse. Once again, this gel confirms that both $\beta_2 m$ and E3/19K are co-precipitated with MHC Cl I molecules in 293.12 cells. This experiment demonstrates that a mature MHC Cl I epitope is very rapidly stabilised in the presence of E3/19K, even though the MHC Cl I molecule is not transported out of the ER.

Figure 19. Time course of maturation of MHC Cl I in 293 and 293.12 cells

293 and 293.12 cells were grown to 95% confluence and labelled using 35S-Met/Cys Pro-Mix. Cells were pulsed for 30 minutes and chased for 0, 30, 60, 120 and 240 minutes (lanes 1-5) respectively. Lysates were precipitated with non conformation-specific sera R426 or the conformation specific monoclonal W6/32. Precipitates were separated by SDS-PAGE. MHC Cl I (a), β_2m (b) and E3/19K (c) are indicated.

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293 and 293.12 precipitation with R426 and W6/32

a)

b)

MHC Cl I

E3/19K

b2m

293   293.12

293   293.12

kD

66 —

43 —

29 —

14 —

100
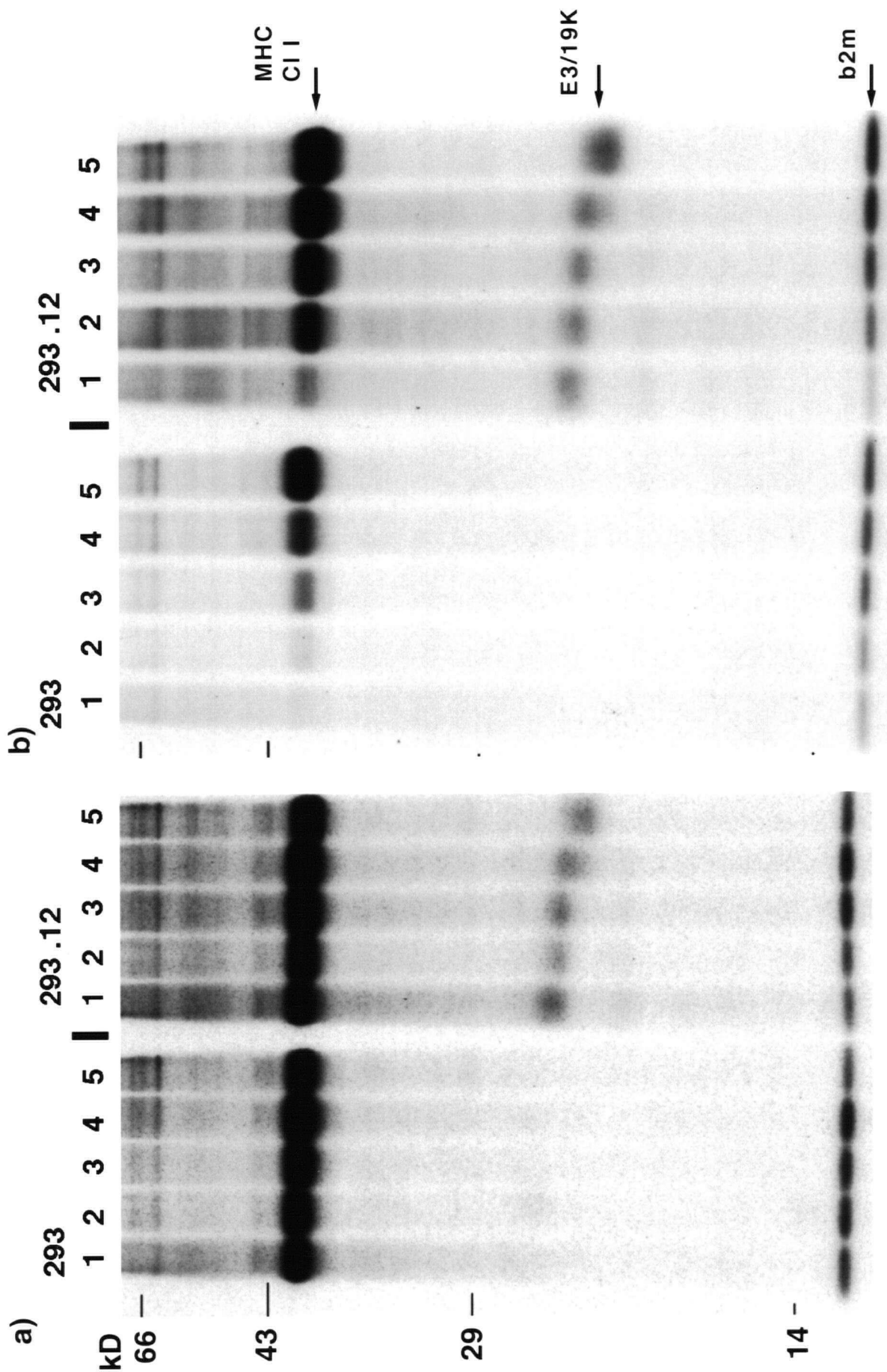
Because E3/19K binds to MHC Cl I so quickly and almost immediately induces it to form a mature conformation, it was suggested that it was acting in a chaperone like manner. It is interesting that E3/19K quickly stabilizes a mature epitope. The following experiments examined whether the association of the 'chaperone' E3/19K would affect the association of other more regular ER resident chaperones such as calnexin. Specifically, an experiment was performed to determine if E3/19K and calnexin binding to MHC Cl I was exclusive.

5.3.3. E3/19K binding to MHC Cl I does not exclude calnexin association

An experiment that tested if E3/19K and calnexin could bind to MHC Cl I at the same time is shown in figure 20. MHC Cl I molecules were immunoprecipitated with either W6/32 or R426. This experiment was done as documented in Materials and Methods except the cells were lysed in the gentle CHAPs buffer. Lysis in NP40 typically abolished any association of calnexin with MHC Cl I molecules. MHC Cl I was precipitated, run on a gel and then transferred to a membrane. The membrane was probed with anti calnexin antisera as described in Materials and Methods.

Figure 20 shows the association of calnexin with the MHC Cl I-β_2m complex. In 293 cells precipitated with W6/32, no calnexin can be found associated with this complex. When precipitated with R426, calnexin is found associated with the complex at all timepoints. In the 293.12 cells, the MHC Cl I-β_2m-E3/19K complex does associate with calnexin at all timepoints using both W6/32 and R426.

Figure 20. **Calnexin association with MHC Cl I precipitated from 293 and 293.12 cells**

293 and 293.12 cells were grown to 95% confluence and labelled using Pro-Mix. Cells were pulsed for 30 minutes and chased for 0, 60, 120 and 240 minutes (lanes 1-4 respectively). Lysates were precipitated with either the conformation specific monoclonal W6/32 or non conformation-specific sera R426. Precipitates were separated by SDS-PAGE. Gels were blotted onto Immobilon PVDF membranes overnight (O/N). Membranes were fixed and western blotted with anti-calnexin antisera (1:10,000) using the glass plate incubation described in materials and methods. Specific antisera was detected using Horse Radish Peroxidase (HRPO) conjugated secondary antibody (Jackson) and the ECL method (Amersham). Kodak XAR film was exposed for 1 to 15 minutes.
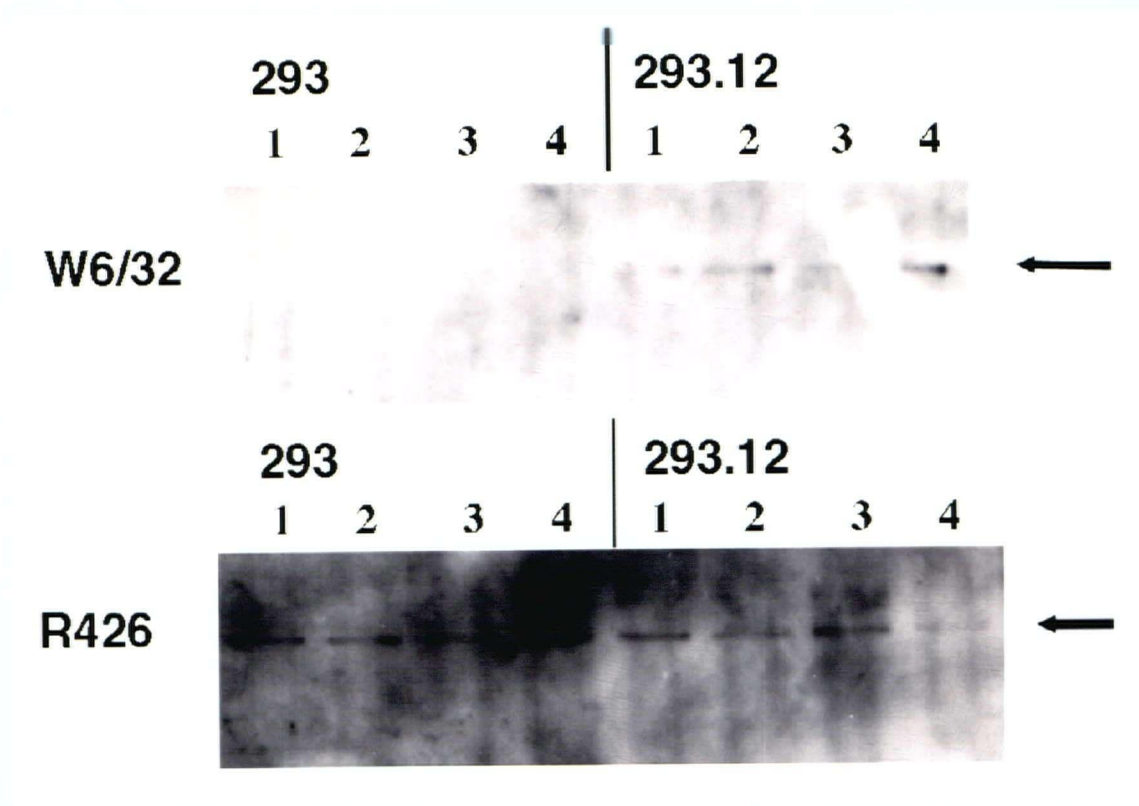
These data confirm that when W6/32 and R426 are used to immunoprecipitate MHC Cl I molecules from 293 and 293.12 cells, different subsets of these molecules are precipitated corresponding to MHC Cl I molecules of different maturity. The R426 precipitate is the total MHC Cl I population including both immature and mature MHC Cl I molecules; the W6/32 precipitate is the subset of those that are mature. Calnexin is not expected to be associated with the mature W6/32 epitope but will associate with the R426 epitope. That calnexin also associates with the MHC Cl I-E3/19K complex is not completely unexpected as this complex is retained in the ER. The observation that E3/19K does not block this association is noteworthy.

5.3.4. MHC Cl I precipitated with anti TAP antisera also associates with calnexin and E3/19K

The role of the TAPs has been intimately linked to empty MHC Cl I molecules. Empty MHC Cl I molecules are retained in the ER until they receive the correct peptide, then they are able to escape the ER and 20-80 minutes later are expressed on the cell surface (152). The association of other ER resident proteins such as tapasin have also recently been observed (153). Precipitation with anti-transporter antisera should bring down those immature MHC Cl I that are accepting peptides.

Figure 21 illustrates 293 and 293.12 lysates precipitated with W6/32 mAb and antitransporter antisera. This experiment consisted of a pulse followed by several chase timepoints. The precipitates were separated on a gel and subsequently transferred to a membrane for western blotting analysis.

This figure demonstrates once again that MHC Cl I in 293.12 cells forms a complex with calnexin. When precipitated with anti MHC Cl I, calnexin still associates with the E3/19K-MHC Cl I-β_2m complex. Moreover, this figure reveals that, when precipitated with

the antitransporter antisera, calnexin can still be found in the western blot of the same precipitate. Additionally, E3/19K can be observed co-immunoprecipitating with this complex. This ER resident complex consists of MHC Cl I, E3/19K, β_2m, calnexin and possibly TAP 1 and TAP 2. This helps establish a clearer picture of the binding process in the ER. It suggests that this is a fairly large protein complex.

Figure 21. Calnexin association with W6/32 and Tap precipitates from 293 and 293.12 cells

293 and 293.12 cells were grown to confluence and labelled as detailed in materials and methods. This figure shows an immunoprecipitation by W6/32 and anti transporter antisera followed by western blotting with anti calnexin antisera. Cells were pulsed 30 minutes and chased for 0, 30, 75, 150 and 240 minutes (lanes 1-5 respectively). a) Lysates were precipitated with the conformation specific monoclonal W6/32 or the anti transporter antisera. Precipitates were separated by SDS-PAGE on the BioRad minigel system according to manufacturers instructions. Co-immunoprecipitated MHC Cl I and E3/19K are denoted by arrows labelled i and ii respectively.

b) Subsequently, membranes were probed with anti calnexin antisera using western blotting techniques detailed in Materials and Methods. Calnexin is denoted by the arrow labelled iii. Visualisation of the anti calnexin antisera utilised secondary goat anti rabbit antisera conjugated to HRPO (Jackson) and the Amersham ECL detection system. ECL treated membranes were exposed to Kodak XAR film for 1 to 15 minutes.

a) Immunoprecipitation

W6/32   α transporter

293

1=0min
2=30min
3=75min
4=150min
5=240min

293.12

97 —
68 —
43 —
29 —

97 —
68 —
43 —
29 —

1 2 3 4 5

i

ii
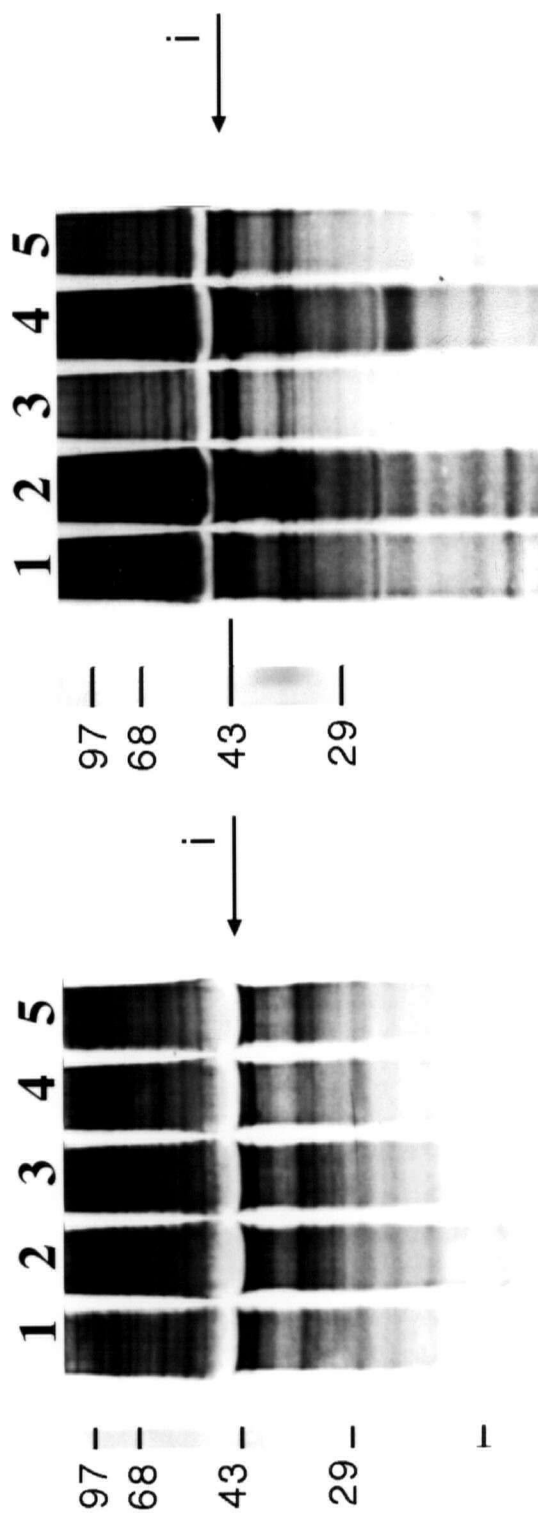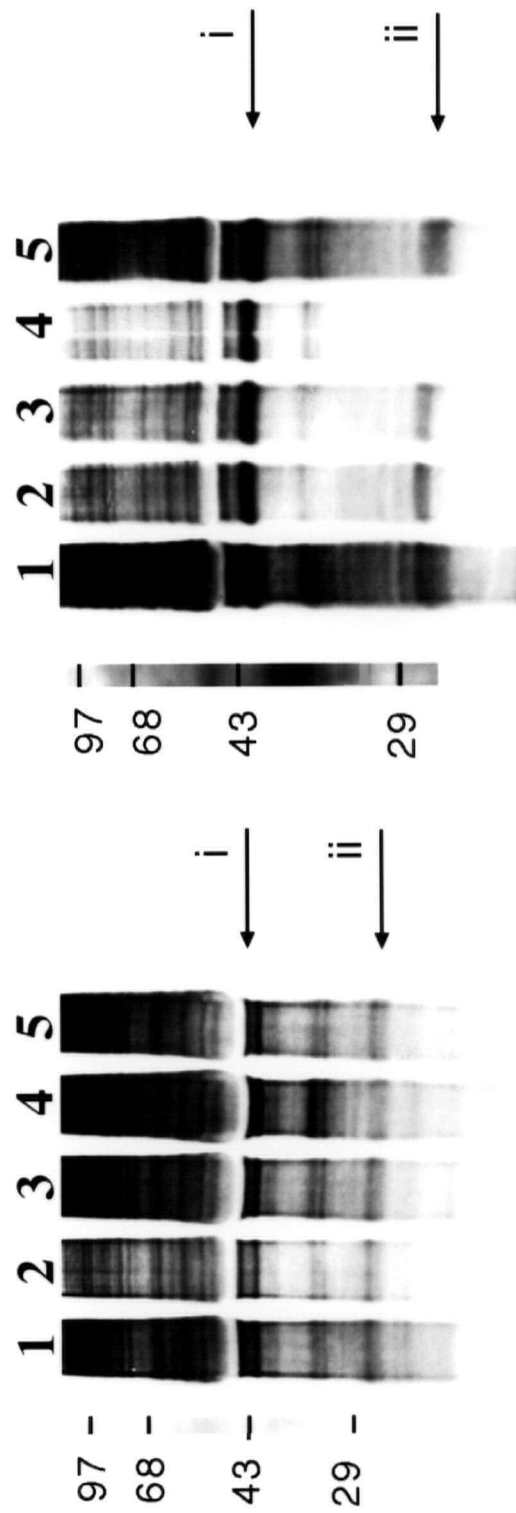
b) anti calnexin western

α transporter

W6/32

293

1=0min
2=30min
3=75min
4=150min
5=240min

293.12

107
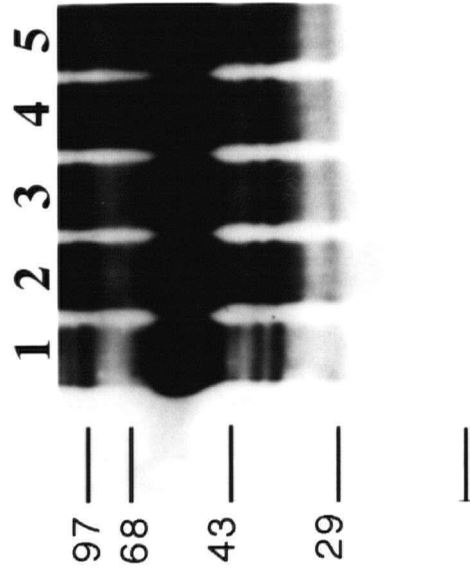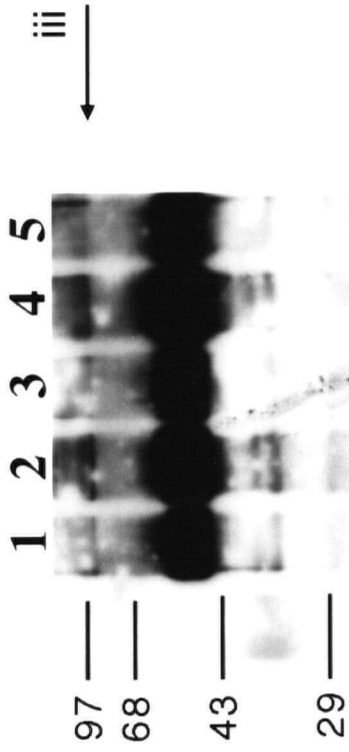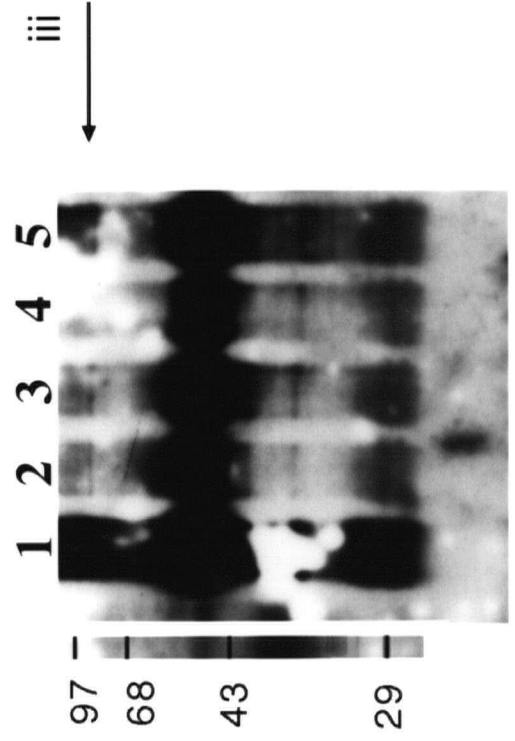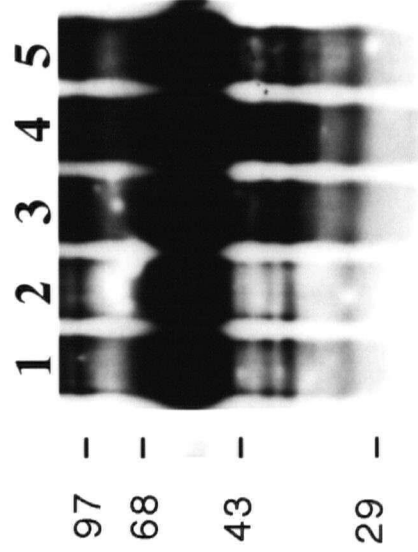
5.3.5. Peptide binding to MHC Cl I does not exclude E3/19K binding

The repertoire of peptides able to associate with specific MHC Cl I proteins has been fairly well characterised. In addition to hundreds of peptides eluted from various MHC Cl I proteins, a set of rules dictating the topography of peptides able to bind specific proteins has been formulated (154). One such peptide that binds the HLA-A2 protein is one derived from the HIV reverse transcriptase (RT) protein. The sequence of the peptide is ILKEPVHGV. While it had been demonstrated that such peptides could be eluted from MHC Cl I and then identified in mass spectroscopy experiments, it had not been shown that these peptides were bound by the peptide binding groove. In an attempt to demonstrate binding of the peptide to the MHC Cl I, the observation that E3/19K co-precipitated with this complex was made.

The HIV RT peptide ILKEVFVG was identified as a peptide that fit the motif of an HLA A2 binding peptide. This peptide was N-terminally biotinylated to generate a marker for binding to MHC Cl I. N-terminally biotinylated peptides were incubated with MHC Cl I and ultimately would be used to precipitate these complexes. The experimental procedure has been described in Materials and Methods. Figure 22 demonstrates that biotinylated peptide was able to precipitate a MHC Cl I enriched lysate. It was also noted that less MHC Cl I was observed when less biotinylated peptide was added. In a competition with cold peptide at 10x excess, binding to the biotinylated peptide was abrogated. Finally E3/19K could still binds peptide bound MHC Cl I molecules. This demonstrated that the association of E3/19K with MHC Cl I molecules did not block the peptide binding groove.

In figure 22 we show an increase in the amount of acid eluted MHC Cl I pulled down with biotinylated peptide corresponding to peptide concentration. A 10 fold increase in biotinylated peptide yielded an increase in the amount precipitated. In a competition with 10 fold excess cold peptide, no MHC Cl I was pulled down, demonstrating the specificity of the peptide for HLA-A2. Finally cold peptide alone did not precipitate MHC Cl I.

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This experiment was performed using lysates from 293.12 cells. In 293.12 cells, the interference of E3/19K with the association of the biotinylated peptide was investigated. If E3/19K was binding to the MHC Cl I molecule through the peptide binding groove, then the binding of the biotinylated peptide should be competitively inhibited. Alternately, E3/19K binding to MHC Cl I molecules might alter the geometry of the peptide binding groove rendering it unable to hold the biotinylated peptide. This did not occur. When the 293.12 lysates were used, the biotinylated peptide continued to precipitate MHC Cl I molecules. In addition, the precipitated MHC Cl I molecules co-precipitated with E3/19K. This shows that peptide binding is not excluded by E3/19K association. It may suggest that MHC Cl I molecules that have associated with E3/19K may be empty facilitating peptide binding in lysate, but this remains to be conclusively shown

Figure 22.   Precipitation of MHC Cl I in enriched suspension using a biotinylated peptide

293.12 cells were grown to 95% confluence and labelled and labelled using ^{35}S-Met/Cys Pro-Mix (Amersham). Cells were pulsed for 30 minutes and chased for two hours. Chase media was alternately supplemented with biotinylated peptide at 5 µM and 50 nM (lanes 1 and 2 respectively), a mixture of peptide (5 µM) and biotinylated peptide (50nM) (lane 3), peptide (5 µM) (lane 4), biotin (25 µM) (lane 5) or mock treated (lane 6). At the end of the chase period, cells were washed and lysed in the presence of the crosslinking reagent DSP (200 µg/ml). Lysates were precipitated with non conformation-specific anti MHC Cl I antisera R426. Precipitates were subjected to a glycine acid solution to remove Prot-A-Sepharose. The supernatant was divided into two aliquots; a 450 µl aliquot and a 50 µl aliquot. The 450 µl aliquot was precipitated with streptavidin-agarose (Pierce) (lanes 1-6 on the left). The 50 µl aliquot was precipitated with R426 a second time (lanes 1-6 on the right). Precipitates were separated on SDS-PAGE.
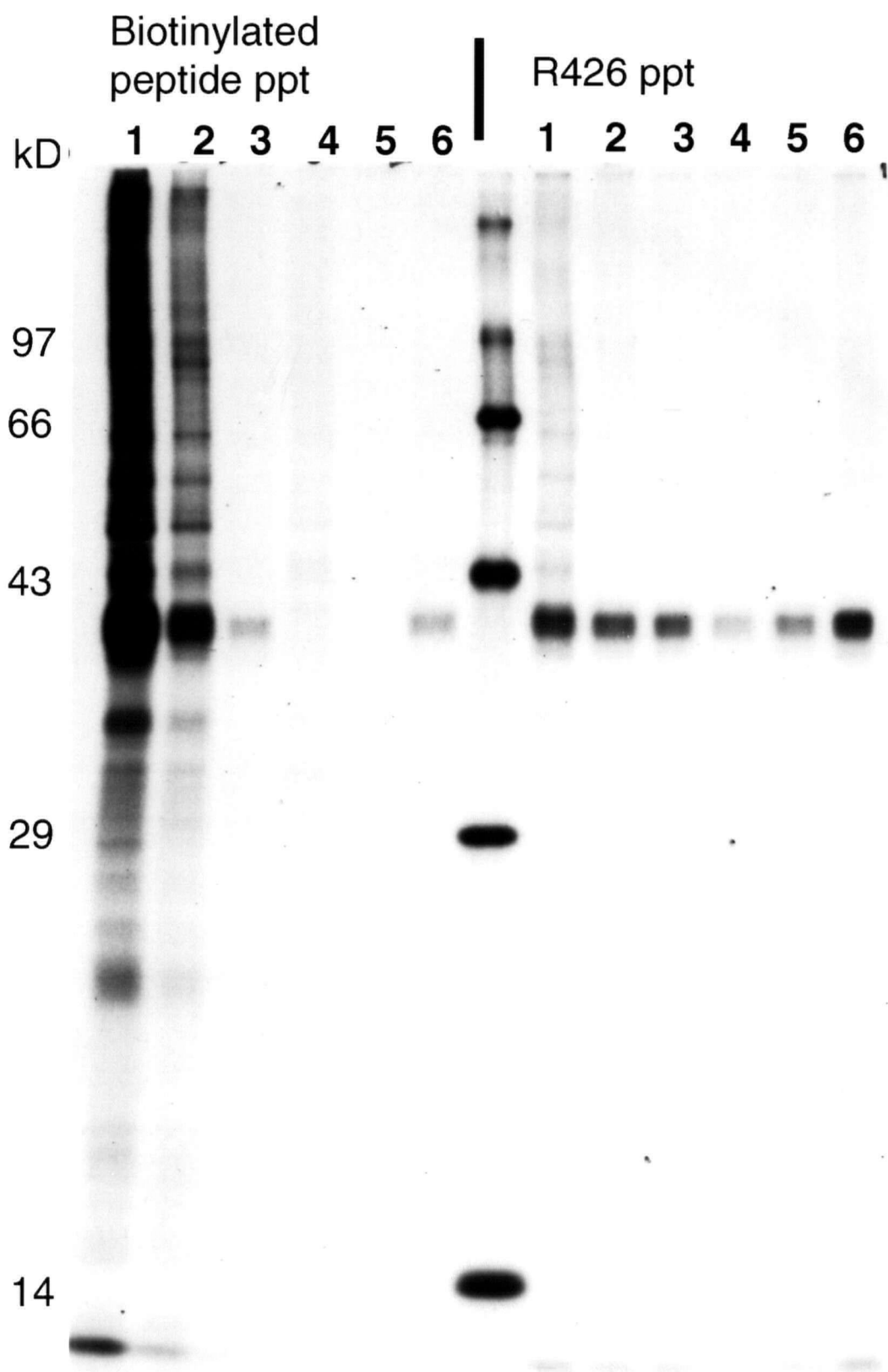
5.4. Discussion

The results in this chapter demonstrated two significant points. The first was that E3/19K in 293 cells rapidly stabilised a mature MHC Cl I epitope. The implications of this are discussed below. Second, the complex involving E3/19K also involves several other ER resident proteins including calnexin and at least one and possibly both TAP proteins.

When testing to determine if E3/19K could bind in the absence of carbohydrates, it was contrary to expectations to observe that in a tunicamycin treatment of 293 and 293.12 cells, the mature MHC Cl I epitope was lost in 293 cells but was retained in 293.12 cells. Tunicamycin blocks the transfer of the dolichol associated carbohydrate to the asparagine residue of the MHC Cl I molecule. In most cases, the lack of this carbohydrate on MHC Cl I molecules prevents any further maturation through the ER and they remain intracellular (124). Some studies have even shown that tunicamycin triggers the onset of apoptosis (155).

293.12 cells stably transfected with the E3/19K gene exhibit some of the characteristics reported in Ad2 infections including reduced cell surface expression of MHC Cl I molecules (156). In 293.12 cells grown in the presence of tunicamycin, E3/19K was able to bind MHC Cl I even when it was lacking a carbohydrate. It was also determined that the mature conformation detected by W6/32 was stabilised in this case. This observation suggested that the binding of E3/19K to the MHC Cl I molecule stabilised a mature conformation.

This study makes the assumption that the W6/32 epitope indicates a mature conformation of MHC Cl I. Previous studies have used a W6/32 conformation as an indication of a mature conformation. However, the fact that this epitope can be precipitated from 293.12 cells which have been incubated in the presence of tunicamycin suggests that there may be many similarities between the structure stabilised by E3/19K and a mature MHC Cl I molecule, but that the two conformations are not identical. It should be noted that the mature conformation referred to in this chapter is likely a stable conformation which resembles that of a mature MHC Cl I protein and as such may differ from the surface bound protein in several

key features.

When using the conformation specific monoclonal W6/32, it was observed that in 293 cells a mature conformation could be detected about 30-60 minutes post chase. This period of time required for maturation includes the participation of many other ER resident proteins including PDI (157, 158), rotamase, calnexin, calreticulin (159, 160), BiP (161-164) and many others. In the presence of E3/19K, MHC Cl I molecules were able to achieve a mature (or W6/32 reactive) conformation almost immediately in 293.12 cells. This suggested that E3/19K binds to nascent MHC Cl I molecules and quickly stabilize them. MHC Cl I molecules could mature faster than in the absence of E3/19K, possibly bypassing the action of the concert of ER chaperones mentioned above.

When precipitated with W6/32 in 293 cells, the mature MHC Cl I epitope is not associated with calnexin molecules. This result was expected as the W6/32 recognizes a mature MHC Cl I molecule which should no longer be accessible to calnexin. Calnexin binds to proteins until they achieve a mature conformation; in the case of MHC Cl I molecules, calnexin release seems to coincide with the addition of peptide and the achievement of the correct conformation for release from the ER. When MHC Cl I molecules are precipitated with R426 from the 293 cell line, MHC Cl I associates with calnexin at all time points. Once again, this can be explained by the fact that R426 recognizes a wide subset of MHC Cl I molecules including those that are immature. Immature MHC Cl I molecules should still be associated with calnexin until they have achieved the correct conformation to leave the ER.

As in 293 cells, the R426 precipitate of 293.12 cells associates with calnexin. This is due to R426 binding both mature and immature MHC Cl I molecules. E3/19K co-immunoprecipitates with this complex demonstrating that E3/19K binding to MHC Cl I molecules does not sterically or in any other way inhibit the association with calnexin. The MHC Cl I protein with the W6/32 epitope is still able to bind calnexin in the presence of E3/19K in 293.12 cells. This is not unexpected as MHC Cl I should be resident in the ER.

Although the initial association of calnexin with glycoproteins is mediated through the carbohydrates (56), the areas that appear to be crucial for continued association are found within the transmembrane region (165) reducing the likelihood of E3/19K sterically interfering with binding.

Calnexin is readily dissociated from the MHC Cl I complex in detergent. Further studies probe the associations between all of the participants of this complex. This complex appears to be loosely associated and fairly large with several participants. Having established this, it was investigated whether other ER resident proteins would also be found in this complex.

Earlier it had been surmised that the binding of E3/19K might involve enveloping the alpha 3 region of the MHC Cl I molecule. Key association points would be the alpha 1 and alpha 2 hypervariable regions. However, it is difficult to understand how calnexin fits into this picture. Calnexin may protrude further into the lumen of the ER than E3/19K and thereby associate with the N terminal portion of the MHC Cl I molecule. One paper suggests that the areas involved in MHC Cl I binding are in the transmembrane region (166). The above scenario raises the question whether calnexin associates with E3/19K as well.

To further elucidate the nature of this calnexin-MHC Cl I-β_2m-E3/19K complex resident in the ER, it was examined whether calnexin binding was preceded by the addition of peptide. Previously it had been shown that MHC Cl I molecules formed ternary complexes with TAP and calnexin and dissociation coincided with the acquisition of a peptide (28). The immunoprecipitation of 293 and 293.12 cells with both an anti transporter antisera and W6/32 addressed this.

In the 293.12 cells precipitated with W6/32 the E3/19K co-immunoprecipitates with MHC Cl I molecules. Surprisingly, precipitation of 293.12 lysates with the anti-transporter antisera precipitates a large complex including E3/19K. This suggests a large complex

involving the peptide transporter, MHC Cl I, β_2m and E3/19K. The association with the peptide transporter is significant because it had been proposed that the nature of E3/19K binding to the MHC Cl I molecule is through the peptide binding groove. That E3/19K and peptide transporter binding to MHC Cl I is not exclusive suggest that E3/19K is may not be binding in the peptide binding groove.

The experiment that shows that biotinylated peptides were able to bind to and cause the immunoprecipitation of MHC Cl I molecules in a lysate demonstrates that when immunoprecipitated with the biotinylated peptide, the E3/19K proteins still associates with MHC Cl I. This is significant in that it demonstrates that E3/19K binding to MHC Cl I does not occur through the peptide binding groove. In addition, the association between E3/19K and MHC Cl I does not sterically interfere with peptide access to the binding groove. In an earlier chapter, the nature of E3/19K binding to MHC Cl I molecules was examined. One of the proposals considered was the possibility of E3/19K binding to the MHC Cl I molecule through the peptide binding cleft. Binding to the cleft could interfere with the transfer of peptides from TAP to MHC Cl I, possibly preventing release from TAP. With the results of this set of experiments this possibility seems unlikely.

These findings also elucidate the order of the sequence of events in the ER. It appears that calnexin binds the MHC Cl I glycoprotein first. Subsequent association with the peptide transporter and loading of the peptide triggers dissociation of both calnexin and the transporter. In cells not infected with Ad , the MHC Cl I molecule is released for further travel through to the cell surface. In Ad infected cells, E3/19K may interfere with the transfer of peptide to the MHC Cl I molecule preventing its subsequent release.

In a previous chapter it is suggested that E3/19K is only able to bind those allelic proteins that remain in the ER to be loaded with peptide. Those that travel empty to the cell surface are not bound as effectively by E3/19K (i.e. K^k). In the case of a slow binder, like D^b,

the length of time that this protein stays associated with the peptide transporter and calnexin in the ER enhances the effectiveness of E3/19K binding. E3/19K binding to D^b seems to take longer than for other proteins. Binding does not occur co-translationally. This means there is a large population of empty MHC Cl I proteins that need to be loaded with peptide present in the ER.

If E3/19K intercepts the MHC Cl I molecules before they are loaded with peptide, does this excess of empty MHC Cl I molecules monopolise all available peptide transporters? Previous results suggest this not to be the case. In the case of D^b, it seems that MHC Cl I molecules which remain in the ER for a long time do not influence the maturation of other proteins. Recent studies implicate a role for tapasin in MHC Cl I loading, stating that one TAP complex can service up to four MHC Cl I molecules (167).

In the last chapter it was noted that E3/19K proteins with the ER retrieval signal are returned to the ER (168). In the case of E3/19K mutants that were missing the ER retention signal, the passage of MHC Cl I through the ER was slowed (114). The ability of E3/19K to slow the passage through the ER, along with the inherent ability to stabilize mature epitopes suggests it shares some of the functions normally attributed to chaperones. Once the ER retention signal is removed, the E3/19K protein still delays the passage of MHC Cl I through the ER (169). In addition, Gabathuler *et al.* (169) are unable to show mutant E3/19K and MHC Cl I associating outside of the ER. In the absence of its ER retention signal, E3/19K association with MHC Cl I molecules may be temporary.

Schekman and his colleagues (83, 170-172) have speculated the existence of a positive signal required for macromolecules to be transported from the ER . The fact that the E3/19K molecule is not readily transported out in the absence of its ER retention signal may be an example of an ER protein that needs a positive signal to leave the ER.

Gabathuler *et al.* (114) show that removal of the ER retention signal does slow the

passage of MHC Cl I molecules through the ER. However, they do not clearly show whether or not the E3/19K protein without its ER retention signal (621 protein) makes its way out of the ER. It is likely that MHC Cl I is not continually bound by 621 since the level of cell surface MHC Cl I is not reduced in FACS experiments (173). There is no evidence to show association in any compartment other than the ER. It is conceivable that when the 621-MHC Cl I complex reaches a subcompartment with physical characteristics different from the ER, dissociation occurs. E3/19K has an affinity for only those MHC Cl I molecules to which it is exposed in the same compartment. This affinity is likely not high and if the MHC Cl I -E3/19K complex moves to a compartment such as the cis Golgi, the complex spontaneously dissociates.

Finally, it is the ER retention/retrieval signal KKMP that plays a role in retrieving the E3/19K and possibly the entire complex to the ER (168, 174), keeping them in an environment favouring association of E3/19K and MHC Cl I. There is no evidence that bound MHC Cl I are retrieved from a distal compartment, possibly because they will not progress past being TAP associated without a peptide. If the association of MHC Cl I and E3/19K is largely dependant on the conditions found within the ER and is unable to bind outside this compartment, then the relationship between these two proteins is similar to that of a chaperone.

In the last chapter it was suggested that at the cell surface there may be a type of regulation of MHC Cl I molecules. A positive signal generated at the cell surface related to the concentration of MHC Cl I molecules present, may feed back to the ER and limit the maturation of more MHC Cl I molecules. The speedy appearance of a 'mature epitope' within the cell may have further implications. The overloading of molecules ready to leave the ER may send a signal to the cell surface that upregulates cell surface turnover. Alternately, the mature epitope of the MHC Cl I molecules within the ER may help avoid the inevitable UPR. It has been speculated that the UPR is stimulated by the levels of free BiP in the ER (175) and vice versa (176). Mature conformations of retained proteins may be less likely to be bound by BiP and

less likely to trigger the UPR. Previously it has been proposed that the UPR helps prevent apoptosis of affected cells (176, 177), but some of the signalling triggers leukotrienes that stimulate inflammation. Infiltration of an afflicted area by immune response cells may allow a body to subsequently mount a more effective immune response to an affliction. Adenovirus infected cells may be less susceptible to such a response not only because of their characteristic ability to evade the immune system but also because of their weak stimulation of the UPR (68).

The complex involving E3/19K, MHC Cl I and transporter does exist. Additionally, it is observed that TAP and calnexin are also associated. The involvement of all of these components yields a very large complex.

6. Discussion

The effect of the adenovirus E3/19K protein on both the cellular machinery involved in antigen presentation and the regular maturation of MHC Cl I molecules has been examined with investigations on the role of the interactions of the many accessory proteins found in the ER, Golgi and other compartments of the cell. This study outlined the participants in the processing of cell surface proteins and helped elucidate the sequence and relative order of many of the steps required for effective antigen presentation. This study also investigated the role of E3/19K in the normal infection process of adenovirus and possibly other related viruses (adeno associated viruses).

The first chapter (three) examined the ability of E3/19K to bind specific protein products of different H-2 proteins in mice. This was the first time that the binding ability of E3/19K has been assessed against a common background (human 293 cells). Previously, pairs of proteins in different cell lines were exposed to E3/19K and the retention compared. The K^d and K^b allelic proteins bind to E3/19K very effectively while the K^k and D^k proteins do not. These proteins were classified into binding and non-binding groups. The D^b and L^d proteins were also found to bind, albeit slowly. Previously they had been shown not to bind to E3/19K. The binding of these proteins had a half life of 3-12 hours, that contrasted greatly with the $t_{1/2}$ for the strong binders K^d and K^b. This new class of binders was designated slow-binders.

E3/19K binding to MHC Cl I is based partly on an innate affinity of specific allelic proteins to E3/19K. More importantly, binding requires that E3/19K and the target protein occupy the same intracellular compartment for an extended period of time. Allelic proteins like K^d bind quickly to E3/19K whereas proteins like D^b bind less quickly, but equally as

effectively, to E3/19K. That E3/19K does not bind K^k and D^k likely reflects the amount of exposure that they have to E3/19K rather than a lack of affinity. As these molecules are quickly transported to the cell surface, they are not in the same compartment with E3/19K long enough for an association to occur.

Unlike in the mouse, when surveying E3/19K binding to human HLA proteins, it is found that all tested allelic proteins bind. The K^k and D^k allelic proteins are the only two shown to date not to bind to E3/19K. It is noteworthy that the $t_{1/2}$ for K^k to travel through the ER and become Endo H resistant is the shortest of all allelic proteins tested. The observation that the K^k protein does not bind may be due to the fact that the allelic protein is transported out of the ER at a very rapid rate and therefore is not physically available to be bound by E3/19K. It follows that if the rate of transport out of the ER for the K^k protein could be retarded, E3/19K binding may be observed.

The observation that the D^b protein binds E3/19K strongly but with a long $t_{1/2}$ suggests that the factors that influence E3/19K binding are closely related to factors that influence transport from the ER and to the cell surface. Normally, MHC Cl I proteins are translated into the ER where they immediately achieve a correct conformation through the interaction of many chaperones and ER resident proteins. Proteins that achieve a correct conformation are quickly shuttled along the ER-Golgi pathway where they are acted upon by other chaperones. Those that do not achieve a correct conformation are retained in the ER.

Previous studies have shown that different allelic proteins are transported with different rates through the ER. One of the factors that influences the rate of transport out of the ER is the availability of peptides that can be bound by the MHC Cl I molecule. The affinity of specific MHC Cl I proteins for existing peptides and the competition for these peptides may greatly influence the rate of transport of MHC Cl I molecules to the cell surface. In the case of

D^b the inability to bind peptides that confer a stable conformation may be the reason for slow transport.

For K^k, which is rapidly transported from the ER, the protein may have ready access to peptides that allow the correct mature conformation and the resulting transport to the cell surface. Another scenario is that K^k may bypass the quality control mechanism of the ER and is transported empty. Several studies have demonstrated that empty MHC Cl I molecules can be found on the cell surface in low numbers. These molecules are unstable and are rapidly turned over at the cell surface. Factors that influence their stability include the allelic protein which is being expressed and the cell line (and therefore cellular machinery) in which it is expressed. In the experiment with transfected 293 cells, the K^k protein may bypass the chaperones responsible for quality control and proceed to the cell surface without peptides. This would explain the quick rate of passage through the ER to the cell surface and could also account for the inability of E3/19K to form any association with this protein.

Unlike the K^k protein, D^b proceeds through the ER with a much longer half life. Comparison with K^d shows that D^b binds E3/19K much more slowly. This may be due to its slow rate of maturation through the ER. It could also reflect a lower affinity of D^b for E3/19K. A molecule with lower affinity for E3/19K may still be able to bind based on the length of time in the same compartment. The greater exposure of D^b to E3/19K offsets its lower affinity. Conversely, the fact that K^d is rapidly transported through the ER is offset by its higher affinity for E3/19K.

The findings of earlier researchers should be reconsidered in light of these observations. Previously, groups determining the affinity of E3/19K for specific allelic proteins may actually have been determining the amount of exposure these allelic proteins had

to E3/19K. When D^b was identified as a non binder, its rate of transport out of the ER limited the binding to E3/19K. The inability to form a complex with E3/19K was dependant on cellular factors that influence the rate of transport through the ER rather than an overall affinity between the two proteins. The ability of D^b to bind has been overlooked by other groups partly because of the longer time required for this association. Perhaps all allelic proteins are either fast binders or slow-binders. Those previously shown not to bind may not have been exposed to the conditions that would allow them to share the compartment with E3/19K for a period long enough to promote binding.

In the following chapter, factors that influence the normal maturation of MHC Cl I molecules were discussed. The $293D^b$ transfectant expressed large amounts of D^b but processed relatively small amounts to the cell surface. Much of the translated product was found within the ER where it remains for an extended period of time.

The $293D^b$ cell line was subjected to conditions that have been demonstrated to restore defective cell surface expression. TAP 2 deficient RMA-S cells grown at 26°C instead of the regular 37°C show increased levels of MHC Cl I molecules expressed at the cell surface . These MHC Cl I molecules were devoid of peptide in the binding cleft. The reduced temperature was believed to lower the kinetic energy of the system and reduce the rate of dissociation of these unstable complexes at the cell surface. The experiment consisted of growing $293D^b$ at 26°C and comparing the cell surface expression to $293D^b$ grown at 37°C. The results showed no increase in cell surface expression in FACS analysis.

To complement this experiment, level of β_2m available to the MHC Cl I molecules in the $293D^b$ cell line was increased. The rationale for this experiment was that if β_2m was in limiting supply, then increasing this supply would result in greater numbers of D^b (and

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presumably all other) MHC Cl I proteins at the cell surface. Increased β_2m caused by the infection of 293D^b with a vaccinia construct did not increase the cell surface expression.

Finally, studies with the CMT-64 cell line showed that high levels of cell surface expression could be achieved by exposing these cells to γ-IFN. γ-IFN acted as a general upregulator of many cellular processes including the expression of TAPs, β_2m and MHC Cl I. When the 293D^b was exposed to γIFN, it was found that the levels of D^b at the cell surface did not increase.

Failed attempts to upregulate the levels of D^b at the cell surface demonstrated an intriguing property of MHC Cl I expression. In a cell with normal levels of MHC Cl I expression (endogenous HLA), levels of foreign MHC Cl I molecule could not be upregulated. Attempts to provide missing cofactors or to reduce the stringency of the quality control (by reducing the temperature) were unsuccessful in increasing the level of expression. This suggests that the level of the D^b protein on the surface of 293 cells is actively regulated. It is assumed that some other accessory protein may have a direct role in controlling the level of surface expression of MHC Cl I.

The bulk flow theory has been the prevailing theory regarding the transfer of membrane and secretory proteins through the ER. More recently, other groups including one led by Schekman have suggested the existence of positive signals that move cargo from the ER to the Golgi (83).

Investigation of the regulation of cell surface molecules may be influenced by the turnover of MHC Cl I molecules at the cell surface. Some investigators (114) have suggested that the number of cell surface molecules is based entirely on the rate of transport to the surface. They suggest that turnover at the cell surface occurs at a set rate; reduced populations

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result when any particular member of the population travels to the cell surface at a rate lower than that of the turnover. The results suggest that the level of expression of any particular species at the cell surface may be concentration dependant. Once the concentration of a particular population of MHC Cl I molecules reaches its saturation point, any increase in expression will not result in increased representation at the cell surface. In the experiments, any excess D^b is retained in the ER.

In the third section, it was observed that association of human HLA proteins with E3/19K caused rapid maturation as assessed by a conformation specific monoclonal antibody. HLA - A, B and C were very quickly recognised by W6/32 in the presence of E3/19K. Due to E3/19K retention in the ER, these molecules never achieved Endo H resistance. In short, E3/19K was binding to a misfolded and unprocessed immature MHC Cl I molecule and forcing it into a conformation that resembled the mature conformation, yet preventing escape from the ER.

Whether this interaction sterically interfered with the ability of other well characterised ER resident chaperones to associate with MHC Cl I molecules was studied. Of particular interest was whether of calnexin and the TAP proteins could still associate with E3/19K bound MHC Cl I. The normal sequence of events for MHC Cl I association with ER resident chaperones was probed with E3/19K.

With experiments described in that chapter, the sequence of events for the E3/19K binding to MHC Cl I molecules can be elucidated. In most cases, MHC Cl I is cotranslationally translocated into the lumen of the ER where it associates with β_2m.

Achievement of a mature conformation permits release by calnexin and egress from the ER is usually coincident with the loading of the correct peptide. With some allelic proteins, release from the ER may occur without peptide loading resulting in rapid transport to the cell surface. E3/19K associates with MHC Cl I molecules in the ER before transport out of the ER. The

"slow-binders" suggest that this association is not necessarily co-translational for all allelic proteins as has been previously reported. E3/19K only will associate with those proteins which stay in the same compartment long enough to permit binding and E3/19K has different affinities for different allelic proteins.

The exact nature of E3/19K association with MHC Cl I molecules has yet to be determined. Differences in the binding affinity of different proteins have led some studies to map those residues in the MHC Cl I molecule that may influence the binding to a greater degree than others. Site directed mutagenesis of E3/19K has shown that mutations in almost any region can abrogate binding. Other studies have mapped the key regions in the MHC Cl I molecule to those hypervariable regions in the alpha 1 and alpha 2 regions. This led researchers in the past to suggest that E3/19K was binding in the peptide binding groove, possibly displacing peptides.

Precipitation of E3/19K in a complex involving peptide transporters suggested that these two proteins were associating with MHC Cl I non-exclusively. It is possible that E3/19K blocked the transfer of peptides to MHC Cl I and their release from the TAP proteins. The experiments that precipitated MHC Cl I with biotinylated peptide further demonstrated that MHC Cl I and E3/19K did not associate with one another via the peptide binding groove.

The role of E3/19K as a protein that was associating with MHC Cl I as a member of a large group of proteins was investigated. The set of experiments demonstrating the association of several ER proteins in a complex with E3/19K indicated that this complex may be very large. Velocity sedimentation gradients were performed to assess the size of the complex.

The sucrose gradients identified several subpopulations of the E3/19K protein with different densities. These subsets suggest that in addition to binding and retaining MHC Cl I in the ER, E3/19K forms complexes with other proteins as well. E3/19K is in excess in studies that assess co-precipitation by alternately precipitating with anti-MHC Cl I antibodies or anti E3/19K antisera, which suggests that much of it never associates with MHC Cl I.

The nature of binding of different MHC Cl I allelic proteins by E3/19K may reflect both the rate of transport and the path of the MHC Cl I protein being bound. Proteins such as K^k are not bound; these may be rapidly transported by bypassing the regular maturation process involving peptide loading. MHC Cl I proteins present in the ER for long periods of time are bound effectively, for example D^b. Like K^k, D^b may not be maturing properly and is possibly susceptible to the quality control mechanisms present in the ER and therefore unable to exit the ER. Both proximity and time promote association with E3/19K.

Human MHC Cl I proteins are quickly bound by E3/19K and a mature conformation quickly stabilises. This mature conformation may result in rescue from the default degradation pathway that degrades improperly formed proteins in the ER. Additionally, this build up of mature MHC Cl I molecules in the ER may trigger a UPR response.

Finally, the experiments with γIFN reveal that the effect of E3/19K in the ER can be abrogated. This is significant because it makes the physiological role of E3/19K uncertain. Previously it was believed that as a result of its retention of MHC Cl I in the ER, E3/19K reduced CTL killing of infected cells. However, the results of this research suggest that MHC Cl I will not be reduced in an antiviral response. The nature of the restoration of MHC Cl I cell surface expression is also interesting. Many groups have shown that E3/19K is normally found in excess in the ER. The response to γ-IFN is unlikely to be a simple upregulation of MHC Cl I levels.

7. Conclusion

In summary, E3/19K served as a good tool to examine the nature of MHC Cl I allele specificity in viral infection and the subversion of antigen presentation by viral machinery. Secondly, E3/19K proved to be invaluable in probing the cellular machinery involved in the manufacture of proteins that are processed through the ER. This includes all cell surface proteins as well as those that remain associated with membranes inside the cell. It has been demonstrated that E3/19K binds to different MHC Cl I allelic proteins with different affinities. It was also demonstrated that different proteins behave differently based on the characteristics of the cell in which they are expressed. Factors such as the inherent ability of an allelic protein to be transported to the cell surface or detained within the cell proved to be as important as the affinity of specific proteins for E3/19K. Finally, examination and postulation of the effects of E3/19K on viral evasion of the immune system have led to a deeper understanding of the many mechanisms employed to this end.

1. McFadden, G. and K. Kane, 1994. How DNA viruses perturb functional MHC expression to alter immune recognition. *Adv Cancer Res, 63:* 117-209.

2. Langman, R., *The Immune System.* 1989, San Diego: Academic Press, Inc.

3. Klein, J., *Immunology: The Science of Self-Nonself Discrimination.* 1982, Toronto: John Wiley & Sons.

4. Zinkernagel, R.M. and P.C. Doherty, 1974. Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis. *Nature, 251:* 547-8.

5. Haskins, K., J. Kappler and P. Marrack, 1984. The Major Histocompatibility Compex-Restricted Antigen Receptor on T Cells. *Annu. Rev. Immunol., 2:* 51-66.

6. Schwartz, R., 1985. Associations in T-cell activation. *Nature, 317:* 284-285.

7. Williams, A.F. and A.N. Barclay, 1988. The Immunoglobulin Superfamily: Domains For Cell Surface Recognition. *Annu. Rev. Immunol., 6:* 381-405.

8. Schatz, D., M. Oettinger and D. Baltimore, 1989. The V(D)J Recombination Activating Gene, RAG-1. *Cell, 59:* 1035-1048.

9. Benacerraf, B. and H.O. McDevitt, 1972. Histocompatibility-linked immune response genes. *Science, 175:* 273-9.

10. Wiertz, E.J., S. Mukherjee and H.L. Ploegh, 1997. Viruses use stealth technology to escape from the host immune system. [Review] [52 refs]. *Molecular Medicine Today, 3:* 116-23.

11. Steinmetz, M. and L. Hood, 1983. Genes of the major histocompatibility complex in mouse and man. *Science, 222:* 727-733.

12. Klein, J., *Natural history of the major histocompatibility complex.* 1986, New York: Wiley-Interscience Publication. 1-775.

13. Bjorkman, P.J., M.A. Saper, B. Samraoui, W.S. Bennett, J.L. Strominger and D.C. Wiley, 1987. Structure of the human class I histocompatibility antigen, HLA-A2.

Nature, 329: **506-12.**

14. Townsend, A., C. Öhlén, J. Bastin, H. Ljunggren, L. Foster and K. Kärre, 1991. Association of class I major Histocompatibility heavy and light chains induced by viral peptides. *Nature, 340:* **443-448.**

15. Garcia, K.C., M. Degano, L.R. Pease, M. Huang, P.A. Peterson, L. Teyton and I.A. Wilson, 1998. Structural basis of plasticity in T cell receptor recognition of a self peptide-MHC antigen. *Science, 279:* **1166-72.**

16. Hunt, D.F., *et al.*, 1992. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry [see comments]. *Science, 255:* **1261-3.**

17. Deres, K., T.N. Schumacher, K.H. Wiesm:uller, S. Stevanovi:c, G. Greiner, G. Jung and H.L. Ploegh, 1992. Preferred size of peptides that bind to H-2 Kb is sequence dependent. *Eur J Immunol, 22:* **1603-8.**

18. Falk, K., O. Rotzschke, K. Deres, J. Metzger, G. Jung and H.G. Rammensee, 1991. Identification of naturally processed viral nonapeptides allows their quantification in infected cells and suggests an allele-specific T cell epitope forecast. *J Exp Med, 174:* **425-34.**

19. Rammensee, H.G., K. Falk and O. Rotzschke, 1993. MHC molecules as peptide receptors. [Review] [111 refs]. *Current Opinion in Immunology, 5:* **35-44.**

20. Rammensee, H.G., K. Falk and O. Rotzschke, 1993. Peptides naturally presented by MHC class I molecules. [Review] [136 refs]. *Annual Review of Immunology, 11:* **213-44.**

21. Lupas, A., P. Zwickl and W. Baumeister, 1994. Proteasome sequences in eubacteria. *Trends in Biochemical Sciences, 19:* **533-4.**

22. Hershko, A. and A. Ciechanover, 1992. The Ubiquitin System for Protein Degradation. *Annu Rev Biochem, .*

23. Ortiz-Navarrete, V., A. Seelig, M. Gernold, S. Frentzel, P. Kloetzel and G. Hammerling, 1991. Subunit of the 20S proteasome (multicatalytic proteinase) encoded by the major histocompatibility complex. *Nature, 353:* **662-664.**

24. Goldberg, A.L. and K.L. Rock, 1992. Proteolysis, proteasomes and antigen presentation. [Review] [92 refs]. *Nature, 357:* 375-9.

25. Peters, J.M., 1994. Proteasomes: protein degradation machines of the cell. *Trends Biochem Sci, 19:* 377-82.

26. Monaco, J.J. and H.O. McDevitt, 1986. The LMP antigens: a stable MHC-controlled multisubunit protein complex. *Hum Immunol, 15:* 416-26.

27. Spies, T., V. Cerundolo, M. Colonna, P. Cresswell, A. Townsend and R. DeMars, 1992. Presentation of viral antigen by MHC class I molecules is dependent on a putative peptide transporter heterodimer. *Nature, 355:* 644-6.

28. Suh, W.K., E.K. Mitchell, Y. Yang, P.A. Peterson, G.L. Waneck and D.B. Williams, 1996. MHC class I molecules form ternary complexes with calnexin and TAP and undergo peptide-regulated interaction with TAP via their extracellular domains. *J Exp Med, 184:* 337-48.

29. Suh, W.K., M.F. Cohen-Doyle, K. Fruh, K. Wang, P.A. Peterson and D.B. Williams, 1994. Interaction of MHC class I molecules with the transporter associated with antigen processing. *Science, 264:* 1322-6.

30. Ortmann, B., M.J. Androlewicz and P. Cresswell, 1994. MHC class I/beta 2-microglobulin complexes associate with TAP transporters before peptide binding. *Nature, 368:* 864-7.

31. Elliott, T., V. Cerundolo and A. Townsend, 1992. Short peptides assist the folding of free class I heavy chains in solution. *European Journal of Immunology, 22:* 3121-5.

32. Cromme, F.V., *et al.*, 1994. Loss of transporter protein, encoded by the TAP-1 gene, is highly correlated with loss of HLA expression in cervical carcinomas. *Journal of Experimental Medicine, 179:* 335-40.

33. Ossevoort, M.A., *et al.*, 1993. Differential effect of transporter Tap 2 gene introduction into RMA-S cells on viral antigen processing. *Eur J Immunol, 23:* 3082-8.

34. Gaskins, H.R., J.J. Monaco and E.H. Leiter, 1992. Expression of intra-MHC

transporter (Ham) genes and class I antigens in diabetes-susceptible NOD mice [letter; comment]. *Science, 256:* **1826-8.**

35. Attaya, M., *et al.,* 1992. Ham-2 corrects the class I antigen-processing defect in RMA-S cells. *Nature, 355:* **647-9.**

36. Momburg, F., J. Roelse, G.J. Hammerling and J.J. Neefjes, 1994. Peptide size selection by the major histocompatibility complex-encoded peptide transporter. *J Exp Med, 179:* **1613-23.**

37. Schumacher, T.N., *et al.,* 1994. Peptide length and sequence specificity of the mouse TAP1/TAP2 translocator. *Journal of Experimental Medicine, 179:* **533-40.**

38. Powis, C.J., J.C. Howard and G.W. Butcher, 1990. Variation in the biosynthesis of the rat RT1.Aa classical class I antigen due to the cim system. *Transplant Proc, 22:* **2517-8.**

39. Lodish, H. and N. Kong, 1990. Perturbation of Cellular Calcium Blocks Exit of Secretory Proteins from the Rough Endoplasmic Reticulum. *J.B.C., 265:* **10893-10899.**

40. Lodish, H. and N. Kong, 1993. The Secretory Pathway is normal in Dithiothreitol-treated cells, but disulfide-bonded proteins are reduced and reversibly retained in the endoplasmic reticulum. *J.B.C., 268:* **20598-20605.**

41. Sadasivan, B., P.J. Lehner, B. Ortmann, T. Spies and P. Cresswell, 1996. Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. *Immunity, 5:* **103-14.**

42. Wada, I., M. Kai, S. Imai, F. Sakane and H. Kanoh, 1997. Promotion of transferrin folding by cyclic interactions with calnexin and calreticulin. *Embo J, 16:* **5420-32.**

43. Sadasivan, B.K., A. Cariappa, G.L. Waneck and P. Cresswell, 1995. Assembly, peptide loading, and transport of MHC class I molecules in a calnexin-negative cell line. *Cold Spring Harb Symp Quant Biol, 60:* **267-75.**

44. Hebert, D.N., B. Foellmer and A. Helenius, 1996. Calnexin and calreticulin

promote folding, delay oligomerization and suppress degradation of influenza hemagglutinin in microsomes. *Embo J, 15:* **2961-8.**

45.   Zhou, X., F. Momburg, T. Liu, M.U. Abdel, M. Jondal, G.J. Hammerling and H.G. Ljunggren, 1994. Presentation of viral antigens restricted by H-2Kb, Db or Kd in proteasome subunit LMP2- and LMP7-deficient cells. *Eur J Immunol, 24:* **1863-8.**

46.   Klar, D. and G. Hammerling, 1989. Induction of assembly of MHC class I heavy chains with beta2-microglobulin by interferon-gamma. *Embo, 8:* **475-481.**

47.   Gabathuler, R., G. Reid, G. Kolaitis, J. Driscoll and W.A. Jefferies, 1994. Comparison of cell lines deficient in antigen presentation reveals a functional role for TAP-1 alone in antigen processing. *Journal of Experimental Medicine, 180:* **1415-25.**

48.   Blobel, G., 1980. Intracellular protein topogenesis. *Proc Natl Acad Sci U S A, 77:* **1496-500.**

49.   Arar, C., V. Carpentier, J.P. Le Caer, M. Monsigny, A. Legrand and A.C. Roche, 1995. ERGIC-53, a membrane protein of the endoplasmic reticulum-Golgi intermediate compartment, is identical to MR60, an intracellular mannose-specific lectin of myelomonocytic cells. *J Biol Chem, 270:* **3551-3.**

50.   Ferreira, L.R., K. Norris, T. Smith, C. Hebert and J.J. Sauk, 1994. Association of Hsp47, Grp78, and Grp94 with procollagen supports the successive or coupled action of molecular chaperones. *J Cell Biochem, 56:* **518-26.**

51.   Bonnerot, C., M.S. Marks, P. Cosson, E.J. Robertson, E.K. Bikoff, R.N. Germain and J.S. Bonifacino, 1994. Association with BiP and aggregation of class II MHC molecules synthesized in the absence of invariant chain. *Embo J, 13:* **934-44.**

52.   Flynn, G.C., J. Pohl, M.T. Flocco and J.E. Rothman, 1991. Peptide-binding specificity of the molecular chaperone BiP. *Nature, 353:* **726-30.**

53.   Anderson, K.S., J. Alexander, M. Wei and P. Cresswell, 1993. Intracellular transport of class I MHC molecules in antigen processing mutant cell lines. *Journal of Immunology, 151:* **3407-19.**

54.   David, V., F. Hochstenbach, S. Rajagopalan and M.B. Brenner, 1993. Interaction with newly synthesized and retained proteins in the endoplasmic reticulum suggests a chaperone function for human integral membrane protein IP90 (calnexin). *J Biol Chem, 268:* 9585-92.

55.   Degen, E. and D. Williams, 1991. Participation of a Novel 88-kD Protein in the Biogenesis of Murine Class I Histocompatibility Molecules. *J Cell Biol, 112:* 1099-1115.

56.   Ware, F.E., A. Vassilakos, P.A. Peterson, M.R. Jackson, M.A. Lehrman and D.B. Williams, 1995. The molecular chaperone calnexin binds Glc1Man9GlcNAc2 oligosaccharide as an initial step in recognizing unfolded glycoproteins. *J Biol Chem, 270:* 4697-704.

57.   Hebert, D.N., B. Foellmer and A. Helenius, 1995. Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. *Cell, 81:* 425-33.

58.   Jackson, M.R., D.M. Cohen, P.A. Peterson and D.B. Williams, 1994. Regulation of MHC class I transport by the molecular chaperone, calnexin (p88, IP90). *Science, 263:* 384-7.

59.   Hammond, C. and A. Helenius, 1994. Folding of VSV G protein: sequential interaction with BiP and calnexin. *Science, 266:* 456-8.

60.   Trombetta, S.E., M. Bosch and A.J. Parodi, 1989. Glucosylation of glycoproteins by mammalian, plant, fungal, and trypanosomatid protozoa microsomal membranes. *Biochemistry, 28:* 8108-16.

61.   Degen, E., M. Cohen-Doyle and D. Williams, 1992. Efficient Dissociation of the p88 Chaperone from Major Histocompatibility Complex Class I molecules Requires Both β_2-Microglobulin and Peptide. *J Exp Med, 175:* 1653-1661.

62.   Fenteany, G., R.F. Standaert, W.S. Lane, S. Choi, E.J. Corey and S.L. Schreiber, 1995. Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science, 268:* 726-31.

133

63. Hughes, E.A., C. Hammond and P. Cresswell, 1997. Misfolded major histocompatibility complex class I heavy chains are translocated into the cytoplasm and degraded by the proteasome. *Proceedings of the National Academy of Sciences of the United States of America, 94:* **1896-901.**

64. Braakman, I., J. Helenius and A. Helenius, 1992. Manipulating Disulfide Bond Formation and Protein Folding in the Endoplasmic Reticulum. *Embo J, 11:* **1717-1722.**

65. Gething, M.J. and J. Sambrook, 1992. Protein folding in the cell. *Nature, 355:* **33-45.**

66. Creighton, T.E., 1997. Protein folding coupled to disulphide bond formation. *Biol Chem, 378:* **731-44.**

67. Cox, J.S., C.E. Shamu and P. Walter, 1993. Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. *Cell, 73:* **1197-206.**

68. Pahl, H.L., M. Sester, H.G. Burgert and P.A. Baeuerle, 1996. Activation of transcription factor NF-kappaB by the adenovirus E3/19K protein requires its ER retention. *Journal.of.Cell Biology., 132:* **511-522.**

69. Swiedler, S., J. Freed, A. Tarentino, J. Plummer and G. Wart, 1985. Oligosaccharide microheterogeneity of the murine major histocompatibility antigens. Reproducible site-specific patterns of sialylation and branching in asparagine-linked oligosaccharids. *The Journal of Biological Chemistry, 260:* **4046-4054.**

70. Swiedler, S.J., G.W. Hart, A.L. Tarentino, T.H. Plummer, Jr. and J.H. Freed, 1983. Stable oligosaccharide microheterogeneity at individual glycosylation sites of a murine major histocompatibility antigen derived from a B- cell lymphoma. *J Biol Chem, 258:* **11515-23.**

71. Ganan, S., J.J. Cazzulo and A.J. Parodi, 1991. A major proportion of N-glycoproteins are transiently glucosylated in the endoplasmic reticulum. *Biochemistry, 30:* **3098-104.**

72.   Sousa, M.C., M.A. Ferrero-Garcia and A.J. Parodi, 1992. Recognition of the oligosaccharide and protein moieties of glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase. *Biochemistry,   31:* **97-105.**

73.   Dorner, A., D. Bole and R. Kaufman, 1987. The Relationship of N-linked Glycosylation and Heavy Chain-binding Protein Association with the Secretion of Glycoproteins. *The Journal of Cell Biology,   105:* **2665-2674.**

74.   Moore, S.E. and R.G. Spiro, 1993. Inhibition of glucose trimming by castanospermine results in rapid degradation of unassembled major histocompatibility complex class I molecules. *J Biol Chem,   268:* **3809-12.**

75.   McCracken, A.A. and J.L. Brodsky, 1996. Assembly of ER-associated protein degradation in vitro: dependence on cytosol, calnexin, and ATP. *J Cell Biol,   132:* **291-8.**

76.   Huppa, J.B. and H.L. Ploegh, 1997. The alpha chain of the T cell antigen receptor is degraded in the cytosol. *Immunity,   7:* **113-22.**

77.   Farquhar, M.G. and G.E. Palade, 1998. The Golgi apparatus: 100 years of progress and controversy. *Trends Cell Biol,   8:* **2-10.**

78.   Schatz, G. and B. Dobberstein, 1996. Common principles of protein translocation across membranes. *Science,   271:* **1519-26.**

79.   Mellman, I. and K. Simons, 1992. The Golgi complex: in vitro veritas?. [Review] [126 refs]. *Cell,   68:* **829-40.**

80.   Pfeffer, S.R. and J.E. Rothman, 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. [Review] [117 refs]. *Annual Review of Biochemistry, 56:* **829-52.**

81.   Schekman, R., 1996. Polypeptide translocation: a pretty picture is worth a thousand words [comment]. *Cell,   87:* **593-5.**

82.   Balch, W.E., J.M. McCaffery, H. Plutner and M.G. Farquhar, 1994. Vesicular stomatitis virus glycoprotein is sorted and concentrated during export from the endoplasmic reticulum. *Cell,   76:* **841-52.**

83.   Nishimura, N. and W.E. Balch, 1997. A di-acidic signal required for selective export from the endoplasmic reticulum. *Science,* *277:* **556-8.**

84.   Scheiffele, P., J. Peranen and K. Simons, 1995. N-glycans as apical sorting signals in epithelial cells. *Nature,* *378:* **96-8.**

85.   Fiedler, K., R.G. Parton, R. Kellner, T. Etzold and K. Simons, 1994. VIP36, a novel component of glycolipid rafts and exocytic carrier vesicles in epithelial cells. *Embo Journal,* *13:* **1729-40.**

86.   Zinkernagel, R.M. and P.C. Doherty, 1979. MHC-restricted cytotoxic T cells: Studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. *Adv.Immunol.,* *27:* **51-177.**

87.   Takeshita, T., *et al.,* 1993. Role of conserved regions of class I MHC molecules in the activation of CD8+ cytotoxic T lymphocytes by peptide and purified cell-free class I molecules. *Int Immunol,* *5:* **1129-38.**

88.   Takahashi, H., R. Houghten, S.D. Putney, D.H. Margulies, B. Moss, R.N. Germain and J.A. Berzofsky, 1989. Structural requirements for class I MHC molecule-mediated antigen presentation and cytotoxic T cell recognition of an immunodominant determinant of the human immunodeficiency virus envelope protein. *J Exp Med,* *170:* **2023-35.**

89.   Sun, R., S.E. Shepherd, S.S. Geier, C.T. Thomson, J.M. Sheil and S.G. Nathenson, 1995. Evidence that the antigen receptors of cytotoxic T lymphocytes interact with a common recognition pattern on the H-2Kb molecule. *Immunity,* *3:* **573-82.**

90.   Bjorkman, P.J., M.A. Saper, B. Samraoui, W.S. Bennett, J.L. Strominger and D.C. Wiley, 1987. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature,* *329:* **512-8.**

91.   van Endert, P.M., 1999. Genes regulating MHC class I processing of antigen. *Curr Opin Immunol,* *11:* **82-8.**

92.   Straus, S., *Adenovirus Infections in Humans,* in *The Adenovirus,* H. Ginsburg, Editor.

1984, Plenum: New York. p. 451-487.

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

94. de Jong, P.J., G. Valderrama, I. Spigland and M.S. Horwitz, 1983. Adenovirus isolates from urine of patients with acquired immunodeficiency syndrome. *Lancet, 1:* 1293-1296.

95. Hierholzer, J.C., R. Wigand, L.J. Anderson, T. Adrian and J.W. Gold, 1988. Adenoviruses from patients with AIDS: a plethora of serotypes and a description of five new serotypes of subgenus D (types 43-47). *Journal.of.Infectious.Diseases., 158:* 804-813.

96. Matsuse, T., S. Hayashi, K. Kuwano, H. Keunecke, W.A. Jefferies and J.C. Hogg, 1992. Latent adenoviral infection in the pathogenesis of chronic airways obstruction. *American Review of Respiratory Disease, 146:* 177-84.

97. Nermut, M., *The Architecture of Adenovirus*, in *The Adenovirus*, H. Ginsburg, Editor. 1984, Plenum: New York. p. 5-32.

98. Philipson, L. and K. Lonberg-Holm, 1969. Fate of adenovirus during the early phase of infection. *J Gen Microbiol, 57:* x-xi.

99. Berk, A.J., 1986. Adenovirus promoters and E1A transactivation. *Annu Rev Genet, 20:* 45-79.

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

101. Bernards, R., P.I. Schrier, A. Houweling, J.L. Bos, A.J. van der Eb, M. Zijlstra and C.J. Melief, 1983. Tumorigenicity of cells transformed by adenovirus type 12 by evasion of T-cell immunity. *Nature, 305:* 776-9.

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

103. Gooding, L.R., L.W. Elmore, A.E. Tollefson, H.A. Brady and W.S. Wold, 1988. A 14,700 MW protein from the E3 region of adenovirus inhibits cytolysis by tumor necrosis factor. *Cell, 53:* 341-6.

104. Gooding, L.R., I.O. Sofola, A.E. Tollefson, P. Duerksen-Hughes and W.S. Wold, 1990. The adenovirus E3-14.7K protein is a general inhibitor of tumor necrosis factor-mediated cytolysis. *J Immunol, 145:* 3080-6.

105. Carlin, C., A. Tollefson, H. Brady, B. Hoffman and W. Wold, 1989. Epidermal growth factor receptor is down-regulated by a 10.400 MW protein encoded by the E3 region of adenovirus. *Cell, 57:* 135-144.

106. Andersson, M., S. Paabo, T. Nilsson and P.A. Peterson, 1985. Impaired intracellular transport of class I MHC antigens as a possible means for adenoviruses to evade immune surveillance. *Cell, 43:* 215-22.

107. Tollefson, A.E., A.R. Stewart, S.P. Yei, S.K. Saha and W.S. Wold, 1991. The 10,400- and 14,500-dalton proteins encoded by region E3 of adenovirus form a complex and function together to down-regulate the epidermal growth factor receptor. *J Virol, 65:* 3095-105.

108. Gooding, L.R., T.S. Ranheim, A.E. Tollefson, L. Aquino, P. Duerksen-Hughes, T.M. Horton and W.S. Wold, 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:* 4114-23.

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

110. Flomenberg, P., J. Szmulewicz, E. Gutierrez and H. Lupatkin, 1992. Role of the adenovirus E3-19k conserved region in binding major histocompatibility complex class I molecules. *J Virol, 66:* 4778-83.

111. Kornfeld, R. and W.S. Wold, 1981. Structures of the oligosaccharides of the

glycoprotein coded by early region E3 of adenovirus 2. *J Virol, 40:* **440-9.**

112. Flomenberg, P., M. Chen and M. Horwitz, 1988. Sequence and Genetic Organization of Adenovirus Type 35 Early Region 3. *Journal of Virology, 62:* **4431-4437.**

113. Sester, M. and H.G. Burgert, 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:* **5423-32.**

114. Gabathuler, R. and S. Kvist, 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:* **1803-10.**

115. Signas, C., M.G. Katze, H. Persson and L. Philipson, 1982. An adenovirus glycoprotein binds heavy chains of class I transplantation antigens from man and mouse. *Nature, 299:* **175-8.**

116. Paabo, S., F. Weber, O. Kampe, W. Schaffner and P.A. Peterson, 1983. Association between transplantation antigens and a viral membrane protein synthesized from a mammalian expression vector. *Cell, 33:* **445-53.**

117. Tanaka, Y. and S. Tevethia, 1988. Differential effect of adenovirus 2 E3/19K glycoprotein on the expresson of H-2Kb and H-2Db Class I antigens and K-2Kb and H-2Db restricted SV40 Specific CTL Mediated Lysis. *Virology, 165:* **357-366.**

118. Severinsson, L., I. Martens and P.A. Peterson, 1986. Differential association between two human MHC class I antigens and an adenoviral glycoprotein. *J Immunol, 137:* **1003-9.**

119. Wold, W.S. and L.R. Gooding, 1989. Adenovirus region E3 proteins that prevent cytolysis by cytotoxic T cells and tumor necrosis factor. [Review]. *Molecular.Biology.&.Medicine., 6:* **433-452.**

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

121.   Graham, F.L., J. Smiley, W.C. Russell and R. Nairn, 1977. Characteristics of a human cell line transformed by DNA from human Adenovirus type 5. *Journal of General Virology,   36:* 59-72.

122.   Persson, H., C. Signas and L. Philipson, 1979. Purification and characterization of an early glycoprotein from adenovirus type 2-infected cells. *J Virol,   29:* 938-48.

123.   Lévy, F., R. Larsson and S. Kvist, 1991. Translocation of peptides through microsomal membranes is a rapid process and promotes assembly of HLA-B27 heavy chain and beta 2-microglobulin translated in vitro. *J Cell Biol,   115:* 959-70.

124.   Ou, W.J., P.H. Cameron, D.Y. Thomas and J.J. Bergeron, 1993. Association of folding intermediates of glycoproteins with calnexin during protein maturation. *Nature, 364:* 771-6.

125.   Powis, S.J., A.R. Townsend, E.V. Deverson, J. Bastin, G.W. Butcher and J.C. Howard, 1991. Restoration of antigen presentation to the mutant cell line RMA-S by an MHC-linked transporter. *Nature,   354:* 528-31.

126.   Bikoff, E.K., L. Jaffe, R.K. Ribaudo, G.R. Otten, R.N. Germain and E.J. Robertson, 1991. MHC class I surface expression in embryo-derived cell lines inducible with peptide or interferon. *Nature,   354:* 235-8.

127.   Newcomb, J.R. and P. Cresswell, 1993. Structural analysis of proteolytic products of MHC class II-invariant chain complexes generated in vivo. *J Immunol,   151:* 4153-63.

128.   Beier, D.C., J.H. Cox, D.R. Vining, P. Cresswell and V.H. Engelhard, 1994. Association of human class I MHC alleles with the adenovirus E3/19K protein. *Journal of Immunology,   152:* 3862-72.

129.   Burgert, H.-G., J. Maryanski and S. Kvist, 1987. "E3/19K" protein of adenovirus type 2 inhibits lysis of cytolytic T lymphocytes by blocking cell-surface expression of histocompatibility class I antigens. *Proceedings of the National Academy of Science of USA,   84:* 1356-1360.

130.   Cox, J.H., R.M. Buller, J.R. Bennink, J.W. Yewdell and G. Karupiah, 1994.

Expression of adenovirus E3/19K protein does not alter mouse MHC class I-restricted responses to vaccinia virus. *Virology,* *204:* **558-62.**

131. Jefferies, W.A. and H.G. Burgert, 1990. E3/19k from Adenovirus 2 is an immunosubversive protein that binds to a structural motif regulating the intracellular transport of major histocompatibility complex class I proteins. *Journal of Experimental Medicine,* *172:* **1653-1664.**

132. Lapham, C.K., I. Bacik, J.W. Yewdell, K.P. Kane and J.R. Bennink, 1993. Class I molecules retained in the endoplasmic reticulum bind antigenic peptides. *J Exp Med,* *177:* **1633-41.**

133. Jefferies, W.A. and G.G. MacPherson, 1987. Expression of the W6/32 HLA epitope by cells of rat, mouse, human and other species: critical dependence on the interaction of specific MHC heavy chains with human or bovine beta 2-microglobulin. *European Journal of Immunology,* *17:* **1257-63.**

134. Korner, H. and H.G. Burgert, 1994. Down-regulation of HLA antigens by the adenovirus type 2 E3/19K protein in a T-lymphoma cell line. *Journal.of.Virology.,* *68:* **1442-1448.**

135. Fellous, M., M. Kamoun, J. Wiels, J. Dausset, J. Clements, J. Zeuthen and G. Klein, 1977. Induction of HLA Expression in Daudi Cells after Cell Fusion. *Immunogenetics,* *5:* **423-436.**

136. Seong, R., C. Clayberger, A. Krensky and J. Parnes, 1988. Rescue of Daudi Cell HLA Expression by Transfection of the Mouse beta2 Microglobulin Gene. *J Exp Med,* *167:* **288-299.**

137. Gillot, D., A. Nouri, S. Compton and R. Oliver, 1993. Accurate and rapid assessment of MHC antigen upregulation following cytokine stimulation. *J. Imm. Methods,* *165:* **231-239.**

138. Boehm, U., T. Klamp, M. Groot and J.C. Howard, 1997. Cellular responses to interferon-gamma. [Review] [282 refs]. *Annual Review of Immunology,* *15:* **749-95.**

139.   Jefferies, W.A., G. Kolaitis and R. Gabathuler, 1993. IFN-gamma-induced recognition of the antigen-processing variant CMT.64 by cytolytic T cells can be replaced by sequential addition of beta 2 microglobulin and antigenic peptides. *J Immunol, 151:* 2974-85.

140.   Seliger, B., S. Hammers, A. Hohne, R. Zeidler, A. Knuth, C.D. Gerharz and C. Huber, 1997. IFN-gamma-mediated coordinated transcriptional regulation of the human TAP-1 and LMP-2 genes in human renal cell carcinoma [In Process Citation]. *Clin Cancer Res, 3:* 573-8.

141.   Townsend, A., T. Elliott, V. Cerundolo, L. Foster, B. Barber and A. Tse, 1990. Assembly of MHC class I molecules analyzed in vitro [published erratum appears in C ell 1990 Sep 21;62(6):following 1233]. *Cell, 62:* 285-95.

142.   Aosai, F., T.H. Yang, M. Ueda and A. Yano, 1994. Isolation of naturally processed peptides from a Toxoplasma gondii- infected human B lymphoma cell line that are recognized by cytotoxic T lymphocytes. *J Parasitol, 80:* 260-6.

143.   Powis, S., J. Howard and G. Butcher, 1991. The major histocompatibility complex class II-linked *cim* locus controls the kinetics of intracellular transport of a classical class I molecule. *J. Exp. Med, 173:* 913-921.

144.   Ljunggren, H.G., *et al.*, 1990. Empty MHC class I molecules come out in the cold. *Nature, 346:* 476-80.

145.   Shamu, C.E., 1998. Splicing: HACking into the unfolded-protein response. *Curr Biol, 8:* R121-3.

146.   Sidrauski, C., J.S. Cox and P. Walter, 1996. tRNA ligase is required for regulated mRNA splicing in the unfolded protein response [see comments]. *Cell, 87:* 405-13.

147.   Menzel, R., F. Vogel, E. Kargel and W.H. Schunck, 1997. Inducible membranes in yeast: relation to the unfolded-protein-response pathway. *Yeast, 13:* 1211-29.

148.   Lehner, P.J., M.J. Surman and P. Cresswell, 1998. Soluble tapasin restores MHC class I expression and function in the tapasin-negative cell line .220. *Immunity, 8:* 221-31.

149. Kahn-Perles, B., C. Boyer, B. Arnold, A. Sanderson, P. Ferrier and F. Lemonnier, 1987. Acquisition of HLA class I W6/32 defined antigenic determinant by heavy chains from different species following association with bovine beta (2)-microglobulin. *The Journal of Immunology, 138:* 2190-2196.

150. Ploegh, H., H. Orr and J. Strominger, 1981. Biosynthesis and cell surface localization of nonglycosylated human histocompatibility antigens. *Journal of Immunology, 126:* 270-275.

151. Andersson, H., I. Nilsson and G. von Heijne, 1996. Calnexin can interact with N-linked glycans located close to the endoplasmic reticulum membrane. *FEBS Lett, 397:* 321-4.

152. Kvist, S. and F. Levy, 1993. Early events in the assembly of MHC class I antigens. [Review] [78 refs]. *Seminars in Immunology, 5:* 105-16.

153. Ortmann, B., *et al.*, 1997. A critical role for tapasin in the assembly and function of multimeric MHC class I-TAP complexes. *Science, 277:* 1306-9.

154. Rammensee, H.G., T. Friede and S. Stevanoviic, 1995. MHC ligands and peptide motifs: first listing. *Immunogenetics, 41:* 178-228.

155. Dricu, A., M. Carlberg, M. Wang and O. Larsson, 1997. Inhibition of N-linked glycosylation using tunicamycin causes cell death in malignant cells: role of down-regulation of the insulin-like growth factor 1 receptor in induction of apoptosis. *Cancer Res, 57:* 543-8.

156. Burgert, H.G. and S. Kvist, 1987. The E3/19K protein of adenovirus type 2 binds to the domains of histocompatibility antigens required for CTL recognition. *EMBO Journal., 6:* 2019-2026.

157. Klappa, P., R.B. Freedman and R. Zimmermann, 1995. Protein disulphide isomerase and a lumenal cyclophilin-type peptidyl prolyl cis-trans isomerase are in transient contact with secretory proteins during late stages of translocation. *Eur J Biochem, 232:* 755-64.

158. Freedman, R.B., T.R. Hirst and M.F. Tuite, 1994. Protein disulphide isomerase: building bridges in protein folding. *Trends Biochem Sci,* *19:* 331-6.

159. van Leeuwen, J.E. and K.P. Kearse, 1996. Calnexin associates exclusively with individual CD3 delta and T cell antigen receptor (TCR) alpha proteins containing incompletely trimmed glycans that are not assembled into multisubunit TCR complexes. *J Biol Chem,* *271:* 9660-5.

160. Otteken, A. and B. Moss, 1996. Calreticulin interacts with newly synthesized human immunodeficiency virus type 1 envelope glycoprotein, suggesting a chaperone function similar to that of calnexin. *J Biol Chem,* *271:* 97-103.

161. Kim, P.S. and P. Arvan, 1995. Calnexin and BiP act as sequential molecular chaperones during thyroglobulin folding in the endoplasmic reticulum. *J Cell Biol,* *128:* 29-38.

162. Nossner, E. and P. Parham, 1995. Species-specific differences in chaperone interaction of human and mouse major histocompatibility complex class I molecules. *J Exp Med,* *181:* 327-37.

163. Sanders, S.L. and R. Schekman, 1992. Polypeptide Translocation Across the Endoplasmic Reticulum Membrane. *J Biol Chem,* *267:* 13791-13794.

164. Haas, I.G., 1994. BiP (GRP78), an essential hsp70 resident protein in the endoplasmic reticulum. *Experientia,* *50:* 1012-20.

165. Margolese, L., G.L. Waneck, C.K. Suzuki, E. Degen, R.A. Flavell and D.B. Williams, 1993. Identification of the region on the class I histocompatibility molecule that interacts with the molecular chaperone, p88 (calnexin, IP90). *J Biol Chem,* *268:* 17959-66.

166. Carreno, B.M., K.L. Schreiber, D.J. McKean, I. Stroynowski and T.H. Hansen, 1995. Aglycosylated and phosphatidylinositol-anchored MHC class I molecules are associated with calnexin. Evidence implicating the class I- connecting peptide segment in calnexin association. *J Immunol,* *154:* 5173-80.

144

167. Solheim, J.C., M.R. Harris, C.S. Kindle and T.H. Hansen, 1997. Prominence of beta 2-microglobulin, class I heavy chain conformation, and tapasin in the interactions of class I heavy chain with calreticulin and the transporter associated with antigen processing. *J Immunol, 158:* 2236-41.

168. Martire, G., *et al.,* 1996. Different fate of a single reporter protein containing KDEL or KKXX targeting signals stably expressed in mammalian cells. *Journal.of.Biological.Chemistry., 271:* 3541-3547.

169. Gabathuler, R., F. Levy and S. Kvist, 1990. Requirements for the association of adenovirus type 2 E3/19K wild-type and mutant proteins with HLA antigens. *J Virol, 64:* 3679-85.

170. Kuehn, M.J., J.M. Herrmann and R. Schekman, 1998. COPII-cargo interactions direct protein sorting into ER-derived transport vesicles. *Nature, 391:* 187-90.

171. Tisdale, E.J., H. Plutner, J. Matteson and W.E. Balch, 1997. p53/58 binds COPI and is required for selective transport through the early secretory pathway. *Journal of Cell Biology, 137:* 581-93.

172. Schekman, R. and I. Mellman, 1997. Does COPI go both ways?. [Review] [20 refs]. *Cell, 90:* 197-200.

173. Lippe, R., E. Luke, Y.T. Kuah, C. Lomas and W.A. Jefferies, 1991. Adenovirus infection inhibits the phosphorylation of major histocompatibility complex class I proteins. *J Exp Med, 174:* 1159-66.

174. Townsley, F.M. and H. Pelham, 1994. The KKXX signal mediates retrieval of membrane proteins from the Golgi to the ER in yeast. *Eur J Cell Biol, 64:* 211-216.

175. Pahl, H.L. and P.A. Baeuerle, 1997. The ER-overload response: activation of NF-kappa B. [Review] [37 refs]. *Trends in Biochemical Sciences, 22:* 63-7.

176. Brewer, J.W., J.L. Cleveland and L.M. Hendershot, 1997. A pathway distinct from the mammalian unfolded protein response regulates expression of endoplasmic reticulum chaperones in non-stressed cells. *Embo Journal, 16:* 7207-16.

177. Cox, J.S., R.E. Chapman and P. Walter, 1997. The unfolded protein response coordinates the production of endoplasmic reticulum protein and endoplasmic reticulum membrane. *Molecular Biology of the Cell,* 8: **1805-14.**

178. Roche, P.A., M.S. Marks and P. Cresswell, 1991. Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature,* 354: **392-4.**

179. Anonymous, *The Handbook of Biochemistry and Biophysics.* **1966,** Cleveland: Cleveland World Publishing.

180. McMaster, W.R. and A.F. Williams, 1979. Identification of Ia glycoproteins in rat thymus and purification from rat spleen. *Eur J Immunol,* 9: **426-33.**

181. Haidl, I., *Characterization of Dendritic Cell and Macrophage Cell Surface Proteins,* . 1996, University of British Columbia.

Appendix 1

Lysates from 293 transfectanst were precipitated with the E3/19K specific monoclonal Tw 1.7 at 30 and 120 minutes post pulse. Arrows denote E3/19K and co-immunoprecipitated MHC Cl I.
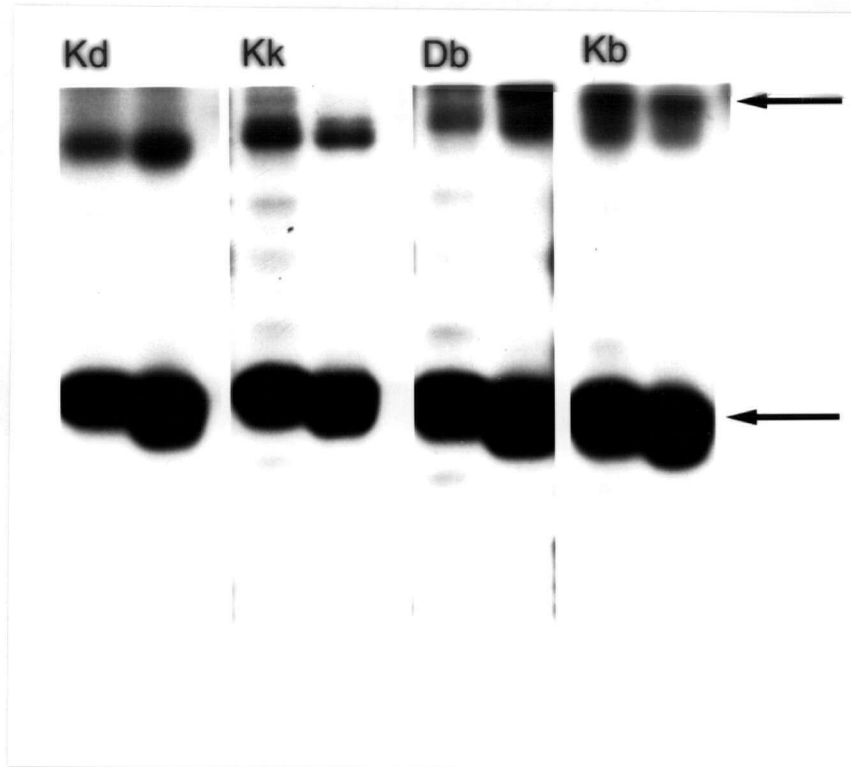
Appendix 2

Velocity Gradient centrifugation

A2.1. Introduction

The work described in the previous chapters suggested a complex interaction between E3/19K, MHC Cl I, β_2m and other ER resident proteins. Additionally, it appeared that the ability of E3/19K to bind to specific MHC Cl I proteins is based partly on affinity and partly on the time resident in the same compartment within the cell. The experiments with 293D^b demonstrated that there might exist different populations of E3/19K which differentially bound D^b (and possibly the other proteins). Finally, there was evidence that the complex that E3/19K formed in the ER involves calnexin and the TAPs, with the possibility of other chaperones being present in the complex.

To address these questions, the size and shape of the MHC-E3/19K complexes was investigated. The use of the velocity sedimentation gradients allowed the opportunity to further divide the intracellular MHC-E3/19K of a cell into subgroups based on the distance it migrated through a sucrose gradient when subjected to centrifugal force for a set period of time. In previous experiments, lysates were solubilized in relatively harsh detergents, effectively destroying anything but the strongest of associations between molecules. By using gentle detergents before separating, protein complexes could be identified by their size. A specific protein such as MHC Cl I likely associates with many different proteins in various complexes throughout its maturation stages through the cell. Identification of these different intermediates would help identify the stages of the maturation of the MHC Cl I molecule and would help in understanding its ability to bind to E3/19K. It was suggested that MHC-E3/19K binding is more complex than first believed.

It was further hypothesised that E3/19K exists in multimers. This would shed some insight into the life cycle of this protein and the optimal conditions for this protein to associate with

MHC Cl I. This in turn would offer insight on the evasion of the immune system by the adenovirus.

A2.2.   Rationale and Goals.

To determine the relative size of the E3/19K complex, cell lysates were run over a continuous sucrose gradient. In a series of experiments that utilized the gentle detergents N-Octyl Glucoside, Chaps and digitonin, lysates were separated according to their size and shape. The proteins were identified with immunoprecipitation. Further experiments resulted in the characterization of attributes of these proteins in an attempt to determine the intracellular compartment they had resided at the time of labelling.

A2.3.   Results

These experiments set out to determine the size of the MHC Cl I complex by identifying the fraction or fractions in which these molecules eluted. Initial results showed that MHC Cl I was present in several different sucrose gradient fractions when precipitated with W6/32. The precipitated MHC Cl I had a different MW when precipitated from different fractions. In 293.12 cells the MHC Cl I complex associated with E3/19K eluted in different sets of fractions than those in which the MHC Cl I - β_2m complex (in 293 cells) alone eluted. These results gave an estimate of the size of the complex consisting of E3/19K bound to MHC Cl I. Furthermore, it was observed that E3/19K also eluted in fractions corresponding to larger complexes than the fractions containing MHC molecules.

A2.3.1.   Sucrose gradients separate cellular components by size

In figure 23, 293 cells were labelled overnight and the lysate run on a continuous 5-20% sucrose gradient as described in Materials and Methods. The purpose of this experiment

was to establish that the sucrose gradient did separate proteins based on size. This experiment allowed the determination of the characteristics and separating qualities of a sucrose gradient. The higher mass proteins travelled further through the sucrose and eluted in different fractions. This was not unexpected and indicated a good method for analyzing the data in the following gels.

Figure 23. Separation of 293 lysates over a 5-20% sucrose gradient

      293 cells were grown to confluence and labelled as above. Cells were labelled for two hours and lysed in N-Octyl Glucoside lysis buffer. 300 µl of the lysate was layered on top of a 5-20% sucrose gradient and spun in a SW41 rotor at 36,800 rpm for 28 hours. Twelve 1 ml fractions were collected (lane 1 is the top fraction; lane 12 the bottom) and separated without precipitation by SDS-PAGE. Subsequent transfer to an Immobilon PVDF membrane followed. a) The membrane was exposed to Kodak XAR film for a period ranging from one day to three months (at -80°C).
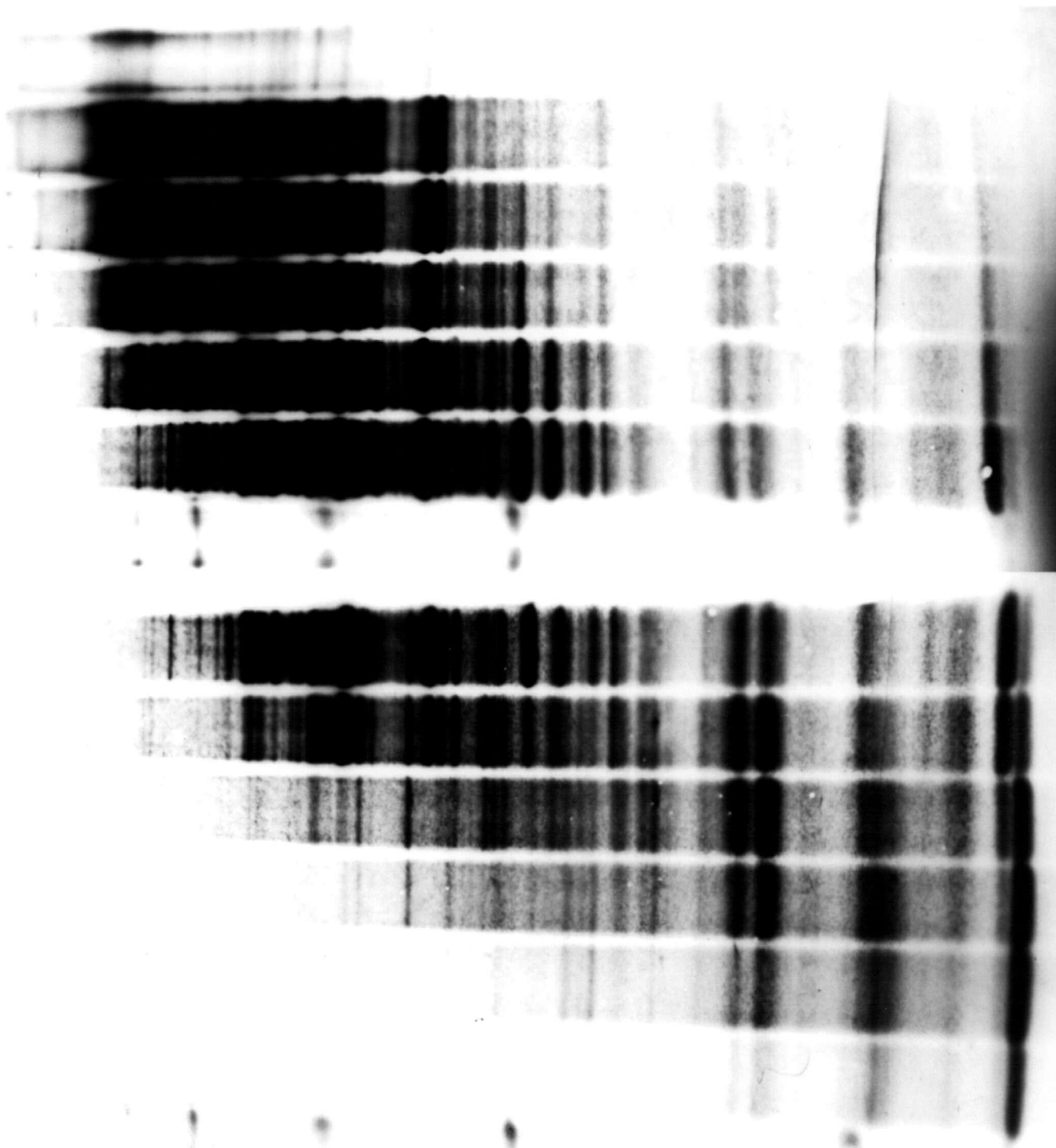
A2.3.2. MHC Cl I complex precipitated from specific sucrose fraction

293 lysates run over 5-20% sucrose gradients were precipitated with W6/32. This yielded MHC Cl I- β_2m complexes in fractions 3, 4 and 5 (figure 24). MHC Cl I was found to have migrated to other fractions as well, but β_2m was not observed associated in these fractions. This suggested that β_2m does not associate with all the MHC Cl I present in a cell, but rather with a subset. The fraction of MHC Cl I that does not associate with β_2m represented a distinct subpopulation not observed before.

To elucidate differences between MHC Cl I complexes with and without E3/19K, 293.12 cell lysates were separated using the same technique and immunoprecipitated with W6/32. In the 293.12 cells, the MHC Cl I complex (MHC Cl I, β_2m, E3/19K) was found in denser fractions (fractions 5, 6 and 7). E3/19K is observed co-immunoprecipitating in these fractions. The difference in migration, and therefore their size, of the complexes in the two cells is very likely due to E3/19K.

Figure 24. Precipitation of MHC Cl I and E3/19K from 293 and 293.12 cell lysates separated over a 5-20% sucrose gradient

293 (a) and 293.12 (b) cells were grown to confluence and labelled as above. Cells were labelled for 2 hours and lysed in N-Octyl Glucoside lysis buffer. 300 µl of the lysate was layered on top of a 5-20% sucrose gradient and spun in an SW41 rotor at 36,800 rpm for 28 hours. Eleven 1 ml fractions were collected (lane 1 is the top fraction; lane 11 the bottom). Fractions were precipitated with W6/32 or the anti E3/19K antisera R418. Precipitates were separated using SDS-PAGE.

a) **293 Sucrose Gradient**

W6/32

R418

kD

97
66
43
29
14
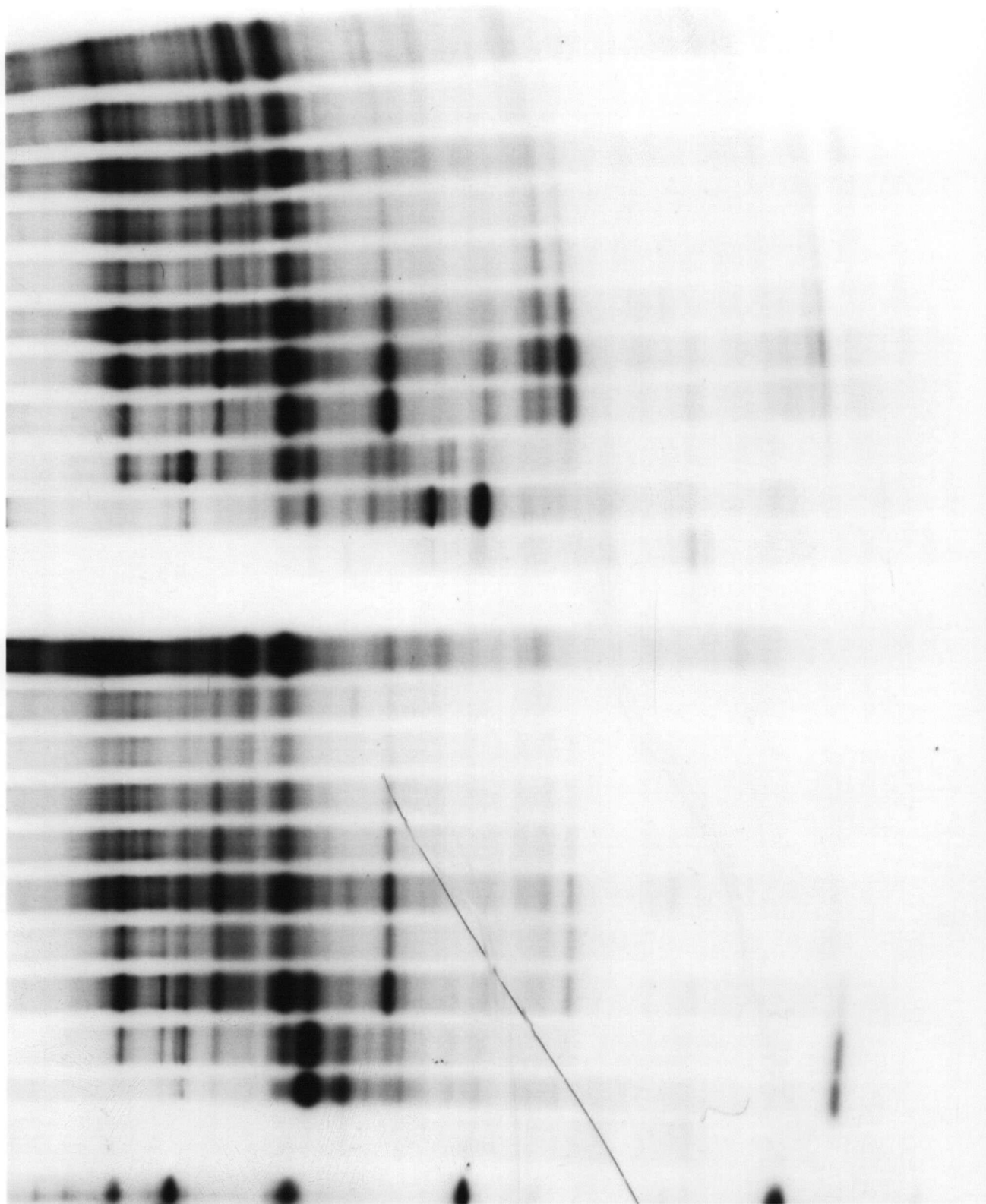
b) 293.12 Sucrose Gradient

W6/32

R418

155
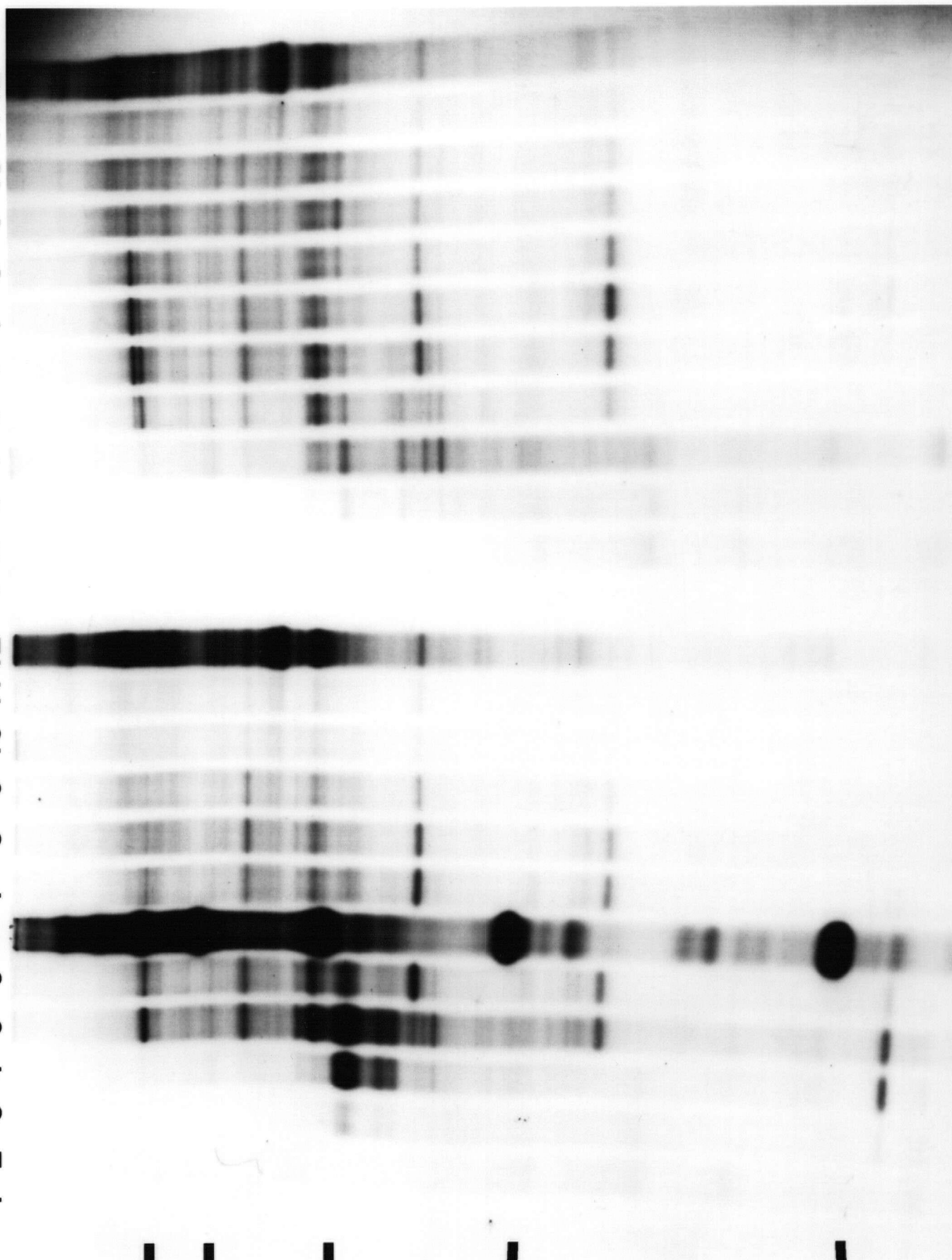
In the 293.12 lysates, E3/19K did not localize exclusively in those fractions with labelled MHC Cl I present. In addition to the fractions with labelled MHC Cl I and β_2m complexes, E3/19K seemed to migrate further through the sucrose gradient ending up in fractions where MHC Cl I was not observed. A band at ~25 kDa and a band at ~50 kDa was also observed in these fractions (8-10). This led to speculation that the sucrose gradient was separating different sub-populations of E3/19K complexes within the 293.12 cells. The standard E3/19K complex observed in earlier experiments consisting of β_2m, MHC Cl I, and E3/19K were localised to fractions 5, 6 and 7. E3/19K complexes without observable MHC Cl I, which could be multimers, were found in the fractions 8, 9 and 10.

The discovery of E3/19K in different fractions suggested that they may be present in different sub-populations. These sub-populations may localise with different ER resident proteins. In some complexes, E3/19K may form multimers that do not include MHC Cl I and β_2m. The observation of different subpopulations of E3/19K suggested these subpopulations may exhibit differing affinities and abilities to form complexes with MHC Cl I. Additionally, MHC Cl I with different mobilities was observed in different fractions. Variance in mobility reflects differences in the maturity of the MHC Cl I; specifically in the post translational modifications of these proteins.

It is possible that E3/19K has differing affinities for MHC Cl I based on its maturity and its corresponding conformation. If E3/19K is in the presence of an MHC Cl I molecule that is in a favourable conformation for binding, then a complex will form. If not, E319K may form complexes with other proteins, including itself, resulting in multimers.

A2.4. Discussion

This set of experiments demonstrated the MHC Cl I complex can be precipitated from different sucrose gradient fractions suggesting that the complex exists in different forms. In 293.12 cells, the complex precipitated by W6/32 consists of MHC Cl I H chain, β_2m, peptide and E3/19K. This complex does elute in a different fraction from the corresponding MHC Cl I complex (without E3/19K) found in 293 cells. In addition finding E3/19K present in complexes with MHC-β_2m complex, populations of E3/19K are likely present in the ER in other complexes. Association in these complexes is both with and more likely without MHC Cl I molecules.

In addition to determining which fractions of the sucrose gradient the different subpopulations of these proteins precipitate in, the sucrose gradient can be used to calculate the sedimentation coefficient (S value) of the protein complexes and the apparent molecular weight of these complexes. These S values are calculated by determining the distance migrated through a gradient of known density by a complex acted on by known centrifugal force. To convert the S value to the MW, estimation or determination of the stokes radius must be incorporated.

The S value was calculated using formulae previously published (178). The sedimentation coefficient was given by the formula:

$$s_{20,w} = s_{T,m}[\eta_{T,m}(\rho_P - \rho_{20,w})/\eta_{20,w}(\rho_P - \rho_{T,m})]$$

where $\eta_{T,m}$ and $\eta_{20,w}$ is the viscosity of sucrose at temperature T or water at 20°C, respectively. $\rho_{T,m}$ and $\rho_{20,w}$ are the densities of the sucrose at temperature T or water at 20°C.

ρ_p is the density of the particle analyised. This can also be the reciprocal of the partial specific

volume, $1/v$. The values for the sucrose viscosity and density were obtained from standard

tables (179). The partial specific volume of the protein-detergent complex was assumed to be

similar to other transmembrane glycoprotein-detergent complexes. It was further assumed that

these proteins bind 0.5 grams of detergent per gram of protein. $S_{T,M}$ is given by the equation

$$S_{T,m} = \ln(r_2/r_1)/(\omega^2 t)$$

Where r_2 and r_1 is the distance travelled from r_1 to r_2, ω is the angular velocity of the rotor and

t is time.

From the S value, the molecular weight of both the protein-detergent complex and the

protein can be calculated according to the following:

$$M_r = (6\pi N_A S_{20,w} R_e)/(1 - v\rho_{20,w})$$

with N_A being Avogadro's number and R_e the stokes radius of the protein. The stokes radius

was estimated to be 4.4 nm (180).

MW estimates obtained using these sucrose gradients showed agreement with

previously published values for MHC Class II complexes (181). The size of the protein and

the radius of the detergent with the proteins contribute to the size of the complex. Substituting

the calculated S value in the formula above, the apparent molecular weight for a complex can be

determined (see table 6). The MHC Cl I-β_2m complex in 293 cells in the third and fourth

fractions give values of 60-75 kDa. This compares well to the literature value of 60 kDa. In

293.12 cells, the MHC Cl I, β_2m - E3/19K complex were calculated to be in the 95-116 kDa

range. This value makes it is quite possible that a second E3/19K protein is present in this

complex. This would make the ratio of MHC Cl I to β_2m to E3/19K to be 1:1:2.

Table 6. **Determination of Molecular Weight (MW) of proteins in sucrose gradient fractions**

Fraction	r^2	S	MW (kD) (1)	MW (kD) (2)
3	9.19	4.18	93	56
4	9.89	5.57	123	75
5	10.6	7.06	156	95
6	11.3	8.62	191	116
7	11.9	10.42	230	140
8	12.7	12.69	281	170

(1) MW of protein and detergent

(2) MW of protein alone

The generalised calculations of the MW for complexes found in 12 different 1 ml fractions from samples layered on top of a continuous 5%-20% sucrose gradient and separated for 28 hours at 36,400 rpm (148,000g) are shown above. The S and MW of proteins found in different fractions was calculated using formulae in text. r is the distance migrated through the gradient. Viscosity and density of fractions (not shown) are derived from standard tables (179). The variables Stokes Radius (Re) was previously determined to be 4.5 nm (180).

The association of E3/19K with MHC Cl I as a dimer is not completely unexpected. In experiments using the E3/19K-Vaccinia vector, E3/19K is overexpressed and many bands with molecular weights approximately twice that of E3/19K are observed (data not shown).

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Additionally, in an earlier chapter that investigates the steric interactions of E3/19K with MHC Cl I, it was proposed that for E3/19K to effectively associate with the specific amino acids of the MHC Cl I sequence shown to influence its binding, E3/19K may act in concert with additional factors that perturbs the 3-D structure of the MHC Cl I molecule. A novel method of membrane perturbation is proposed for E3/19K to extend far enough into the lumen of the ER for E3/19K to reach the residues on the MHC Cl I molecule that affect its binding. Multimers of E3/19K could achieve this.

E3/19K is also present in the denser fractions. E3/19K appeared in fractions corresponding to MW of 116-170kD in fractions precipitated with MHC Cl I antisera. Unlike the less dense fractions, in these fractions labelled MHC Cl I is not apparent. Also present is the band that correlates to a protein approximately double the size of E3/19K. This complex could be a multimer of E3/19K.

In the denser fractions, it is important to note that these complexes are still being precipitated with anti-MHC Cl I antisera. While labelled MHC Cl I is not visible under these conditions, it is likely still present. These fractions could be illustrating a novel interaction between multimers of E3/19K and MHC Cl I that may precede labelling in the pulse/chase. E3/19K may have associated with MHC Cl I molecules prior to labelling and entered into a very stable conformation. E3/19K may form very long-lived associations with MHC Cl I molecules. The appearance of a band at approximately double the size of E3/19K (about 50 kDa) further suggests that the MHC Cl I, β_2m, E3/19K complex is larger than the one normally observed with MHC Cl I and β_2m and that multimers of E3/19K may be involved.

In an earlier chapter, it was observed that the D^b protein in 293D^b does not bind to E3/19K until several hours post chase. The earlier results showed that one of the features of the D^b protein is that it needs a longer time to bind to E3/19K. Additionally, in 293 D^b cells,

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several differently glycosylated subsets of the E3/19K population were observed. In the 293 D^b it is the fully glycosylated version of E3/19K that first binds to the D^b protein. Later time points reveal that all the other subsets are found associated with D^b. This could represent a cooperative binding of E3/19K to MHC Cl I.

Identification of the slowbinding protein, D^b, allows the proposal that in the case of some proteins such as L^d, binding to E3/19K may take a very long time to occur. While the cell surface expression of L^d is markedly reduced, very little co-immunoprecipitation throughout the time course of the experiments is observed. E3/19K may be a very long lived resident of the ER, and it may bind to different subsets of MHC Cl I dependant on its maturity.

The existence of E3/19K in the denser fractions of the sucrose gradient could be due to its participation in a larger complex with other ER resident proteins or chaperones. This may represent portions of the total E3/19K population that hitherto go undetected in the MHC Cl I co-precipitation. Both these findings and the literature report that the amount of E3/19K precipitated by anti E3/19K antisera is much greater than the E3/19K that co-precipitates with MHC Cl I. There probably exists a pool of E3/19K that never associates with MHC Cl I, or at least does not associate within the time constraints of the experiments reported here. The E3/19K found in the denser fractions may be this subset of the total E3/19K population. E3/19K in this fraction may also be associated with the ER resident proteins such as calnexin and others.

The E3/19K complex with MHC Cl I is a little more complicated than originally thought. Previously it had been assumed that E3/19K bound to the MHC Cl I complex as a monomer. A sub population of E3/19K is present in a larger complex that may involve multimers of E3/19K. Additionally, the ER resident chaperones such as calnexin and TAP may be present. These complexes are originally observed when precipitated with anti MHC Cl

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I sera and antibodies.   However, labelled MHC Cl I is not observed in these fractions. This suggests that the MHC Cl I component of these complexes has not been labelled. This may reflect an association of MHC Cl I with E319K that is of longer duration than the length of the pulse-chase.