MODULATION OF THE MHC CLASS I ANTIGEN PROCESSING AND PRESENTATION PATHWAY

by

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to the required standard

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ABSTRACT

Antigen processing and presentation are central to the complex immune response elicited upon infections by pathogens. They result in the generation of peptides that can be presented to T cells and elicit specific immune responses. Two distinct pathways process and present foreign antigens and are respectively mediated through MHC class I and class II molecules. Although much has been learned about these pathways in recent years, their details remain to be resolved. The present study further examines the MHC class I pathway. To this end, two distinct approaches were undertaken. First, we examined the ability of Adenovirus type 2 to block this pathway. Thus far, this phenotype had solely been attributed to the E3/19K viral protein. Since past characterizations were unexpectedly done in the presence of other viral proteins, including E3/6.7K, we sought to determine the role of E3/6.7K on this phenotype and to further characterize its properties. Using a bank of viral deletion mutants, lacking either E3/6.7K or E3/19K, we found a specific interaction between E3/6.7K and E3/19K. Despite this interaction, E3/6.7K did not affect MHC class I surface expression or phosphorylation. However, we report the novel finding that at least one additional viral protein was necessary for the optimal down regulation of MHC class I surface expression by the virus. Unexpectedly, the detection of disulfide bonds in E3/6.7K suggested that it likely had a peculiar orientation in the ER membrane.

In the second approach, we examined the ability of a mutant cell line, gro29, deficient for HSV-1 viral egress, to process and present peptides to T cells. The results indicated that gro29 had a slight, but likely inconsequential, deficiency in the processing of molecules through its secretory pathway and had a normal complement of proteins at its surface.

Surprisingly, this mutant was deficient for the presentation of HSV-1 peptides, but normally processed and presented allogeneic and Influenza peptides. Most important, this dichotomy indicated the existence of distinct MHC class I processing and presentation pathways.

Overall the data revealed a complex interaction between Adenovirus type 2 and the MHC class I processing and presentation pathway. They also underlined the usefulness of the gro29 variant cell line as mutant antigen processing and presentation cells.

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CHAPTER 1

GENERAL INTRODUCTION

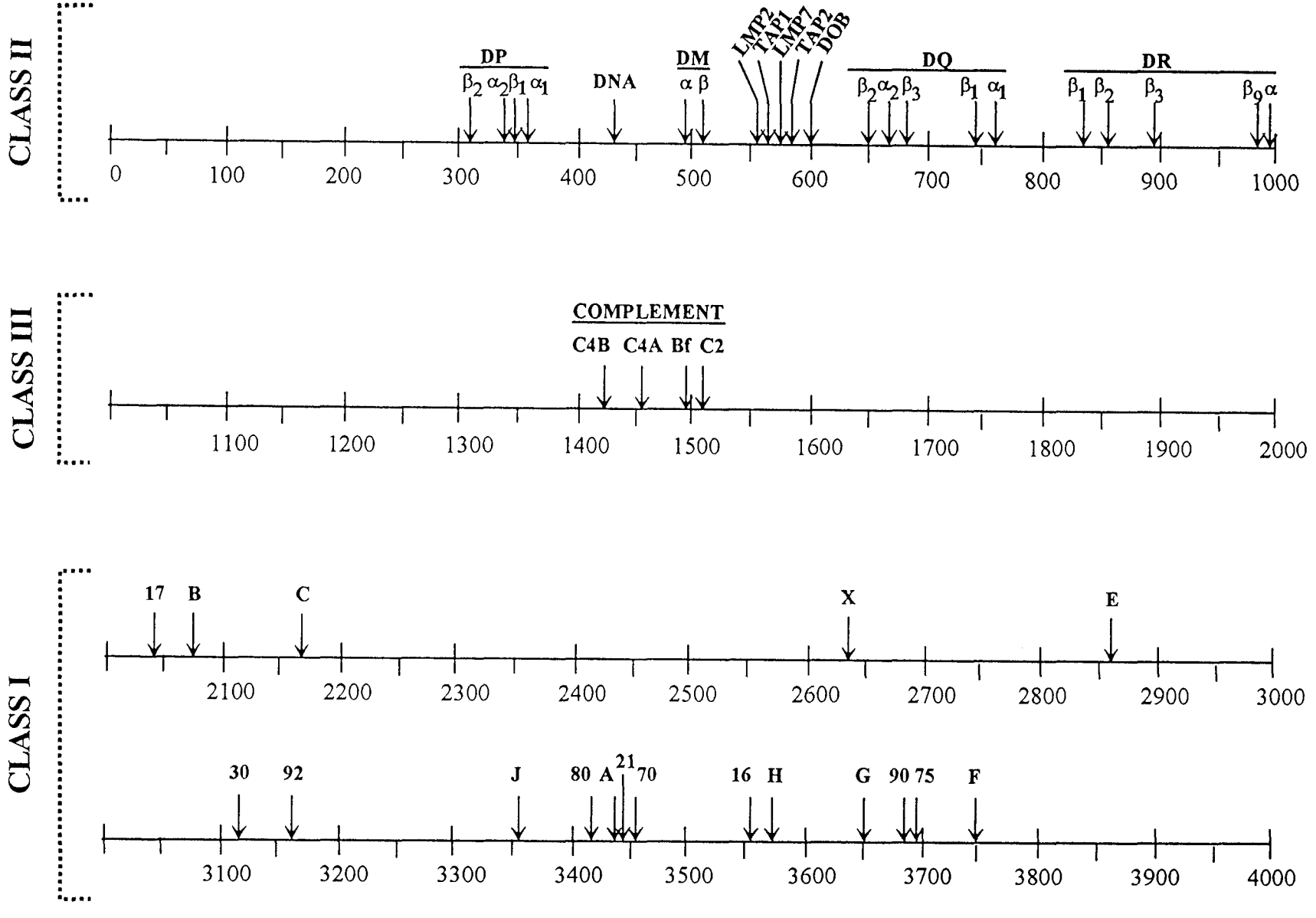
1.1- Immune responses and histocompatibility antigens

Immune responses play a crucial role in the protection of hosts against pathogens. They also maintain their integrity by rejecting transplants from non compatible donors. Since the traditional classification of immune responses into humoral or cellular immunity, it has become clear that these pathways are interconnected and are generally both involved against a given pathogen or foreign protein (1). Humoral immunity is mediated by molecules circulating in the blood and includes antibodies produced by B lymphocytes and members of the complement cascade. In contrast, as its name implies, cellular immunity is mediated by cells and involves T lymphocytes, macrophages, dendritic cells, natural killer cells (NK), lymphocyte activated killer cells (LAK), killer cells (K), platelets, and granulocytes (2). Initially identified as transplantation antigens (3), major histocompatibility complex (MHC) molecules have since been shown, among other proteins, to be essential players in the specificity of some of the cellular immune responses (4-6). Three categories of MHC molecules, namely classes I to III, have been identified and are distinct on several bases, as discussed below. In humans, the MHC class I molecules are encoded by the classical A, B, and C genes, while the MHC class II molecules are encoded by the DR, DP, and DQ genes. These molecules, collectively referred to as histocompatibility locus antigens (HLA) (7,8), also include numerous non classical MHC class Ib genes (Fig. 1) (9,10). In

Fig. 1: Map of the human major histocompatibility complex

The location of the genes mapped to the MHC locus on chromosome 6 is indicated. Please note that the centromere is located at 0 map unit while the telomere is at 4000 map units. Three classes of molecules (I, II, and III) are present in the MHC locus, as indicated to the left of the figure. The map represents the current knowledge accumulated as of July 1993. Only the molecules of interest are shown.

Adapted from (9).


CLASS II
DP
β_2 α_2 β_1 α_1
DNA
DM
α β
LMP2
TAP1
LMP7
TAP2
DOB
DQ
β_2 α_2 β_3 β_1 α_1
DR
β_1 β_2 β_3 β_9 α
0 100 200 300 400 500 600 700 800 900 1000
CLASS III
COMPLEMENT
C4B C4A Bf C2
1100 1200 1300 1400 1500 1600 1700 1800 1900 2000
CLASS I
17 B C X E
2100 2200 2300 2400 2500 2600 2700 2800 2900 3000
30 92 J 80 A 21 70 16 H G 90 75 F
3100 3200 3300 3400 3500 3600 3700 3800 3900 4000

mice, the MHC class I and II antigens are respectively encoded by the murine MHC (H-2) D, K, and L genes and the I-A and I-E genes (7,8,11). Interestingly, the MHC class I and II molecules are functionally distinct. Hence, MHC class I proteins normally present cytosolic peptides to $CD8^+$ cytolytic T lymphocytes (CTL), whereas MHC class II proteins typically present exogenous peptides to $CD4^+$ T helper cells. The outcome of these distinct pathways is generally the lysis of the presenting cells in the context of class I presentation and to B-T cell cooperation and antibody production in the context of class II presentation. Finally, the MHC class III genes encode proteins of the complement cascade in both humans and mice. These gene products are important players of the immune response and can lead to the opsonization of antigens and their phagocytosis by macrophages or neutrophils. They also participate in the lysis of cells by NK, LAK, neutrophils, and macrophages (2). Besides these major antigens, the existence of many minor histocompatibility complex antigens has also been documented (3). Their genomic localization and the minor graft rejections they mediate set them apart from the MHC antigens.

1.2- Structure of MHC proteins

1.2.1- Organization of the MHC class I proteins

MHC class I molecules consist of two chains; a highly polymorphic (12) heavy 45 kilodaltons (kDa) chain non covalently coupled to a monomorphic light 12 kDa chain called β_2 microglobulin (β_2M) (7). The heavy chains are encoded on a single locus on chromosome 6 in humans and 17 in mice (3,13). They are composed of three extracellular domains, called

α_1, α_2, and α_3 domains, of approximately 90 amino acids each, a transmembrane domain of approximately 30 amino acids, and a short cytoplasmic tail approximately 30-40 amino acids long (Fig. 2) (14,15). The amino acid sequences of the α_1 and α_2 domains are very polymorphic, whereas the transmembrane domain, α_3, and the cytoplasmic tail are fairly conserved among the different MHC haplotypes (14,15). Interestingly, the structural organization of the heavy chains is consistent with their genomic organization. Hence, each of the extracellular domains, the transmembrane domain as well as a leader sequence is encoded by a separate exon (Fig. 3) (8). Curiously, the cytoplasmic tail is encoded by 3 distinct exons of approximately 10 amino acids each. The light chain is a 12 kDa protein of 99 amino acids encoded by 4 exons (3,16). It is a highly conserved chain among mammalian cells and is unique within each species. Unlike the heavy chains, it is not encoded in the MHC locus, but on chromosome 15 in humans (17) and 2 in mice (3).

The crystal structures of soluble forms of HLA-A2.1 (18), HLA-Aw68 (19), HLA-B27 (20), H-2 K^b (21), and H-2 D^b (22) have been examined and show structural similarities (Fig. 4). In all cases, the polymorphic α_1 and α_2 domains are distal to the plasma membrane and interact to form a cleft. This cleft is composed of two α helices at the top and a β-pleated sheet composed of eight antiparallel β strands at the bottom (18-23). The β_2M protein contributes to the stability of the heavy chain by interacting non covalently with the membrane proximal α_3 domain and the floor of the α_1-α_2 β-pleated sheet (Fig. 4). All four extracellular domains (α_1-α_3 and β_2M) are globular, while three of them are stabilized by a disulfide bond (Fig. 2, 4). These crystallographic analyses also revealed that the majority of the polymorphic

Fig. 2: Structure of MHC class I proteins

The structure of a typical MHC class I protein is depicted. The heavy chain is composed of 3 extracytoplasmic domains (α_1, α_2, α_3), a transmembrane domain, and a cytoplasmic tail. The light chain (β_2M) is a soluble protein which interacts non covalently with the α_1, α_2, and α_3 domains and stabilizes the heavy chain. The α_2, α_3 domains are each stabilized by a disulfide bond, as is the β_2M protein. Production of soluble forms of MHC class I proteins can be achieved by cleavage with papain, as indicated on the figure. "CHO" represents the highly conserved N-linked glycosylation site at position 86 of MHC class I molecules.

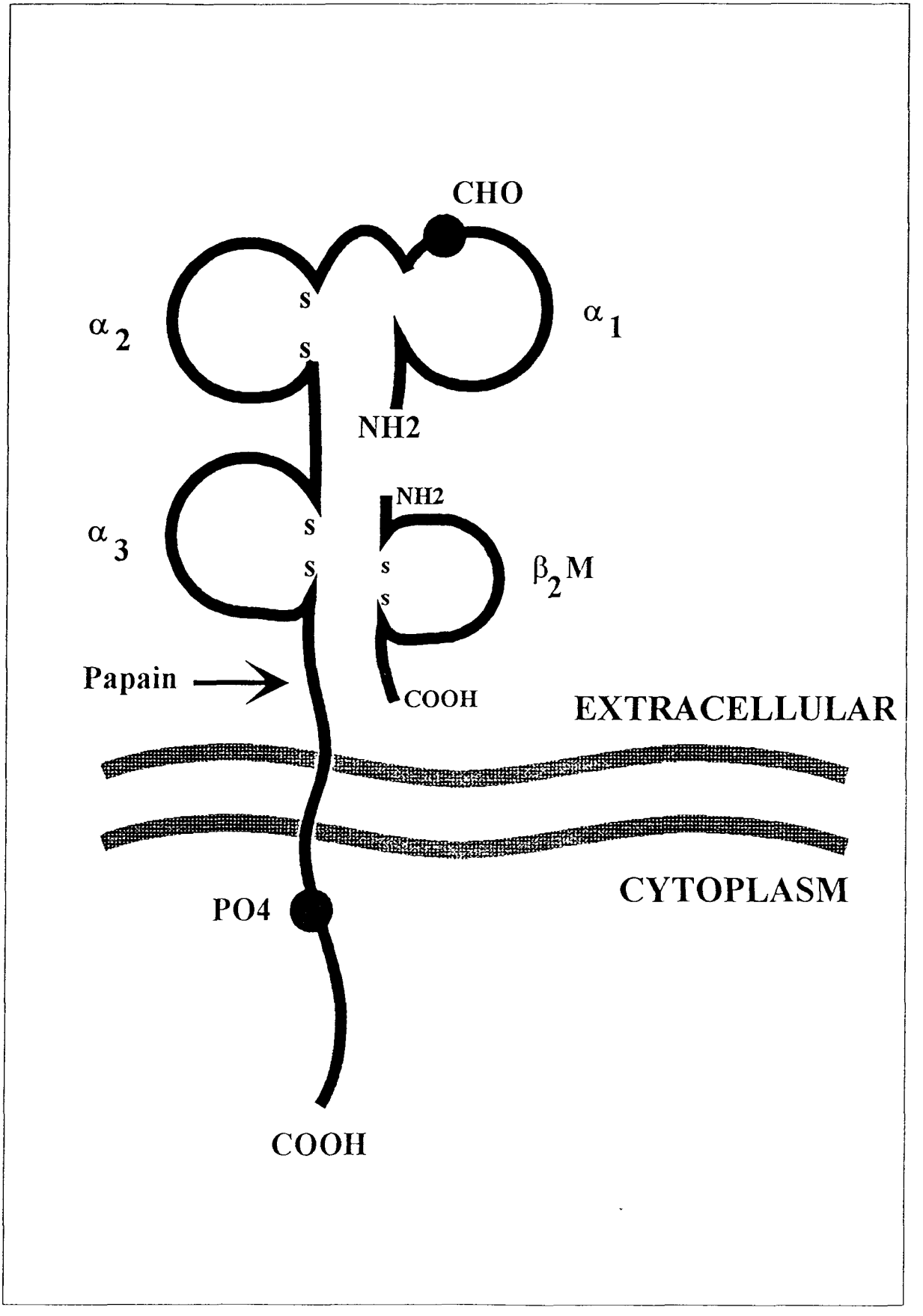
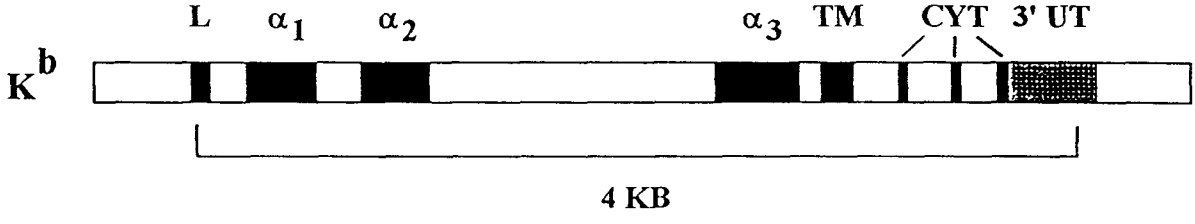

CHO
α2
α1
S
S
NH2
NH2
α3
S
S
S
S
β2M
Papain
COOH
EXTRACELLULAR
CYTOPLASM
PO4
COOH

Fig. 3: Genome organization of MHC molecules

The genomic arrangement of the exons encoding the MHC class I heavy chain (Panel A) and MHC class II α and β chains (Panel B) are shown. Each exon is depicted by a solid bar, while the introns are indicated by open bars. L: Leader sequence, TM: Transmembrane domain, CYT: Cytoplasmic domain, 3'UT: 3' untranslated sequences.

Adapted from (8).

A) MHC CLASS I



B) MHC CLASS II

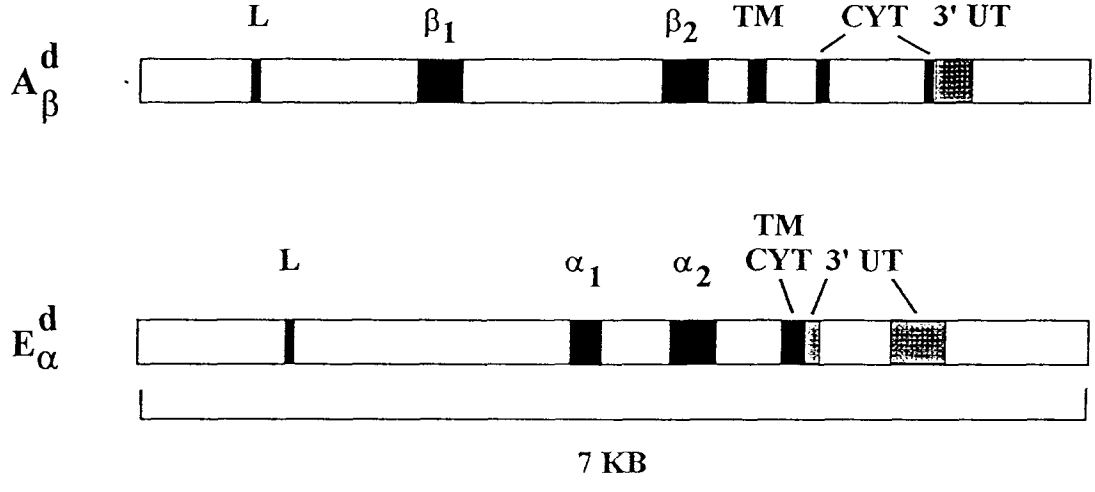
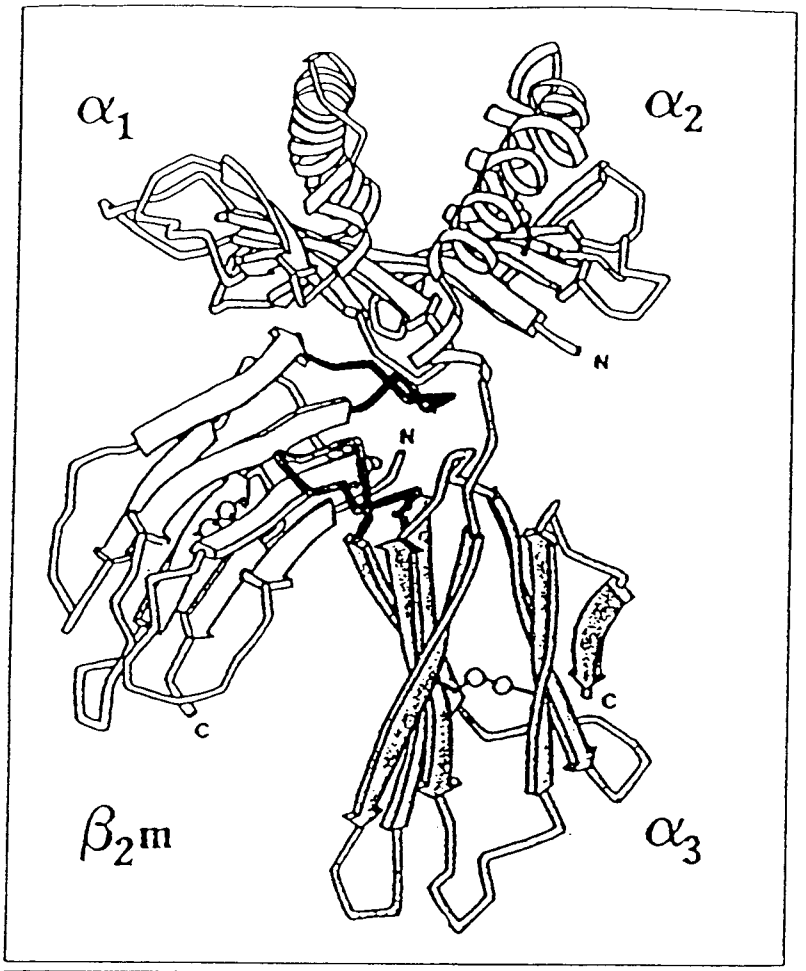
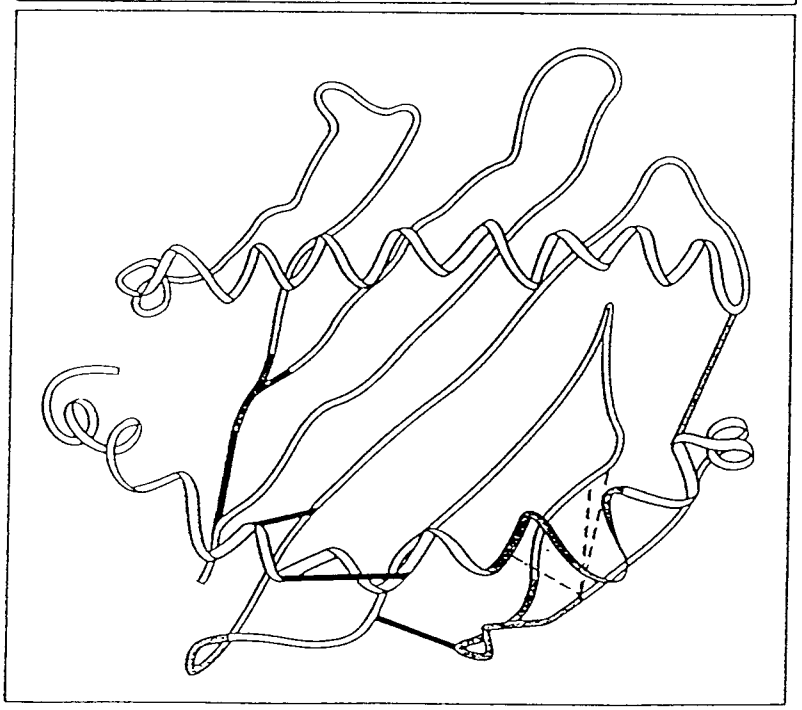


Fig. 4: Crystal structure of MHC class I molecules

The side view of the crystal structure of the HLA-A2 protein (soluble form) is shown in the upper panel. The α_1 and α_2 membrane distal domains of the heavy chain form a deep cleft where peptides can bind. The α_3 domain of the heavy chain and the β_2M protein are proximal to the plasma membrane. Please note the interaction of the light chain with both the α_3 domain and the β-pleated sheet of the peptide binding groove. Below is the top view of the peptide binding cleft. The top of the cleft is composed of two α helices formed by the α_1 and α_2 domains, whereas the bottom of the cleft is made of eight antiparallel β strands.

Adapted from (18).


α_1
α_2
N
N
C
C
β_2m
α_3



amino acids among the various MHC class I molecules examined reside in the cleft formed by the α_1-α_2 domains.

1.2.2- Organization of the MHC class II proteins

MHC class II molecules are composed of two chains, α and β, with molecular masses of 32 and 28 kDa respectively (3). Both chains encode approximately 230 amino acids but differ in their molecular masses due mainly to differential glycosylation. The 31-34 kDa α chain includes two extracytoplasmic domains termed α_1 and α_2 of approximately 85 and 94 amino acids respectively. It also contains a transmembrane domain and an intracytoplasmic tail of approximately 51 amino acids in total (3). The 26-29 kDa β chain has the same configuration with domains of approximately 94 (β_1), 94 (β_2), and 45 (anchor domain and cytoplasmic tail) amino acids respectively (3). Unlike MHC class I polypeptides, both chains are polymorphic and the bulk of the polymorphism resides in the α_1 and β_1 domains (8). The arrangement of the extracellular domains of the MHC class II proteins is very similar to that of the MHC class I molecules (Fig. 5). However, unlike MHC class I proteins, the exon structure of the MHC class II chains vary somewhat and can be encoded by 4 to 6 exons (Fig. 3) (3,8). In addition, both heavy and light MHC class II chains are encoded in the MHC locus (Fig. 1).

Consistent with their analogous protein organization, the three dimensional crystal structures of MHC class I and II molecules are remarkably similar (Fig. 6). For instance, the MHC class II α and β chains bind non covalently to each other to form four extracellular globular domains, of which three are stabilized by a disulfide bond (24). Furthermore, the α_1 and β_1 domains form a cleft distal to the plasma membrane and the α_2 and β_2 domains

Fig. 5: Structure of MHC class II proteins

The topology of MHC class II proteins closely resemble that of MHC class I proteins. Both are composed of two non covalently interacting chains that together form four extracellular globular domains. In both cases, three of those four domains are stabilized by a disulfide bond. Furthermore, both molecules are type I transmembrane proteins and are attached to the plasma membrane by 1 or 2 anchoring domains.

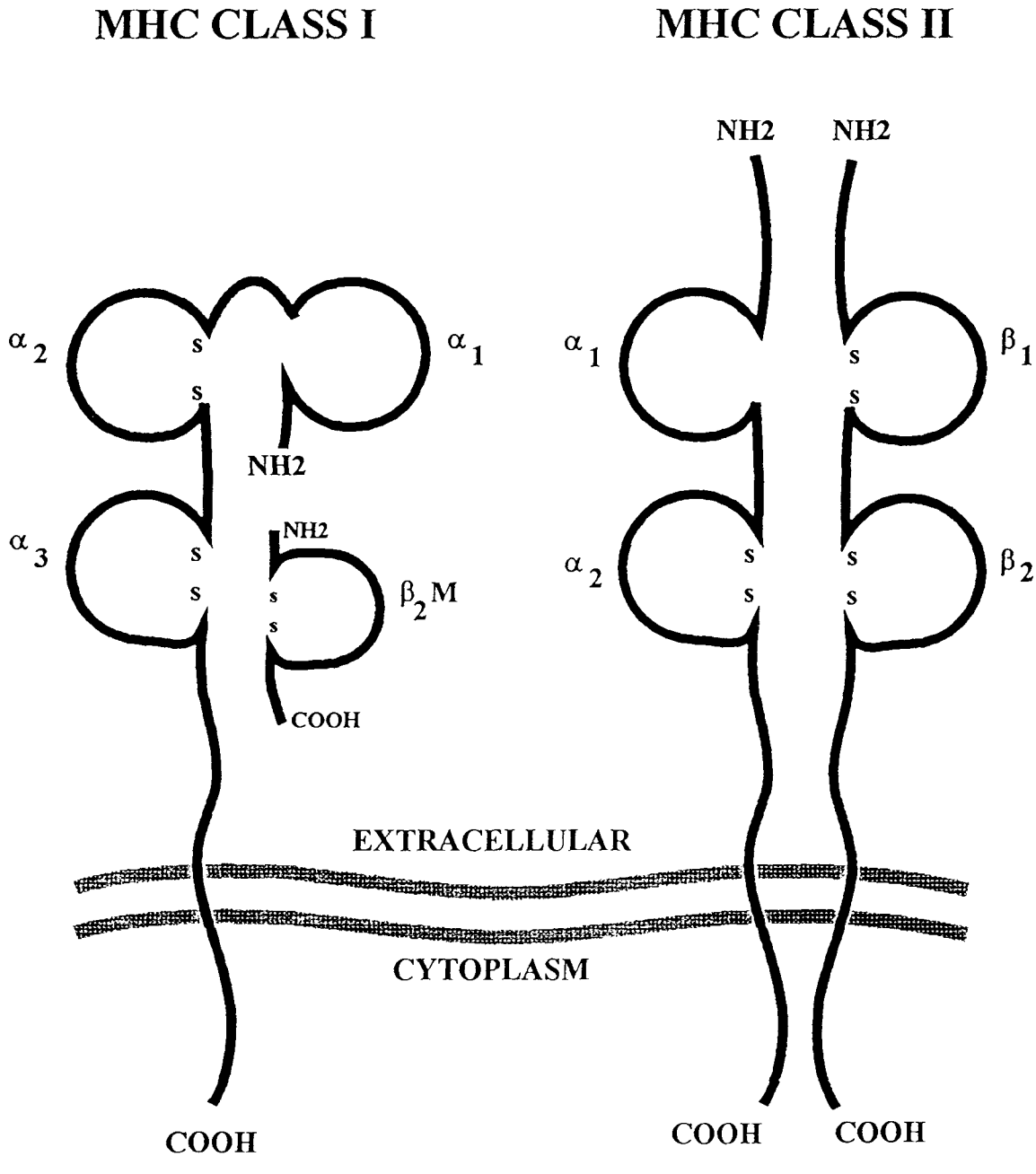
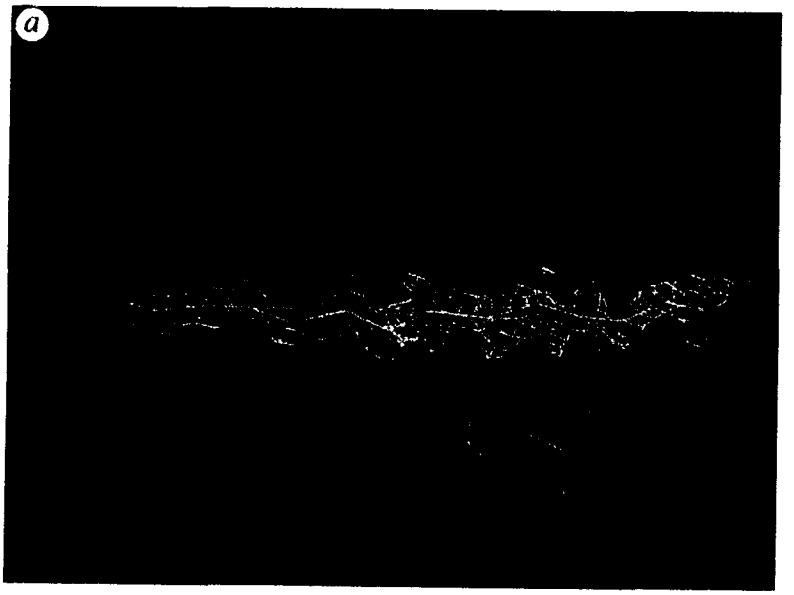

MHC CLASS I
MHC CLASS II
NH2
NH2
α2
α1
S
S
NH2
α3
NH2
β2M
COOH
α1
β1
α2
β2
EXTRACELLULAR
CYTOPLASM
COOH
COOH
COOH

Fig. 6: Crystal structure of the MHC class II molecule

Top views of the tridimensional structure of the peptide binding cleft formed by the α_1 and β_1 domains of MHC class II proteins of the HLA-DR1 molecule are shown. In the upper panel, the presence of a peptide (in red-yellow) in the MHC class II cleft (in blue) is shown. Note that both ends of the cleft are open and can thus accommodate peptides longer than the MHC class I cleft. In the lower panel, the MHC class I (in red) and II (in blue) peptide binding grooves are compared. The structural similarity between these two proteins is further shown by the overlapping segments (in white).

Reprinted from (24).


a


b

interact non covalently near the cell surface. An important difference, however, resides in the shape of the MHC class I and II clefts. Whereas the MHC class I cleft is closed at both ends, the MHC class II cleft is open at both its ends. The functional relevance of this difference is discussed below.

1.2.3- Binding of peptides to MHC class I and II molecules

The acid elution and the analysis of peptides from whole cells or purified MHC class I and class II molecules indicate that MHC molecules are somewhat promiscuous and bind a broad range of peptides (25-32). Such studies have further been confirmed by binding studies on purified MHC molecules (33-40). This would account for the large combination of MHC-peptide complexes dealt with the immune system. This promiscuity is not however unlimited and it has therefore been possible to formulate peptide binding motifs for a number of MHC alleles, particularly for MHC class I proteins (23,26,32,41-47). This has resulted in the identification of anchor residues which are important for the interactions between peptides and MHC molecules. Nonetheless, this binding is not solely limited to these anchor residues, since the binding affinity of the peptides can be affected by neighboring peptide residues (45,48-52).

Upon the analysis of MHC class I and II crystals, the presence of electron dense material in the membrane distal extracellular cleft can be detected (Fig. 6). Initially, the heterogeneity of this material precluded its identification (18,19). However, the crystallization of soluble MHC proteins expressed in SF9 drosophila cells (20,21,24) or *Escherichia coli* (22) and their incubation with homogenous synthetic peptides have resulted in the identification of this electron dense material as peptides. Thus, the heterogeneity of the

peptides in the MHC cleft can be explained by the promiscuity of the MHC proteins for different peptides. Detailed crystallographic analyses have also revealed that the anchor residues found on each peptide interact with "pockets" of varying depths and sizes found on the floor of the MHC cleft (20-24). The shape of the MHC peptide binding cleft appears important in determining the size of the peptides that it can bind. For instance, the closed architecture of the MHC class I α_1-α_2 groove limits the binding to peptides of 8-10 amino acids (21). In contrast, the open MHC class II α_1-β_1 cleft binds 13-25 amino acid long peptides (24). Thus both peptide size and sequence are dictated by the MHC peptide binding groove. This selectivity is reflected by the few peptides that are generated from a given antigen in a given cell and presented by a given MHC molecule (41).

1.3- Biosynthesis of MHC proteins

The biosynthesis of glycoproteins such as MHC class I and II proteins can biochemically be assessed due to their maturation throughout the secretory pathway (53). Hence, the addition of N-linked sugars to the proteins occurs en bloc in the lumen of the ER. These sugars are then trimmed in the ER and the cis Golgi. They are further modified by the addition of complex sugars and sialic acid residues in the medial and trans Golgi. The presence and maturation of these N-linked sugars can readily be monitored by analysis with endoglycosidases. For instance, the immature sugars found in the ER and the cis Golgi are sensitive to digestion by the endoglycosidase H (endo H), whereas the processed sugars found in downstream compartments are resistant (53). Since cleavage of the sugars by endo H can be detected by SDS-PAGE, the processing of the glycoproteins can be monitored fairly easily.

Thus, the acquisition of endo H resistance is indicative of the transport of the N-linked glycoproteins out of the ER and past the cis Golgi. Such acquisition of endo H resistance has therefore been a useful tool to examine the biosynthesis of MHC proteins.

1.3.1- Biosynthesis of MHC class I proteins

MHC class I molecules are integral proteins found at the cell surface of most cells. Their translation is targeted to the rough endoplasmic reticulum (RER) by the presence of a signal sequence at their amino termini (3,14,15). The β_2M protein also contains a signal sequence but no anchor domain or glycosylation site (3,16). The heavy chain contains 2 to 3 N-linked glycosylation sites and no O-linked sugars (3,14,15). It is modified with immature N-linked carbohydrates in the lumen of the ER and further processed through the Golgi en route to the plasma membrane, as indicated above. The heavy chain can also be further modified by the addition of phosphate residues at serine 335 and possibly at serine 332 (54-56) and tyrosine 320 (54,57). The exact intracellular location of this phosphorylation event is not known, but it has been suggested to take place at the cell surface (56-58). Stoichiometry analyses indicate that 10-60% of all MHC class I molecules are phosphorylated (54,56,59). It is presently unclear what the role of phosphorylation might be. Interestingly, MHC molecules lacking exon 7, which includes the two potential phosphorylation sites 332 and 335, are expressed at the cell surface, but undergo increased endocytosis (60,61). Together these observations suggest that phosphorylation of MHC class I heavy chains may not be essential for their transport to the cell surface, but may modulate their recycling. Thus differential phosphorylation could regulate the steady state surface expression of MHC class I molecules.

Binding of light and heavy chains is required for their mutual transport to the cell surface (62-72). Failure to oligomerize primarily results in the secretion of the light chain (69) and the ER retention of the heavy chain (63). Calnexin, also referred to as IP90 or p88, has been implicated in this ER retention. Calnexin is an ER resident protein that interacts with various molecules in the ER lumen including MHC class I heavy chains (73-80). Although its function is unknown, it has been proposed to promote MHC class I assembly or retain improperly assembled or folded MHC class I heavy chains in the ER.

Although essential, the formation of the heavy chain-β_2M heterodimers is not sufficient for egress out of the ER. Recent studies have shown that binding of peptides to the heavy chain is also needed for the stable assembly of light and heavy chains, their egress out of the ER, and their stable expression at the cell surface (62,70,71,81-91). This is caused by a stabilizing conformational change of the heavy chain induced by β_2M (63,92-94) and peptide (21,62,70,71,83,85,86,88,95-97). A notable exception is the efficient transport of the D^b and L^d heavy chains without β_2M (92,98). Furthermore, the expression of "empty" MHC class I proteins devoid of peptides has also been documented. However, these molecules are highly unstable and rapidly disassemble (70,71,85,91,93,97,99).

1.3.2- Biosynthesis of MHC class II proteins

MHC class II proteins are expressed on a few select cell types, namely B cells, macrophages, activated human T cells, and dendritic cells (3). The biosynthesis of MHC class II proteins resembles that of MHC class I molecules in that they are type I transmembrane proteins synthesized on the RER, are N-linked glycosylated, and need to assemble to exit the

ER (24,100). However, despite these similarities, MHC class I and II proteins differ on several counts. In contrast to class I proteins, the assembly and transport of class II molecules do not require binding of peptides in the ER but that of a third chain called the invariant chain (Ii) (100-102). This chain is a type II transmembrane protein that exists in different isoforms due to alternative start codons and alternative splicing (100). The functional significance of these various Ii isoforms is currently unknown but it has been proposed that they play distinct roles (103). Upon their translation and insertion in the ER membrane, the Ii chains oligomerize into trimers. Each of the Ii chains can then interact with a MHC class II α-β dimer and thus form a heterogeneous nonamer (104). The ER interaction of MHC class II proteins with Ii chains is essential for the routing of MHC class II proteins to the plasma membrane. However, MHC class II proteins do not travel through the default secretory route but migrate to the plasma membrane via the endosomal pathway (101,105-109). This active transport is dictated by a targeting signal present at the amino terminus of the Ii chain (107,110,111). It also appears that a targeting signal may be present on the MHC class II molecules themselves (112-115). Besides its targeting function, it has been suggested that the Ii chain can prevent the binding of class I peptides present in the ER to the class II molecules (116-118). However, this may not be the case since class II molecules do not appear to readily bind the peptides found in the ER lumen in the absence of Ii (119). Furthermore, the selectivity of the MHC molecules for some peptides and not others argues against the need for such a function (see above). In contrast, binding of the MHC class II restricted peptides to the class II proteins is inhibited by the Ii chain (118,120,121).

Expression of Ii chains without MHC class II proteins results in the localization of Ii in endosomes (106-108,111) suggestive of the presence of a targetting signal. The long delay of the MHC class II proteins in the endosomes of normal cells suggests that the Ii chain also has an endosomal retention signal, possibly identical to its endosomal targeting signal. It is along the endosomal-lysosomal pathway that MHC class II proteins encounter and bind class II peptides (100,105,109). Although the exact intracellular location of this interaction is still under investigation, a specialized compartment called MHC class II containing compartment (MIIC) has been proposed (105,109). Peptide binding and transport of the MHC class II molecules to the cell surface are achieved by the removal of the Ii chain (122,123). This appears to be a two-step process. First, the proteolysis of the Ii chain leaves a residual class II Ii associated invariant chain (CLIP) bound to the MHC class II molecules (120,121,124,125). Second, the CLIP peptide, which inhibits the binding of class II restricted peptides, is removed from the MHC class II molecules (126). Analysis of mutant cells indicates that two gene products, DMA and DMB, encoded in the MHC class II locus may be important for this latter function (120,121,127,128). These proteins associate with each other and are structurally analogous to MHC class II proteins. It has been suggested that they may participate in the removal of CLIP from MHC class II molecules by binding to CLIP themselves (129,130). The resulting MHC class II-peptides complexes are then transported to the cell surface by vesicular transport to the plasma membrane, where they can interact with $CD4^+$ T cells. The pathway by which they reach the cell surface is currently unknown.

1.4- Antigen processing

1.4.1- Processing of MHC class I antigens

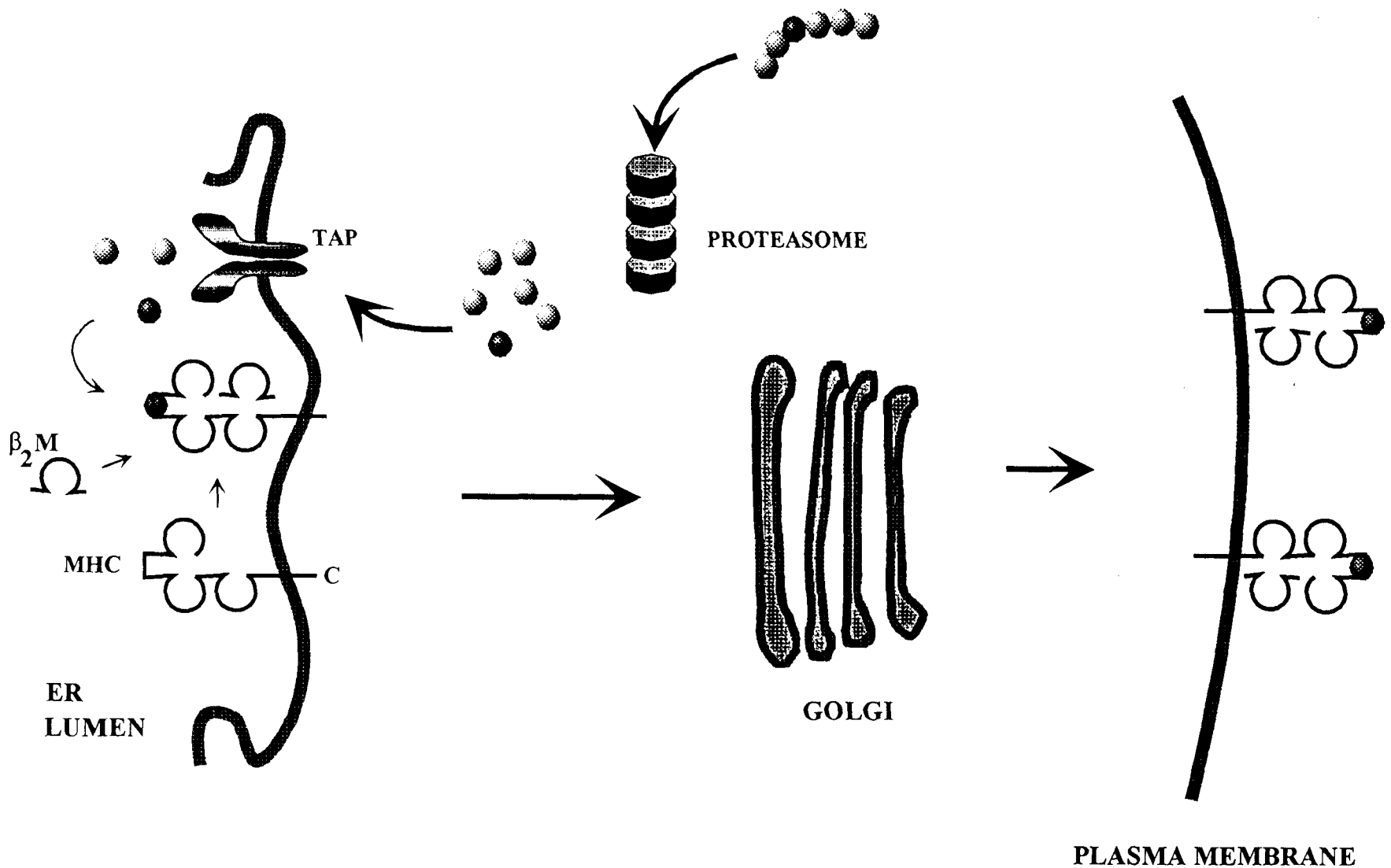
Tremendous headway has been made in the understanding of the machinery involved in the processing of MHC class I restricted antigens. An overall view of antigen processing is depicted in figure 7. Importantly, antigenic proteins are not presented "as is" at the cell surface but rather are denatured and cleaved into antigenic peptides that can then associate with MHC molecules and be presented to T cells (27-30,81,88,131-143). Recent technologic advances have allowed the characterization of those peptides. For instance, the isolation of naturally processed viral peptides from infected cells is currently common procedure and indicates that the class I antigenic determinants are on average 8-10 mers (26,32,41-43). Peptide sequencing has further revealed that they are generally proteolytic fragments of cytosolic proteins (144). A notable exception is the presentation of peptides resulting from the processing of signal sequences upon protein synthesis and translocation (145).

1.4.2- Role of proteasomes in MHC class I antigen processing

Although it is still unclear how MHC class I peptides are generated, it is generally assumed that their generation is done by the ubiquitin degradation pathway (146-148). This pathway is the major non lysosomal degradation pathway in cells (146,149-151). It is a complex ATP dependent mechanism that tags proteins with the polypeptide ubiquitin (146). The consequence of this tagging is the rapid degradation of the tagged proteins by a large proteolytic complex called 26S proteasome (148,150,152,153). This 1300 kDa molecular mass complex is composed of a multiunit proteolytic component, referred to as 20S

Fig. 7: MHC class I antigen processing and presentation pathway

The overall processing and presentation MHC class I pathway is depicted. It includes the biosynthesis of the MHC class I heavy and light chains on the RER and the degradation of antigens by the 26S proteasome. Subsequently, the peptides are transported to the ER lumen by the TAP transporters. Finally, the properly assembled heavy chain, light chain, and peptide trimers are transported to the cell surface through the Golgi apparatus. See text for details.


TAP
PROTEASOME
β_2M
MHC
C
ER
LUMEN
GOLGI
PLASMA MEMBRANE

proteasome, and two presumed regulatory 19S cap complexes which exhibit ATPase activity (147). The 26S proteasome is not only involved in the degradation of ubiquitin labeled protein but also in that of non ubiquitinated substrates (149,152,154,155). In all, over 25 subunits ranging in molecular mass from 21 to 110 kDa compose the 26S proteasome (156). The 700 kDa 20S proteasome itself is a multicatalytic proteolytic complex composed of 12-15 subunits of molecular mass between 19 and 36 kDa (147,148). It is highly conserved from yeast to man and contains unique proteases not found elsewhere. It is a cylindrical hollow structure that preferentially cleaves at the carboxyl terminus of acidic, basic, and hydrophobic amino acids (147-150,157). Interestingly, the majority of the peptides binding to MHC class I proteins have hydrophobic residue at their carboxyl termini, corresponding well with the enzymatic activity of the 20S proteasome (42,43). The 26S proteasome has, along with these activities, an isopeptidase activity that removes the ubiquitin polypeptides and allows their recycling. The 20S proteasome is an essential but insufficient component for the degradation of ubiquitinated substrates, since only the assembled 26S complex is enzymatically active (148). Both the assembly and the function of the 26S proteasome appear to be an ATP dependent process.

The low molecular mass polypeptides (LMP) complex is composed of multiple units and exhibits proteolytic activity (158-160). The LMP complex and proteasomes share both serological and biochemical properties. It was further shown that the LMP complex is a constituent of the 20S proteasome (156,161,162). This led to the hypothesis that proteasomes are involved in antigen processing (163). Interestingly, two of the LMP gene products, LMP2 (161,164) and LMP7 (165), were mapped to the highly polymorphic MHC

class II locus (158,161,162,165). Furthermore, these gene products have been isolated from 20S proteasomes (156) and they alter their enzymatic activity (157,166-168). The location of the LMP2 and LMP7 genes in the MHC locus and the properties of the proteasomes have been considered evidence for proteasomes specialized in antigen processing (156,161,169). The polymorphism of the LMP2 and LMP7 proteins (159,160,162,164,170,171) and their influence on antigen presentation (157,166-168,172,173) also support this idea. Finally, the variation in the subunits composition of the proteasomes suggests that they may contribute to the selectivity of the peptides that are presented by MHC class I proteins to CTL. However, this may not be the case since the LMP proteins are not essential to antigen processing and presentation. Hence, mutant cell lines devoid of the LMP2 and LMP7 genes efficiently present peptide loaded MHC class I molecules at their surface (174-177). Despite these apparent contradictions, the current belief is that the 26S proteasome is responsible for the generation of MHC class I restricted peptides.

1.4.3- Role of the transporter associated with MHC class I antigen processing

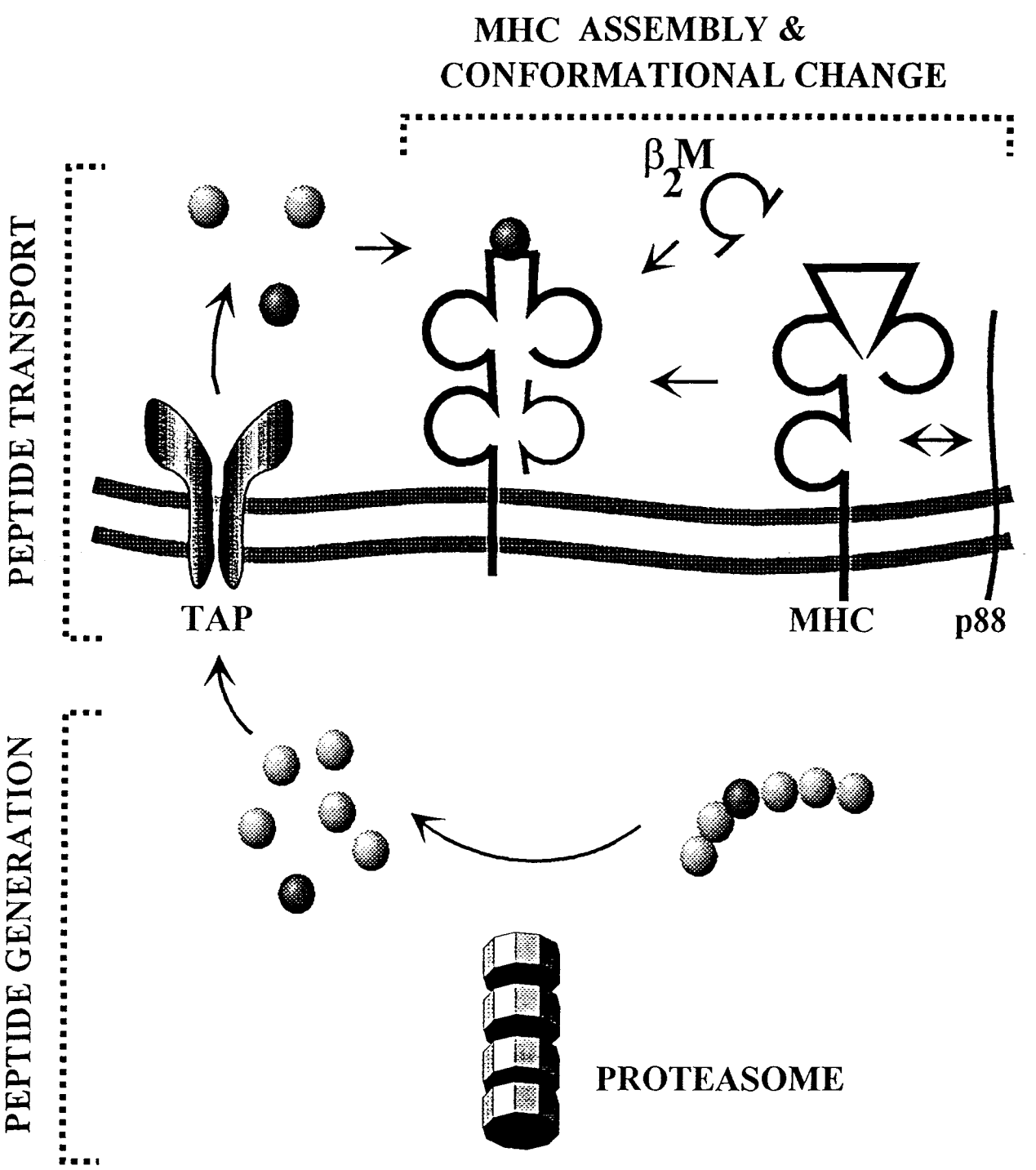
Proteasomes are located in the cytosol and in the nucleus (178,179). Since most peptides presented by MHC class I proteins are of cytosolic origin (144), it is likely that these peptides are produced in the cytosol. Interestingly, binding of cytosolically produced peptides to MHC class I molecules can induce transport of the MHC proteins out of the ER (62,70-72,81-90). It has therefore been postulated that the binding of peptides to MHC class I proteins takes place in the ER, as first suggested by Elliott *et al.* (180). This hypothesis is supported by studies using the antibiotic brefeldin A (BFA), which blocks both egress of

molecules out of the ER as well as MHC class I presentation (181,182). It is also supported by the block of antigen presentation by the ER resident 19 kDa protein encoded by the E3 region (E3/19K) of Adenoviruses (183). Finally, the ER retention of MHC class I proteins in mutant cell lines deficient in the processing of antigens is also consistent with this hypothesis (62,88). Thus binding of cytosolically produced peptides to the ER resident MHC class I peptide binding cleft is dependent on the transport of those peptides across the ER membrane (Fig. 8).

The signal sequence independent transport mechanism of MHC class I restricted peptides across the ER membrane is mediated by the transporter associated with antigen processing (TAP) proteins. These transport proteins are composed of two chains, TAP1 and TAP2 (184,185). Both chains have a molecular mass of approximately 65 to 70 kDa and are encoded in the MHC class II locus, near the LMP2 and LMP7 genes (Fig. 1) (83,184-186). The TAP proteins are structurally related to the multidrug resistance family of ATP binding cassette (ABC) proteins (184,185,187). In particular, they share multispanning amino terminal transmembrane domains and an ATP binding domain at their carboxyl terminus. Unlike the TAP polypeptides, these ABC proteins typically contain two such structural units in eukaryotes and have molecular masses approximately twice as big as the TAP proteins. This suggests that the TAP1 and TAP2 proteins cooperate to form dimers. Coimmunoprecipitation (188,189), complementation (190) and transfection studies using transport deficient cell lines (189,191) all support the view that the TAP proteins form heterodimers. Interestingly, it has also been suggested that TAP homodimers might be functional (174,192).

Fig. 8: Selectivity of the processing and transport of MHC class I peptides

The role of the 26S proteasome and of TAP1 and TAP2 transporter proteins on MHC class I processing is shown. The first step involves the controlled degradation of immunogenic proteins in the cytosol by the proteasome. The peptides generated by this proteolytic event might be influenced by the inclusion of particular subunits in the proteasome, for example LMP2 and LMP7. Subsequently, these peptides are transported, possibly selectively, by TAP1-TAP2 heterodimers or homodimers from the cytosol to the lumen of the ER. Finally, empty MHC class I proteins will select from this pool of transported peptides and specifically bind some of them. Binding of the peptide and the light chain to the heavy chain induces a conformational change and results in the egress of the MHC class I proteins out of the ER and to the cell surface. This binding also results in the release of the calnexin (p88) molecule from the misfolded and free MHC class I proteins.


ER LUMEN
MHC ASSEMBLY &
CONFORMATIONAL CHANGE
β2M
PEPTIDE TRANSPORT
TAP
MHC
p88
PEPTIDE GENERATION
PROTEASOME
CYTOSOL

The ABC proteins are a family of transporters involved in the transport of proteins, oligopeptides, peptides, and ions (193,194). Thus, by analogy to these transporters, it was originally proposed that TAP can transport peptides across the ER membrane (184,185). This has since been documented by the localization of the TAP proteins in the ER membrane (195) and by *in vitro* peptide transport assays using permeabilized cells or microsomes and synthetic peptides (196-202). The mapping of the class I modifier (cim) effect to the TAP genes is also evidence for TAP involvement in antigen presentation (203). The cim phenotype is a property of different rat strains to present the RTA.A^a MHC class I alloantigen to A^{a+} or A^{a-} specific CTL (186,204,205). Furthermore, the requirement for TAP proteins to transport peptides to the ER lumen has been demonstrated with minichromosomes encoding peptides modified to contain a signal sequence (206,207). Finally, the role of TAP proteins in MHC class I presentation has been shown with knockout mice (208) and by the analysis of various TAP deficient cell lines (62,174,188-192,209,210). Consistent with their homology with the ABC family of transporters, there is evidence that indicates that the function of the TAP proteins is ATP dependent (197,198,202). However, there is also evidence that some peptides may be transported through the ER membrane by an ATP independent (211,212) or even TAP independent manner (211-217). Interestingly, analysis of TAP genes in rats, humans, and mice (203,218-221) has indicated that they are polymorphic molecules. This polymorphism appears to have some functional relevance, since in rats it can result in the selective transport of peptides (171,196-198,200-203) and their presentation to CTL (171,203). However, this has not been documented in other systems and there are reports indicating that TAP polymorphism may not be important for peptide transport (222,223).

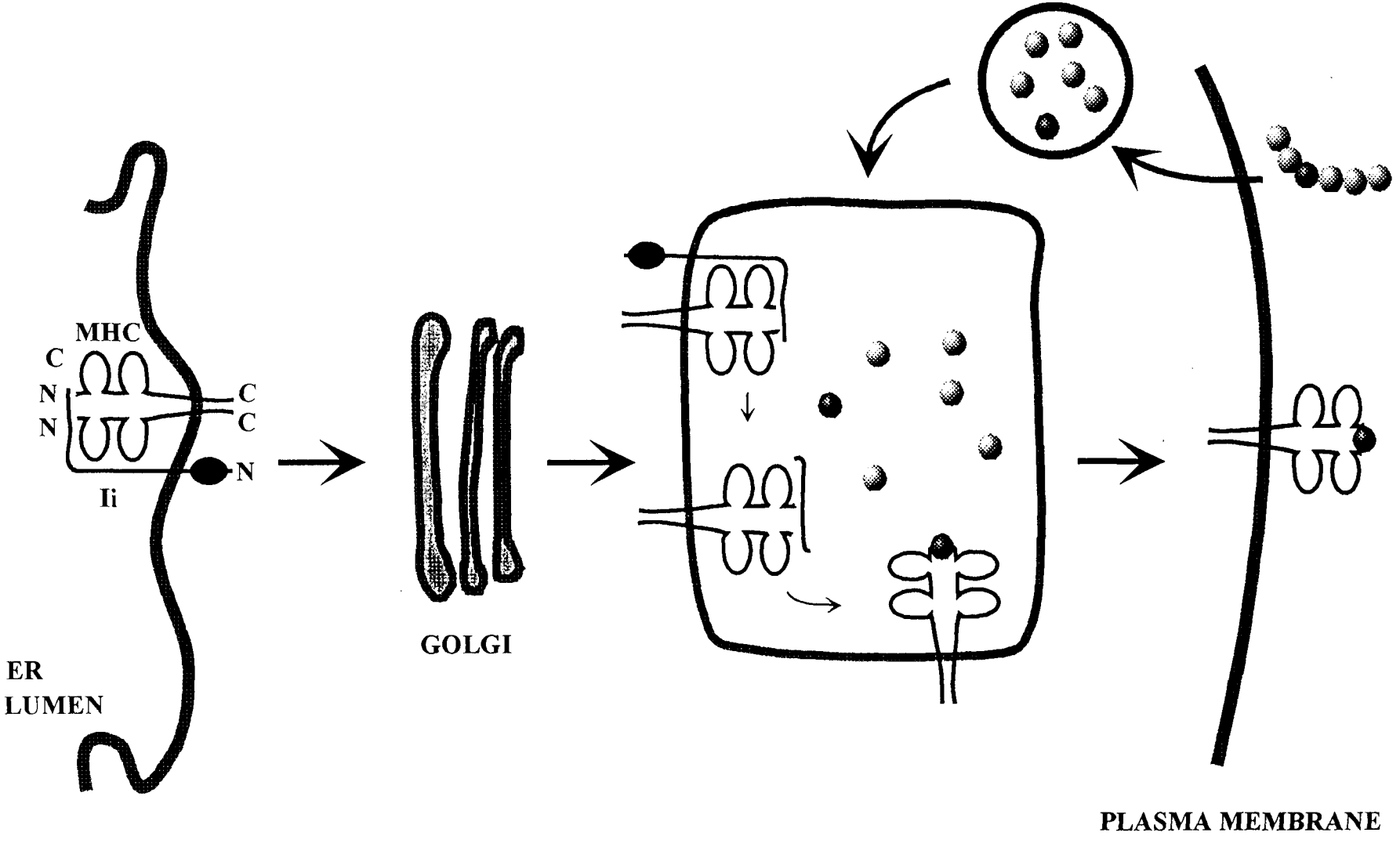
Taken together, the above information suggests that the few peptides presented from immunogenic proteins possibly result from the potential selectivity of the proteasome, TAP, and MHC class I molecules. Reports of TAP independent presentation of peptides to CTL further suggests an additional level of complexity of the antigen processing and presentation pathway. This overall heterogeneity likely accounts for the differential presentation of peptides by different individuals. Interestingly, this may be necessary for the survival of species, given the various pathogens and foreign proteins they encounter.

1.4.4- Processing of MHC class II antigens

Figure 9 shows an overall view of antigen processing in the context of MHC class II proteins. Similarly to MHC class I presentation, T cell recognition of MHC class II epitopes is dependent on the degradation of antigens into peptides (36,224-226). Hence, proteolytic fragments of 13-25 amino acids are presented to $CD4^+$ T cells by class II antigen presenting cells (APC) (41,117,124,125,227). However, the processing of class II antigens is different from that of class I peptides. Proteins presented by MHC class II proteins are usually acquired by endocytosis (41,117,124,125,227) and are degraded into peptides in the endocytic pathway (228). The exact site of the proteolytic cleavage of the antigens is unclear (100,144), but possibly involves degradation by the prelysosomal or lysosomal proteolytic compartments (229-231). As discussed previously, it is also unknown where peptides and MHC class II proteins meet on the endosomal pathway. Various colocalization and biochemical studies have implicated that the processed antigenic determinants meet and bind the class II molecules in early endosomes, late endosomes, or the recently identified MIIC compartment (100,144).

Fig. 9: MHC class II antigen processing and presentation pathway

The overall MHC class II mediated antigen processing and presentation pathway is shown. It includes the biosynthesis of the MHC class II α and β chains, their assembly in the ER and their binding to the Ii chain. For simplicity, the oligomerization of the Ii trimers with MHC class II α and β chains into nonamers is not shown. The assembled molecules are routed to the endocytic pathway by the Ii chain through the Golgi apparatus. The cleavage of the Ii chain in a compartment of the endosomal-lysosomal pathway leaves the residual Ii CLIP fragment bound to the MHC class II protein. Antigens acquired by the endocytic pathway are presumably degraded into peptides by enzymes in prelysosomes or lysosomes. They then come across the MHC class II protein in an internal compartment, likely the MIIC compartment. Binding of peptides by the MHC class II molecules requires the removal of CLIP, possibly by proteins encoded by the DM genes (not shown). Stable peptide loaded MHC class II proteins are then transported to the plasma membrane for presentation to T cells. The exact route used for this transport is unclear at the moment.


MHC
C
N
N
C
C
N
Ii
ER
LUMEN
GOLGI
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1.5- Distinct class I and II processing and presentation

As outlined above, differences exist between MHC class I and II mediated immune responses. They include MHC protein structure, assembly, biosynthesis, and transport. They also differ by the pathways by which they acquire peptides and the source of those peptides. These antigen processing differences are also mirrored by their sensitivity to various agents. For example, class I presentation requires protein synthesis and transport through the Golgi secretory pathway and is thus sensitive to the protein synthesis inhibitor cycloheximide (214) and to the transport inhibitor BFA (181,182). Furthermore, certain peptide analogs can block the processing of proteins into peptides by the proteasome (173). In contrast, class II processing is specifically sensitive to lysosomotropic agents such as chloroquine, emetine and ammonium chloride. These agents act by raising the pH of endosomes and lysosomes and interfere with endosomal trafficking (122,232-235). MHC class II processing is also sensitive to protease inhibitors, such as leupeptin and pepstatin, that perturb the activity of the endosomal proteolytic enzymes (123,234,236-240). Other differences between the MHC class I and II pathways include the MHC class II restricted processing and presentation of whole proteins added to the medium (232). In contrast, the cytosolic loading of whole proteins or proteolytic fragments leads to MHC class I presentation (131).

1.6- Distinct allogeneic and viral processing and presentation

As discussed above, normal cells express at their plasma membrane MHC molecules that contain peptides. The transfer of cells between individuals expressing different MHC haplotypes results in the recognition and the destruction of the introduced cells by CTL (3).

This presentation has been coined allogeneic presentation and has been a very important aspect of cellular immunity for transplants and autoimmune diseases. Unfortunately, the mechanisms of allorecognition are still fairly poorly understood. It was initially thought that allogeneic CTL recognized surface MHC molecules devoid of peptides. However, the discovery by crystallography of electron dense material in the α_1-α_2 groove of MHC class I molecules suggested that allogeneic responses involve the presentation of self peptides (18-22). The presence of allopeptides bound to MHC molecules has since been documented by various laboratories (134,241-248). Thus it seems that the presentation of allogeneic and viral antigens to CTL follows the same rules.

Despite their functional analogy, presentation of allogeneic peptides to CTL can nevertheless be distinguished from viral specific recognition and lysis by CTL by two accounts. First, the frequency of unprimed CTL capable of killing allogeneic targets is much greater than that of viral specific CTL (3,249-251). Two hypotheses have been put forward to justify this predisposition of CTL for allogeneic targets. According to one hypothesis, the high allogeneic CTL precursor frequency could be explained by the high density of MHC molecules at the cell surface. In this model, the T cell receptor (TCR) would have a low affinity with its ligand, but this would be compensated by a high probability of contact (252). The second hypothesis is the altered self model which suggests that the MHC molecules are altered in some way and are thus recognized as foreign proteins by CTL (253,254). This model does not rely on the density of MHC molecules but rather on their high affinity interaction with TCR. The binding and presentation of self peptides by MHC molecules (134,241-248) support the altered self hypothesis. The promiscuity of MHC molecules and

the constitutive expression of allopeptides that can bind to MHC molecules could result in the presentation of a vast combination of different MHC-peptide complexes and would thus elicit many T cell clones. However, the observation that few allopeptides can be eluted from MHC proteins suggests that the high density hypothesis may be correct (43). It is therefore still unclear why allogeneic CTL are so common. The allorecognition of apparently empty MHC class I molecules by CTL (32,246,255) underlines an additional level of complexity for allogeneic presentation. It also underlines our current lack of understanding of allogeneic presentation.

A second and significant potential difference between allogeneic and viral recognition by T cells might be the use of distinct processing and presentation pathways. This has been well illustrated with the RMA/S mutant cell line. This cell line has a defective TAP2 gene (210) that affects the assembly and presentation capacity of MHC class I molecules (190,191,210). Interestingly, this cell line is deficient for the presentation of Influenza epitopes to class I restricted T cells (62), but not for the presentation of allogeneic (214,246) or Vesicular stomatitis virus (VSV) (214) epitopes. This suggests that alternative MHC class I pathways exist and that the processing and presentation of particular peptides may be dependent on either or both of the TAP transporters. The results presented in Chapter 4 of this thesis support this view.

1.7-Objectives and approaches

Although much has been learned in the past decade about antigen processing and presentation, the exact details of these pathways remain to be clarified. Identification of alternative pathways, including allogeneic presentation, and the clarification of the rules and conditions that result in the processing and the presentation of a given antigen via a particular pathway need closer examination. Furthermore, the molecular characterization of the mechanisms by which pathogens modulate these pathways is also of interest. Such studies would not only yield information pertaining to the functioning of the immune system but also to potential antiviral therapies. A better understanding of these details will also hopefully allow the precise prediction of epitopes for given MHC haplotypes. Such knowledge would potentially have important clinical implications.

As indicated above, the presentation of antigens by MHC molecules is a complex phenomenon. The general objectives of this study are to better define the antigen processing and presentation pathway(s) in the context of MHC class I proteins and examine their modulation by pathogens. To address these objectives, two approaches were used. The first approach consisted in the investigation of antigen processing and presentation in a system that is naturally hindered by viral infection, in particular by Adenoviruses. These viruses are believed to evade the immune response of their host by inhibiting the MHC class I antigen processing and presentation pathway. As discussed in details in Chapter 3, the E3/19K of Adenovirus type 2 (Ad2) binds and retains MHC class I molecules in the ER. In doing so, it blocks their transport to the plasma membrane and protects the infected cells from lysis by

CTL. The specific objective was to examine the role of the newly characterized adenoviral 6.7 kDa protein of the region 3 (E3/6.7K) in this process.

The second approach involved the characterization of the processing and presentation potential of a mutant cell line called gro29 (Chapter 4). Unlike most antigen processing and presentation mutants, this cell line was not selected for loss of MHC epitopes at its surface but rather for resistance to Herpes simplex virus type 1 infection. This mutant synthesizes viral specific gene products, but slowly processes and transports them through the cell, thus constituting a good candidate as an antigen processing and presentation mutant.

CHAPTER 2

MATERIALS AND METHODS

2.1- Tissue culture

Cell lines were used and grown as follows. The human HeLa and low passage 293 cells were propagated in alpha minimum essential medium (αMEM) supplemented with 10% fetal calf serum (FCS). These low passage 293 cells (under passage 70) grew relatively slowly and were exclusively used for the titration of the adenoviral stocks. The human A549, 293.12, 621.13, and 293 cell lines were grown in Dulbecco minimum essential medium (DMEM) containing 10% calf serum (CS). Finally, the human MRC5 and murine Ltk$^-$, gro29, NIH/3T3, and IT22.6-K^d cell lines were cultivated in DMEM supplemented with 10% FCS. The Ltk$^-$ and gro29 cell lines are of H-2^k origin whereas the NIH/3T3 cell line is of H-2^d origin. The IT22.6-K^d cell line is an H-2^q cell line transfected with the K^d gene (256). All media were obtained from Gibco Laboratories (Burlington, Ont.) and were supplemented with 100 units/ml of penicillin, 100 μg/ml of streptomycin sulfate, and 2 mM L-glutamine. All sera were also bought from Gibco Laboratories and were heat inactivated 30 minutes at 56°C. Low passage 293 cells were removed from dishes using versene (137 mM NaCl, 2.7 mM KCl, 0.5 mM EDTA, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1.1 mM glucose). All other cell lines were treated with 0.25% trypsin-1 mM EDTA to detach them from the dishes.

2.2- Animals

Mice were used for priming of allogeneic and virus specific CTL. For this purpose, six to twelve weeks old CBA/J or CBA/ca ($H\text{-}2^k$) and BALB/c ($H\text{-}2^d$) mice were bred at the University of British Columbia Microbiology and Immunology departmental breeding facility. They were maintained according to the regulations of the Canadian Council on Animal Care.

2.3- Viruses

Two distinct sets of viruses were used. First, in Chapter 3, wild type (wt) and deletion mutant Adenoviruses were used. Ad2, dl327, and dl801 were obtained from Dr. H.S. Ginsberg (Columbia University, New York, NY). Rec700, dl701, dl703, dl704, dl708, dl710, dl739, and dl754 were generous gifts from Dr. W.S.M. Wold (St. Louis University, St. Louis, MO). Finally, Ad5 was kindly provided to us by Dr. F.L. Graham (McMaster University, Hamilton, Ont.). These viruses were all grown and titrated on low passage 293 cells according to Harrison *et al.* (257). The deletions in the mutant viruses remove various genes in the E3 region of the viral genome (Fig. 10). The impact of these deletions on the E3/6.7K and the E3/19K proteins can be found in figure 11 and figure 12. Unless otherwise indicated, 293 or HeLa cells were infected at a multiplicity of infection (MOI) of 50. The infection of cells in subconfluent 60 mm dishes was performed according to Wold *et al.* (258,259). For this purpose, the viruses were diluted to the desired concentration in phosphate buffered saline (PBS) and 0.25 ml of virus was added to each dish. Following a 40 minute adsorption period, the inoculum was then replaced with normal medium. Control samples (mock) were treated with PBS alone.

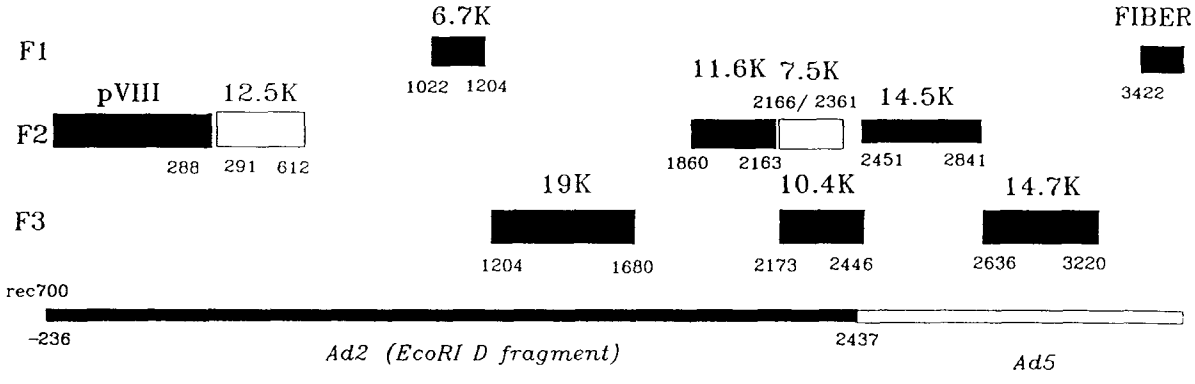
Fig. 10: Map of the E3 region of the Ad2 genome and viral mutants used in this study

Panel A shows the map of the Ad2 E3 region in all three reading frames (F1-F3). The numbers under each open reading frame (ORF) indicate the boundary of the gene in basepairs, basepair # 1 being the first nucleotide of the major RNA transcript of E3. The genes are identified by the molecular weight of the predicted encoded proteins. Please note that both the pVIII and the fiber genes extent beyond the map to the left and right respectively.

Ad2 and Ad5 are wt viruses of the serotype 2 and 5 respectively. Rec700 is a recombinant virus that encodes a wt Ad5 genome, except for most of its E3 region, which is derived from wt Ad2 (260). The mutant viruses dl327 (261,262), dl701 (263), dl703 (264), dl704 (263), dl708 (261,264-266), dl710 (265), dl739 (267), and dl754 (261) are recombinant viruses derived from rec700. They contain various deletions within E3/6.7K, E3/19K, or most of the E3 region. The dl801 mutant virus is not a recombinant virus, but rather is an Ad2 virus containing a large deletion within the E3 region (268). The virus rec700 is the wt recombinant virus and is equivalent to Ad2.

The deletions contained in the various viral mutants are indicated below by hatched bars (Panel B). The resulting effects on the expression of the E3/6.7K and E3/19K proteins are indicated along side. -: indicates that no protein is made; wt: wild type protein generated; aa: amino acids; del: deletion; no signal seq: no signal sequence; no CYT tail: no cytoplasmic tail. See figure 11 and figure 12 for further details on the mutant viruses.

A) E3 REGION



B) VIRUSES

	Proteins	
	E3/6.7K	*E3/19K*
Ad2	wt	wt
Ad5	wt	wt
rec700	wt	wt
dl327	-	
dl701	-	wt
dl703	wt	no signal seq
dl704	wt	51 aa del
dl708	wt	no CYT tail
dl710	wt	extra 5 aa
dl739	-	wt
dl754	extra 28 aa	90 aa del
dl801	-	-

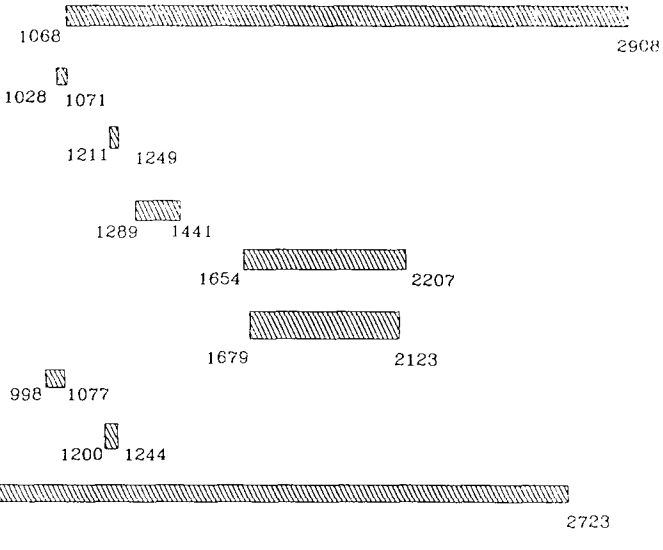


Fig. 11: Sequences of the E3/6.7K and E3/19K proteins encoded by the viral mutants

The impact of the viral deletions on the sequences of the E3/6.7K and the E3/19K proteins is shown. The deletions of amino acids are depicted by boxes, whereas the generation of new amino acids resulting from frame shifts or the deletion of stop codons is indicated by underlined sequences. The Ad2 and Ad5 wt sequences are shown for comparison. The lengths and predicted molecular masses of the mutated proteins are indicated as well. Please note that the predicted molecular masses of the E3/19K are for full length proteins before cleavage of the signal sequence.

The dl327 and dl801 mutants contain deletions that encompass most of the E3 region and do not encode any functional E3/6.7K or E3/19K protein. Dl703 encodes an E3/19K protein devoid of its ER signal sequence. Dl704 and dl754 respectively lack 51 and 90 amino acids within the E3/19K protein. Neither mutants produce functional E3/19K proteins (Wold, personal communication). The dl708 mutant has lost most of the E3/19K cytoplasmic tail, which contains the ER retention signal. The deletion in dl710 removes the E3/19K stop codon, resulting in the addition of 5 extra amino acids at the carboxyl end of the molecule. Dl701 and dl739 do not encode any E3/6.7K protein.

A) E3/6.7K MUTANTS

	SEQUENCE	*Length*	*MW*
wt Ad2:	MSNSSNSTSLSNFSGIGVGVILTLVILFILILALLCLRVAACCTHVCTYCQLFKRWGQHPR	61 aa	6,671
wt Ad5:	MNNSSNSTGYSNSGFSRIGVGVILCLVILFILILTLLCLRLAACCVHICIYCQLFKRWGRHPR	63 aa	7,059
rec700 :	wild type E3/6.7K	61 aa	6,671
dl327 :	MSNSSNSTSLSNFSGTS*ERERLKQEVEDMVNLHQCKRGIFCVVKQAKLTYEKTTTGNRLSYKL PTQRQKLVLMVGEKPITVTQHSAETEGCLHFPYQGPEDLCTLIKTMCGIRDLIPFN*	119 aa	13,456
dl701 :	MS[redacted]*GRGYPYSCNSVYSYTSTSVP*	22 aa	2,407
dl703 :	wild type E3/6.7K	61 aa	6,671
dl704 :	wild type E3/6.7K	61 aa	6,671
dl708 :	wild type E3/6.7K	61 aa	6,671
dl710 :	wild type E3/6.7K	61 aa	6,671
dl739 :	no protein made	0 aa	-------
dl754 :	MSNSSNSTSLSNFSGIGVGVILTLVILFILILALLCLRVAACCTHVCTYCQLFKRWGQHP[redacted]*AAL PKRLSLRNQLAMLHLNQKLMNALLL*	88 aa	9,682
dl801 :	no protein made	0 aa	-------

B) E3/19K MUTANTS

	SEQUENCE	*Length*	*MW*
wt Ad2:	MRYMILGLLALAAVCSAAKKVEFKEPACNVTFKSEANECTTLIKCTTEHEKLIIRHKDKIGKYA VYAIWQPGDTNDYNVTVFQGENRKTFMYKFPFYEMCDITMYMSKQYKLWPPQKCLENTGTFCST ALLITALALVCTLLYLKYKSRRSFIDEKKMP	159 aa	18,438
wt Ad5:	MIRYIILGLLTLASAHGTTQKVDFKEPACNVTFAAEANECTTLIKCTTEHEKLLIRHKNKIGKY AVYAIWQPGDTTEYNVTVFQGKSHKTFMYTFPFYEMCDITMYMSKQYKLWPPQNCVENTGTFCC TAMLITVLALVCTLLYIKYKSRRSFIEEKKMP	160 aa	18,502
rec700 :	wild type E3/19K	159 aa	18,438
dl327 :	no protein made	0 aa	-------
dl701 :	wild type E3/19K	159 aa	18,438
dl703 :	MR[redacted]*C*AAKKVEFKEPACNVTFKSEANECTTLIKCTTEHEKLIIRHKDKIGKYA VYAIWQPGDTNDYNVTVFQGENRKTFMYKFPFYEMCDITMYMSKQYKLWPPQKCLENTGTFCST ALLITALALVCTLLYLKYKSRRSFIDEKKMP	146 aa	17,121
dl704 :	MRYMILGLLALAAVCSAAKKVEFKEPAC[redacted] [redacted]TVFQGENRKTFMYKFPFYEMCDITMYMSKQYKLWPPQKCLENTGTFCST ALLITALALVCTLLYLKYKSRRSFIDEKKMP	108 aa	12,555
dl708 :	MRYMILGLLALAAVCSAAKKVEFKEPACNVTFKSEANECTTLIKCTTEHEKLIIRHKDKIGKYA VYAIWQPGDTNDYNVTVFQGENRKTFMYKFPFYEMCDITMYMSKQYKLWPPQKCLENTGTFCST ALLITALALVCTLLYLKYKSRR[redacted]*L*	151 aa	17,474

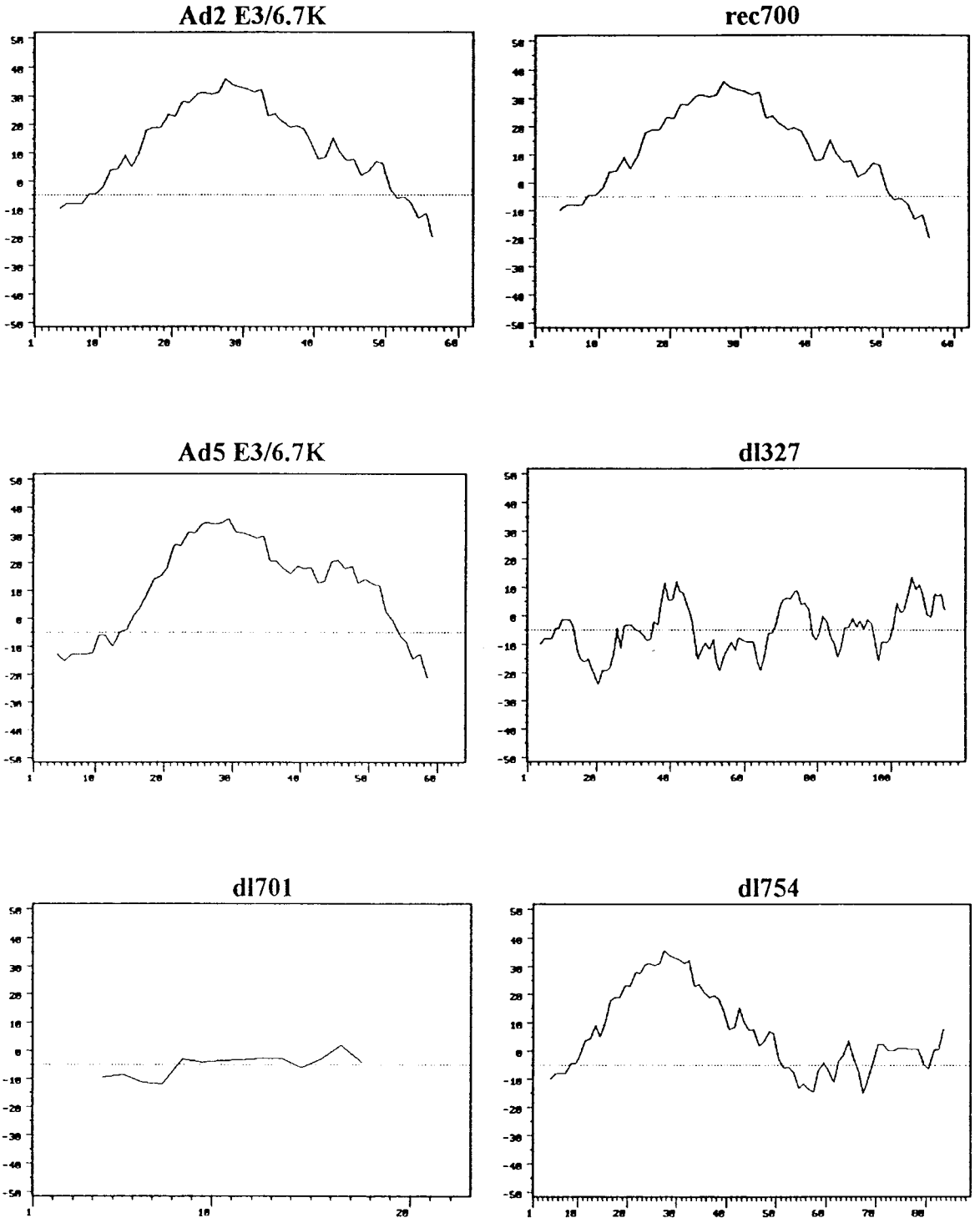
B) E3/19K MUTANTS (con't)

	SEQUENCE	*Length*	*MW*
wt Ad2:	MRYMILGLLALAAVCSAAKKVEFKEPACNVTFKSEANECTTLIKCTTEHEKLIIRHKDKIGKYA VYAIWQPGDTNDYNVTVFQGENRKTFMYKFPFYEMCDITMYMSKQYKLWPPQKCLENTGTFCST ALLITALALVCTLLYLKYKSRRSFIDEKKMP	159 aa	18,438
dl710 :	MRYMILGLLALAAVCSAAKKVEFKEPACNVTFKSEANECTTLIKCTTEHEKLIIRHKDKIGKYA VYAIWQPGDTNDYNVTVFQGENRKTFMYKFPFYEMCDITMYMSKQYKLWPPQKCLENTGTFCST ALLITALALVCTLLYLKYKSRRSFIDEKKMP*GSRTV*	164 aa	18,938
dl739 :	wild type E3/19K	159 aa	18,438
dl754 :	[redacted] [redacted]MYKFPFYEMCDITMYMSKQYKLWPPQKCLENTGTFCST ALLITALALVCTLLYLKYKSRRSFIDEKKMP	69 aa	8,237
dl801 :	no protein made	0 aa	-------

Fig. 12: Hydrophobicity of the E3/6.7K and E3/19K proteins encoded by the Adenoviruses used in this study

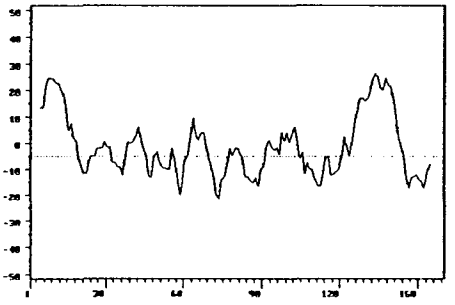
The hydrophobicity plots of the E3/6.7K and E3/19K proteins encoded by the wt and mutant viruses used in this study (see Fig. 10 and 11) are depicted. The predicted sequences were analyzed with the PCGENE program "SOAP" using the algorithm of Kyte and Doolittle (269). Residues above the median at y=0 were hydrophobic whereas the residues below the median were hydrophilic. In all cases, the default parameters suggested by the PCGENE software were used. The hydrophobicity plots for the E3/6.7K protein encoded by the dl703, dl704, dl708, and dl708 mutants as well as for the E3/19K protein encoded by the dl701 and dl739 mutants are not shown since these mutants express wild type proteins. Similarly, the hydrophobicity plots for the E3/6.7K proteins encoded by the dl739 and dl801 mutants as well as for the E3/19K proteins encoded by the dl327 and dl801 mutants are not shown since they do not express that particular protein due either to the deletion of the entire gene or its start codon.

A) E3/6.7K

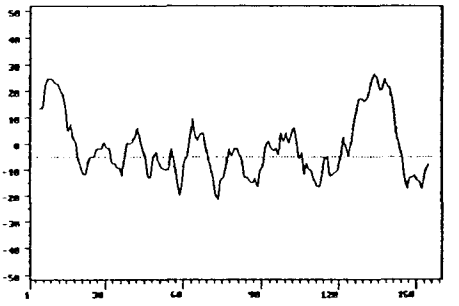


B) E3/19K

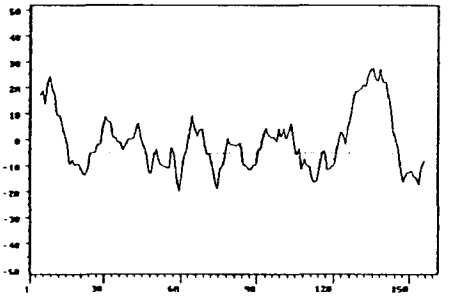
Ad2 E3/19K



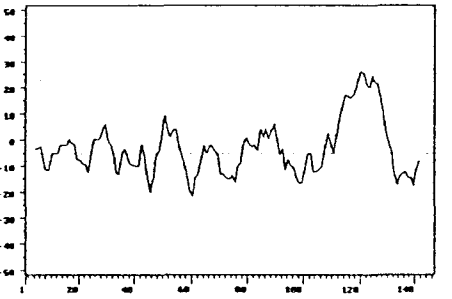
rec700



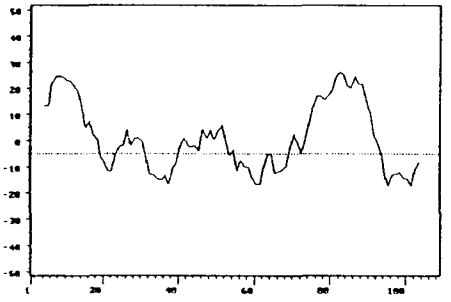
Ad5 E3/19K



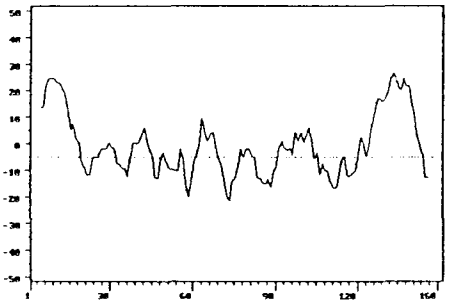
dl703



dl704



dl708



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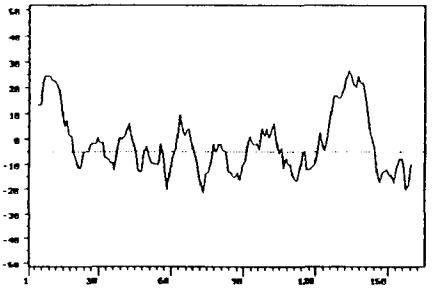
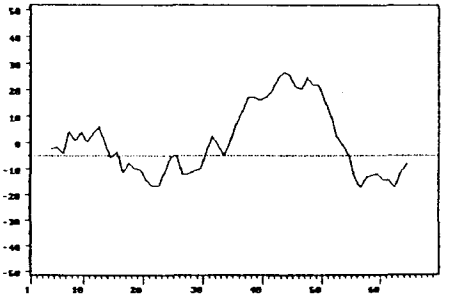


dl754



The second set of viruses was used in Chapter 4. The Herpes simplex type 1 (HSV-1) Kos strain was obtained from Dr. F. Tufaro (University of British Columbia, Vancouver, B.C.) and was propagated and titrated on Vero cells. Subconfluent gro29 and Ltk^- cells were infected at a MOI of 5 for both ^{51}Cr release assays and for surface expression analyses (see below). Stocks of titered Influenza virus strain A/PR/8/34 were obtained from the Laboratory for Disease Control (Ottawa, Ont.). Four hundred hemagglutination units per cell was used to infect the gro29 and Ltk^- target cells for the ^{51}Cr release assay (see below).

2.4- Antibodies

Various antibodies were used and are described below. W6/32 and PA2.1 are monoclonal antibodies directed against HLA-A,B,C and HLA-A2 respectively. H100.27.55, 16.3.22, 11.4.1s, and 15.5.5s are monoclonal antibodies reactive against H-2 K^kD^k, H-$2K^k$, H-$2K^k$, and H-$2D^k$ respectively. All the hybridomas producing these antibodies were bought from the American Tissue Culture Collection (ATCC; Rockville, MD). OKT9 and yE1/9.9.3 are two monoclonal antibodies specific for the human and the murine transferrin receptors (TR) and were obtained from ATCC and Dr. F. Takei (Terry Fox Laboratories, Vancouver, B.C.) respectively. All hybridomas were grown in DMEM supplemented with 10% FCS and 5×10^{-5} M β_2-mercaptoethanol (2-ME). In each case, the unpurified supernatant was used as a source of antibodies. Anti-fibronectin receptor (αFNR) is a rabbit polyclonal antiserum specific for the human fibronectin receptor, but cross reacts with its murine homologue. This antibody was bought from Telios Pharmaceuticals (San Diego, CA). Anti-precursor glycoprotein 1 (αPGP1), also referred to as αCD44, is a rat antiserum reacting against CD44

and was a kind gift of Dr. B. Hyman (Salk Institute, LaJolla, CA). αAd2 E3/6.7K, αAd2 E3/19K, and αAd5 E3/19K are rabbit antisera against E3/6.7K or E3/19K as their names imply. They were a generous gift of Dr. W.S.M. Wold (St. Louis University, St. Louis, MO). Both αAd2 E3/6.7K and αAd5 E3/19K are peptide antisera directed against the C-terminal 15 amino acids of the respective molecule. In contrast, the αAd2 E3/19K antibody is specific for the N-terminal 15 amino acids of the mature Ad2 E3/19K. The αAd5 E3/19K antibody also immunoprecipitates Ad2 E3/19K proteins. Anti-Thy-1 is a rabbit anti-mouse Thy-1 antiserum, which was obtained from Dr. R. McMaster (University of British Columbia, Vancouver, B.C.) and Dr. A. Williams (Oxford University, Oxford, England). Finally, the anti-proteasome antibody is a rabbit polyclonal antiserum against rat proteasome and was obtained from Dr. J. Driscoll (Harvard Medical School, Boston, MA). This antibody cross reacts against its murine homologue but not against its human homologue.

2.5- Flow cytometry

To measure the surface expression of MHC class I proteins and other molecules, 60 mm dishes were treated with versene to remove the cells from the dishes. For the experiments involving Adenoviruses (Chapter 3), the cells were examined at 20 hours post infection (hpi), unless otherwise indicated. In contrast, the infected gro29 and Ltk^- cells were examined at various times post infection as indicated in the respective figures. In all cases, the cells were washed with fluorescence activated cell sorter (FACS) buffer (DMEM, 20 mM hepes, 20 mM sodium azide, 0.5% bovine serum albumin (BSA)). They were then incubated for 45 minutes at room temperature, or 60 minutes on ice, with 200 ul of primary antibody (supernatant or

diluted 1:25 if it was an antiserum). The samples were subsequently washed twice with FACS buffer and incubated with 100 ul of a 1:25 dilution of the proper secondary antibody coupled to fluorescein isothiocyanate (FITC) -- for example goat α mouse IgG (GAM-FITC, Jackson ImmunoResearch Laboratories, Mississauga, Ont.) -- for a further 45 minutes at room temperature or 60 minutes at 4°C. Finally, the cells were washed once in FACS buffer and once in cold PBS without calcium and magnesium (PBS^{--}) and were fixed in 1.5% paraformaldehyde in PBS^{--}. Five thousand cells were analyzed on a Becton-Dickinson FACS machine using either the FACSCAN or the LYSYS II software for analysis. For the negative control (no first antibody (NFA)), the cells were incubated with FACS buffer instead of the primary antibody. The relative level of protein surface expression, when compared to the control sample, was calculated as follows using linear fluorescence values:

$$\% \text{ Mock} = \frac{(\text{Infected} - \text{NFA}) \times 100}{(\text{Mock} - \text{NFA})}$$

or

$$\% \text{ Ltk}^{-} = \frac{(\text{gro29} - \text{NFA}) \times 100}{(\text{Ltk}^{-} - \text{NFA})}$$

2.6- Generation of cytolytic T lymphocytes

2.6.1- Allogeneic CTL

To elicit allogeneic CTL, mouse spleens were excised from healthy CBA/ca or CBA/J ($H\text{-}2^k$) and Balb/c ($H\text{-}2^d$) animals and teased in petri dishes in DMEM/10% CS. The cell clumps were then removed by filtering through a funnel lined with cotton cloth. The resulting cell suspension was submitted to flash lysis to remove the red blood cells by resuspension in 1 ml of distilled water rapidly followed by the addition of 10 ml of PBS. The cells were subsequently washed in DMEM/10% CS and resuspended to 5×10^7 cells/ml in PBS^{--}. To stimulate the production of allogeneic CTL, 10^7 $H\text{-}2^k$ splenocytes were injected intraperitoneally into $H\text{-}2^d$ animals ($\alpha H\text{-}2^k$ CTL) and vice versa for $\alpha H\text{-}2^d$ CTL. Finally, the animals were boosted with a second injection approximately 1 month later.

Nine to eighteen weeks after the initial priming of the mice; spleens were harvested from 2-3 primed mice and 2-3 unimmunized allogeneic mice per assay. Single cell suspensions were prepared as described above and the filtered cells washed in PBS^{--} supplemented with 20 mM hepes and 0.5% BSA (PBS-BSA). The cells were finally resuspended in 20 ml of complete medium (0.5X RPMI, 15 mM hepes, 0.11% $NaHCO_3$, 8.5 mN NaOH, 2.5×10^{-5} M 2-ME, 10% FCS, 2 mM L-glutamine, 100 mg/L sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, 0.5 x NCTC 109 medium). The naive splenocytes were irradiated on ice with 2200 rads and incubated with the primed splenocytes to further stimulate the latter cells *in vitro*. Both cell populations were mixed at a ratio of 1:1 in complete medium in 250 ml flasks at a total concentration of 2×10^6 cells/ml. To maximize

the contact between the two cell populations, the flasks were incubated vertically at 37°C in a tissue culture incubator. These secondary stimulated CTL were assayed 5 days later as described below.

2.6.2- Viral specific CTL

HSV-1 specific CTL were generated by the injection of 5 x 10^6 tissue culture infectious dose ($TCID_{50}$) in the foot pads and ears of CBA/J mice. The resulting effector population was isolated from the draining lymph nodes (retropharyngeal and popliteal) at day 5 post immunization. These primary CTL were grown for 3 days *in vitro* without restimulation at an initial concentration of 4 x 10^6 cells/ml in complete medium. The ^{51}Cr release assay was then performed as described below.

The generation of Influenza specific CTL was performed by the injection of 400 hemagglutination units intraperitoneally in CBA/J mice. Four weeks post immunization, the spleens were harvested and single cell suspensions were prepared as for allogeneic CTL. They were restimulated with Influenza infected syngeneic splenocytes at a total concentration of 3 x 10^6 cells/ml in complete medium. These cells were assayed 7 days later.

2.7- ^{51}Cr release assay

2.7.1- Allogeneic CTL

On the fifth day of the *in vitro* stimulation, the CTL (effector population) were used in a ^{51}Cr release assay as follows. They were spun down and resuspended in 9 ml of PBS-BSA.

This suspension was layered on top of 3 ml of Ficoll-Paque (Pharmacia, Piscataway, NJ) and spun at 750 x g for 20 minutes at room temperature. The cells located at the PBS-Ficoll interface were harvested and washed twice in PBS-BSA to remove the Ficoll. The cells were finally resuspended to 1 x 10^7 cells/ml in assay medium (1 x RPMI, 30 mM hepes, 0.22% $NaHCO_3$, 17 mN NaOH, 5 x 10^{-5} M 2-ME, 2.5% FCS).

The target cells, grown in petri dishes, were trypsinized, washed once in PBS-BSA and once in assay medium without serum. They were resuspended to 1-5 x 10^7 cells/ml in assay medium without serum and 10^6 cells were labeled for 1.5 hour at 37°C with 100 μCi $Na^{51}Cr0_4$ (350-600 mCi/mg Cr; Amersham, Oakville, Ont.). Unincorporated label was removed by 4 washes in PBS-BSA. Aliquots of the cells were removed before and after the washes and were counted in a LKB 1282 gamma counter to determine the level of incorporation of the label. The labeled cells were resuspended at a final concentration of 1 x 10^5 cells/ml in assay medium.

The ^{51}Cr release assay was performed in triplicates in U-shaped 96 well plates using 10^4 labeled target cells per well (100 μl) and various effector to target (E:T) ratios in a total volume of 200 μl. Before incubation at 37°C with 5% CO_2, the plates were spun down at 200 x g for 5 minutes to allow immediate contact between effector and target cells. Five hours later, the plates were spun at 900 x g for 5 minutes, and 100 μl of the supernatant was counted to monitor the chromium release. Control wells included target cells (100 μl) incubated without effector cells and treated with either 100 μl of medium or 3% Triton X-100 to determine the minimal (min) and maximal (max) releases of label respectively. The

spontaneous release of ^{51}Cr and the percentage of ^{51}Cr release for each sample were determined as follows:

$$\text{Spontaneous release} = \frac{(\text{Min} \times 100)}{\text{Max}}$$

$$\%\ \text{Specific}\ ^{51}\text{Cr release} = \frac{(\text{Sample - min}) \times 100}{(\text{Max - min})}$$

2.7.2- Viral specific CTL

Three days after the *in vitro* culture of the primary stimulated αHSV-1 CTL, 10^6 target Ltk$^-$ or gro29 cells were infected for 4 hours with HSV-1 at a MOI of 5 in the presence of 100 μCi ^{51}Cr. All other steps for both the target and effector populations were as described for the allogeneic assays.

Two million gro29 or Ltk$^-$ cells were infected with 400 hemagglutination units of Influenza for 3 hours in the presence of 200 μCi ^{51}Cr 7 days after the restimulation of the anti-Influenza CTL. Alternatively, they were incubated with the Influenza "SDYEGRLIQNSLTI" NP50-63 peptide (single amino acid codes) at 50 μM and labeled as above. The labeled target cells and the Influenza specific CTL were then treated as for the allogeneic assays, except that the release assay was for 4 hours instead of 5 hours. Where indicated, the target cells were

incubated for 30 minutes on ice with a 50 fold dilution of the H100.27.55 antibody (ascites) in assay medium before addition of CTL.

2.8- ^{35}S metabolic labeling

2.8.1- Adenovirus infected cells

Mock treated or infected cells (see Viruses above) were pulse-labeled with ^{35}S L-cysteine (>1000 Ci/mmol, New England Nuclear, Mississauga, Ont.). To this end, the cycloheximide (CH) enhanced protocol derived from Wold *et al.* (258,259,270) and Wilson-Rawls and Wold (271) was used. The protocol makes use of a CH enhancing step, to improve early adenoviral mRNA yields, and release from the CH block of protein translation in the presence of the 1-β-arabinofuranosyl cytosine drug (araC). AraC blocks DNA replication and prevents the viruses from shifting from early to late gene expression (272,273). In particular, 25 μg of CH (Sigma, St. Louis, MO) per ml of tissue culture medium was added at 4 hpi. At 7 hpi, the cells were washed twice with labeling medium (serum free αMEM lacking cysteine and supplemented with 2 mM L-glutamine). They were then incubated with 2 ml of labeling medium containing 20 μg/ml of araC (Sigma, St. Louis, MO) and 200 μ Ci of ^{35}S cysteine. At 11 hpi, the cells were lysed for 15 minutes on ice in 800 μl of iso-high-pH buffer (10 mM Tris pH 8.5, 140 mM NaCl, 1 mM $MgCl_2$, 0.5% Nonidet P40 (NP40), 20 μg/ml phenylmethylsulfonylfluoride (PMSF)).

Cell debris was removed by centrifugation at 38,400 x g for 30 minutes at 4°C. Five microliter aliquots were taken from the supernatants and dried on Whatman's filter paper. The

aliquots were precipitated in 10% trichloroacetic acid (TCA) for 30 minutes. The filter paper was rinsed successively in 100% acetone, 50% acetone-50% ether, and finally 100% ether. The air dried samples were counted in 5 ml of Beckman's READY SAFE scintillation fluid using a Beckman LS 5000 TA counter. The supernatants were usually frozen at this point. They were ultimately analyzed by electrophoresis and autoradiography (see below).

2.8.2- Ltk⁻, gro29 and 293 transfectants

Ltk⁻, gro29, or 293 transfectant cells seeded in 60 mm dishes were starved for 1 hour in labeling medium (L-methionine, L-cysteine and serum free DMEM). Unless otherwise indicated, the cells were labeled for 30 minutes in fresh labeling medium containing 200 μCi/dish of ^{35}S Pro-Mix (>1000 Ci L-methionine/mmol, 70% ^{35}S L-methionine - 30% ^{35}S L-cysteine; Amersham, Oakville, Ont.) and subsequently washed twice in DMEM/10% CS. When indicated, the samples were chased in normal medium containing serum and cold L-methionine and L-cysteine. The samples were ultimately lysed in 800 μl of ice cold lysis buffer (20 mM Tris pH 7.6, 120 mM NaCl, 4 mM $MgCl_2$, 1% NP40, and 20 μg /ml PMSF) and treated as described above.

2.9- ^{32}P metabolic labeling

2.9.1- 293 transfectants

Labeling of cells with ^{32}P orthophosphate (>9000 Ci/mmol; Amersham, Oakville, Ont.) was identical to the ^{35}S metabolic labeling protocol used to label gro29 cells or 293

transfectants, except that the cells were starved in phosphate and serum free DMEM and the labeling was done for 2 hours in 2 ml of phosphate and serum free DMEM supplemented with 500 μCi ^{32}P per dish.

2.9.2- Adenovirus infected cells

293 or HeLa cells were infected at a MOI of 50 as above (see Viruses section). Nineteen hours post infection, the cells were starved for an hour in phosphate and serum free DMEM. The medium was then replaced with 2 ml of fresh labeling medium supplemented with 500 μCi ^{32}P orthophosphate and the cells incubated for 2 hours. The cells were subsequently lysed and treated as for ^{35}S metabolically labeled cells.

2.10- Immunoprecipitation and electrophoresis

The ^{35}S or ^{32}P metabolically labeled samples were thawed on ice and normalized to contain equivalent TCA precipitable counts. Two microliters of a 20 μg/ml PMSF stock in isopropanol was added to each sample. They were then precleared with 2 μl of preimmune rabbit antiserum and 100 μl of protein A Sepharose 4CL (Pharmacia, Piscataway, NJ). The beads were subsequently removed by centrifugation at 38,400 x g for 30 minutes at 4°C. Specific immunoprecipitations were performed using 2-5 μl of αAd2 E3/6.7K, 20-25 μl of a 1:10 dilution of αAd5 E3/19K, 2 μl of αAd2 E3/19K, 100 μl of W6/32 or PA2.1, or 100-150 μl of yE1/9.9.3 antibody and 50 μl of protein A Sepharose 4CL. The beads were successively washed 3 times in buffer B (10 mM Tris pH 7.5, 2 mM EDTA, 150 mM NaCl, 0.2% NP40), twice in buffer C (10 mM Tris pH 7.5, 2 mM EDTA, 500 mM NaCl, 0.2% NP40), and once

in buffer D (10 mM Tris pH 7.5). Unless otherwise indicated, the immunoprecipitates were reduced prior to analysis by boiling 5 minutes in 1 x sample buffer (154 mM Tris pH 8.8, 769 mM sucrose, 3.8 mM EDTA, 0.008% bromophenol blue, 3% SDS, 38 mM 1.4-dithiothreitol (DTT)) and by the subsequent addition of iodoacetamide to a final concentration of 50 mM (Sigma, St. Louis, MO). The samples were analyzed by 10-18% (Chapter 3) or 10-15% (Chapter 4) gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (274). ^{14}C protein molecular mass standards were obtained from either Bethesda Research Laboratories (low molecular mass marker) or Amersham (high molecular mass marker). Both high and low prestained molecular mass standards were bought from Bethesda Research Laboratories. Following electrophoresis for approximately 16 hours at 14 mA, the gels were fixed for 45-60 minutes in 10% glacial acetic acid-25% isopropanol and treated for 30-45 minutes with Amplify (Amersham, Oakville, Ont.). Alternatively, 10% glacial acetic acid-30% methanol and 1 M sodium salicylate in 30% methanol were used. The gels were finally dried in a Bio-Rad model 583 gel dryer for 2 hours at 80°C and put on Kodak's XAR-5, Kodak's XRP-1, or Amersham's Hyperfilm MP X-ray film.

2.11- Densitometry analysis

The quantification of signals obtained by autoradiography, when appropriate, was determined by densitometry using a Molecular Dynamics laser densitometer and the Image Quant Software. A rectangular scanning window was set to encompass the entire width of the bands so to as not obtained biased results due to uneven signal across the bands. The integration of each signal was expressed in arbitrary units and usually expressed as a

percentage of a control band contained on the same gel, as indicated. In some cases, different exposures of the same gels were scanned and averaged. They, however, were not considered separate experiments ('n' value).

2.12- Endoglycosidase H analysis

When desired, the immunoprecipitated proteins were treated with endo H. Following the wash in buffer D (see Immunoprecipitation above), the bead bound immunoprecipitated proteins were split in two and incubated overnight in 12 µl of 70 mM sodium citrate pH 5.5 without (mock) or with 2.5 mU of endo H (Boehringer, Laval, Qc). The digestion was boosted with an equivalent amount of enzyme and digestion buffer for a total digestion time of 22-30 hours. The addition of 25 µl of 2 x sample buffer stopped the reaction. The samples were boiled 5 minutes and loaded as usual on SDS-PAGE gels. Digestion of the ^{14}C high molecular mass standards provided a positive control for the enzyme digestion since the 43 kDa marker (ovalbumin) was endo H sensitive.

2.13- 2D gel electrophoresis

When suitable, the samples were analyzed by 2D gel electrophoresis. Separation of the radiolabeled proteins was first achieved by isoelectrofocusing (IEF) gel electrophoresis in a GT Series gel electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, CA) as follows. The samples were loaded in 50 µl of lysis buffer (9.8 M urea, 2% NP40, 2% carrier ampholytes pH 7-9, 100 mM DTT) and electrophoresed for 20 hours at 400 volts. The first dimension gels were removed from the apparatus and put in equilibration buffer

(0.001% bromophenol blue, 60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT). If required, these first dimension gels were kept at -20°C. The proteins were further resolved by SDS-PAGE by layering the first dimension gels on top of 10% polyacrylamide gels. The samples were submitted to electrophoresis at 14 mA for approximately 16 hours. The resulting 2D gels were fixed, amplified, dried and put on X-ray film as for 1D gels (see above).

2.14- Statistical analysis

Where appropriate, the statistical significance of the results was calculated with the Student's T test, corrected for small samples ($n<30$). The p values expressed were for bilateral tests. Error bars on the histograms represent the standard deviation of each mean.

CHAPTER 3

MODULATION OF THE MHC CLASS I ANTIGEN PROCESSING AND PRESENTATION PATHWAY BY ADENOVIRUS TYPE 2

3.1- INTRODUCTION

As discussed in detail in Chapter 1, the immune system is a complex mechanism which detects and eliminates pathogens and foreign proteins. Survival of pathogens can thus be intimately linked to their ability to evade, at least temporarily, recognition and destruction by the immune system. The examination of such modulation can not only yield information on the mechanism by which a given pathogen circumvents immune responses but also on the immune system itself. The study related herein examined the interaction of one such pathogen, Ad2, with the MHC class I processing and presentation pathway.

3.1.1- Adenoviruses

Since the discovery of Adenoviruses in 1953 by Rowe *et al.* (275) and in 1954 by Hilleman and Werner (276), forty-seven distinct antigenic types of human Adenoviruses have been identified (277). A number of Adenoviruses that infect other species are also known (278) and belong, with the human Adenoviruses, to the Adenoviridae family which is composed of two genera: 1- Mastadenovirus (Adenoviruses infecting mammals) and 2- Aviadenovirus (Adenoviruses with avian hosts). These genera are further subdivided based on the adenoviral hosts (ex.: Human, ovine, bovine, etc.) (278). There may also be

Adenoviruses growing in poikilotherms that would represent a group distinct from the above two (278).

Numerous classification schemes exist for human Adenoviruses including classifications based on hemagglutination properties (279), oncogenicity (280), restriction cleavage patterns (281), molecular masses of the polypeptides V, VI and VII (282), GC content (283), and DNA homology (284). Table 1 shows an overall classification of human Adenoviruses based on DNA homology.

3.1.2- Clinical implications

Adenoviruses are prevalent pathogens in infants (285,286). They are associated with a variety of diseases, primarily respiratory, ocular, and gastrointestinal. The most common ailments include acute respiratory diseases, epidemic keratoconjunctivitis, and pharyngoconjunctival fevers (285). Adenoviruses manifest their presence at the cellular level by causing a cytopathic effect (cpe). This consists in the rounding of the infected cells, a significant increase in the refraction of the plasma membrane, and the appearance of rough and convoluted nuclear membranes. This is followed by the lysis of the infected cells and the release of the virus ((285) and personal observations). At another level, the establishment of animal models (287-293) has allowed the clinical characterization of the pathological effects of Adenoviruses. Hence, infiltration of the respiratory tissues by monocytes, macrophages, lymphocytes, and polymorphonuclear cells is observed in humans (294) and in experimentally infected mice (287) or cotton rats (289,292,293). While the initial pathologic effects of Adenoviruses are possibly non specific, the presence of T cells in the lungs at later stages indicates a specific immune response (287,289,292). However, replication of the virus does

Table 1: Classification of Adenoviruses

The classification of the 47 currently known Adenoviruses is shown. The classification scheme is based on DNA homology, as indicated in the table. The oncogenicity and pathogenicity of the various serotypes are also indicated.

Reprinted from (277).

Group	Types	DNA homology	Tumors in newborn† hamsters	Pathogenicity and epidemiology‡
A	12, 18, 31	48–69% within group; 8–20% with other groups	High: tumors in most animals in 4 months	Associated with upper respiratory infections and diarrhea; isolated from stools of children
B	3, 7, 11, 14, 16, 21, 34, 35	89–94% within group; 9–20% with other types	Weak: tumors in a few animals in 4–18 months	Common (3,7); moderate to severe infections; acute respiratory disease epidemics among military recruits (7, also 3, 14, 21); "bad colds" and severe illness (7); summer pharyngoconjunctival fever (3, 7); pharyngitis (3, 7); febrile pneumonia (3, 7); acute hemorrhagic cystitis (11, occasionally 21); isolated from kidneys and lungs of immunocompromised patients (35, 35)
C	1, 2, 5, 6	99–100% within group; 10–16% with other types	Nil	Most common (esp. 2, 1); mild to severe infections of upper respiratory tract (especially nasopharynx) of infants and children; latent infection of lymphoid tissue; readily isolated from throat and anal swabs of children
D	8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 33, 36, 37, 38, 39, 42, 43, 44, 45, 46, 47	94–99% within group; 4–17% with other types	Nil, Ad9 induces mammary fibroadenomas in rats	Ad8 and Ad19 commonly cause epidemic keratoconjunctivitis; isolated from stool; Ad37 is associated with urethritis and cervicitis
E	4	4–23% with other types	Nil	Acute respiratory disease epidemics in military recruits; cause of epidemic keratoconjunctivitis, severe pharyngoconjunctival fever and pneumonia
F	40, 41	?	?	Fastidious in culture; gasteroenteritis in infants

Taken from Green *et al.* (1979*a*), Straus (1984) and Hierholzer *et al.* (1988*a*).
† Types in groups A to E have been shown to transform cells *in vitro*.
‡ Numbers in parentheses indicate group types.

not appear to be essential for the pathology of the virus, since abortive infections do not affect the pathologic outcome of the infection (287,292). The recent production of transgenic mice encoding adenoviral DNA should prove useful in further defining the effect of Adenoviruses on their hosts (295).

Although adenoviral specific CTL are generated upon infection (296), there are various observations that point to the conclusion that Adenoviruses can resist, at least for some time, clearance by the immune system. For instance, while seroconversion against Adenoviruses generally occurs at an early age, Adenoviruses can be shed for months or years following infection (286) and latency has been observed (285,297,298). Immunocompromised patients such as neonates, transplant patients, immune-depressed cancer patients, or individuals infected with the Human immunodeficiency virus (HIV) are often infected with Adenoviruses (285,299,300). Furthermore, the isolation of Adenoviruses from patients is often accompanied by the concomitant isolation of bacteria, fungi, or other viruses (301,302). Finally, it has been shown that some of the human adenoviral strains can induce tumors in animals (303) or transform cells in tissue culture (303,304). Thus, these viruses survive in their hosts long enough to complete their life cycle and sometimes much longer. Interestingly, Adenoviruses appear to have devoted many genes to this task, as discussed in the next section.

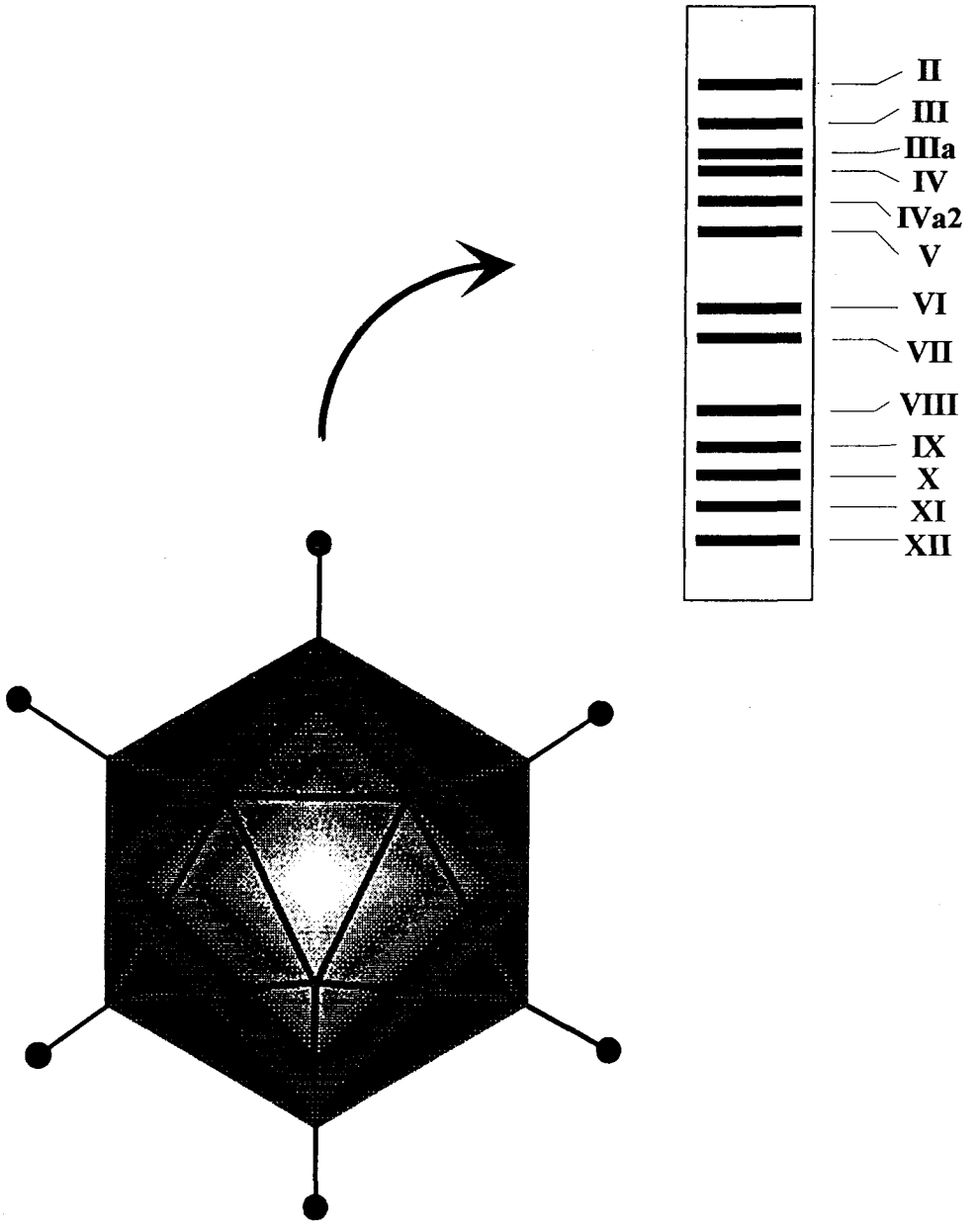
3.1.3- Arrangement of the Ad2 genome and its E3 region

Adenoviruses are non-enveloped viruses containing double-stranded linear DNA (305-308) with an icosahedral capsid (Fig. 13) (309). All human and non-human

Fig. 13: Structure of the Adenovirus virion

The architecture and composition of the adenoviral virion is shown. The virion is a non enveloped and icosahedral capsid that contains double stranded DNA. It is composed of numerous viral proteins, which can be resolved by gel electrophoresis as schematically depicted here. These proteins, with the exception of the fiber proteins found on the surface of the virions, are numbered in Roman numerals.

Adapted from (310).


II
III
IIIa
IV
IVa2
V
VI
VII
VIII
IX
X
XI
XII

serotypes studied so far share the same general genetic organization (311). They have a molecular weight of 16-30 x 10^6 (278,311), contain six early transcription regions (E1A, E1B, E2A, E2B, E3, E4), which are expressed prior to DNA synthesis, and five late transcription blocks (L1-L5) expressed following initiation of replication (311). Utilization of both strands, translation of the three coding frames, and differential splicing allow an economical and efficient use of the coding capacity of the virus (Fig. 14).

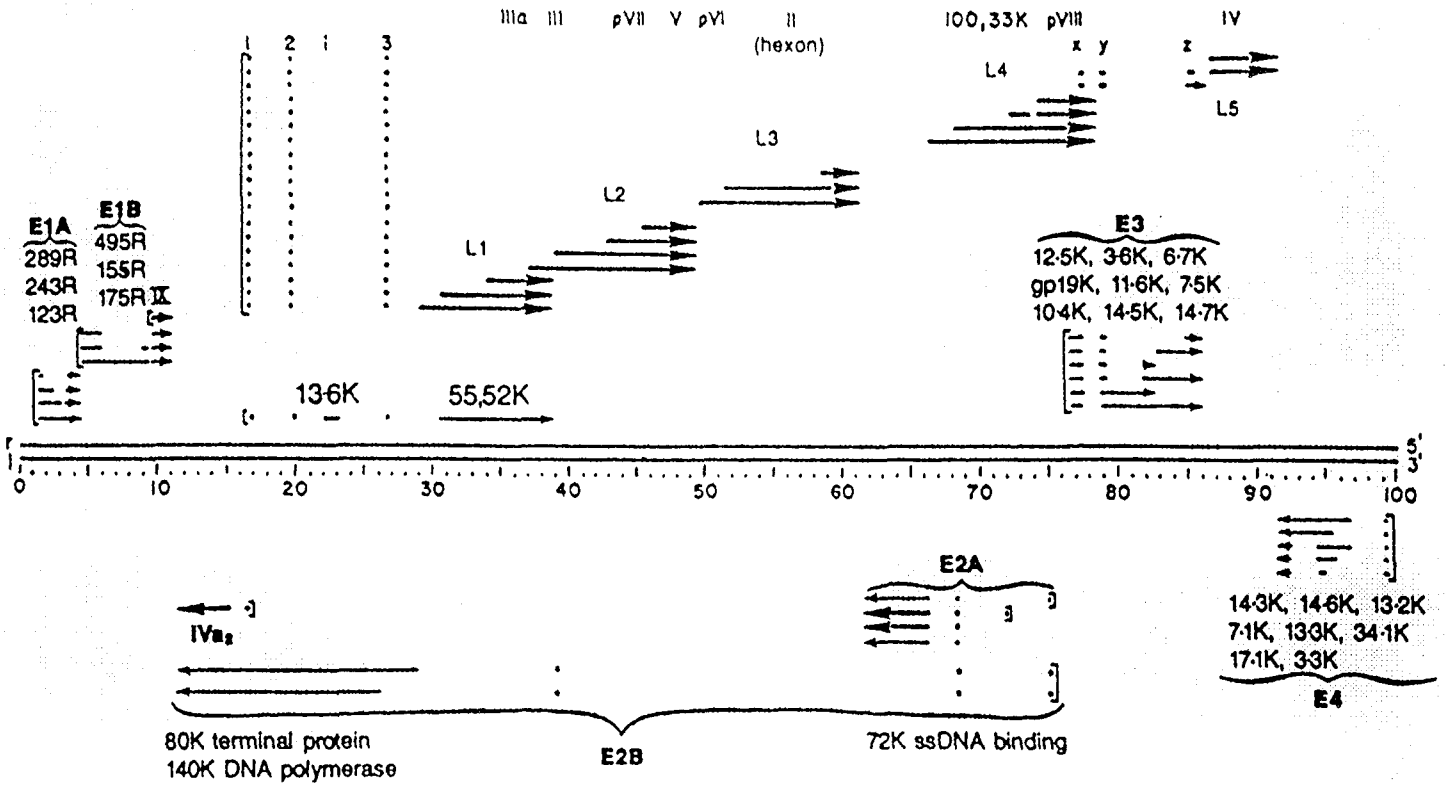
Interestingly, the adenoviral genome appears to be arranged functionally. The E1 region encodes the 243R and the 289R proteins which are involved in gene regulation and transformation (303,304,311). The E2 region encodes three proteins involved in DNA replication, namely the 140 kDa adenoviral DNA polymerase, the 72 kDa DNA binding protein, and the 87 kDa precursor of the 80 kDa terminal protein involved in initiation of DNA replication (311). The E4 region has only been poorly characterized to date. In Ad2, it encodes for approximately seven proteins (312,313) and appears to be non essential for the replication of the virus in tissue culture, except for the 34.1K protein (314). The late regions (L1-L5) mostly encode structural components of the adenoviral virions (310). Finally, the E3 region contains nine ORF named according to the molecular mass of the proteins they are predicted to encode (Fig. 14). The gene products for the E3/6.7K (315), E3/19K (258,316), E3/11.6K (259), E3/10.4K (317), E3/14.5K (318), and E3/14.7K (319) ORF have been detected. So far, no proteins corresponding to the E3/12.5K, E3/3.6K, and E3/7.5K ORF have been found. The function of the E3 region appears to protect infected cells from destruction by the immune system (310,320,321) and is discussed in greater details below.

Fig. 14: Transcription map of group C Adenoviruses

The various mRNA transcribed from the Adenovirus genome are represented. To this end, the viral genome is divided into 100 map units. The DNA strands designated R and L are transcribed rightward and leftward respectively. E1A, E1B, E2A, E2B, E3, and E4 mRNA are detected early in infection (before the onset of DNA replication), whereas the L1-L5 mRNA are late RNA species (detected only after the onset of DNA replication). However, the L1 mRNA are synthesized at early as well as at late times. The capped 5' ends of the cytoplasmic mRNA indicate the positions of transcriptional promoters while the arrowheads represent the 3' polyadenylation sites. Gaps in arrows indicate intervening sequences, which are removed from the mRNA by splicing. The proteins translated from these mRNA are designated by their molecular masses in kDa, by the number of amino acid residues they encode (E1 region), or by roman numerals (virion components). The particular protein sizes and masses indicated refer to the Ad2 genome.

This particular map applies to group C Adenoviruses and may not be directly assumed to be correct for other Adenoviruses. For instance, group B Adenoviruses differ from group A in that they contain two additional ORF in the E3 region (20.1K and 20.4K) (322). Finally, group A Adenoviruses have not been fully sequenced.

Adapted from (277).


IIIa III pVII V pVI II (hexon) 100,33K pVIII IV
1 2 i 3
x y z
L4 L5
L3
L2
L1
E1A E1B
289R 495R
243R 155R
123R 175R
E3
12·5K, 3·6K, 6·7K
gp19K, 11·6K, 7·5K
10·4K, 14·5K, 14·7K
13·6K
55,52K
5'
3'
0 10 20 30 40 50 60 70 80 90 100
E2A
14·3K, 14·6K, 13·2K
7·1K, 13·3K, 34·1K
17·1K, 3·3K
E4
IVa2
80K terminal protein
140K DNA polymerase
E2B
72K ssDNA binding

3.1.4- Immune evasiveness of Adenoviruses

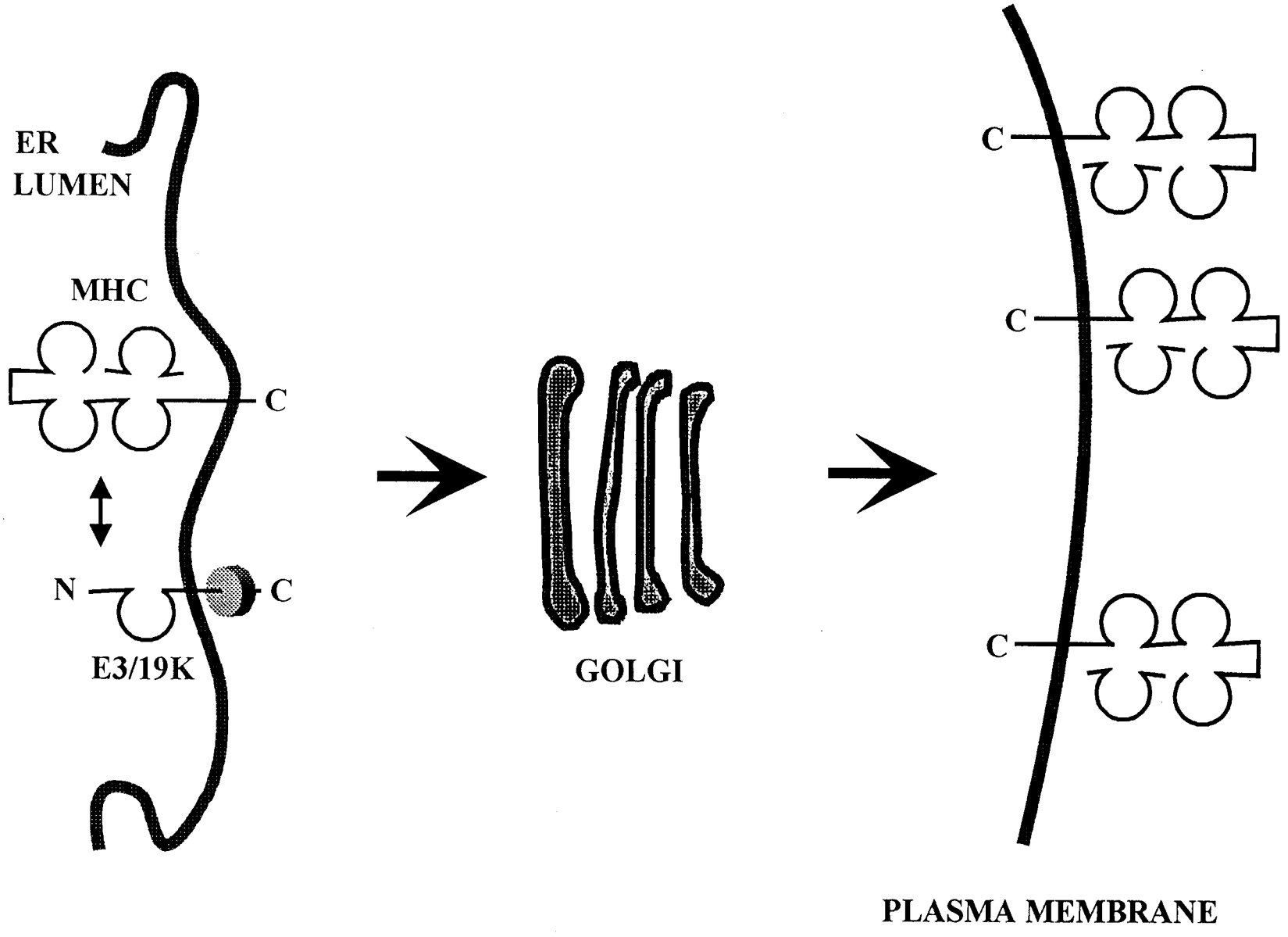
As indicated above, it appears Adenoviruses benefit from the evasion of immune surveillance. It is therefore not surprising that they have evolved mechanisms to actively modulate the immune response of their hosts. So far, a number of such mechanisms have been documented and are described below. Interestingly, a number of such mechanisms involve genes of the E3 region of the adenoviral genome.

3.1.4.1- Block of MHC class I surface expression by Adenoviruses

The modulation of MHC class I surface expression by Adenoviruses has been documented for group A and C Adenoviruses. The best studied case relates to the ability of group C Ad2 and Ad5 to block the surface expression of MHC class I proteins. As suggested above, this property has been mapped to the E3 region of the viral genome, and in particular to the E3/19K protein. This protein is a type I transmembrane protein (258,316,323) localized in the ER membrane (324). It blocks MHC class I surface expression by binding to the heavy chain of some MHC class I proteins and by retaining them in the ER (Fig. 15) (324-332). Binding of E3/19K to MHC class I molecules is independent of species barriers and has been observed with MHC class I proteins from mice (326,328,330,333,334), humans (324,325,329,331,334-338), rats (332,336,339), and monkeys (337). However, the strength of the binding of E3/19K to various MHC alleles varies considerably. In the murine system, Ad2 E3/19K efficiently binds to K^d (326,328,330,340,341), D^b (333), L^d (326), and possibly to K^b (333). However, it does not bind to the K^k (330,340,341), D^k (330,341), D^d (326,340), or the $H\text{-}2^q$ (328) alleles. Similarly, in humans, Ad2 E3/19K binds more strongly to HLA-A2 than to B7 (331,334,338) and only moderately to HLA-Aw68, B27, and Bw58 (331). In

Fig. 15: Block of MHC class I transport by the E3/19K protein

The interaction of Ad2 or Ad5 E3/19K with MHC class I molecules is shown. This interaction occurs by the binding in the ER lumen of E3/19K to the heavy chain of MHC class I proteins. This interaction blocks the egress of the heavy chains to the plasma membrane. The block results in the ER retention of the E3/19K-MHC class I complex and is due to the presence of an ER retention signal located in the cytoplasmic tail of E3/19K. N and C indicate the amino and carboxyl terminus respectively.


ER
LUMEN
MHC
C
N
C
E3/19K
GOLGI
C
C
C
PLASMA MEMBRANE

addition, an intermediate binding was found for HLA-A3, A1, and Aw69 proteins (331). In contrast to the H-2 alleles, E3/19K binds to all the HLA alleles that have been examined. However, this difference need not be considered fundamental since the binding of the HLA alleles quantitatively reflects the qualitative differences seen in the murine system. Furthermore, only a hand full of human alleles have been tested thus far (331,334,335,342).

The detailed examination of the binding between E3/19K and MHC class I molecules is currently under study. It appears that the E3/19K residues essential for its binding to MHC class I proteins are localized in the ER lumenal domain of the protein (266,343) and possibly include most of those residues (344). This suggests that the overall three dimensional conformation of the E3/19K protein is critical for its interaction with MHC class I molecules. This conclusion is further supported by the lack of interaction between E3/19K and MHC class I proteins when the E3/19K ER lumenal cysteine residues, likely involved in disulfide bonds, are replaced by serine residues (345). On the other hand, the MHC class I residues mediating binding to E3/19K have been localized in the α_1 and α_2 domains (330,331,340). Finally, the binding of E3/19K and MHC class I proteins is not affected by the N-linked carbohydrates present on either molecule (325) and is independent of the presence of β_2M (334,346).

The mechanism of ER retention of MHC class I proteins by E3/19K is only partially understood. Mutational studies indicate that deletion of part or all the E3/19K cytoplasmic tail results in the egress of E3/19K out of the ER (266,326,327,346-348). In addition, transposition of the E3/19K cytoplasmic tail onto non ER retained transmembrane proteins results in their ER retention (327,348). Comparison of the E3/19K ER cytoplasmic sequences

with other ER retained molecules indicates that E3/19K does not contain the well-characterized KDEL ER retention signal (349). Although the precise boundaries of the E3/19K ER retention signal have yet to be agreed upon, the current data points to the need for most of the E3/19K cytoplasmic tail.

Significantly, the block of MHC class I surface expression by Ad2 results in a lower susceptibility of Adenovirus infected cells to killing by Adenovirus specific CTL (328,332,344,350). Interestingly, this protection is not limited to Adenoviruses, since Ad2 also protects against the presentation of SV40 (333), Influenza (326), Vaccinia (326), and allogeneic peptides (342). It is believed that this represents a significant mechanism by which group C Adenoviruses evade immune surveillance (277,287,292,320,324,328,342,351). Surprisingly, *in vivo* studies indicate that the presence of E3/19K diminishes the severity and delays the pathogenesis caused by Adenoviruses (292), indicating that the immune response is partly responsible for the pathogenesis observed. This suggests that the function of E3/19K may not be to hide the virally infected cells from the immune system but to limit the damage done by the immune system to the host. Consistently, the replication efficiency of Adenoviruses in animals (287,292) and the priming of naive CTL in mice (350) is not affected by the E3/19K protein.

Apart from the block of the MHC class I transport by Ad2 and Ad5, Adenoviruses also modulate the surface expression of MHC class I proteins by interfering with their synthesis. This mechanism is particular to the group A Adenoviruses, of which the Ad12 serotype is the best characterized. It is unclear why groups A and C have evolved different mechanisms, but it has been suggested that this may relate to their different oncogenicity

(352,353). For instance, it has been shown that the transformation of primary rat, mouse, or human cells by Ad12 results in the block MHC class I surface expression (353-355). This block has been observed for all alleles observed and crosses the species barrier (354). Consistent with group C Adenoviruses, this down regulation of MHC class I surface expression by group A Adenoviruses results in the protection of Ad12 transformed primary cells against allogeneic CTL (352,355). It also protects Ad12 transformed and Influenza infected cells against Influenza specific CTL (356). Contrary to group C Adenoviruses, the ability of group A Adenoviruses to modulate the surface expression of MHC class I proteins maps to the E1A region of the genome, and in particular to the viral protein encoded by the 13S mRNA (352). The exact mechanism of action of the Ad12 E1A is still under debate. Proposed explanations include the down regulation of MHC class I transcription (353-355,357-360), the post-transcriptional regulation of MHC class I mRNA (360), and the down regulation of the TAP2 peptide transporter protein (361).

3.1.4.2- Other mechanisms of immune evasiveness

In addition to the mechanisms described above, Adenoviruses can modulate the immune system of their hosts by a variety of other means. Interestingly, this modulation often involves the E3 region of their genome. For instance, the expression of the E1A gene products, essential for the efficient replication of the virus (362), also results in the presentation of E1A peptides that are presented to CTL in some MHC class I backgrounds (26,42,296,363,364). To counteract this effect, the virus timely modulates its E1A expression to reduce the expression of E1A (363,365-367). Mapping studies indicates that, among other

viral proteins, two E3 viral proteins, E3/10.4K and E3/14.5K, may be responsible for this phenotype (367).

An additional mechanism that Adenoviruses have evolved to modulate immune responses relates to the tumor necrosis factor (TNF) cytokine. TNF is secreted by activated macrophages and monocytes in response to an inflammatory stimulus (368,369). Although, cells are usually resistant to TNF (369,370), infection by Adenoviruses induces sensitivity to the cytokine (371). To impede this effect, the Adenovirus genome encodes four proteins that protect infected cells from TNF. Again, the E3 region of the genome appears to play an important role in the regulation of this immune function. Thus, three E3 proteins, E3/14.7K, E3/10.4K, and E3/14.5K mediate resistance to TNF (261,350). Although the E1B/19K protein also confers resistance against this cytokine, the E3/14.7K is the main mediator of TNF resistance (261,350). Interestingly, TNF expression up regulates the expression of the E3 region (372), thus suggesting that the viruses have evolved a finely tuned mechanism to negate the effect of the cytokine.

Finally, to successfully infect and replicate in their hosts, Adenoviruses may have evolved yet another mechanism. It has been documented that Adenoviruses can persist in lymphoid cells (291). It has also been shown that the adenoviral E3 promoter, directing the expression of immunologically relevant viral proteins (see above), binds transcription factors found in lymphoid cells and not in HeLa cells (373). In particular, it has been shown that the transcription factor Nf_k B can bind the E3 promoter and induce E1A independent E3 transcription (374). Assuming that this interaction results in the enhancement of the immune

evasiveness of Adenoviruses, this could contribute to the prevalent persistence of the viruses in lymphoid cells.

3.1.5- Rationale to study E3/6.7K

As discussed above, Adenoviruses have evolved a number of complementary mechanisms to help them evade the immune system. Many of the proteins implicated are located in the E3 region of the viral genome. However, this region also contains genes for which no function has been found so far. They include the E3/6.7K protein and the putative E3/3.6K, E3/12.5K and E3/7.5K proteins. The general grouping of the adenoviral genome by function (see above) suggests that those gene products might be immunomodulators. The contiguous arrangement of E3/19K and E3/6.7K on the same mRNA indicates cotranscriptional regulation of the two viral proteins, thus suggesting a functional complementarity. Moreover, previous studies involving E3/19K transfectants also included other ORF including the E3/6.7K gene (325,328,330,335,340,346). This prompted us to speculate that E3/6.7K may play a role in circumventing the host immune response.

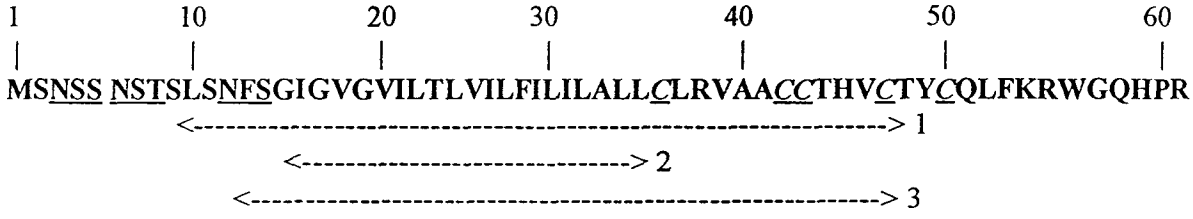
3.1.6- Characteristics of the Ad2 E3/6.7K protein

Although the function of the E3/6.7K protein is a complete mystery, it has over the past few years been partly characterized. Hence, the Ad2 genome encodes a predicted E3/6.7K protein of 61 amino acids with a molecular mass of 6671 (Fig. 16). Despite the presence of an initial methionine residue in the predicted E3/6.7K protein sequence, the E3/6.7K protein has never been detected in experiments using ^{35}S methionine as a metabolic label. Consistently, Wilson-Rawls *et al.* (315) showed, using ^{35}S cysteine as label, that the

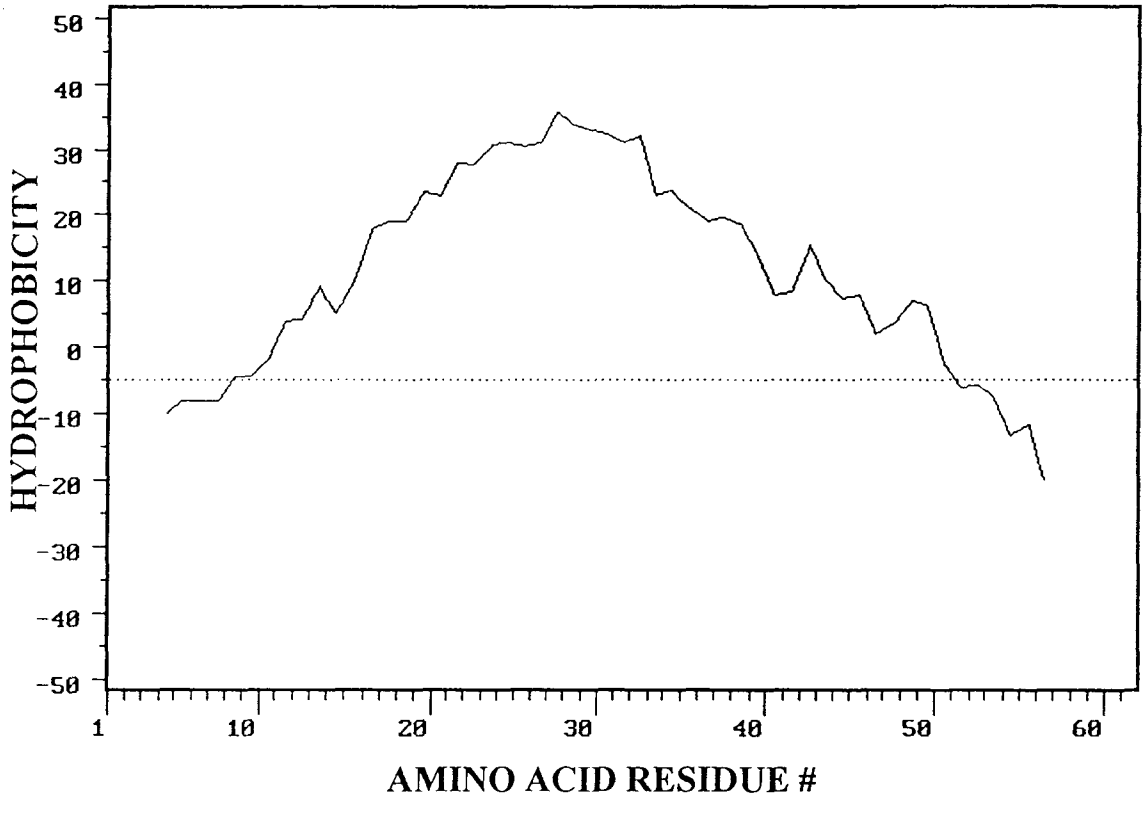
Fig. 16: Properties of the Ad2 E3/6.7K protein

The sequence of the Ad2 E3/6.7K protein was analyzed by computer analysis using the software PCGENE from Intelligenetics. The protein sequence was deduced by translation of its ORF from the known sequence of the Ad2 genome. Potential N-linked glycosylation sites and cysteine residues possibly involved in the formation of disulfide bonds are underlined (upper panel). E3/6.7K has no signal sequence but is predicted to be an integral protein (dotted arrows) according to the three algorithms used (1- Klein, Kanehisa, and DeLisi ((375), SOAP program), 2- Eisenberg, Schwarz, Komaromy, and Wall, ((376), HELIXMEM program) and 3- Rao and Argos ((377), RAOARGOS program)). In the lower panel, the hydrophobicity plot of Ad2 E3/6.7K indicates that this protein is largely hydrophobic, according to the PCGENE program "SOAP" using the algorithm of Kyte and Doolittle (269). Residues above the median at y=0 are hydrophobic whereas the residues below the median are hydrophilic. In all cases, the default parameters suggested by the PCGENE software were used.

Ad2 E3/6.7K



1- Klein, Kanehisa, and DeLisi
2- Eisenberg, Schwarz, Komarony, and Wall
3- Rao and Argos



E3/6.7K ORF does indeed encode a gene product of the predicted molecular mass. It has since been shown that E3/6.7K is an N-linked glycosylated (271) transmembrane protein (315). Immunoprecipitation studies reveal two polypeptides, the unglycosylated 7-8 kDa E3/6.7K polypeptide and the glycosylated E3/6.7K 15-16 kDa polypeptide (315). Wilson-Rawls *et al.* (271) also showed by immunofluorescence and by endo H sensitivity that E3/6.7K is an ER localized protein. Unfortunately, the E3/6.7K molecule does not have any protein or DNA homology to other known molecules, besides E3/6.7K proteins found in other Adenovirus serotypes. It shares, however, structural features with some proteins. These include the Influenza M2 matrix protein (378), the IsK protein (379), Ad2 E3/11.6K (380), the Influenza B NB protein (381), the Rotavirus glycoprotein NS28 (382), and the sialoglycoprotein β (383). All of these molecules are fairly short proteins with a signal anchor and no signal sequence, and do not assume a type I or II transmembrane orientation (Table 2). Interestingly two of these proteins, M2 and IsK, have ion channel activity (384,385). Despite these discoveries, the function of the E3/6.7K protein has not been elucidated.

3.1.7- Objectives

Given the role of the E3 region in the modulation of immune responses and the cotranscriptional regulation of E3/6.7K with the immunologically relevant E3/19K protein, the objective of this study was to study the properties and function of the E3/6.7K protein. In particular, the role of E3/6.7K on the surface expression of MHC class I proteins was examined.

Table 2: Proteins structurally related to Ad2 E3/6.7K

The proteins structurally homologous to E3/6.7K are depicted. The length of both the E3/6.7K and E3/11.6K proteins is for the Ad2 serotype. M2 is the Influenza A Matrix 2 protein. NB is the Influenza NB protein. NS28 is the non structural glycoprotein 28 from Rotavirus. β is the sialoglycoprotein β protein. IsK is the slow potassium ion channel protein, also referred to as Mink.

The characteristics of those homologues are indicated as follows. TM: Number of predicted transmembrane domains; aa: amino acids; Sig. Seq.: Cleavable signal sequence at the amino terminus; Homology: Protein homology to the Ad2 E3/6.7K (calculated with the PCGENE program 'PALGN' (386) using the default parameters suggested by the program).

Protein	Length	TM	Sig. Seq.	Homology
E3/6.7K	61 aa	1	none	N/A
E3/11.6K	101 aa	1	none	7%
M2	97 aa	1	none	13%
NB	99 aa	1	none	25%
NS28	175 aa	1	none	2%
β	128 aa	1	none	13%
IsK	129 aa	1 or 2	none	3%

3.1.8- Approach

Because wt Ad2 encodes both E3/19K and E3/6.7K (along with a variety of other viral proteins), it was difficult to specifically analyze the role of the E3/6.7K protein. To directly assess the effect of E3/6.7K, and of E3/19K without E3.6/7K, on MHC class I cell surface expression, we made use of mutant viruses bearing various deletions in the E3 region of the genome. The deletions encompass the E3/6.7K and/or the E3/19K genes or the entire E3 region. The processing and the surface expression of MHC class I molecules were thus examined in cells infected with these mutants.

3.2- RESULTS

3.2.1- Optimization of the block of MHC class I surface expression by Ad2

As discussed in details in the Introduction to this chapter, infection of cells by Ad2 can block the surface expression of MHC class I proteins. However, this block is not absolute as some MHC proteins can still be detected at the plasma membrane. Thus, to study the role of E3/6.7K on MHC class I surface expression, the determination of the parameters maximizing the inhibition of MHC class I surface expression was important to enable the most sensitive monitoring possible. This could presumably allow the detection of more subtle changes caused by the virus. Therefore, in a first step, MHC class I surface expression was examined in wt Ad2 infected 293 cells (MOI = 50) and analyzed late in the replication cycle (20 hpi). Infection of this cell line yielded a 66% reduction of the MHC class I surface expression compared to mock treated cells (Fig. 17). In an attempt to further reduce MHC class I plasma membrane expression, the kinetics of MHC modulation by wt Ad2 was examined by FACS analysis. An initial MOI of 50 was used to ensure that all cells were infected. By 10 to 12 hpi, Ad2 significantly reduced MHC class I surface expression. By 20 hpi, this down regulation was stable at 44% $\pm$ 1 of mock levels (Fig. 18). In a further attempt to reduce MHC surface expression, 293 cells were infected at higher MOI and analyzed as above. Despite the higher amount of viruses, no further significant reduction of MHC surface expression was obtained (Fig. 19). Thus, the lowest MHC class I surface expression achieved was between 41 and 49% of mock levels. Since high MOI did not result in a decreased MHC

Fig. 17: Down regulation of MHC class I surface expression by Ad2

Subconfluent dishes of 293 cells were infected with Ad2 at a MOI of 50 as indicated in Materials and Methods. The infected cells were removed from the dishes with versene at 20 hpi and the level of MHC class I surface expression estimated by FACS analysis. The monoclonal W6/32 antibody and secondary GAM-FITC or GAM-FITC alone were used. The samples were then analyzed by flow cytometry. The data are expressed as the percentage of MHC class I surface expression relative to the mock infected cells, arbitrarily set at 100%, and calculated as in Materials and Methods. Typical FACS profiles are shown. The logarithmic fluorescence values were converted to linear values and are shown in the top left corner of each profile. For this particular experiment, the level of MHC class I surface expression was 34% (the average of 23 separate experiments was 40% $\pm$ 7; Table 4). The background fluorescence for the no first antibody (NFA) is indicated in panel a.

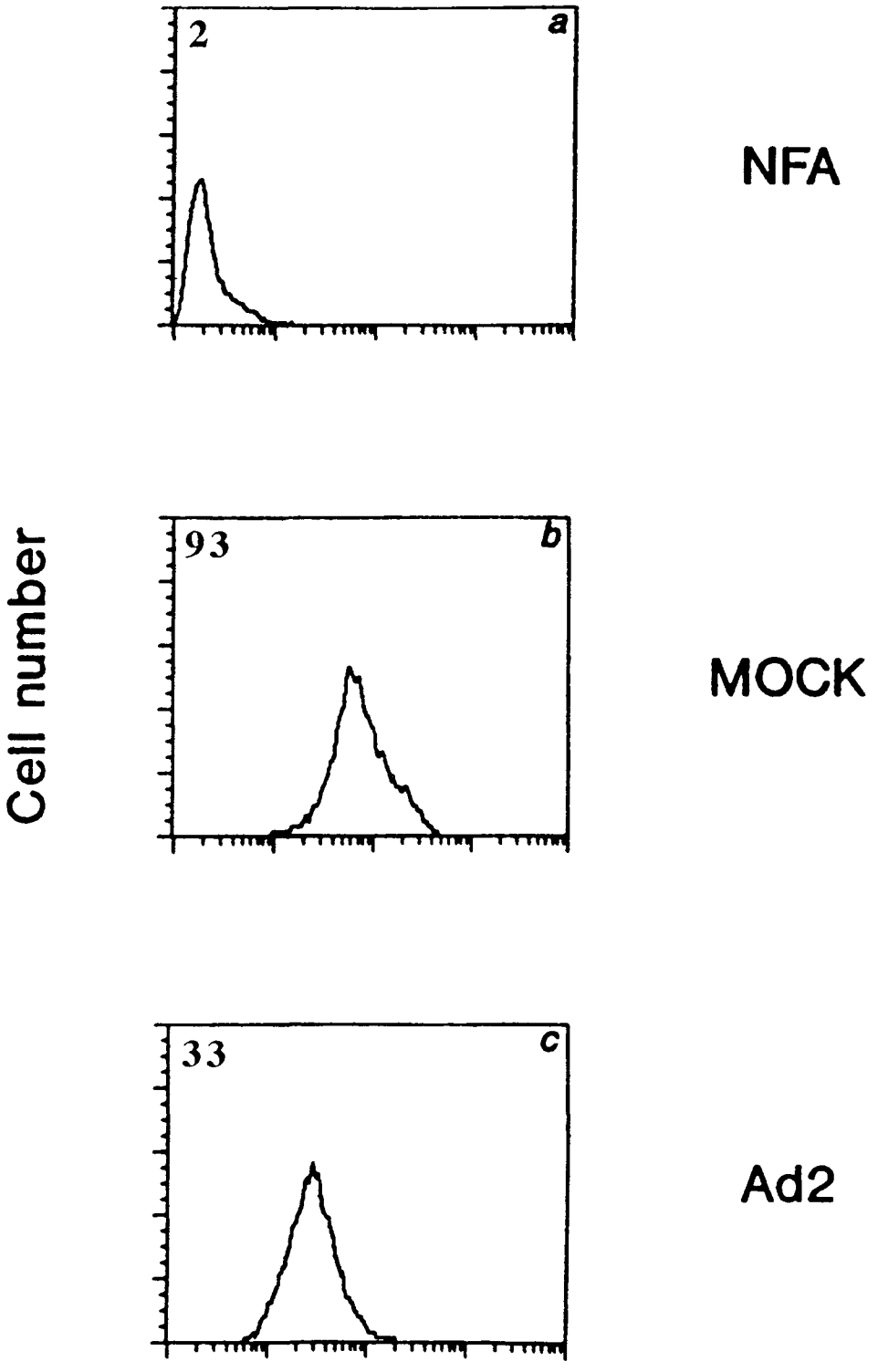

2
a
NFA
93
b
MOCK
33
c
Ad2
Cell number
Log of fluorescence intensity

Fig. 18: Kinetics of the inhibition of MHC class I surface expression by Ad2

A FACS analysis was performed on Ad2 infected cells to determine the expression of MHC class I molecules on their cell surface at different times post infection. The 293 cell line was mock treated or infected with Ad2 at a MOI of 50. At the indicated times, the cells were removed from the dishes and incubated with W6/32 and GAM-FITC or GAM-FITC only. The samples were then analyzed by flow cytometry as in figure 17. By 20 hpi or so the level of surface expression of class I molecules in infected cells reached a plateau and was on average 44% $\pm$ 1 (n=2) compared to mock infected cells.

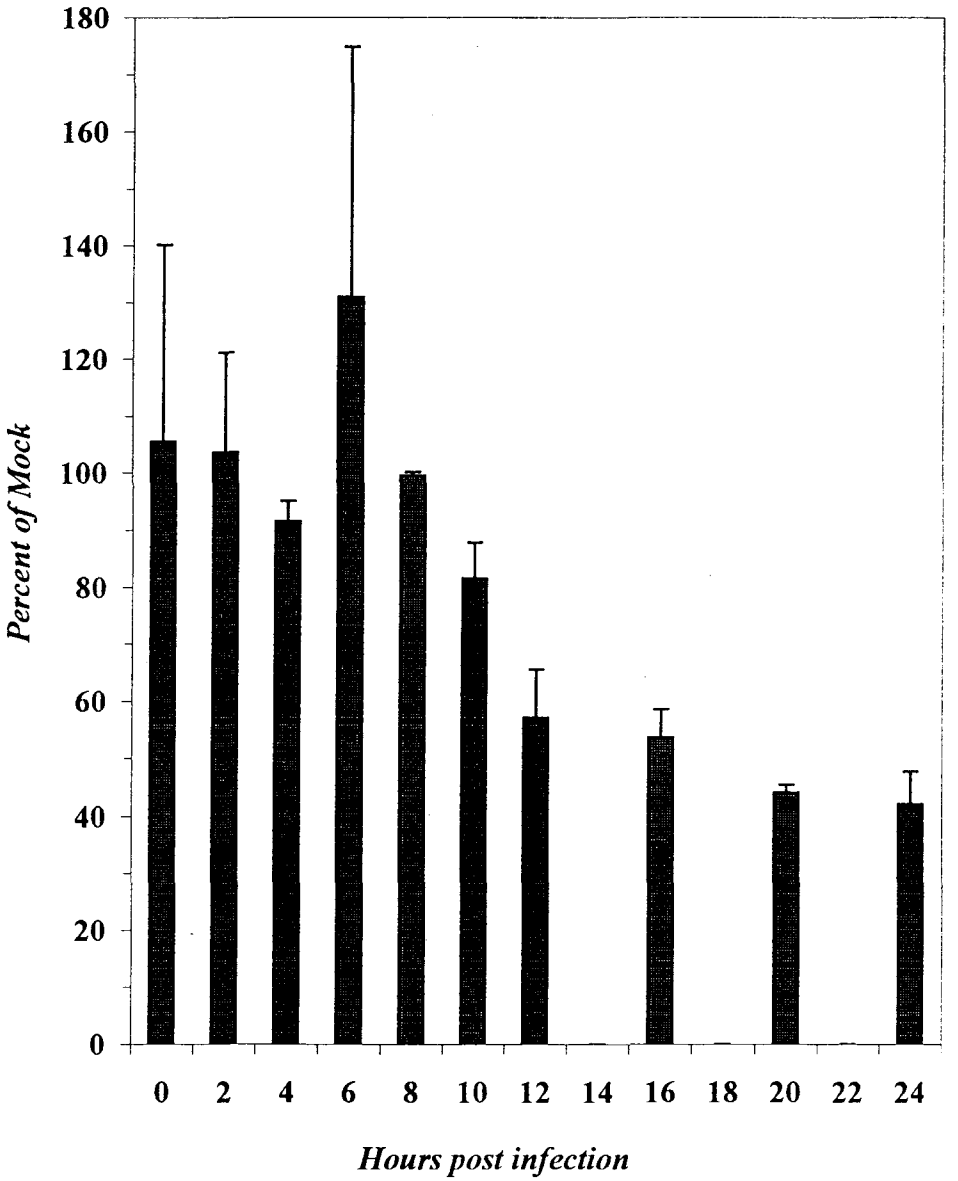
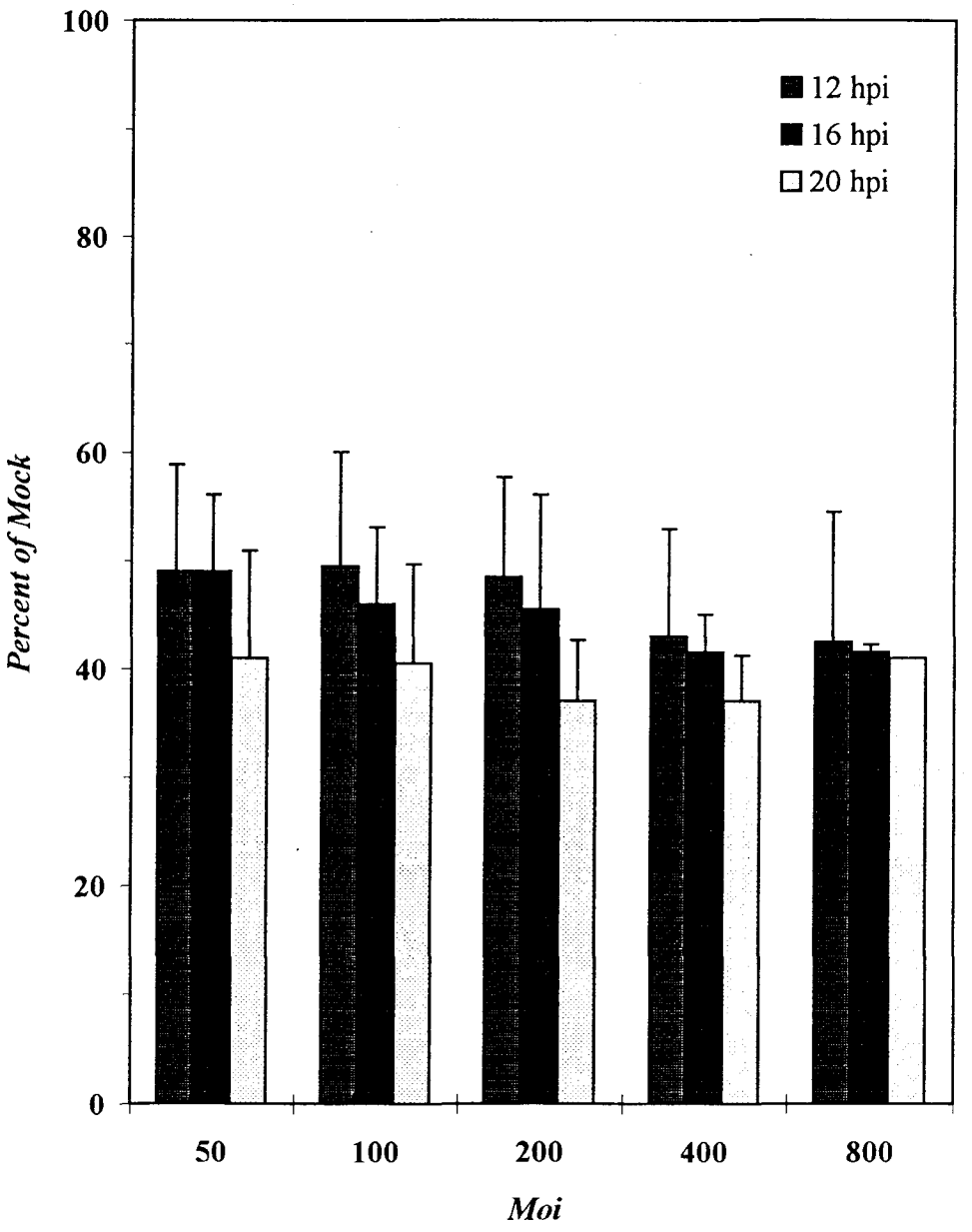

180
160
140
120
100
80
60
40
20
0
Percent of Mock
0
2
4
6
8
10
12
14
16
18
20
22
24
Hours post infection

Fig. 19: Effect of MOI on MHC class I surface expression

Subconfluent 293 cells were infected with Ad2 as in figure 17 at MOI ranging from 50 to 800. MHC class I surface expression was evaluated at 12, 16, and 20 hpi by FACS analysis as before using the W6/32 primary antibody. The data represent the means and standard deviations of two independent experiments. Statistical analyses by the Student's T test indicated that there were no significant differences ($p>0.05$) at all the time points examined between MHC class I surface expression at MOI of 50 and 800 ($n=2$).


100
80
60
40
20
0
Percent of Mock
12 hpi
16 hpi
20 hpi
50
100
200
400
800
Moi

class I surface expression ($p>0.05$), most assays were subsequently done at 20 hpi using a MOI of 50.

3.2.2- Cell type specificity of the MHC class I down regulation by Ad2

The 293 cell line was originally derived from human embryonic kidney cells immortalized with DNA fragments from Ad5. It contains the E1 region of the virus in its genome and is highly permissive for Ad2 and Ad5 (387). This raised the possibility that the modulation by Ad2 of MHC class I surface expression seen in infected 293 cells required the endogenous E1 viral proteins and was therefore specific for this cell line. To examine this, the unrelated HeLa cell line, free of adenoviral sequences, was analyzed. As seen in figure 20, Ad2 did affect the surface expression MHC class I proteins in infected HeLa cells. However, its effect was much reduced compared to 293 cells (Fig. 19). As for the 293 cell line, the use of a higher MOI did not influence the level of surface expression of the MHC class I proteins on HeLa cells. To ascertain that the difference between the two cell lines was not due to a slower kinetics of Ad2 infection in HeLa cells, the MHC class I surface expression was monitored over 40 hours. The data revealed that Ad2 could indeed modulate the expression of MHC class I molecules at the surface of HeLa cells, starting at approximately 12-16 hpi and reaching a plateau by 36 hpi (Fig. 21). Significantly, MHC class I surface expression in infected HeLa cells at 40 hpi ($46\% \pm 1$) was reduced to a level comparable to that of infected 293 cells at 24 hpi ($38\% \pm 9$).

Since Adenoviruses are lytic viruses, it was possible that the MHC class I down regulation was caused by the imminent lysis of the infected cells rather than the specific modulation of MHC class I expression by Ad2. Therefore cell death in 293 and HeLa infected

Fig. 20: Modulation of MHC class I surface expression by Ad2 in HeLa cells

Subconfluent HeLa cells were infected with Ad2 and analyzed as in Fig 19. Student's T tests indicated that there were no significant differences ($p>0.05$) at all time points examined between MHC class I surface expression at MOI of 50 and 800 ($n=2$).

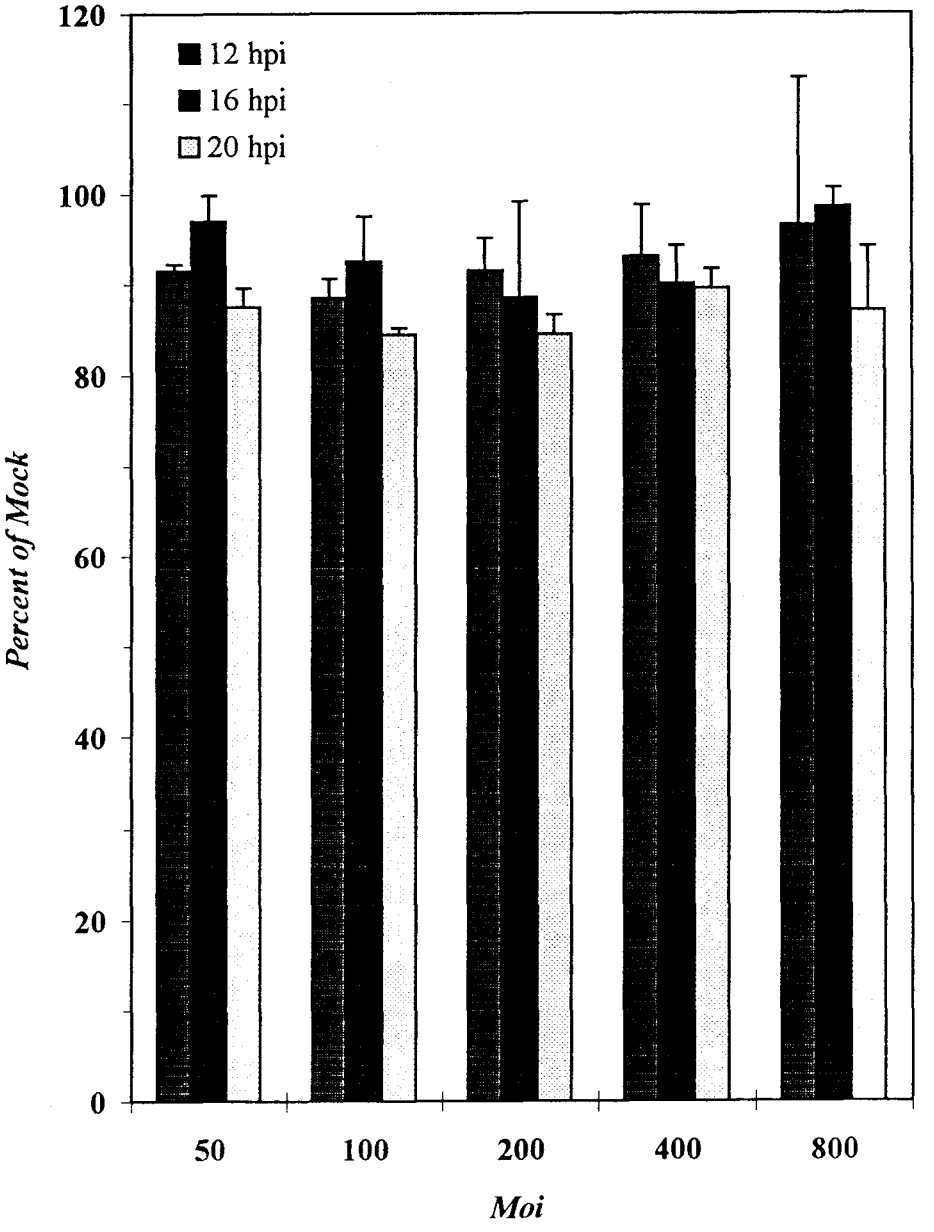
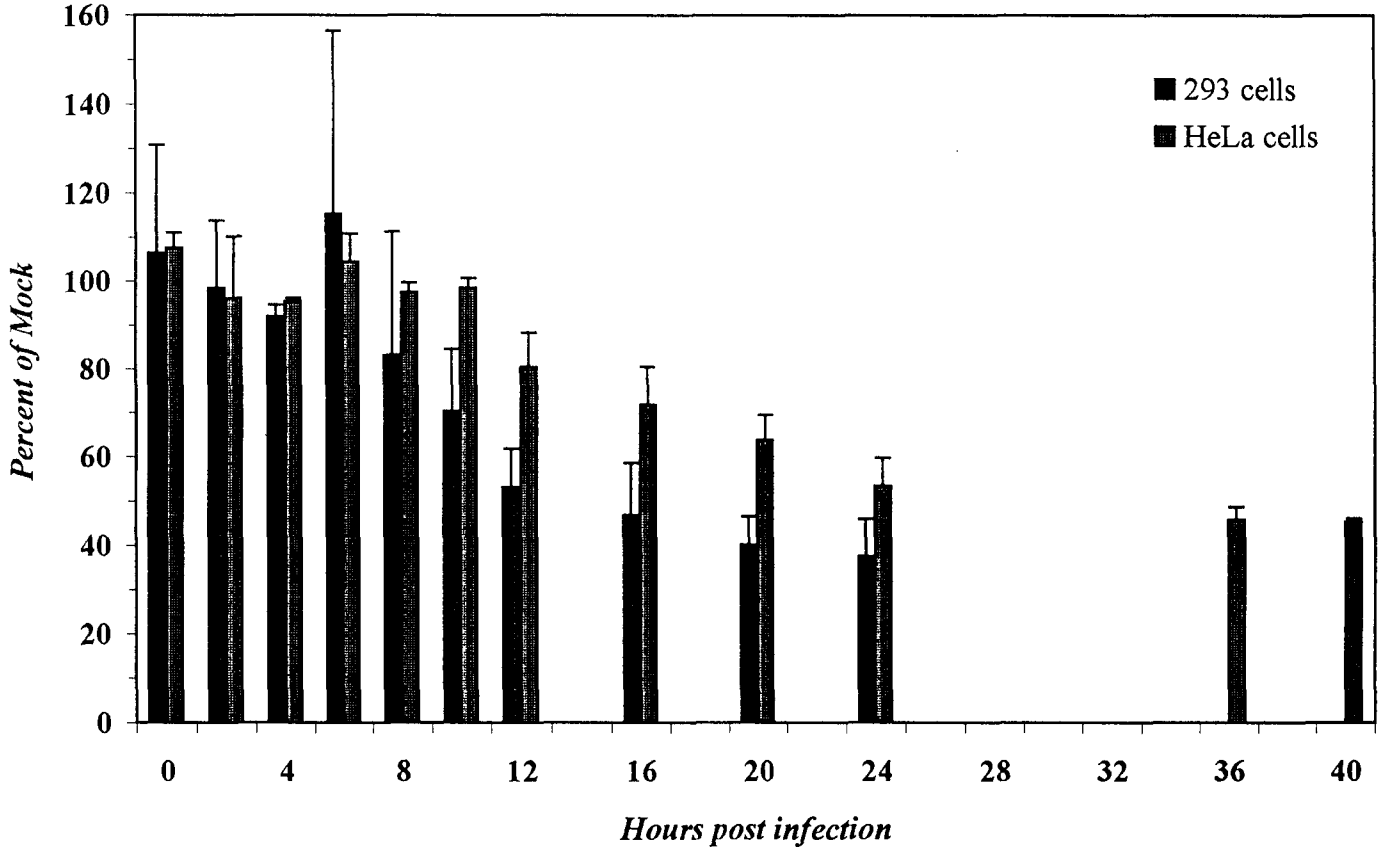

120
100
80
60
40
20
0
12 hpi
16 hpi
20 hpi
Percent of Mock
50
100
200
400
800
Moi

Fig. 21: Cell specificity of MHC class I down regulation

The surface expression of MHC class I was monitored by FACS analysis with W6/32 and GAM-FITC or GAM-FITC alone over a 40 hour period. 293 or HeLa cells were infected with Ad2 at a MOI of 50. The 293 cell line was not analyzed beyond 24 hpi, since under the condition used (MOI = 50) it exhibited significant cpe thereafter (50% cpe by 25-30 hpi, 95% cpe by 35-40 hpi; data not shown). Similarly, significant lysis of the HeLa cells due to the infection occurred by 40 hpi and 95% of the HeLa cells were lysed by 40-65 hpi (data not shown). See figure 17 and Materials and Methods for the experimental conditions.


160
140
120
100
80
60
40
20
0
Percent of Mock
293 cells
HeLa cells
0
4
8
12
16
20
24
28
32
36
40
Hours post infection

cells was crudely measured in parallel with MHC class I surface expression. Other cell lines, namely MRC5 and A549, were also used to avoid cell specific results. The tested hypothesis was that if cell death was solely responsible for the reduction of MHC class I expression at the cell surface, there should be a direct correlation between cpe and MHC class I surface inhibition. The results indicated that there was no correlation between inhibition of MHC class I surface expression and cpe in 293 cells (Table 3). This suggested that in 293 cells the effect of Ad2 on MHC class I molecules was not due to its lytic activity. Ad2 also modulated MHC class I surface expression on A549 cells beyond the cytopathic effect of the virus. However, in HeLa and MRC5 cells, the down regulation of MHC class I correlated fairly well with the cpe caused by the virus (Table 3). Therefore, Ad2 could modulate MHC class I surface expression irrespective of its lytic activity in some cells, but this effect was cell type dependent.

3.2.3- Detection of the E3/6.7K protein in infected cells

The functional analysis of E3/6.7K required an approach that could distinguish it from its bicistronic E3/19K partner. We took advantage of a bank of deletion mutant viruses lacking the gene for either E3/6.7K or E3/19K (Fig. 10). Hence, the E3/6.7K protein was examined with an αAd2 E3/6.7K antibody in 293 cells infected with wt or mutant viruses. Using the CH enhancing protocol (see Materials and Methods) and ^{35}S cysteine to allow the labeling of E3/6.7K proteins, we found that E3/6.7K migrated as two polypeptides; a 7-8 kDa polypeptide and a 15-16 kDa polypeptide (Fig. 22), thus confirming previously published results (315). Importantly, the detection of E3/6.7K in two distinct Ad2 infected human cell

Table 3: Impact of cell lysis on MHC class I surface expression

Four cell lines were examined to determine the role of cell lysis on the modulation of MHC class I surface expression. A549, HeLa, MRC5, and 293 cells were infected with Ad2 at a MOI of 50 and MHC class I surface expression determined by FACS analysis using the W6/32 antibody, as described in figure 17. Concurrent monitoring of the viral infection and its resulting cell lysis was crudely monitored by measurement of the cpe. In each case, the background cpe (mock dish) was subtracted to obtain the specific cpe caused by the viral infection. All the data are expressed as a percentage of the mock dish for comparison. Student's T tests were performed to analyze the data. Please note that for the 293 cell line, the reported surface expression was the average of all experiments done (from Table 4).

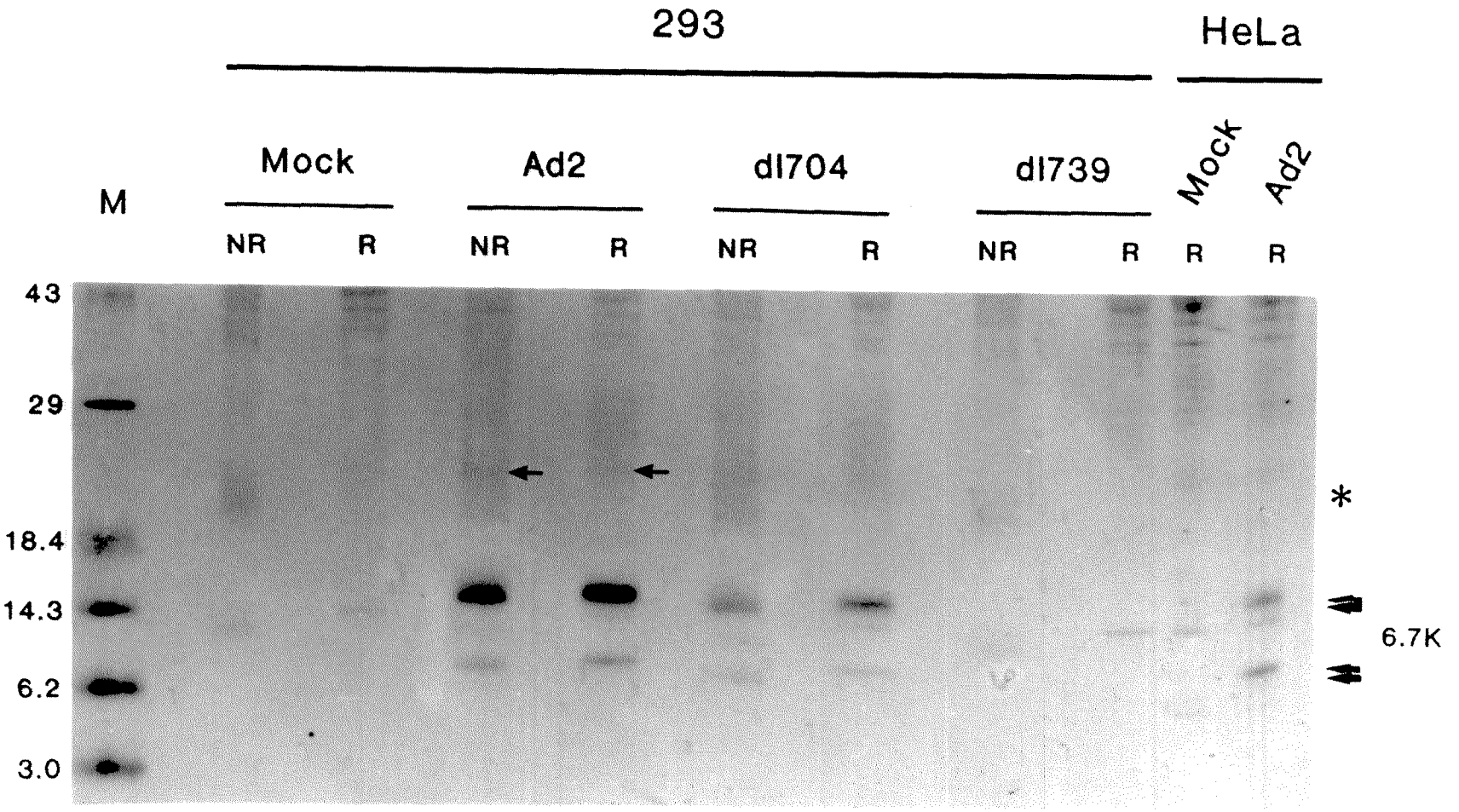
Please consult the Appendix for additional details.

Cell line	*Surface expression (% Mock)*	*Surface inhibition (100% - %Mock)*	*n*	*% CPE*	*n*	*T test*
A549	23 ± 3	77 ± 3	2	31 ± 18	3	0.001<p<0.05
HeLa	27 ± 4	73 ± 4	2	80 ± 16	3	p>0.05
MRC5	85 ± 7	15 ± 7	2	14 ± 8	3	p>0.05
293*	40 ± 7	60 ± 7	23	28 ± 2	2	p<0.001

*: Assay done at 40 hpi except for 293 cells, for which the assay was done at 24 hpi

Fig. 22: Detection of the Ad2 E3/6.7K protein

Mock treated or virally infected 293 and HeLa cells (MOI = 50) were metabolically labeled with ^{35}S cysteine using the CH enhancing procedure, as described in Materials and Methods. The pulsed cells were subsequently lysed and the lysates immunoprecipitated using the αAd2 E3/6.7K specific rabbit antiserum. The samples were analyzed on a 10-18% SDS-PAGE gradient gel under reducing (R) and non reducing (NR) conditions and by autoradiography. The molecular masses of the ^{14}C standards (M) are indicated on the left in kDa. For non reducing conditions, distilled deionized water replaced the DTT contained in the 1 x sample buffer and the subsequent addition of IAA (see Materials and Methods). The migration of the E3/6.7K polypeptides under reducing and non reducing conditions is marked by arrows on the right of the gel. A faint signal with a molecular mass corresponding to that of the E3/19K protein was detectable and is indicated to the right of the gel. The viruses used were as follows: Ad2: wt virus; dl704: E3/19K$^-$ deletion mutant; dl739: E3/6.7K$^-$ deletion mutant.


293
HeLa
Mock
Ad2
dl704
dl739
Mock
Ad2
M
NR
R
NR
R
NR
R
NR
R
R
R
43
29
18.4
14.3
6.2
3.0
*
6.7K

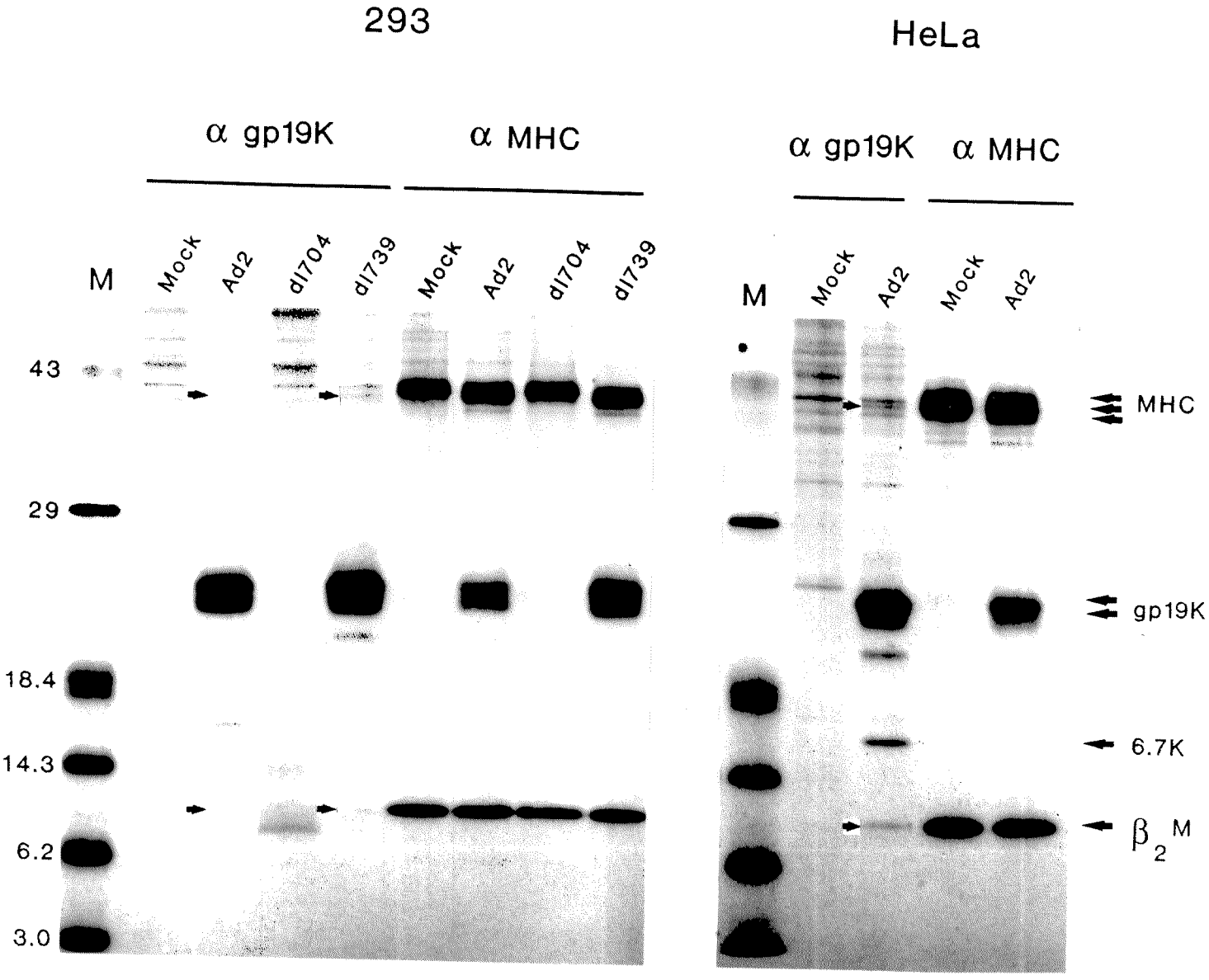
lines indicated that it was not an artifact of the 293 cell line (Fig. 22). The lack of the two polypeptides in the mock treated and dl739 (E3/6.7K$^-$) samples confirmed their E3/6.7K identity (Fig. 22). Further analysis under reducing and non reducing conditions showed that both E3/6.7K polypeptides had a slightly higher apparent molecular mass under reducing conditions, indicating that both molecules had at least one disulfide bond (Fig. 22).

3.2.4- Coimmunoprecipitation of E3/6.7K and E3/19K

Given the mechanism by which E3/19K blocks the surface expression of MHC class I molecules, it was of interest to determine if E3/6.7K proteins could also interact with HLA molecules or with the E3/19K protein itself. Furthermore, it was important to determine if E3/6.7K was required for the binding of E3/19K to MHC class I. To achieve this goal, 293 cells were infected with control wt virus or viral mutants and labeled with ^{35}S cysteine as above. Coimmunoprecipitation studies were then performed using antibodies specific for E3/19K or MHC class I molecules. The results showed that the αAd5 E3/19K antibody coimmunoprecipitated the MHC class I heavy and light chains, the upper E3/6.7K polypeptide, and an unknown polypeptide of approximately 21-23 kDa (αE3/19K in Ad2 lanes, Fig. 23). Curiously, the lower E3/6.7K polypeptide was not detectable in those lanes. The results also demonstrated that the binding of E3/19K to MHC class I molecules could occur in the absence of E3/6.7K (αMHC in dl739 lane (E3/6.7K$^-$); Fig. 23). The coimmunoprecipitation of E3/6.7K, E3/19K, and HLA could also be detected in HeLa cells under these conditions (Fig. 23). In contrast, immunoprecipitations with the αHLA class I antibodies coimmunoprecipitated E3/19K but not E3/6.7K (αMHC, Ad2 lane in Fig. 23). The

Fig. 23: Coimmunoprecipitation of E3/6.7K and E3/19K

Subconfluent 293 and HeLa cells were mock treated or infected with wt or mutant viruses (MOI = 50) and labeled with ^{35}S cysteine as in figure 22. The samples were then immunoprecipitated with the αAd5 E3/19K or the αMHC class I (W6/32) antibody. They were then analyzed under reducing conditions on SDS-PAGE and by autoradiography. The positions of E3/19K (gp19K), E3/6.7K (6.7K), and the heavy (MHC) and light HLA class I chains (β_2M) are shown to the right of the gels. Please note the E3/19K dependent shift of the molecular mass of the MHC class I heavy chain. As before, the molecular masses of the ^{14}C standards (M) are indicated in kDa on the left. The viruses used were as follows: Ad2: wt virus; dl704: E3/19K$^-$ deletion mutant; dl739: E3/6.7K$^-$ deletion mutant.


293
HeLa
α gp19K
α MHC
α gp19K
α MHC
M
Mock
Ad2
dl704
dl739
Mock
Ad2
dl704
dl739
M
Mock
Ad2
Mock
Ad2
43
29
18.4
14.3
6.2
3.0
MHC
gp19K
6.7K
β2M

αMHC antibody also coimmunoprecipitated the 21-23 kDa polypeptide but not as efficiently as the αAd5 E3/19K antibody (αMHC, Ad2 lane in Fig. 23). It is worth noting that immunoprecipitations with the αE3/6.7K antibody resulted in the detection of a weak signal possibly corresponding to the E3/19K polypeptide (Ad2 lanes in Fig. 22).

To confirm the interaction between E3/6.7K and E3/19K, a bank of viral mutants lacking either or both genes for these proteins was used to infect 293 or HeLa cells. The coimmunoprecipitation of E3/6.7K in Ad2 infected 293 (Fig. 24, Panel A) or HeLa (Fig. 24, Panel C) cells with the αAd5 E3/19K antiserum was consistent with the previous results (Fig. 23). For instance, no E3/6.7K protein was coimmunoprecipitated in 293 (Fig. 24, Panel A) or HeLa (Fig. 24, Panel C) infected cells without functional E3/19K proteins (mock, dl704, dl708, dl703, and dl754) or functional E3/6.7K (mock, dl701, and dl739). Surprisingly, E3/6.7K was coimmunoprecipitated from wt Ad2 infected cells but not from cells infected with wt Ad5 or the wt recombinant rec700 virus (Fig. 24). This discrepancy was not due to an inactive E3/6.7K gene in rec700, since it expressed similar amounts of E3/6.7K proteins as the wt Ad2 virus (Fig. 24, Panel B). In contrast, there were no detectable Ad5 E3/6.7K proteins using the αAd2 E3/6.7K antiserum (Fig. 24, Panel B).

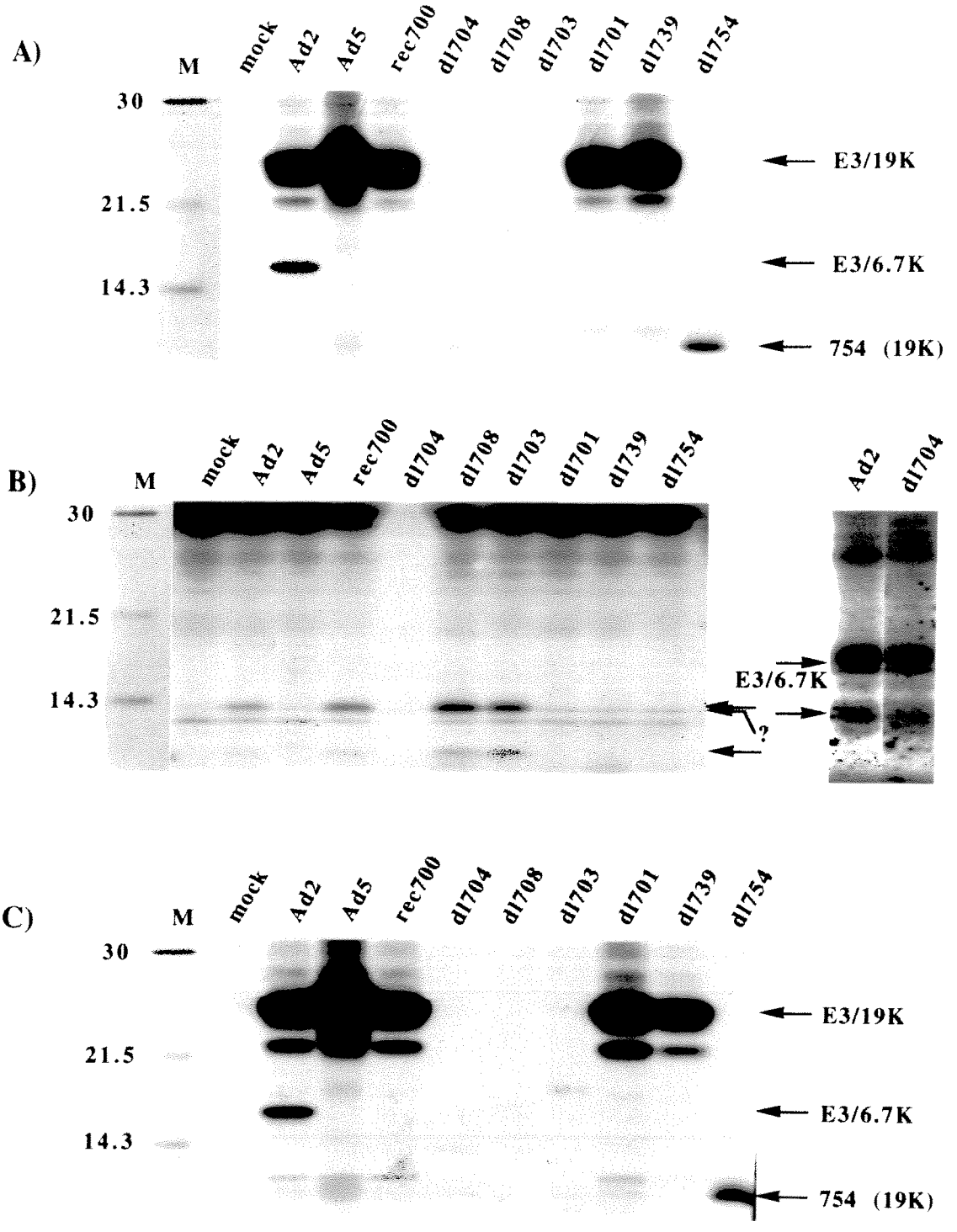
3.2.5- Down regulation of MHC class I molecules by E3/6.7K and E3/19K

3.2.5.1- Modulation of MHC class I surface expression in transfected cell lines

With the determination of the optimal infection parameters being established, the functional analysis of the E3/6.7K protein was undertaken. Given the ability of Ad2 to modulate MHC class I surface expression and the interaction of E3/6.7K with the E3/19K

Fig. 24: Specific coimmunoprecipitation of E3/6.7K and E3/19K

To confirm the interaction of E3/6.7K with E3/19K, mock treated or virus infected cells were metabolically labeled with ^{35}S cysteine using the CH enhancing protocol described in Materials and Methods. The samples were analyzed with the αAd2 E3/6.7K or the αAd5 E3/19K antiserum. Panel A shows the αAd5 E3/19K immunoprecipitations using 293 infected cell lysates and panel B the αE3/6.7K immunoprecipitations from the same cell line. In panel C, infected HeLa cells and the αAd5 E3/19K antibody were used. The positions of the proteins in the gels and the molecular masses of the marker are shown as before. The presence of a polypeptide migrating just below the upper E3/6.7K polypeptide is also indicated (see arrow and question mark). Please note the presence of the truncated form of E3/19K in the dl754 infected cells (labeled 754 (19K)). Also note that the lack of detectable E3/6.7K proteins in dl704 infected cells (Panel B) was simply due to underloading of the lane in this particular experiment. To prove this point, the expression of E3/6.7K by dl704 in another experiment is shown to the right of panel B. The viruses used included wt viruses (Ad2, Ad5, rec700), mutant viruses lacking E3/6.7K (dl739, dl701) or E3/19K (dl704), and mutant viruses expressing different forms of E3/19K (dl708, dl703, dl754).


A)
M
mock
Ad2
Ad5
rec700
dl704
dl708
dl703
dl701
dl739
dl754
30
21.5
14.3
E3/19K
E3/6.7K
754 (19K)
B)
M
mock
Ad2
Ad5
rec700
dl704
dl708
dl703
dl701
dl739
dl754
Ad2
dl704
30
21.5
14.3
E3/6.7K
?
C)
M
mock
Ad2
Ad5
rec700
dl704
dl708
dl703
dl701
dl739
dl754
30
21.5
14.3
E3/19K
E3/6.7K
754 (19K)

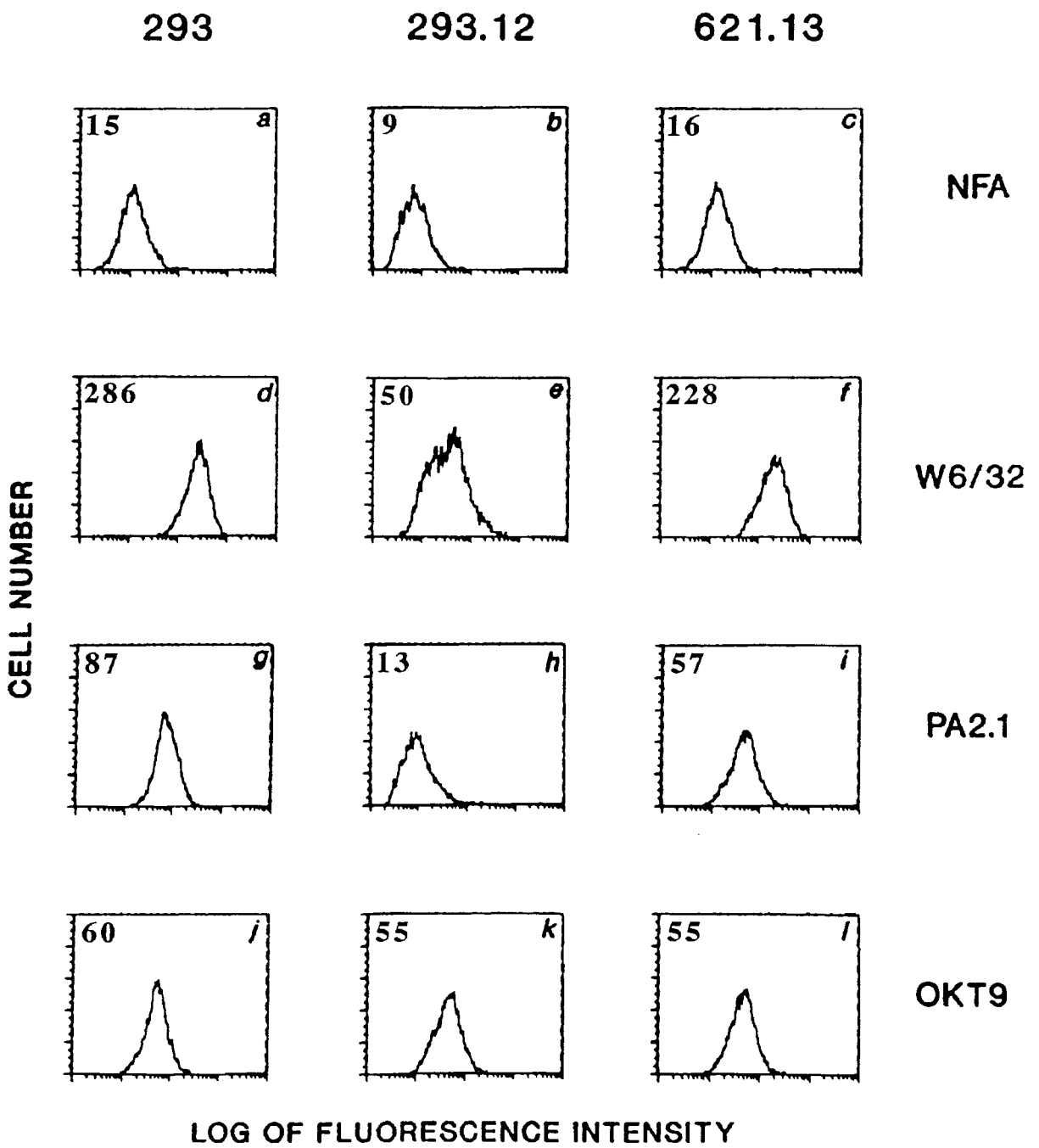
protein (Fig. 23, 24), the function of E3/19K and E3/6.7K was examined by FACS analysis. As a first step, the well characterized 293.12 and 621.13 cell lines were used. Both cell lines are 293 derived and are transfected with the EcoRI D fragment of Ad2 containing, among other genes, the E3/6.7K and E3/19K genes (Fig. 10). While the former cell line encodes a wt E3/19K gene product (325), the 621.13 cell line expresses a truncated form of E3/19K referred to as M621. The transmembrane M621 protein still binds MHC class I molecules but lacks the E3/19K ER retention sequences and is thus transported to the plasma membrane along with the MHC class I proteins (346,347). The analysis of these cell lines indicated that, in the presence of wt E3/6.7K and wt E3/19K, the 293.12 cell line had a lower MHC class I surface expression than the 293 cell line (Fig. 25). In contrast, the expression of a non functional E3/19K, but wt E3/6.7K, in the 621.13 cell line resulted in a partially restored MHC class I surface expression. This was consistent with the published role of E3/19K on MHC class I surface expression (324-327,330). The data also showed that this effect was specific for the MHC class I molecule, since the expression of the TR was unaltered by the transfected gene products (Fig. 25).

3.2.5.2- Modulation of MHC class I surface expression by E3/6.7K and E3/19K

Since E3/19K modulates MHC class I surface expression, the question still remained whether the observed binding of E3/6.7K to E3/19K was functionally relevant. Therefore, to investigate the role of E3/6.7K on MHC class I surface expression, 293 cells were infected using the optimal parameters previously determined (see above) and using a bank of viral mutants lacking functional E3/6.7K, E3/19K, or both. The surface expression of MHC class I molecules was then determined by FACS analysis using the HLA class I specific W6/32

Fig. 25: Modulation of MHC class I surface expression in transfected cells

The cell lines 293 (no transfection), 293.12 (wt E3/6.7K and wt E3/19K), and 621.13 (wt E3/6.7K and truncated E3/19K) were grown to subconfluence and removed from the dishes with versene. They were then analyzed by FACS analysis using either one of the two MHC class I specific monoclonal antibodies W6/32 and PA2.1. Controls included the OKT9 antibody reacting against the TR or the secondary antibody alone (NFA). The linear fluorescence values are shown in the top left corner of each profile. In two separate experiments, the expression of the W6/32, PA2.1, and OKT9 epitopes on the surface of 293.12 cells, relative to the control 293 cell line, were 16% $\pm$ 3, 6% $\pm$ 2, and 89% $\pm$ 12 respectively. For the 621.13 cell line, the averages were respectively 74% $\pm$ 5, 55% $\pm$ 6, and 81% $\pm$ 9.


293
293.12
621.13
15
a
9
b
16
c
NFA
286
d
50
e
228
f
W6/32
87
g
13
h
57
i
PA2.1
60
j
55
k
55
l
OKT9
CELL NUMBER
LOG OF FLUORESCENCE INTENSITY

antibody. Without functional E3/6.7K (dl739 and dl701), the mutant viruses reduced MHC class I surface expression as efficiently as the wt controls (Ad2, Ad5, rec700; Table 4). In contrast, the lack of wt E3/19K in the dl704, dl754, and dl703 viral mutants resulted in a significant increase ($p<0.001$) of MHC class I at the plasma membrane when compared to Ad2 infected cells. However, infection of 293 cells with dl708 or dl710 resulted in a MHC class I surface expression comparable to wt Ad2 infected cells ($p>0.05$; Table 4). Interestingly, the deletion of all or most of the Ad2 E3 region resulted in a MHC class I surface expression intermediate between the E3/19K$^-$ mutants and mock treated cells. Statistical analysis revealed that the results with dl327, but not with dl801, were significantly higher than the results obtained for either dl704 or dl754 ($0.001<p<0.01$). Despite this discrepancy, there was no statistical difference between the results obtained with dl327 and dl801 ($p>0.05$). It should be noted that, in eight independent experiments, both viruses consistently yielded a higher MHC class I surface expression than the E3/19K$^-$ mutant viruses.

The different modulation of MHC class I surface expression observed for the various mutant viruses raised the possibility of a non specific but differential effect by the different mutants on MHC class I surface expression. To address this issue, the presence of the TR at the plasma membrane was examined by FACS analysis using the optimal infection conditions. A second MHC class I specific antibody, PA2.1, was also used to verify that the effects seen with the W6/32 monoclonal antibody were not epitope specific. The expression of the TR, although variable, remained substantially the same and did not correlate with the expression of MHC class I proteins in the cells infected with the various viral mutants (Fig. 26).

Table 4: Modulation of MHC class I surface expression by E3/6.7K and E3/19K

Mock treated and virally infected 293 cells (MOI = 50) were analyzed by FACS analysis at 20 hpi as before. The MHC class I surface expression was determined with the W6/32 monoclonal antibody. The various viruses are classified according to their genotype. In each case, the name of the virus, its genotype, and the resulting average MHC class I surface expression in infected 293 cells are shown. The background fluorescence (NFA) was subtracted from the linear fluorescence for each sample. The results are expressed as a percentage of the normal level of MHC class I surface expression on mock treated samples (see Materials and Methods). The figure is the cumulative result of all FACS analyses done with each given virus. Each value represents no less than 6 independent experiments. Statistical comparisons between the results obtained with the various viruses and Ad2 (p^1) or either dl704 or dl754 (p^2) are shown and were performed as outlined in Materials and Methods. STDEV: Standard deviation; n: number of independent experiments performed. Wt viruses (Ad2, Ad5, rec700), mutant viruses lacking E3/6.7K (dl739, dl701), E3/19K (dl704), or E3 (dl327, dl801) and mutant viruses expressing different forms of E3/19K (dl703, dl708, dl710, dl754) were used.

		%Mock			T test	
Wild type		Average	STDEV	n	p^1	p^2
Ad2	-	40	7	23	-	-
Ad5	-	35	5	9	p>0.05	-
rec700	-	36	5	8	p>0.05	-
E3/6.7K	***mutants***					
dl739	deletion	32	6	25	p<0.001	-
dl701	deletion	29	5	8	0.001<p<0.01	-
E3/19K	***mutants***					
dl704	deletion	58	11	13	p<0.001	-
dl754	deletion	56	10	8	p<0.001	p>0.05
dl703	no sig. seq.	52	10	11	p<0.001	p>0.05
dl708	no CYT tail	38	12	6	p>0.05	0.001<p<0.01
dl710	extra 5 aa	42	6	11	p>0.05	0.001<p<0.01
E3	***mutants***					
dl327	deletion	75	15	8	p<0.001	0.001<p<0.01
dl801	deletion	66	17	8	p<0.001	p>0.05

Fig. 26: Specificity of MHC class I down regulation by the mutant viruses

Subconfluent dishes of 293 cells were infected at a MOI of 50 with the various adenoviral mutants as indicated. The surface expression of both TR (TR) and MHC class I proteins (W6/32 or PA2.1) were monitored by FACS analysis at 20 hpi, as detailed in the Materials and Methods. In each case, the NFA control was subtracted and the linear fluorescence values expressed as a percentage of the mock treated cells. The averages and standard deviation bars represent two independent experiments. Wt viruses (Ad2, Ad5, rec700), mutant viruses lacking E3/6.7K (dl739, dl701), E3/19K (dl704), or E3 (dl327, dl801) and mutant viruses expressing different forms of E3/19K (dl703, dl710, dl754) were used.

Percent of Mock
0
20
40
60
80
100
120
Ad2
Ad5
rec700
dl739
dl701
dl704
dl754
dl703
dl710
dl327
dl801
Viruses
W6/32
PA2.1
TR

Furthermore, the results using the PA2.1 antibody confirmed the data obtained with the W6/32 antibody.

3.2.6- Modulation of MHC class I phosphorylation

3.2.6.1- Block of MHC class I phosphorylation by wt Ad2

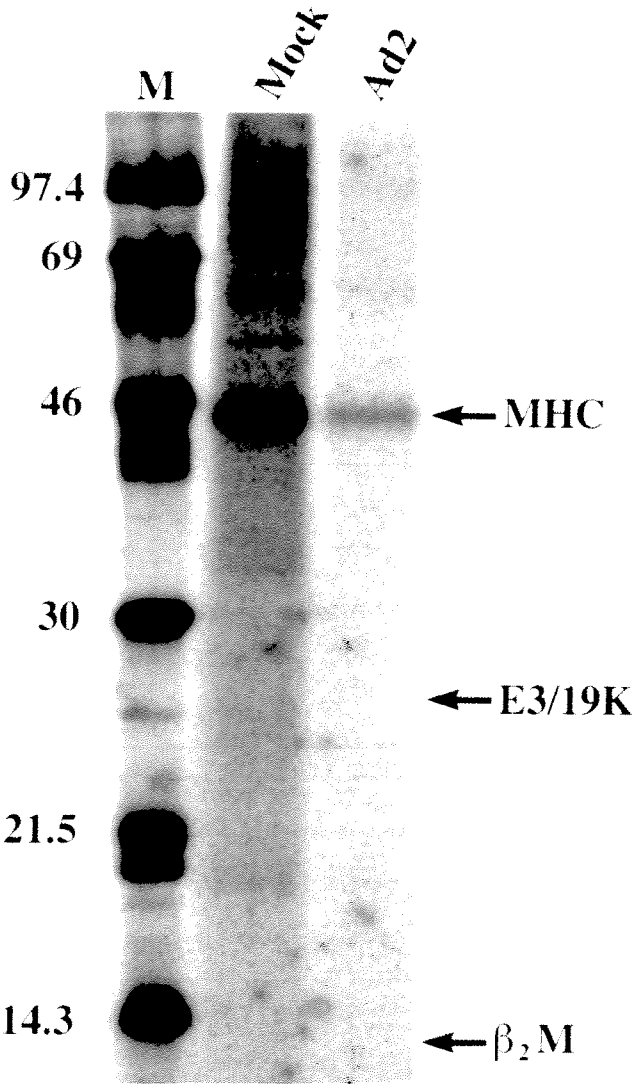
So far, the above results clearly indicated that Ad2 did not express E3/6.7K as a means to directly modulate MHC class I surface expression. However, as mentioned briefly in the Chapter 1, the phosphorylation of MHC class I proteins may modulate their steady state expression at the plasma membrane. Therefore, it is possible that Ad2, and perhaps E3/6.7K, could regulate MHC class I phosphorylation and possibly use such a mechanism to further reduce MHC class I surface expression. To investigate this, we first infected cells with Ad2 under optimal conditions and metabolically labeled them with ^{32}P orthophosphate. The phosphorylation level of MHC class I was then determined by immunoprecipitation. As shown in figure 27, Ad2 significantly reduced the phosphorylation of the MHC class I heavy chains in 293 cells whereas neither β_2M nor E3/19K were phosphorylated. Densitometry analysis further indicated that the level of phosphorylation of MHC class I proteins in Ad2 infected cells (34% $\pm$ 4 of mock; Fig. 27) was comparable to their surface expression (40% $\pm$ 7 of mock; Table 4).

3.2.6.2- Block of MHC class I phosphorylation in E3/6.7K and E3/19K transfected cells

Since Ad2 could indeed block MHC class I phosphorylation (Fig. 27), the role of E3/6.7K and E3/19K on MHC class I phosphorylation was estimated. As for the analysis of

Fig. 27: Down regulation of MHC class I phosphorylation by Ad2

The extent of phosphorylation of MHC class I molecules upon Ad2 infection was examined. 293 cells were starved for an hour and infected with Ad2 (MOI = 50) or mock treated. The cells were labeled at 20 hpi for two hours with ^{32}P orthophosphate (see Materials and Methods). The cells were then lysed, MHC class I molecules immunoprecipitated using the W6/32 antibody, and the immunoprecipitates analyzed by SDS-PAGE and autoradiography. Equal TCA acid precipitable counts were used for the immunoprecipitations. The position of the ^{32}P labeled heavy chain of HLA class I (MHC) as well as the virtual migration positions of E3/19K (E3/19K) and the light chain (β_2M) are shown on the right side. The molecular masses of the protein standards (M) are indicated in kDa on the left side. Densitometry analysis indicated that the level of phosphorylation of MHC class I in Ad2 infected cells was 34% $\pm$ 4 (n=5) compared to mock treated cells.


M
Mock
Ad2
97.4
69
46
30
21.5
14.3
MHC
E3/19K
β2M

MHC class I surface expression, the 293.12 and 621.13 transfected cell lines were first analyzed. These cells were metabolically labeled with ^{32}P orthophosphate and the level of MHC class I phosphorylation evaluated as before. Consistent with the above data, the presence of wt E3/6.7K and wt E3/19K genes in the 293.12 cell line resulted in a reduced MHC class I phosphorylation (Fig. 28). Densitometry analysis of repeat experiments indicated that the level of phosphorylation, when compared to the mock treated cells, was 20% $\pm$ 12 for the 293.12 cell line. In the 621.13 cell line, which encodes a truncated E3/19K protein, MHC class I phosphorylation was restored to normal levels (134% $\pm$ 74; Fig. 28). Importantly, the reduced MHC class I phosphorylation observed in 293.12 cells was not the result of a lower MHC class I protein expression in the cell line (left panel Fig. 28).

3.2.6.3- Respective role of E3/6.7K and E3/19K on MHC class I phosphorylation

The next step in the analysis of the block of MHC class I phosphorylation by Ad2 was to evaluate the respective roles of the E3/6.7K and E3/19K proteins. Since the DNA used to generate the transfectants also included the E3/6.7K gene, viral mutants were used to infect 293 or HeLa cells and MHC class I phosphorylation monitored by immunoprecipitation. The data confirmed the block of phosphorylation of MHC class I molecules by wt Ad2 in 293 cells (30% $\pm$ 6; Fig. 29). They also showed that the deletion of E3/19K (dl704) resulted in normal levels of phosphorylation of the MHC class I proteins (83% $\pm$ 22; Fig. 29). In contrast, the absence of E3/6.7K molecules (dl739) had little effect on the ability of the virus to down regulate MHC class I phosphorylation (41% $\pm$ 11; Fig. 29). Finally, the phosphorylation of MHC class I molecules in infected HeLa cells was not affected under the conditions used.

Fig. 28: Modulation of MHC class I phosphorylation in transfected cells

The level of MHC class I phosphorylation in 293, 293.12 and 621.13 cells was investigated. These cell lines were grown to subconfluence, starved for an hour, and pulse-labeled for 30 minutes with ^{35}S methionine/cysteine or 2 hours with ^{32}P orthophosphate and lysed. MHC class I (αMHC) or E3/19K (αAd5 E3/19K) molecules were immunoprecipitated from equal TCA counts and analyzed by SDS-PAGE and autoradiography. Labeling of the gels is as before. The truncated E3/19K produced by the 621.13 cell line (labeled 621) cannot be detected by the αAd5 E3/19K antiserum because the antibody reacts against the carboxyl tail of wt E3/19K which is not present in the M621 deletion mutant molecule. For this particular experiment, the level of MHC class I phosphorylation was 24% (293.12) and 239% (621.13) compared to the untransfected 293 cells. The densitometry analysis of various gels from separate experiments was performed. Overall, the results were 20% $\pm$ 12 (n=5) and 134% $\pm$ 74 (n=4) respectively for the 293.12 and 621.13 cell lines.

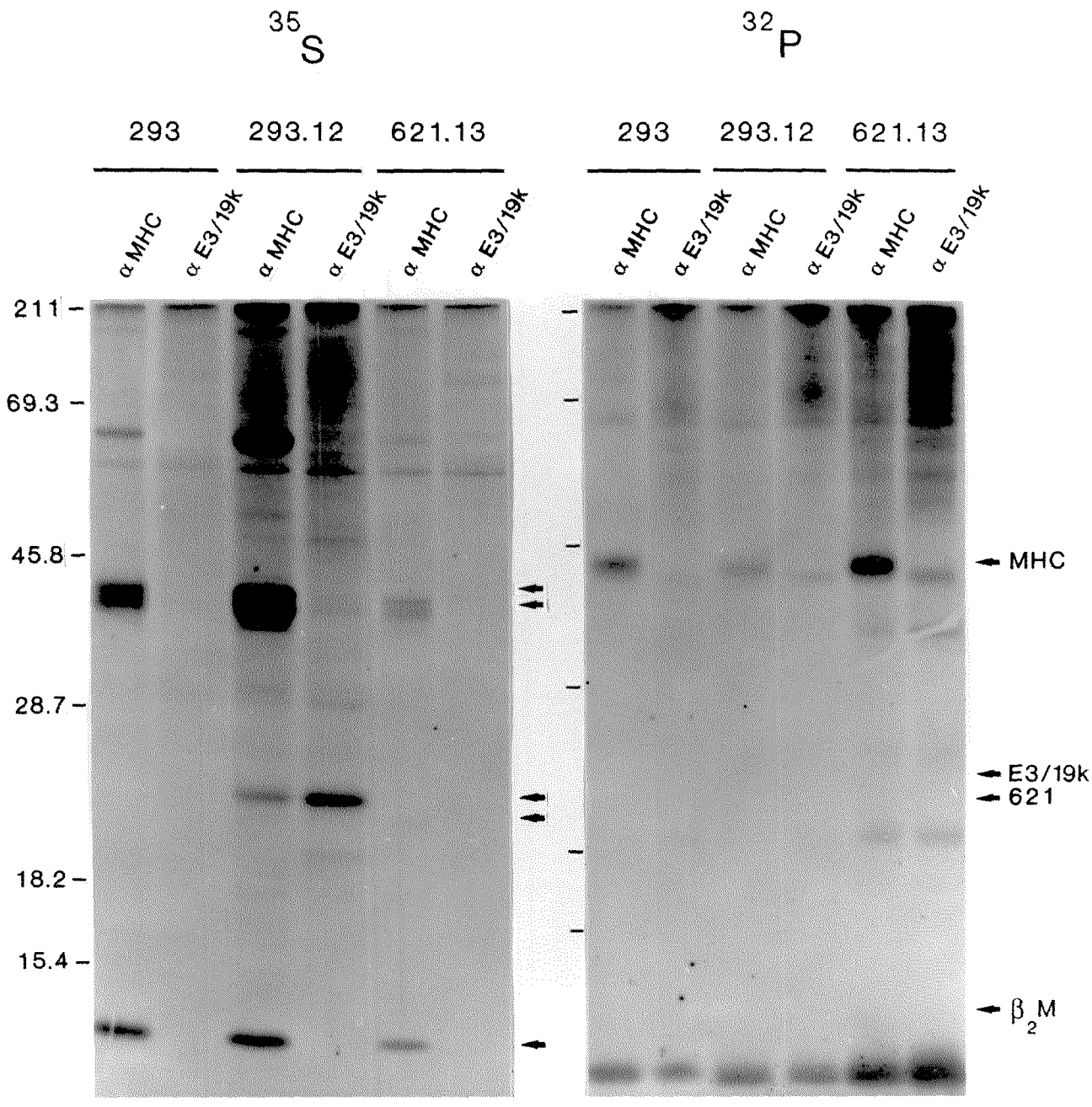
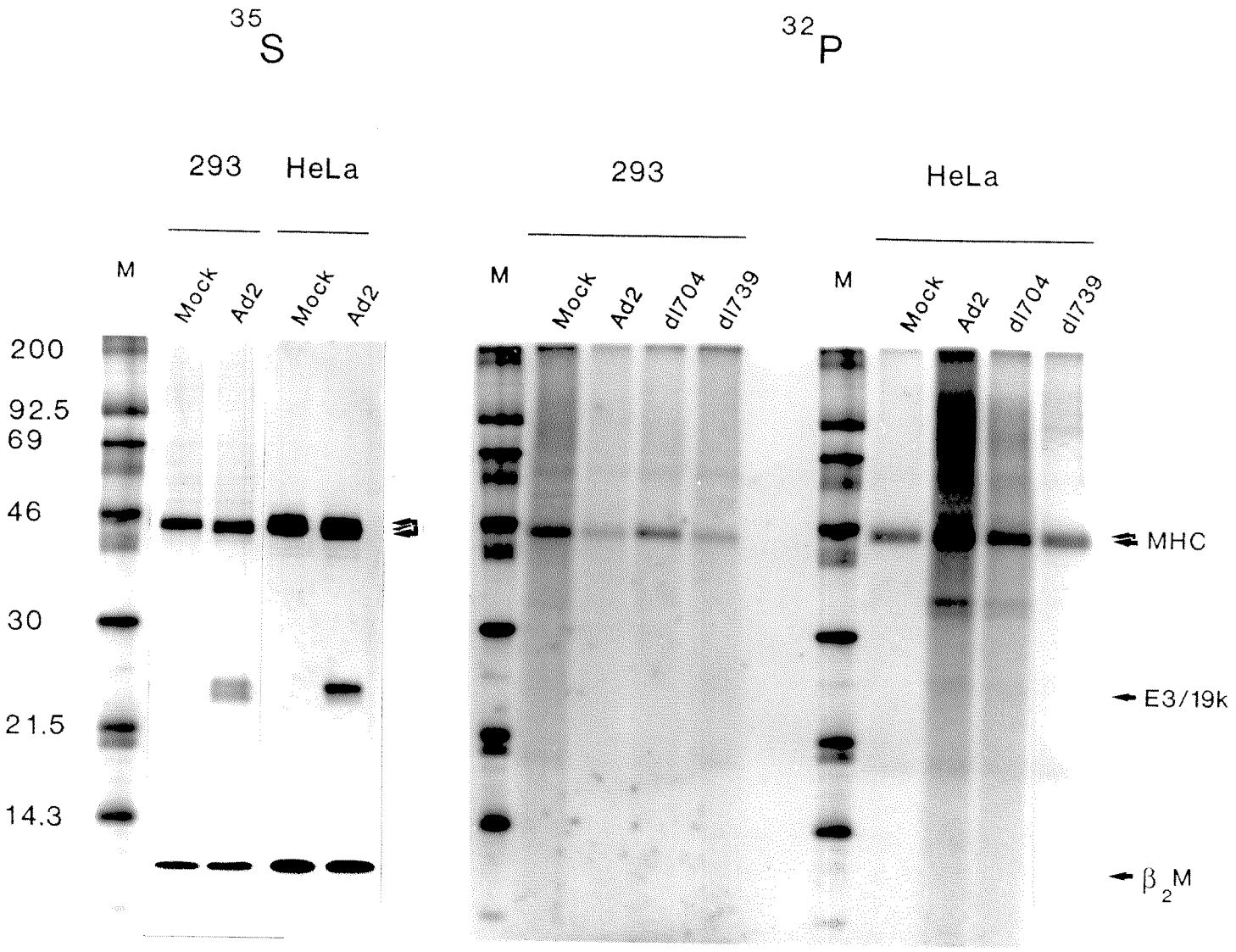

35S
32P
293
293.12
621.13
293
293.12
621.13
α MHC
α E3/19k
α MHC
α E3/19k
α MHC
α E3/19k
α MHC
α E3/19k
α MHC
α E3/19k
α MHC
α E3/19k
211
69.3
45.8
28.7
18.2
15.4
MHC
E3/19k
621
β2M

Fig. 29: Role of E3/6.7K and E3/19K on MHC class I phosphorylation

Mock treated, wt or mutant infected 293 and HeLa cells (MOI = 50) were pulsed with ^{35}S cysteine as before using the CH enhancing procedure (see Fig. 22). Alternatively, the cells were pulsed with ^{32}P orthophosphate as in figure 27. The MHC class I molecules were then immunoprecipitated as usual using the W6/32 monoclonal antibody and analyzed by electrophoresis and autoradiography. The level of phosphorylation of the MHC class I proteins was determined for each virus by densitometry. They were 30% $\pm$ 6 (Ad2), 83% $\pm$ 22 (dl704), and 41% $\pm$ 11 (dl739) respectively. The molecular masses of the marker (M) and the migration of β_2M and the MHC class I heavy chain are shown as before. The viruses used were as follows: Ad2: wt virus; dl704: E3/19K$^-$ deletion mutant; dl739: E3/6.7K$^-$ deletion mutant.

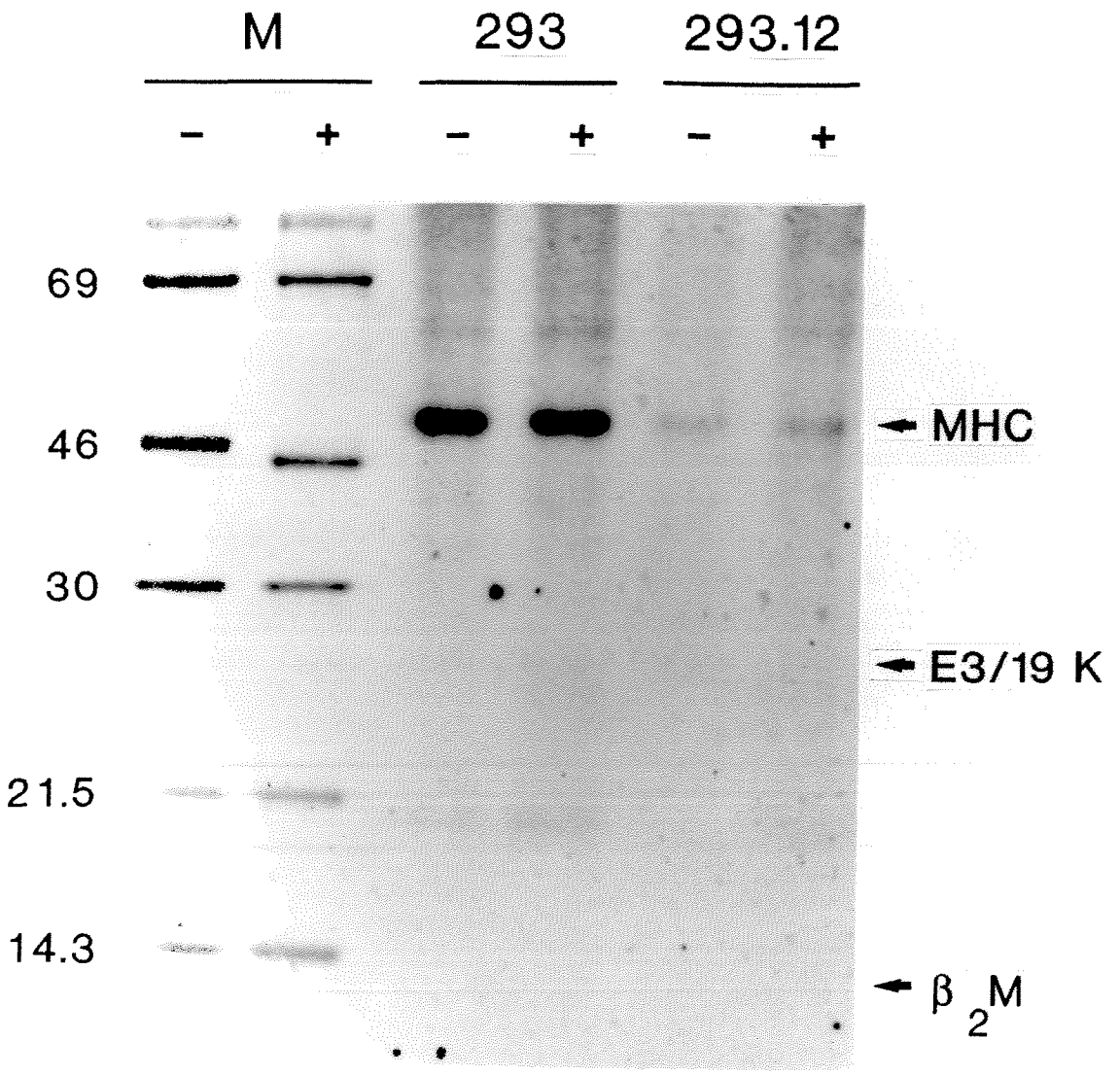

35S
32P
293
HeLa
293
HeLa
M
Mock
Ad2
Mock
Ad2
M
Mock
Ad2
dl704
dl739
M
Mock
Ad2
dl704
dl739
200
92.5
69
46
30
21.5
14.3
MHC
E3/19k
β2M

3.2.6.4- Intracellular site of MHC class I phosphorylation

The finding that Ad2 infections could modulate MHC class I phosphorylation in 293 cells supported the hypothesis that the virus did so to block MHC class I plasma membrane expression, as suggested above. However, it was equally possible that the observed reduction of MHC class I phosphorylation was the consequence, rather than the cause, of the block by Ad2 of MHC class I transport. This was an important issue as E3/19K retains MHC class I proteins in the ER. The availability of the 293.12 and 621.13 cell lines readily allowed the examination of this issue. The differential location of the MHC class I molecules in these cells affects their sensitivity to endo H digestion. This was therefore used to determine if phosphorylated MHC class I molecules could be found in the ER or the cis Golgi or whether they only were localized in downstream compartments. The susceptibility of phosphorylated MHC class I proteins to endo H was therefore evaluated. Figure 30 shows that in both cell lines, the results indicated that all the MHC class I phosphorylated molecules were endo H resistant and were therefore mature glycoproteins.

Fig. 30: MHC class I phosphorylation takes place in a post-ER compartment

293 and 293.12 cells were pulse-labeled for 2 hours with ^{32}P orthophosphate as in figure 28 and MHC class I molecules immunoprecipitated with the W6/32 antibody. The immunoprecipitates were mock treated (-) or digested with endo H (+) overnight as detailed in Materials and Methods and analyzed by SDS-PAGE and autoradiography. The ovalbumin (46 kDa) in the molecular mass standards was sensitive to endo H digestion and was used as a control for the efficiency of the digestion. The figure symbols are as previously noted.


M
293
293.12
-
+
-
+
-
+
69
46
30
21.5
14.3
MHC
E3/19 K
β 2M

3.3- DISCUSSION

3.3.1- Block of MHC class I surface expression by Ad2

This study examined the role of Adenoviruses, and in particular E3/6.7K, on the MHC class I antigen processing and presentation pathway. As discussed below, numerous findings were made. In a first step, the parameters yielding the maximal block of MHC class I surface expression by Ad2 were determined. It was found that the infection of 293 cells at a MOI of 50 was sufficient to maximally reduce MHC class I surface expression (Fig. 19). Unless the CH enhancing procedure was used, the optimal time to analyze the samples was at 20 hpi (Fig. 18). However, the inhibition was not complete as approximately 40% of the MHC class I molecules were still expressed at the plasma membrane (Fig. 17, 18, 19 and Table 4). It has been shown that the efficiency of ER retention of MHC class I proteins by E3/19K is dependent on the level of expression of E3/19K (325,329,335). However, a lack of E3/19K proteins did not likely contribute to the residual MHC class I surface expression observed, since infection of the cells at MOI ranging as high as 800 did not lower this residual MHC class I surface expression (Fig. 19). At that MOI each cell was infected by many viruses and should therefore have expressed significant amounts of E3/19K, particularly since E3/19K is a major adenoviral protein (258,316,323). Clearly the ER retention of MHC class I molecules by E3/19K was leaky and could not simply be improved by over expressing E3/19K, either by infection (Fig. 19) or transfection (Fig. 25 and (325)). This escape from ER retention may have been influenced by various factors including the kinetics of folding of the MHC class I molecules, the availability of peptides that can bind MHC class I molecules, and the local

concentrations of E3/19K and MHC class I heavy and light chains in the ER. It is unclear what impact these factors played in this phenotype.

3.3.2- Cell type specificity of MHC class I down regulation by Ad2

A second interesting aspect of the optimization of the Ad2 modulation of MHC class I surface expression is the apparent cell specificity of this phenotype. This was initially suggested by the minimal block of MHC class I expression at the surface of HeLa cells (Fig. 20) compared to 293 cells (Fig. 19). The 293 cell line is highly permissible to Ad2 infection due to the presence of Ad5 E1 DNA sequences integrated in its genome (387). In the virus, these sequences encode gene products that transactivate various adenoviral genes (362). It has indeed been suggested that those sequences are implicated in the block of MHC class I surface expression by Ad2 (388). In addition, it has also been suggested that the occurrence of this phenotype in 293 cells is solely specific for this cell line. However, the present data indicated that this was not the case. They show that the distinct effect of Ad2 on 293 and other cell lines could partly be explained by the fact that viruses have different kinetics of replication in different cell types. Thus, when analyzed at 40 hpi, Ad2 down regulated the expression of MHC class I molecules at the surface of A549, HeLa, and MRC5 cells, albeit to different extents (Table 3). In fact, the inhibition of the expression of MHC class I at the plasma membrane of infected HeLa cells reached a level similar to the infected 293 cells (Fig. 21, Table 3).

The final stage of a permissive Ad2 infection is lysis of its host cell. It was therefore possible that the reduction of MHC class I surface expression was the indirect result of this

lysis rather than the specific down regulation of MHC class I proteins by Ad2. Examination of this issue in different cell types revealed that, in HeLa and MRC5 cells this may have been the case. The data indicated that there was a direct correlation between MHC class I surface expression and cpe (Table 3). In contrast, the block of MHC class I surface expression in both 293 and A549 cells was significantly greater than the cytopathic effect of Ad2 on these cells. This indicated that the down regulation of MHC class I proteins was not specific for the 293 cell line, but that it was nevertheless cell type dependent. This implied that Ad2 possibly needed host specific components found in 293 and A549 cells but absent in HeLa and MRC5 cells. The down regulation of MHC class I proteins in A549 cells indicated that the Ad5 E1 region contained in 293 cells was not essential for this phenotype, as suggested by Routes *et al.* (388). Importantly, the ineffective MHC class I down regulation by Ad2 in HeLa cells was not due to the lack of expression of E3/6.7K (Fig. 22) or E3/19K (Fig. 23) in these cells. Furthermore, it was not due to a lack of interaction among E3/6.7K, E3/19K, and MHC class I molecules either (Fig. 23). Furthermore, Ad2 efficiently replicates in HeLa cells (personal observations).

3.3.3- Detection of E3/6.7K from infected cells

The objective of this study was to investigate the role of Ad2 E3/6.7K on the inhibition of MHC class I surface expression and to characterize its properties. Hence, the analysis of E3/6.7K by immunoprecipitation yielded 2 polypeptides: a 7-8 kDa polypeptide and a 15-16 kDa polypeptide (Fig. 22). The identity of those polypeptides was confirmed using a specific αE3/6.7K antiserum. It was also confirmed by the absence of the two

polypeptides in mock treated cells and in cells infected with viral mutants lacking the gene for E3/6.7K (Fig. 22). The results were consistent with published observations by others using *in vitro* translation of E3/6.7K mRNA and Western analysis with the same αE3/6.7K specific antiserum (315). Finally, the E3/6.7K polypeptide was not an artifact of the host 293 cell line used since it was also found in the unrelated and infected HeLa cell line (Fig. 22). As indicated in the Introduction of this chapter, the upper polypeptide is the N-linked glycosylated form of the lower polypeptide.

3.3.4- Specific interaction between E3/6.7K and E3/19K

Coimmunoprecipitation studies suggested that the E3/6.7K protein interacted with the E3/19K protein (Fig. 23, 24). The coimmunoprecipitation of E3/6.7K in HeLa cells indicated that this interaction was not cell type dependent (Fig. 23). Furthermore, the absence of the E3/6.7K polypeptide in the viral mutant dl739 (E3/6.7K$^-$) and the mock treated cells strongly suggested that the interaction between E3/6.7K and E3/19K was specific. This was confirmed by the lack of cross reactivity against the E3/6.7K protein by the αAd5 E3/19K antibody (see dl704 lanes in Fig. 23, 24). The observation that only the upper E3/6.7K polypeptide was coimmunoprecipitated first suggested that the presence of the sugars on E3/6.7K was important for its interaction with E3/19K. However, if translocation of E3/6.7K in the ER lumen was not cotranslational (see below), the apparent need for N-linked carbohydrates could have been circumstantial. Thus, incorporation of E3/6.7K in the ER membrane, possibly in the correct orientation, could be necessary for binding to membrane bound E3/19K.

It could not be inferred from the data if E3/6.7K directly interacted with E3/19K or the E3/19K-HLA complex, nor could the importance of MHC class I peptides on this binding be determined. Given that E3/6.7K was detectable with the αAd5 E3/19K antibody and vice versa, but not the αMHC class I antibody (Fig. 23), it seems more likely that E3/6.7K directly interacted with E3/19K rather than the HLA molecules. In fact, it is quite possible that the interaction of E3/19K with either E3/6.7K or MHC class I proteins was mutually exclusive. However, numerous other scenarios could explain these results. For instance, the lack of immunoprecipitation of E3/6.7K with the W6/32 antibody (Fig. 23) could have been due to the extremely low amounts of E3/6.7K proteins detected in the infected cells. For instance, the detection of E3/6.7K proteins by fluorography, following their amplification by the CH enhancing procedure and labeling with ^{35}S cysteine for four hours, required a minimum of two weeks to obtain a weak signal. Nonetheless, it routinely required two to three months to obtain a clear signal. It is also possible that some of the antibodies used, for instance W6/32, were binding to sites important for interactions among E3/6.7K, E3/19K and MHC class I, and therefore competed with the binding molecules. This hypothesis is supported by the lack of detectable coimmunoprecipitated E3/6.7K using an αAd2 E3/19K antibody in comparison to the αAd5 E3/19K antibody used in these studies (data not shown). Experiments that use milder detergents may be useful to keep potentially weakly associated proteins together. Other scenarios relate to the potential presence of free E3/6.7K proteins. Hence, the αMHC class I antibody immunoprecipitated fewer E3/19K proteins than did the αAd5 E3/19K antibody (Fig. 23), suggesting that not all HLA class I molecules and E3/19K proteins were bound to each other. The opposite was also true (Fig. 23) indicating that many E3/19K and

MHC class I molecules were free of interaction with each other. Therefore, it is conceivable that a putative interaction between E3/6.7K and the E3/19K-HLA complex could not be detected if a significant portion of the E3/6.7K proteins were not bound to HLA. This is particularly relevant given the low amount of E3/6.7K protein detectable in the first place. In addition, the low detection level of coimmunoprecipitated E3/6.7K and the presence of free E3/6.7K, E3/19K, and MHC class I molecules in infected cells (Fig. 23) also supported the possibility that the E3/6.7K binding was transient and therefore more difficult to detect. Finally, since the W6/32 antibody is conformation dependent (68), it could also be that E3/6.7K only associated with immature MHC class I proteins and therefore dissociated from them before the W6/32 epitope was formed.

The failure to detect E3/6.7K in the Ad5 infected cells (Fig. 24) could perhaps be explained by the absence of E3/6.7K proteins in Ad5 infected cells. More likely, the epitope recognized by the αAd2 E3/6.7K antiserum is not present in the Ad5 E3/6.7K protein despite the high amino acid identity between the Ad5 and Ad2 E3/6.7K proteins. Only 2 of the 15 carboxyl terminal E3/6.7K residues, against which the antiserum was generated (315), are different between the two adenoviral serotypes. There is currently no αAd5 E3/6.7K antibody available. However, it is possible that the lack of coimmunoprecipitation of E3/6.7K was due to a lack of interaction between E3/6.7K and E3/19K.

As described in Materials and Methods, rec700 is an Ad5 recombinant virus encoding an Ad2 E3 cassette (Fig. 10). Although not definitive, the lack of coimmunoprecipitation of E3/6.7K in rec700 infected, and possibly Ad5, cells (Fig. 24) suggested that another function, present in Ad2 but not Ad5, was required for this interaction. There is a high homology

between the Ad2 and Ad5 E3 DNA (83% identity for the Ad2 fragment present in rec700) and each of the E3 proteins (65 - 91% identity). The crossover point in the rec700 recombinant virus is within the E3 region, and in particular in the E3/10.4K protein (Fig. 10) (260). The crossover event, however, has no effect on E3/10.4K, since the E3/10.4K protein is encoded by the Ad2 genome in the rec700 virus up to the crossover point. Furthermore, the remainder of the E3/10.4K sequence is identical between the two serotypes past the crossover point. Thus the E3/10.4K protein was not responsible for this phenotype. It is also unlikely that the Ad5 encoded 14.5K and 14.7K proteins encoded by rec700 were involved in this process. They appear to mediate resistance to TNF and/or the endocytosis of the epidermal growth factor receptor but do not affect MHC class I surface expression (277,350). Therefore, the requirement for additional Ad2 sequences pointed to non E3 proteins. An alternative possibility is that binding of E3/6.7K to E3/19K required that the E3/6.7K protein be post translationally modified in some fashion or that it first interacted with an additional molecule. This possibility was substantiated by the observation that the E3/6.7K proteins detected with the αAd2 E3/6.7K antibody were of slightly lower molecular mass than the E3/6.7K coimmunoprecipitated with the αAd5 E3/19K antibody (data not shown). Interestingly, this difference of molecular mass was also noted by Wilson-Rawls and Wold (271), but was attributed to residual N-acetylglucosamine moieties on the endo H cleaved proteins in their study. This explanation seems unlikely, however, since the difference in molecular mass was also observed without endo H treatment in our study.

3.3.5- Interaction of E3/19K with other proteins

The immunoprecipitation of E3/19K with the αAd5 E3/19K antibody also immunoprecipitated a polypeptide of 21-23 kDa (Fig. 23, 24). This polypeptide migrated just below the E3/19K polypeptide, which migrated at approximately 25 kDa due to the presence of N-linked sugars. Interestingly, this polypeptide was only detected if E3/19K was present but was also detectable when the W6/32 antibody was used, albeit it was not as abundant (Fig. 23). The identity of this polypeptide is currently unknown. The data suggested that it specifically interacted with E3/19K as did E3/6.7K. However, at this point, we cannot exclude the possibility that it was a degradation product of E3/19K that still reacted with the αAd5 E3/19K antibody.

3.3.6- Functional analysis of E3/6.7K

Analyses of cells infected with the mutant viruses showed that a functional E3/19K was required for the efficient down regulation of MHC class I surface expression (Table 4), as noted many times before (324-327,330). The effect of the viral mutants on MHC class I surface expression was specific, since the expression of the TR cell surface marker was relatively unaffected by the infections (Fig. 26). In contrast, viruses with deleted E3/6.7K were as effective as wt virus in blocking MHC class I surface expression, indicating that this viral protein did not play a role in this phenotype. The results also demonstrated that the down regulation of MHC class I surface expression by the E3/19K protein did not require the presence of E3/6.7K. This was consistent with the fact that the binding of E3/19K to MHC class I molecules occurred without E3/6.7K (Fig. 23). Furthermore, the effective down

regulation of surface MHC class I by Ad5 and rec700 (Table 4), despite the lack of detectable interaction between E3/6.7K and E3/19K, confirmed that E3/6.7K was not required to affect MHC class I surface expression. Consistent with these findings, the molecular mass of the MHC class I proteins slightly differed when E3/19K was expressed (compare the Ad2 or dl739 lanes with the mock or dl704 lanes in Fig. 23). This likely represented the immature and mature N-linked glycosylation forms of MHC class I proteins and reflected their E3/19K ER retention or expression at the cell surface respectively. Finally, preliminary data indicated that the E3/6.7K viral protein did not significantly affect the recognition and killing of adenovirally infected cells by virus specific CTL (Gooding *et al.*, personal communication).

3.3.7- Regulation of MHC class I surface expression by E3/19K mutants

The analysis of 293.12 transfected cells showed that the presence of E3/19K and possibly other viral protein(s) interfered with the surface expression of MHC class I proteins (Fig. 25). The use of a second HLA specific monoclonal antibody, PA2.1, showed that the results were not due to a steric inhibition of the W6/32 epitope by the viral proteins (Fig. 25). Furthermore, labeling with OKT9, which recognizes the transferrin receptor, demonstrated that the reduced MHC class I surface expression seen in 293.12 cells was specific (Fig. 25). Expression of the M621 protein in the 621.13 cell line resulted in a partially reduced MHC class I cell surface expression (Fig. 25). This confirmed the published observations that the ER retention of MHC class I molecules by E3/19K is responsible for their reduced expression at the plasma membrane (324-327,330).

As mentioned above, the expression of wt E3/19K resulted in the block of MHC class I surface expression. Consistently, infection of cells with the mutant viruses dl704 or dl754 resulted in an increase of MHC class I plasma membrane expression compared to wt Ad2 (Table 4). Both of these mutants have an internal deletion (Fig. 10 and 11) which results in completely non functional E3/19K proteins (Wold, personal communication). Furthermore, the analysis of cells infected with the dl703 viral mutant gave the same results (Table 4). This mutant expresses E3/19K proteins lacking their signal sequence (Fig. 10 and 11) and were therefore not likely inserted in the ER membrane (Fig. 12). In addition, the MHC class I surface expression found in dl710 infected cells (Table 4) indicated that the additional 5 amino acids found at the carboxyl end of the dl710 mutant (Fig. 16) did not alter the function of E3/19K.

Curiously, the dl708 mutant virus, which lacks most of the E3/19K cytoplasmic tail (Fig. 10 and 11), was as efficient as wt Ad2 in blocking MHC class I surface expression (Table 4). This deletion should delete most or all the E3/19K ER retention signal. However, there is currently no consensus on the exact sequence of this ER retention signal (266,326,327,346-348). The present data suggested that the deletion of the last 9 amino acids was insufficient for abrogation of ER retention. Gabathuler *et al.* (346) showed by transfection that the E3/19K mutant M621, which also lacks the last 9 carboxyl terminal amino acids, interacts with HLA proteins. In this case, some of the M621 proteins can be detected at the plasma membrane, while most of the M621 proteins remain in the ER (Gabathuler, personal communication). Surprisingly, in that system MHC class I surface expression is only slightly reduced, compared to untransfected cells. Furthermore, treatment

of the immunoprecipitated HLA molecules with endo H showed that M621 does not significantly block the transport of MHC class I proteins (346). The apparent discrepancy between the M621 transfected cells and the dl708 infected cells could be explained various ways. First, it may be due to the presence of additional adenoviral proteins in the infected cells. This scenario was supported by the apparent involvement of additional adenoviral proteins to optimally down regulate MHC class I surface expression (see below). Second, it could be that the ER retention was indeed lost, but the mutated E3/19K molecules encoded by dl708 were expressed at the plasma membrane and blocked recognition of HLA protein by the W6/32 antibody. This seemed unlikely, since it was shown that the reduced surface expression of the MHC class I molecules in cells infected with wt virus was not due to the block of the W6/32 epitope (Fig. 26). Third, MHC class I surface expression is dependent on the amount of E3/19K proteins made (325,329,335). It may therefore be that the transfected cells were less efficient in inhibiting MHC class I surface expression than the infected cells because they produced rate limiting amounts of E3/19K proteins. Although this may be the case the optimization data argue otherwise (Fig. 19). Finally, the dl708 and 621.13 encoded E3/19K proteins differ by one amino acid at their carboxyl termini. While both proteins lack the last 9 amino acids, the former E3/19K protein had an additional leucine residue at its carboxyl terminus (Fig. 11). Therefore, the presence of this additional leucine residue at the carboxyl tail of dl708, but absent in M621, may account for the different results. It has been suggested that the presence of basic lysine residues at positions -3 and -4 at the carboxyl tail of wt E3/19K is important for its ER retention signal (327). The deletion in M621 is such that two basic arginine residues are located at positions -1 and -2. The presence of the additional

leucine residue in dl708 shifts those dibasic residues to positions -2 and -3 (Fig. 11). Therefore, the exact location of those dibasic residues was perhaps important but not as critical as suggested by Jackson *et al.* (327).

3.3.8- Need for other viral proteins to optimally block MHC class I surface expression

Various experiments point to the need for an additional molecule other than E3/19K to optimally down regulate MHC class I surface expression. This additional molecule is unlikely the E3/6.7K protein, since the data clearly indicated that it did not participate in this process. The need for such an additional protein was first suggested by the ability of Ad2 to down regulate MHC class I surface expression in some cells but not others (see above and Table 3). In addition, the residual inhibition of MHC class I plasma membrane expression observed in the 621.13 cell line expressing a defective E3/19K protein (Fig. 25) also supports this conclusion. Similarly, the surface expression of MHC class I proteins was only partially restored when cells were infected with viral mutants lacking functional E3/19K (Table 4). Furthermore, deletion of the entire E3 region resulted in an intermediate phenotype between the E3/19K$^-$ infected cells and the mock treated cells (Table 4). This suggested that in fact possibly two or more additional viral molecules were required for the optimal inhibition of MHC class I surface expression. It also crudely mapped the genes encoding for these additional proteins in the Ad2 genome. First, the difference between E3/19K$^-$ and E3$^-$ infected cells suggested that one of these molecules was encoded in the E3 region, but was different from E3/19K and E3/6.7K. However, it is unclear what this protein might be. The 21-23 kDa polypeptide detected by coimmunoprecipitation (Fig. 23) may represent one of these

additional proteins. Second, the difference between E3⁻ infected cells and mock treated cells suggested that a second protein encoded outside the Ad2 E3 region of the genome was also involved. Although the identity of this protein was also unknown, the Ad5 E1 sequences contained in the 293 cell line are potential candidates for this non E3 protein. This hypothesis is particularly attractive given the transcriptional activator function of this region of the viral genome (389). As mentioned above, the involvement of this region on MHC class I surface expression was also suggested by Routes *et al.* (388,390). Thus, the E1 region contained in the 293 cell line was not absolutely essential but could nevertheless have contributed to this phenotype. The 21-23 kDa polypeptide is, of course, also a potential candidate for this function.

The data fully supported the need for an additional viral protein or two to optimally block MHC class I surface expression. However, an alternative explanation is the complex regulation of the various mRNA transcribed from the E3 region. The 9 mRNA species contained in the Ad2 E3 region are under the control of a unique promoter, as reviewed by Wold and Gooding (277). These various mRNA likely compete for transcriptional, splicing and translational factors since they are produced in different amounts (277). In addition, the deletions in the mutant viruses used in this study alter the mRNA expression pattern ((263,265,267,391-394), and Wold, unpublished observations). The deletion viruses could thus produce different amounts of the many E3 proteins and perhaps this accounts for the complex phenotypes observed. To address this possibility, a detailed and careful analysis of the expression of the viral proteins would be required. However, this analysis would be difficult since the E3 region modulates E1A expression (365-367), which is essential for the

replication of the virus. In addition, these E1A gene products regulate the level of transcription of many other viral genes including the E3 genes (362).

3.3.9- Down regulation of MHC class I phosphorylation

As reported in the literature, Adenoviruses can evade recognition by CTL by down regulating HLA class I surface expression (328,332,344,350). So far, two distinct mechanisms have been documented and differ for group A and C Adenoviruses. Given that the MHC class I cell surface expression might be influenced by their phosphorylation (60,61), the effect of Ad2 on MHC class I phosphorylation was examined. Interestingly, infection of 293 cells with Ad2 caused a significant reduction of MHC class I phosphorylation (Fig. 27). Furthermore, analysis of 293 cells transfected with E3/19K also resulted in a reduced MHC class I phosphorylation (Fig. 28). This reduction was significant and could not be explained by a parallel reduction of MHC class I protein expression (Fig. 28). In addition, the MHC class I phosphorylation level observed for the 621.13 cell line (Fig. 28) suggested that this reduced phosphorylation was due to ER retention. It also indicated that the E3/19K protein itself could not actively dephosphorylate MHC class I proteins, unless the last 8 amino acids missing in M621 were crucial to such an activity.

As the 293.12 and 621.13 transfected cell lines also contained the genes encoding other viral proteins, including E3/6.7K, it was unclear which gene products were responsible for this phenotype. The analysis of MHC class I phosphorylation in virally infected cells indicated that E3/19K was involved in this process (Fig. 29). In contrast, the E3/6.7K protein did not participate in the block of HLA class I phosphorylation (Fig. 29), consistent with the

inhibition of MHC class I surface expression data (Table 4). Furthermore, the results also implied that E3/19K did not require the concomitant expression of E3/6.7K to exert its effect. Once again, the results could not be accounted for by a differential effect on MHC class I protein expression, since it was unaffected by the viruses (Fig. 23, 29). In infected HeLa cells, Ad2 had little or no impact on MHC class I phosphorylation levels. However, this was consistent with the minimal effect of Ad2 on MHC class I surface expression in these cells under the conditions used. (Fig. 20, 21).

3.3.10- Intracellular site of MHC class I phosphorylation

The blocks of MHC class I phosphorylation and surface expression were suspiciously comparable in both wt Ad2 infected cells (expression: 40% of mock; Fig. 17 vs phosphorylation: 34% of mock; Fig. 27) and wt transfected cells (expression: 16% of mock; Fig. 25 vs phosphorylation: 20% of mock; Fig. 28). This suggested that the residual phosphorylated MHC class I proteins likely represented the MHC class I molecules that escaped ER retention. This was substantiated by the normal phosphorylation of MHC class I proteins in M621.13 cell line expressing the E3/19K devoid of its ER retention signal (Fig. 28). The availability of the wt E3/19K transfected cell lines readily enabled the examination of the intracellular site of MHC class I phosphorylation. Endo H analysis of the phosphorylated MHC class I molecules indicated that most or all the phosphorylated MHC class I proteins escaped ER retention (Fig. 30). The resistance of the phosphorylated MHC class I proteins to endo H thus confined the site of phosphorylation to a post ER/cis Golgi compartment. This strongly implied that the block of MHC class I phosphorylation was not a mechanism used by

Ad2 to further reduce MHC class I surface expression. Rather, it was likely the indirect consequence of ER retention by E3/19K.

3.3.11- Orientation of E3/6.7K in the ER membrane

In analyzing the biochemical characteristics of the E3/6.7K protein, an important fact was unveiled. Hence, the immunoprecipitation of E3/6.7K and its analysis under reducing and non reducing conditions revealed that the protein contained at least one disulfide bond (Fig. 22). Although only the upper E3/6.7K polypeptide is glycosylated (271,315), both E3/6.7K polypeptides contained a minimum of one disulfide bond (Fig. 22). Since the smaller polypeptide is the precursor of the larger polypeptide (271), this suggested that the formation of the disulfide bonds occurred before the addition of N-linked carbohydrates. These findings were important and have implications for the orientation of E3/6.7K in the ER membrane.

A computer analysis revealed that E3/6.7K had no signal sequence, but had a large transmembrane domain (Fig. 16). In addition, it showed that the potential N-linked glycosylation sites were located near the amino terminus of the protein. In contrast, it indicated that all the cysteine residues were found at the opposite end of the molecule (Fig. 16). Considering that the formation of both disulfide bonds (395) and the addition of N-linked sugars (53) usually occurs in the lumen of the ER, the data suggested that both ends of the E3/6.7K protein reached the ER lumen at some point. There are however, some examples of cytosolic proteins containing disulfide bonds. These include thioredoxin (396), nef (397), and glutathione reductase (398). Nonetheless, it is unclear whether these rare examples of cytosolic disulfide bonds are relevant for the E3/6.7K protein. For two of these molecules,

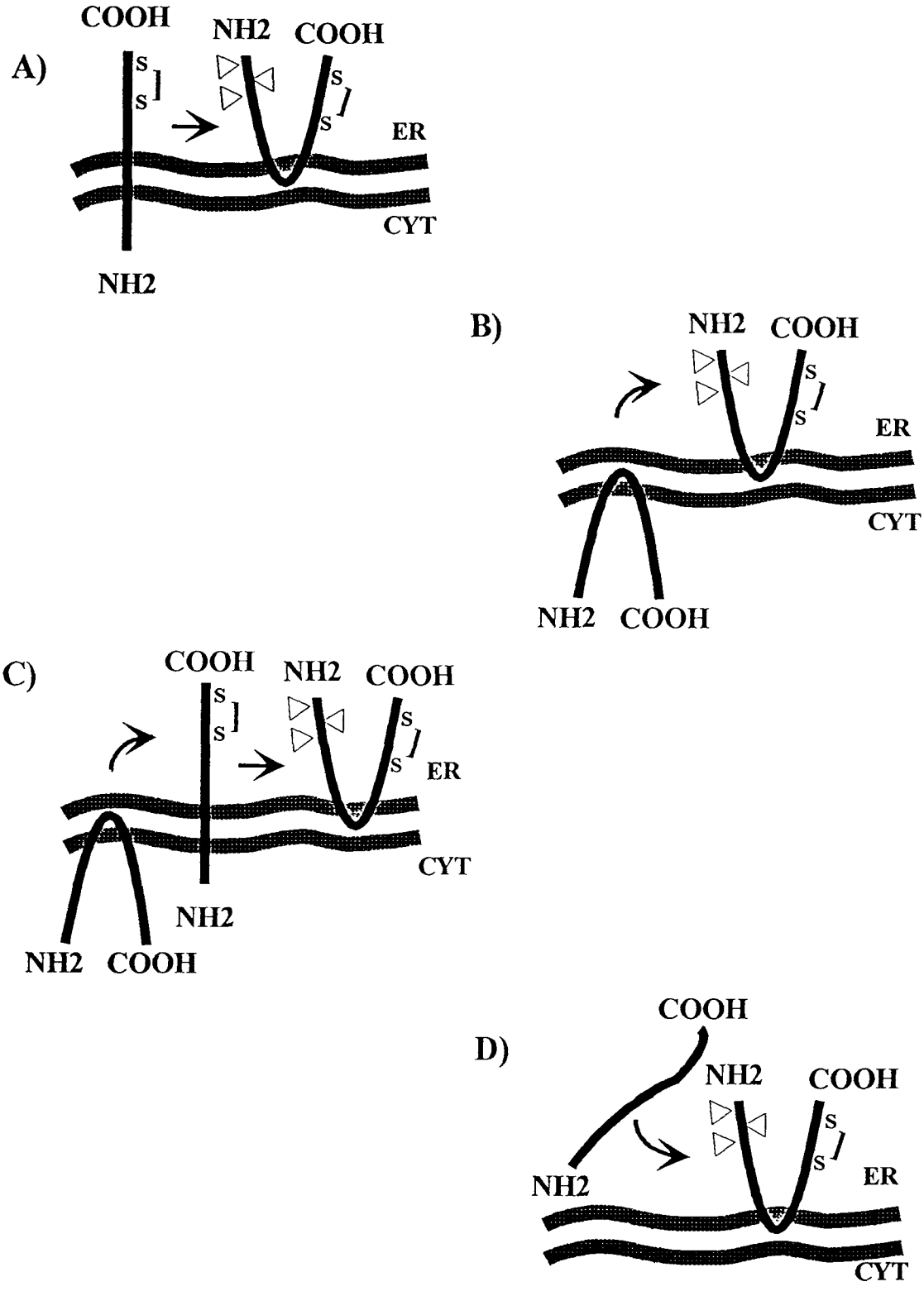
thioredoxin and glutathione reductase, the disulfide bonds are involved in redox reactions and are not essential for the secondary structure of the proteins (396,398). Since E3/6.7K did not share any homology with these proteins (data not shown), it is therefore most likely that the formation of the disulfide bonds occurred in the ER lumen.

Thus, the formation of the disulfide bonds before the addition of the N-linked sugars (Fig. 22 and (271)) indicated that the carboxyl terminus of E3/6.7K was modified before the amino terminus. Interestingly, Wilson-Rawls *et al.* (271,399) suggested that the E3/6.7K protein has its amino terminus in the ER lumen and its carboxyl terminus in the cytoplasm. Considering the presence of N-linked sugars and disulfide bond(s) at the opposite ends of the E3/6.7K protein, the orientation of E3/6.7K was unlikely as previously proposed. So what is the orientation of the E3/6.7K protein in the ER membrane? First, if the E3/6.7K protein assumed an orientation with its amino terminus in the cytoplasm and its carboxyl terminus in the ER lumen, this could not allow the glycosylation of the protein. Second, the possibility that some E3/6.7K molecules were in one orientation while others were in the opposite orientation was unlikely. Most, if not all, glycosylated E3/6.7K proteins contained at least one disulfide bond (Fig. 22). Therefore, the most likely possibility is that both ends of the E3/6.7K protein were the ER lumen. Four scenarios are possible to achieve this configuration and are illustrated in figure 31. First, E3/6.7K may be inserted in the ER membrane as a linear molecule with its carboxyl terminus in the lumen of the ER (Fig. 31, Panel A). This would account for the formation of disulfide bonds before the addition of N-linked sugars. The subsequent addition of sugars would require that the amino terminus flipped inside the lumen of the ER. The amino terminus would likely remain in the oxidizing environment of the ER

lumen, which can form and maintain disulfide bonds (400). Second, E3/6.7K may be inserted in the ER membrane with both its ends in the cytoplasm, which would subsequently be translocated into the ER lumen (Fig. 31, Panel B). The appearance of the disulfide bonds before the addition of the N-linked sugars would possibly reflect a different accessibility of these sites to the respective modifying enzymes. Third, E3/6.7K may be inserted in the ER membrane as above and its carboxyl terminus translocated to the ER lumen before its amino terminus (Fig. 31, Panel C). Finally, it may be that the whole of E3/6.7K is translocated into the ER lumen. This might be possible given the short lengths of its hydrophilic termini (Fig. 31, Panel D). Oligomerization of the E3/6.7K proteins would be necessary to conceal the large E3/6.7K hydrophobic segments and avoid aggregation in the lumen of the ER. Those oligomers would then fuse with the ER membrane. The formation of disulfide bonds before the addition of N-linked sugars could be explained by differential accessibility of the termini, as mentioned above. In all cases, translocation of E3/6.7K in these models could be cotranslational or post translational. Interestingly, Wilson-Rawls and Wold (271) suggested that the E3/6.7K protein may be translocated after it is fully translated or that it may be glycosylated post translocationally. This was suggested on the basis of pulse chase experiments where some E3/6.7K proteins were not chased to the upper glycosylated form of the protein. Thus, the data support the hypothesis that the E3/6.7K protein was oriented in the ER membrane with both its ends in the ER lumen. However, additional experiments are needed to clarify this issue. *In vitro* transcription and translation studies are currently in progress to define the orientation of E3/6.7K in the ER membrane (Jefferies, personal communication).

Fig. 31: Orientation of E3/6.7K in the ER membrane

The likely orientation of E3/6.7K in the ER membrane is depicted. Both the amino (NH2) and carboxyl (COOH) termini are present in the ER lumen. N-linked glycosylation (3 potential sites) and disulfide bonds are depicted by open triangles and brackets respectively. For simplicity, only one of the two potential bonds among the five cysteine residues present in E3/6.7K is shown. In panel A, E3/6.7K is inserted in the ER membrane as a linear molecule with its carboxyl terminus in the lumen. The amino terminus is subsequently translocated into the lumen to allow glycosylation of the E3/6.7K protein. In panel B, E3/6.7K is first inserted in the ER membrane with both its ends in the cytoplasm. Both ends are then translocated into the lumen. Panel C is a variation of panel B where translocation of the two ends is sequential, with the carboxyl end of E3/6.7K being translocated first. In panel D, the E3/6.7K is first entirely translocated in the ER lumen and subsequently inserted in the ER membrane.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CHAPTER 4

ANALYSIS OF THE ANTIGEN PROCESSING AND PRESENTATION POTENTIAL OF THE GRO29 MUTANT CELL LINE

4.1- INTRODUCTION

The previous chapter examined the ability of a natural pathogen, namely Ad2, to modulate the MHC class I antigen processing and presentation pathway. This approach yielded a significant amount of information pertaining to the molecular interactions between Ad2 and MHC class I molecules. A distinct approach to further characterize this pathway consists in the analysis of variant cell lines deficient in the processing or the presentation of peptides to CTL. Therefore, the ability of the gro29 mutant cell line to process and present antigenic determinants in the context of MHC class I molecules was evaluated.

4.1.1- Origin of the gro29 mutant cell line

The Ltk$^-$ cells are fibroblasts of H-2^k murine origin that were adapted to growth in tissue culture from primary connective tissues. As its name implies, the Ltk$^-$ cell line lacks the thymidine kinase gene and is therefore resistant to bromodeoxyuridine (401). It is permissive to HSV-1 infection and was used to generate the gro29 mutant cell line. More specifically, this mutant cell line was derived from Ltk$^-$ cells by ethyl methanesulfonate (EMS) mutagenesis and by subsequent selection for survival after HSV-1 exposure (402). The gro29 cell line is

thus resistant to HSV-1 infection and has been useful to study the role of host proteins for the life cycle of HSV-1.

4.1.2- Characteristics of the gro29 cell line

Despite its origin, the deficiency that gro29 harbors is not limited to HSV-1 but has far reaching implications. These effects are discussed below along with their potential implications for MHC class I antigen processing and presentation.

4.1.2.1- Susceptibility to Herpes simplex virus type 1 infection

Consistent with the selection procedure used to isolate the gro29 cell line, the release of infectious HSV-1 particles by infected gro29 cells is severely impaired when compared to its parental Ltk^- cell line (402,403). However, there is only in a slight reduction in the ability of HSV-1 to infect these cells (404). This is somewhat surprising since all of the other cell lines isolated in the same manner as gro29 have defects in viral entry but can otherwise efficiently produce HSV-1 viruses once infected (404). The gro29 mutant cell line is therefore unique in this regard. The characterization of the gro29 cell line has indicated that this reduced release of HSV-1 particles is not due to a deficiency in HSV-1 replication or assembly of HSV-1 nucleocapsids (402,403). In contrast, the mutation severely affects the intracellular transport and maturation of the virions, which results in their accumulation in perinuclear vacuoles (403).

Parallel to the block of HSV-1 virion egress, the post translational processing and transport of HSV-1 proteins is deficient in gro29. Hence, the analysis of the gB and gD viral

proteins, which both contain N- and O-linked sugars, by pulse chase metabolic labeling revealed the aberrant accumulation of immature viral glycoproteins in gro29 (403). However, this is not due to a deficient production of HSV-1 glycoproteins, since infected gro29 cells synthesize viral glycoproteins in normal amounts. Thus, gro29 is defective in the processing of both HSV-1 virions and proteins. Interestingly, the defect in gro29 hinders the egress of HSV-1 virions to a much greater extent that the processing of the individual HSV-1 proteins (see below).

4.1.2.2- Susceptibility to Pseudorabies virus infection

Pseudorabies virus (PRV), like HSV-1, is a member of the alphaherpesviruses family and shares a similar life cycle to HSV-1 (405). Consistently, the production of PRV virions in gro29 cells is severely reduced in comparison to the PRV infected Ltk^- cells (406). This is accompanied by a reduced transport and maturation rate of three of the PRV proteins, namely gII, gIII, and g50 (406). These glycoproteins undergo different post translational modifications. Unlike gIII, g50 has no N-linked sugars but contains O-linked carbohydrates. Since its processing is also slower in gro29 cells (406), this extends the impact of the gro29 mutation to both N and O-linked glycoproteins. However, the gro29 deficiency is not limited to the post translational processing of carbohydrates as the proteolytic cleavage of the gII glycoprotein is also impaired (406). Together, these data suggest a relatively broad impact of the mutation generated by EMS in gro29 cells.

4.1.2.3- Susceptibility to Vesicular stomatitis virus infection

The deficiency in the gro29 cell line does not specifically interfere with the production of mature alphaherpesviruses and proteins, since it also affects VSV. Hence, the release of infectious VSV particles is significantly reduced in VSV infected gro29 cells compared to infected Ltk^- cells (402). This inhibition is not due to an inefficient entry, replication, or assembly of the VSV virions. However, consistent with the HSV-1 and PRV data, the transport and maturation of the viral proteins are impaired in gro29 (402). For instance, the VSV G protein, the only glycoprotein encoded by the viral genome, is produced in normal amounts but is processed more slowly in gro29 and accumulates in the Golgi apparatus (402). Furthermore, novel forms of VSV G proteins are detectable in these cells (402,407), suggesting the aberrant processing of the proteins. In addition, the fewer VSV virions released in the medium contain a smaller proportion of G proteins than the virions released from infected Ltk^- cells and mostly contain immature G proteins (402). Thus both the production of VSV virions and the processing of the viral G protein are reduced in gro29.

4.1.2.4- Processing of non viral proteins

As indicated above, the deficiency in the gro29 cell line perturbs both the maturation and the transport of viral proteins and the release of infectious particles. The HSV-1 and PRV alphaherpesviruses and the unrelated VSV Rhabdovirus are all affected in gro29 cells, suggesting that the gro29 phenotype has wide ranging implications. This view was further supported in transfection studies using a plasmid construct expressing the human growth hormone gene. These studies indicate that the transport of the human growth hormone and its

secretion into the extracellular medium is hampered in gro29 (407). As for the g50 PRV protein, the human growth hormone is O- but not N-linked glycosylated, confirming the observation that the effect of the gro29 lesion is not limited to N-linked carbohydrates. Finally, analysis of metabolically labeled whole cell lysates reveals that the secretion of numerous host gro29 proteins is reduced. Many of the polypeptides secreted by gro29 also appear more heterogeneous than those secreted by its parental cell line (402). These data therefore suggest that the deficiency in gro29 has far ranging effect on protein and viral metabolism.

4.1.3- Potential impact of the gro29 deficiency on the MHC class I pathway

The published observations concerning the gro29 cell line suggest a generally reduced rate of protein transport varying between approximately 2 and 8 fold (402,403,407). In contrast, unlike the mild block of protein transport, the release of virions into the medium by gro29 cells is sometimes severely impaired. For instance, the release of HSV-1 is reduced by 2000 fold in gro29 cells (403), whereas the release of PRV is reduced by approximately 250 fold (406). This clearly indicates that the production of infectious particles is affected to an extent far greater than the transport of the individual viral components in gro29. In contrast, the release of VSV virions by infected gro29 cells is only reduced by approximately 3 fold (402). It has been suggested that the relatively low impediment on VSV production may relate to its assembly in the cytoplasm rather than the nuclear assembly of the HSV-1 and PRV alphaherpesviruses (402). It is unclear whether the gro29 phenotype is the result of an inefficient transport of proteins and virions, the result of inefficient enzymes involved in their

post translational modifications, or the combined effect of both these possibilities. The detailed analysis of the gro29 mutant indicated that it is deficient in the synthesis of chondroitin sulfate (404). It also suggested that enzymes involved in the metabolism of N-acetylgalactosamine may be defective in gro29 (404).

However, despite these observations, it is currently unknown what the exact mutation might be in the gro29 cell line. Importantly, since the maturation of many proteins is affected, it was likely that this deficiency also affected the transport and maturation of MHC class I proteins. This scenario therefore raised the possibility that the mutation in gro29 may affect the overall MHC class I presentation pathway. Furthermore, it is unclear what effect the severe block of release of the above alphaherpesviruses, and to a lesser extent VSV, might have on MHC class I presentation. It was conceivable that this difference could be functionally important for the presentation of viral peptides by the MHC class I processing and presentation pathway. Finally, the gro29 cell line was isolated to elucidate the role of host molecules in the life cycle of HSV-1 (402). It is therefore distinct from the majority of the antigen processing and presentation mutants currently available. These have generally been selected for the loss of MHC class I or II epitopes (65,408-411). Thus the selection of gro29 cells was not restricted to the loss of MHC epitopes and could therefore have properties different from the existing MHC class I antigen processing and presentation mutants. Taken together, these observations suggested that the gro29 cell line constituted a good candidate as an antigen processing and presentation variant cell line and was therefore examined in more detail.

4.1.4- Objectives and approach

The objectives of this study were to analyze the effect of the gro29 lesion on the MHC class I antigen processing and presentation pathway. More specifically, the effect of the mutation on MHC class I transport and maturation was examined. In addition, the ability of the cell line to process and present various MHC class I restricted antigens was assessed. To achieve these objectives, the surface expression of MHC class I molecules was determined by FACS analysis. Comparison of the processing, the transport, and the surface expression of MHC class I proteins in gro29 and Ltk^- was also determined in pulse chase experiments and by endo H sensitivity. Finally, the impact of the gro29 mutation on antigen presentation was investigated for allogeneic and viral antigenic determinants in standard ^{51}Cr release assays.

4.2- RESULTS

4.2.1- Modulation of MHC class I surface expression

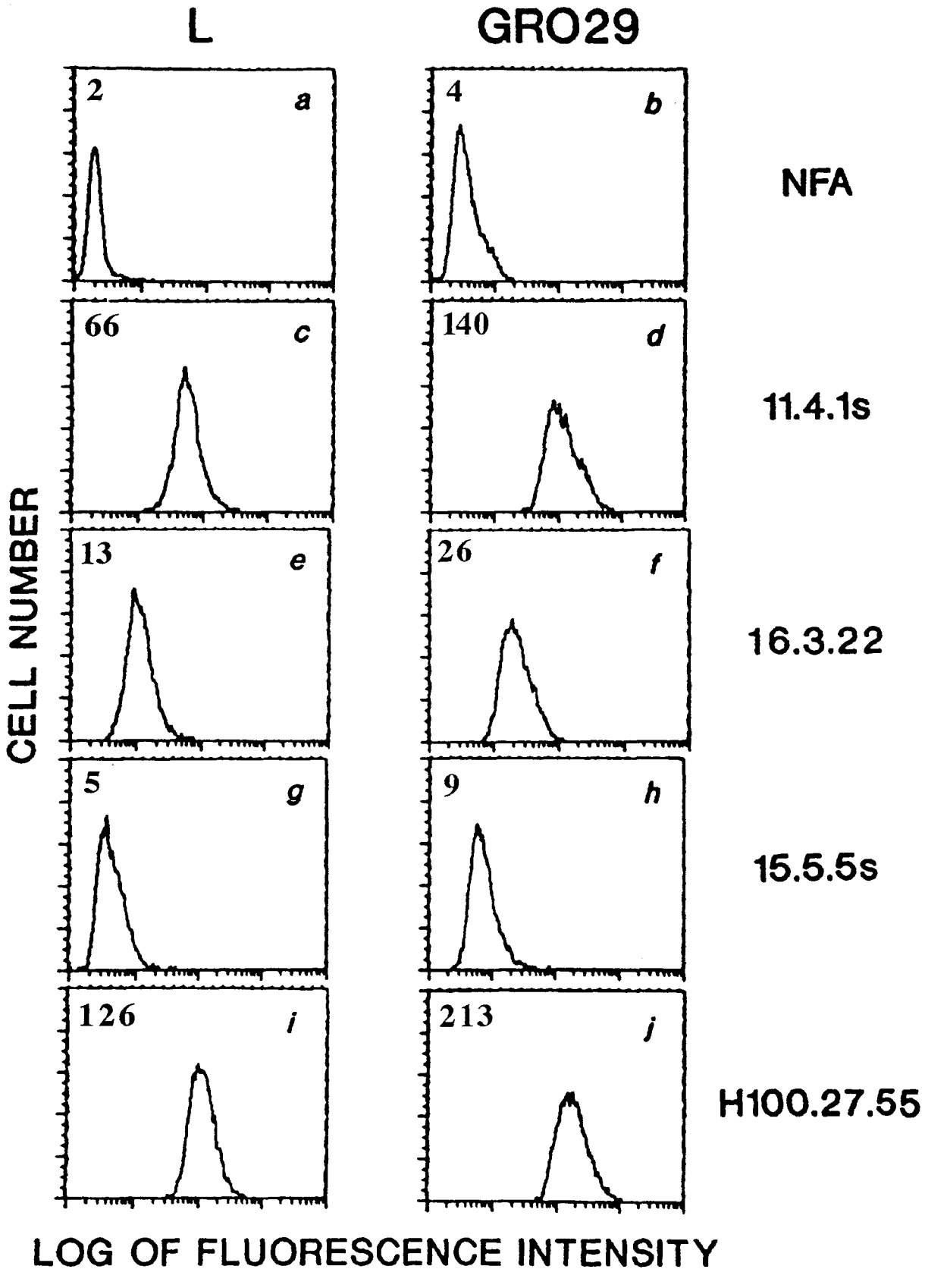
Since the mutation in the gro29 cell line affects the processing of many proteins, the MHC class I molecules were examined to see if they were also affected. In a first step, the surface expression of MHC class I was determined by FACS analysis using H-2^k specific antibodies. Despite the generally slower processing of proteins in gro29 cells, MHC class I expression at the surface of the cells was not reduced when compared to the parental Ltk$^-$ cell line (Fig. 32). In fact, the values obtained for gro29, including the NFA control, were approximately twofold higher on average than for Ltk$^-$ cells. It is worth nothing that both cell types were approximately of the same size. To examine if other cell surface markers were affected by the gro29 mutation, the plasma membrane expressions of FNR, Thy-1, PGP1, and TR were also determined. Consistent with the data on MHC class I protein expression, the level of surface expression of these various proteins was not reduced on the gro29 cell line compared to its parental cell line (Fig. 33).

4.2.2- Intracellular protein transport

The presentation of peptides is not normally dependent of the MHC class I proteins already on the surface of cells but on newly synthesized MHC class I molecules (Chapter 1). Thus, although the surface expressions of MHC class I and other proteins on gro29 were equal to or in excess of that found on Ltk$^-$ cells (Fig. 32, 33), it was of interest to evaluate if the transport rate of MHC class I molecules was normal in those cells. This was particularly important given the reported slower transport rate of various proteins in gro29 cells. Pulse

Fig. 32: Surface expression of MHC class I antigens on gro29 and Ltk$^-$ cells

The surface expression of the H-2K^k and H-2D^k molecules on Ltk$^-$ and gro29 cells was examined. To this effect, subconfluent cells were treated with versene and analyzed by FACS using monoclonal antibodies against H-2K^k (11.4.1s or 16.3.22), H-2D^k (15.5.5s) or H-2K^{k}D^k (H100.27.55). The value in the top left corner of each histogram represents the linear scale fluorescence. To allow comparisons among separate experiments, the gro29/Ltk$^-$ ratio of protein expression was calculated after subtraction of the NFA negative control (see Materials and Methods). As a result, the average of 2 independent experiments yielded the following ratios of gro29 to Ltk$^-$ cell expression: 2.4 $\pm$ 0.6 (16.3.22), 1.9 $\pm$ 0.3 (11.4.1s), 2.5 $\pm$ 1.1 (15.5.5s), and 1.9 $\pm$ 0.3 (H100.27.55).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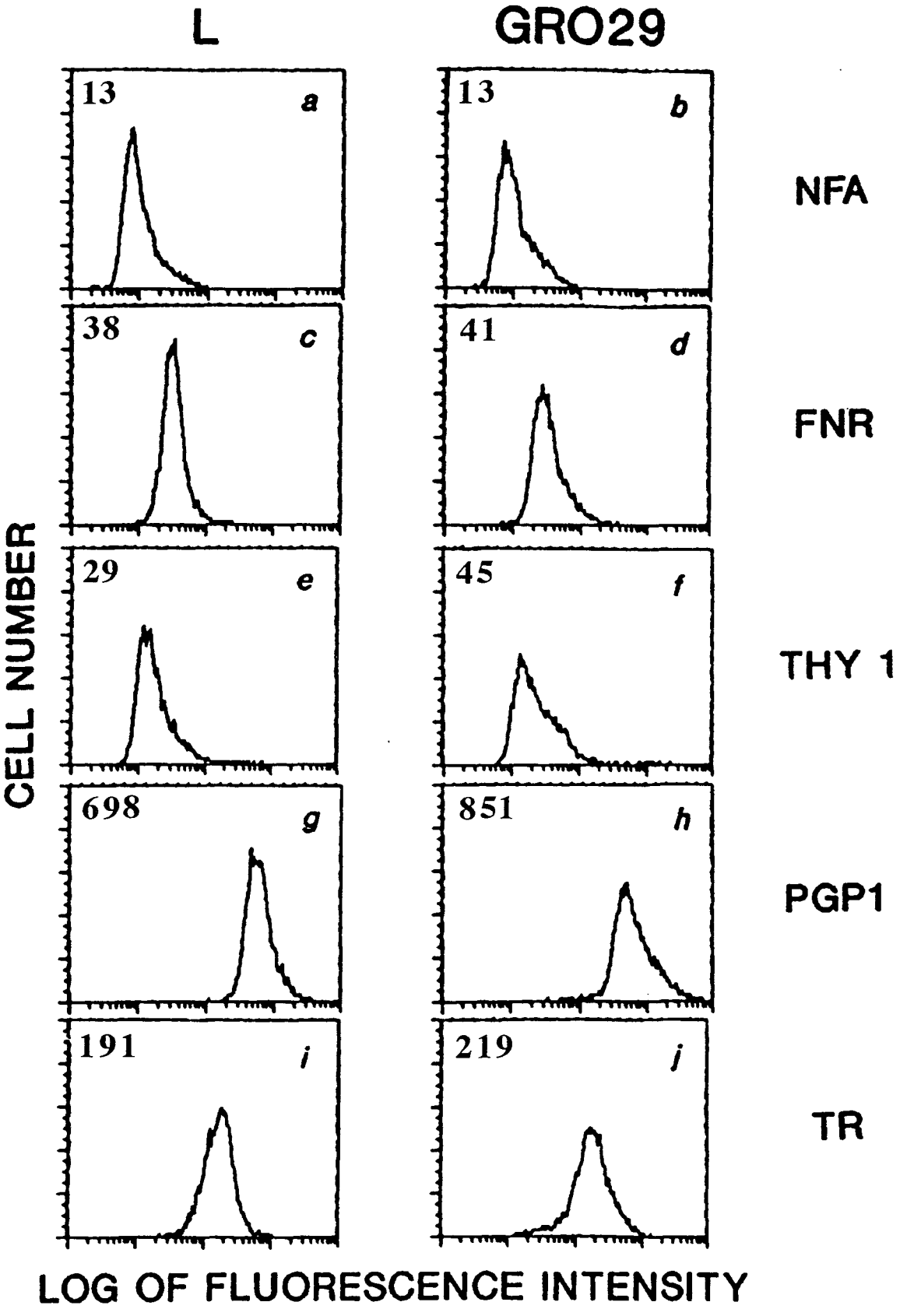
LOG OF FLUORESCENCE INTENSITY

Fig. 33: Surface expression of various cell surface markers

The level of surface expression of various cell markers was examined on gro29 and Ltk$^-$ cells. In particular, the plasma membrane expressions of the FNR, Thy-1, PGP1, and TR were evaluated with the following antisera: FNR: rabbit α human fibronectin receptor which cross reacts with the mouse fibronectin receptor; Thy-1: rabbit α mouse Thy-1; PGP1: rat α mouse precursor glycoprotein 1 (CD44); and TR: yE1/9.9.3 rat α mouse transferrin receptor. As appropriate, the secondary reagent was either goat α rabbit FITC (Panels c-f) or goat α rat FITC (Panels g-j). The NFA samples shown (Panels a-b) are the ones obtained with the goat α rabbit FITC antibody. The NFA results obtained using the goat α rat FITC were respectively 24 for Ltk$^-$ cells and 17 for gro29 cells (not shown). The value in the top left corner of each histogram represents the linear scale fluorescence. On average, the ratios of gro29 to Ltk$^-$ cell expression were: 1.0 ± 0.6 (n=6; TR), 2.3 ± 1.6 (n=4; FNR), 3.1 ± 2.0 (n=4; Thy-1), and 1.2 ± 0.3 (n=4; PGP1).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chases and endo H analyses of MHC class I proteins using the H-2K^k specific 16.3.22 and 11.4.1s monoclonal antibodies were therefore performed. The data indicated that the transport rate of MHC class I molecules was slightly slower in gro29 cells compared to Ltk$^-$ cells (Fig. 34, Panel A). This was most noticeable after a 45 minute chase when the majority of the MHC class I molecules were endo H resistant in Ltk$^-$ cells, but when many molecules still were endo H sensitive in gro29. This indicated that the maturation of the N-linked carbohydrates, which takes place in the medial/trans Golgi, had not been completed yet. Densitometry analysis of the autoradiograms further indicated that there was a two and a half fold difference in the transport rate of MHC class I proteins between the two cell lines. To examine if this difference was specific for MHC class I molecules, the transport of TR molecules was also determined. Consistent with the MHC class I data, the rate of transport of TR proteins was reduced in gro29 by three and a half fold (Fig. 34, Panel B). A statistical analysis showed that there was no significant difference between the transport rates of these two molecules ($p>0.05$). Taken together and consistent with the literature, the data indicated that the transport of MHC class I and TR proteins appeared to be generally slower in gro29 cells.

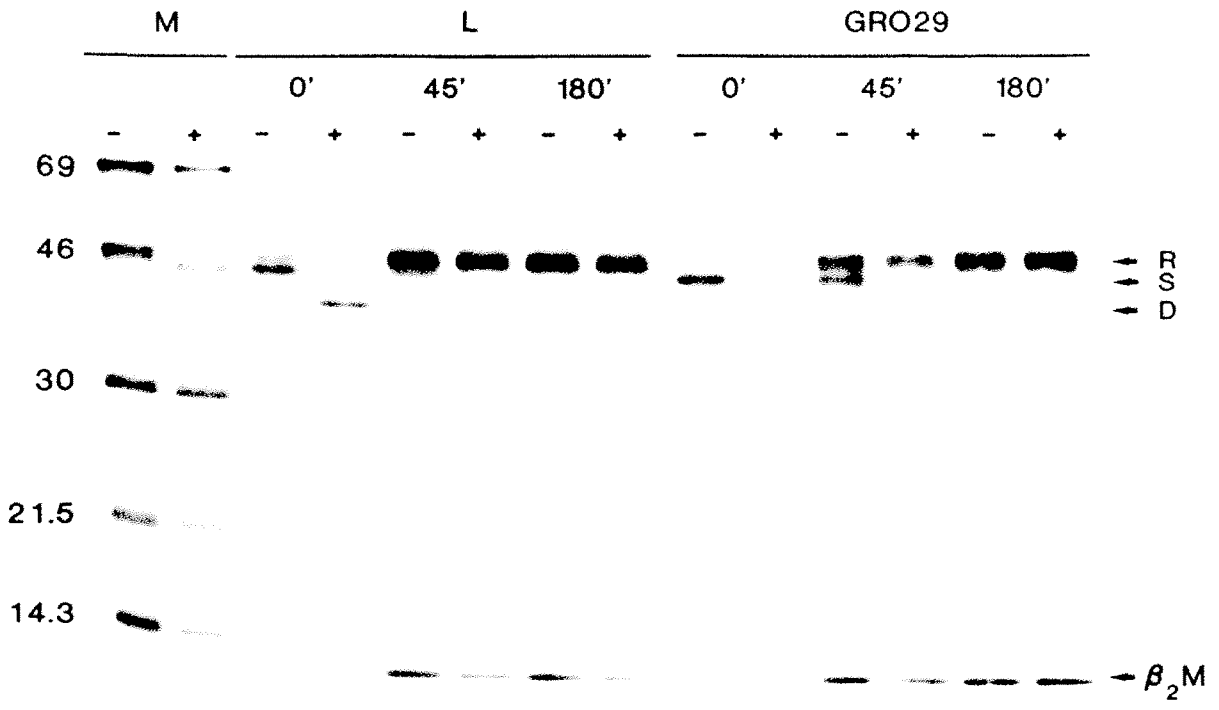
4.2.3- Presentation of allogeneic peptides

The ability of gro29 to process and present antigenic determinants was examined to ascertain whether the slower MHC class I transport rate had an impact on CTL recognition and killing of gro29 targets. First, allogeneic CTL were generated and tested in a ^{51}Cr release assay to measure the efficiency of gro29 to present allopeptides. Thus, the killing of gro29 cells by αH-2^k CTL was indistinguishable from the killing of Ltk$^-$ cells (Fig. 35, Panel A).

Fig. 34: Intracellular protein transport in gro29 cells

Ltk$^-$ or gro29 cells were analyzed by pulse chase to examine the intracellular transport of MHC class I molecules. They were pulse-labeled with ^{35}S methionine/cysteine (Pro-Mix) for 30 minutes and chased for 0, 45, and 180 minutes (see Materials and Methods). The cell lysates were subsequently used for the immunoprecipitation of the H-2K^k molecules with the 11.4.1s and 16.3.22 monoclonal antibodies (Panel A). As a control, the transport of TR proteins was monitored with the yE1/9.9.3 monoclonal antibody (Panel B). The samples were incubated in the presence (+) or absence (-) of endo H, as described in Materials and Methods. They were then analyzed on 10-15% gradient SDS-PAGE and by autoradiography. The migrations of the various MHC class I and TR species are indicated as follows: R: endo H resistant; S: endo H sensitive; D: endo H digested. The ovalbumin (46 kDa) contained in the ^{14}C molecular mass standards (M) was sensitive to endo H digestion and was used as a control for the efficiency of the digestion. The masses of the standards are indicated in kDa on the left side.

A) H-2 K^k



B) TRANSFERRIN RECEPTOR

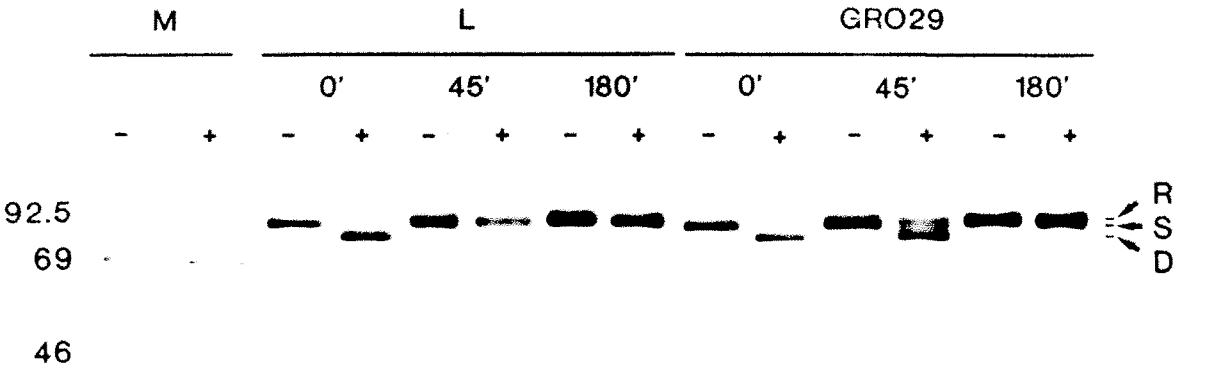
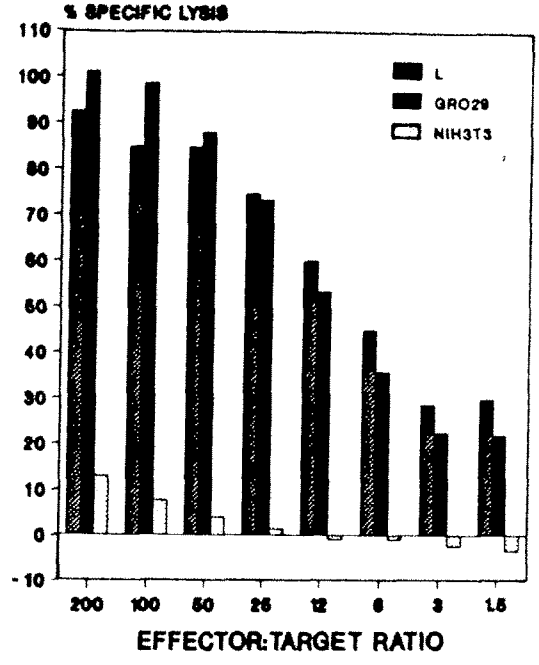


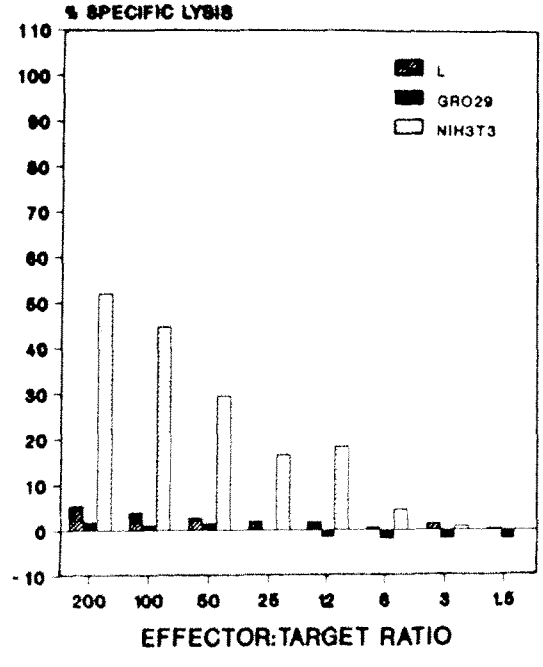
Fig. 35: Susceptibility of gro29 cells to lysis by allogeneic CTL

The lysis of gro29 or Ltk$^-$ cells by αH-2^k (Panel A) or αH-2^d (Panel B) allogeneic CTL was determined to assess their respective ability to process and present allogeneic determinants. NIH/3T3 cells (H-2^d) were also included in the assay as a control cell line. Target gro29 or Ltk$^-$ cells were labeled with ^{51}Cr as described in Materials and Methods and assayed in triplicates in a 5 hour release assay at different effector to target ratios (10^4 targets per well). In all cases, the spontaneous release for gro29 and Ltk$^-$ cells never exceeded 17% of the maximum release. For this particular experiment (Panel A), $p>0.05$ at E:T ratios of 50:1, 25:1, and 12:1 and $p<0.05$ otherwise. In all, five separate experiments were performed and no overall statistically significant differences between gro29 and Ltk$^-$ cells were found.

A) ANTI H-2^{k} ALLOGENEIC EFFECTORS



B) ANTI H-2^{d} ALLOGENEIC EFFECTORS



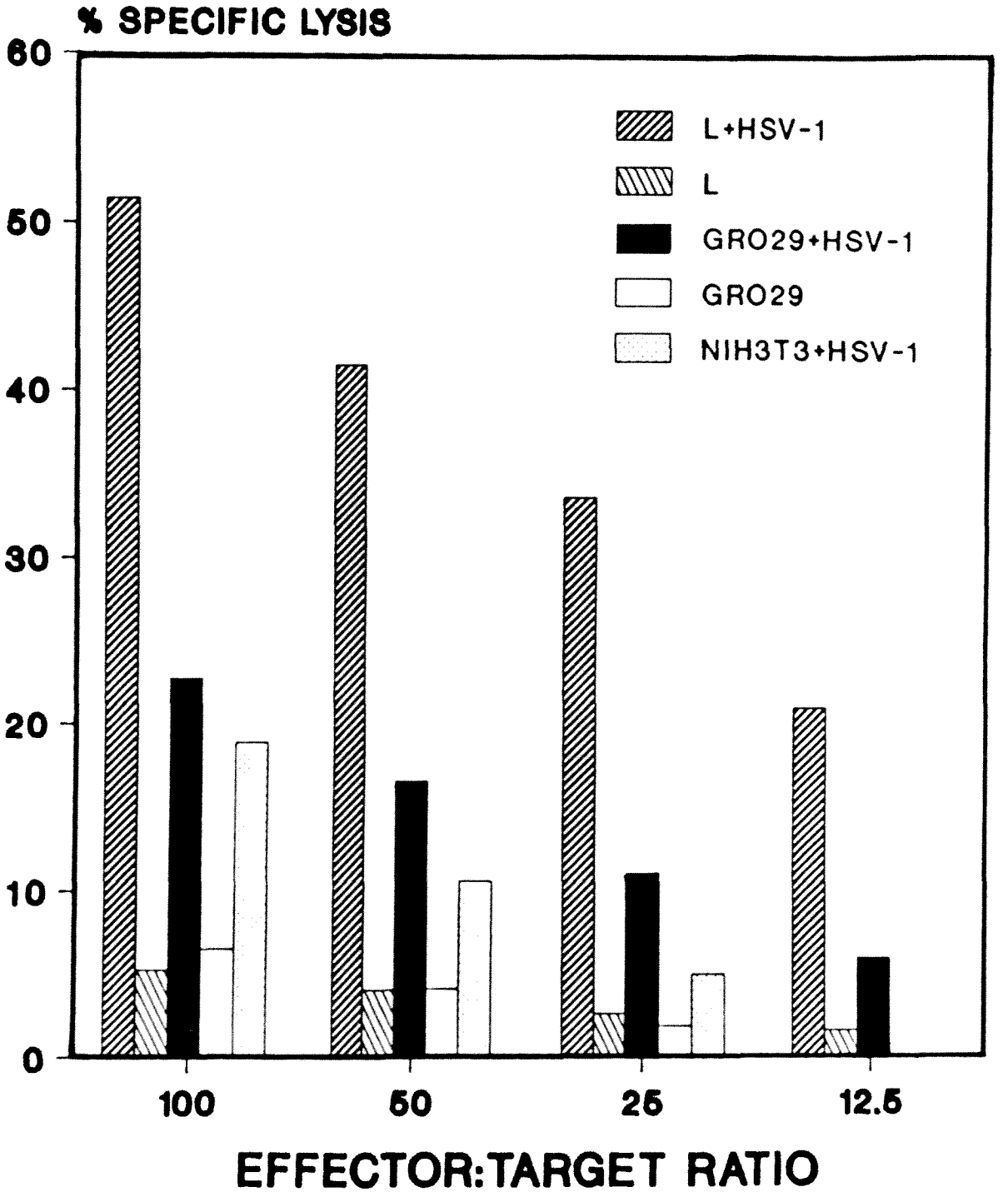
Importantly, the killing was specific as indicated by the very low chromium release from the NIH/3T3 H-2^d control targets. Furthermore, MHC class I restriction was confirmed with the use of αH-2^d CTL (Fig. 35, Panel B). Therefore, the gro29 cell line efficiently and specifically presented allopeptides to CTL.

4.2.4- Presentation of HSV-1 peptides

Given the severe deficiency of gro29 to efficiently replicate or assemble infectious HSV-1, the presentation of HSV-1 antigenic determinants by gro29 cells to CTL was evaluated. The data showed that the αHSV-1 CTL specifically targeted and lysed infected Ltk^- cells but not the mock treated Ltk^- cells (Fig. 36). However, unlike allopeptides, HSV-1 peptides were not efficiently processed and/or presented by gro29. In fact, an eight fold increase in the effector cell population was necessary to obtain a killing of the infected gro29 targets equivalent to the infected Ltk^- cells. For instance, infected Ltk^- cells incubated with the CTL at an E:T ratio of 12.5:1 were killed to the same extent as infected gro29 cells at an E:T ratio of 100:1. The reduced killing of the infected gro29 cells was nevertheless specific and MHC class I restricted. It was significantly greater than the killing of the mock treated gro29 cells or the allogeneic HSV-1 infected NIH/3T3 control cell line. Preliminary experiments further indicated that the killing of the HSV-1 infected gro29 cells was not simply due to a slower infection as it was also reduced as late as 24 hpi (G. Kolaitis, personal communication). Importantly, this lower lysis was not caused by a reduced MHC class I surface expression in the mutant gro29 cell line itself (Fig. 32). In addition it was not due to a reduction of MHC class I surface expression by HSV-1 over the course of the assay (Fig. 37).

Fig. 36: CTL response against HSV-1 infected gro29 cells

The ability of the gro29 cell line to process and present HSV-1 peptides was determined using αHSV-1 specific CTL. Ltk^- or gro29 cells were thus treated with or without HSV-1 (MOI = 5) for 4 hours in the presence of ^{51}Cr (see Materials and Methods for details). They were subsequently incubated with the αHSV-1 effector CTL at E:T ratios of 100:1 to 12.5:1. A 5 hour ^{51}Cr release assay was performed as for the allogeneic CTL assay (Fig. 35). The spontaneous release never exceeded 15% of the maximum release. Statistical analysis of the data revealed that the lysis of HSV-1 infected Ltk^- cells was significantly greater than that of the infected gro29 cells at all E:T ratios tested ($p<0.001$). In addition, the HSV-1 infected gro29 cells were significantly lysed more efficiently than the mock treated gro29 cells ($p<0.01$ to $p<0.001$ depending on the E:T ratio). They were also significantly lysed more efficiently than the HSV-1 infected NIH/3T3 allogeneic control at the 12.5:1 ($p<0.001$), 25:1 ($0.01<p<0.05$) and 50:1 ($0.01<p<0.05$) ratios.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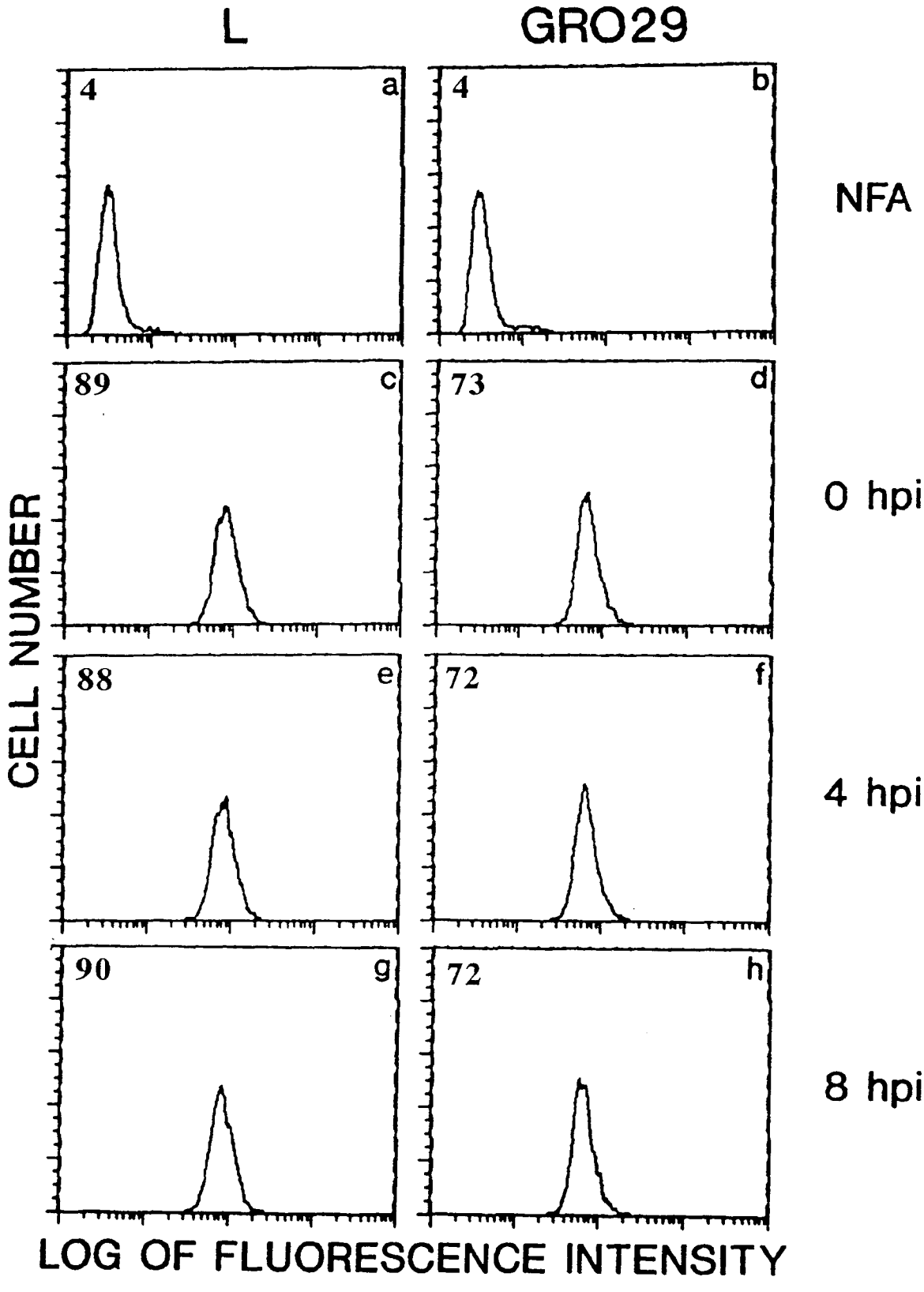
L
GRO29+HSV-1
GRO29
NIH3T3+HSV-1
100
50
25
12.5
EFFECTOR:TARGET RATIO

Fig. 37: Modulation of MHC class I surface expression by HSV-1

The ability of HSV-1 to modulate MHC class I surface expression on gro29 and Ltk^- cells was determined by FACS analysis. To achieve this goal, the cells were infected with HSV-1 at a MOI of 5 and the MHC class I plasma membrane expression monitored by FACS analysis at 0, 4, and 8 hpi with the monoclonal antibody H100.27.55 (αH-$2K^kD^k$). The labels on the figure are as before (see Fig. 32). On average (n=2), the gro29/Ltk^- expression ratios were 0.9 ± 0.1 (0 hpi), 0.9 ± 0.2 (4 hpi), and 1.0 ± 0.2 (8 hpi) respectively.


L
GRO29
4
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89
c
73
d
0 hpi
88
e
72
f
4 hpi
90
g
72
h
8 hpi
CELL NUMBER
LOG OF FLUORESCENCE INTENSITY

4.2.5- Presentation of Influenza peptides

The gro29 cell line efficiently presented allogeneic (Fig. 35) but not HSV-1 (Fig. 36) peptides. To examine the specificity of the presentation defect in gro29 cells, their ability to present other viral peptides was evaluated. Unfortunately, the presentation of VSV to CTL could not be achieved due to the unsuccessful generation of H-2^k restricted αVSV CTL (G. Kolaitis, personal communication). However, H-2^k restricted αInfluenza CTL could be obtained. The presentation of Influenza peptides by gro29 was therefore evaluated. In agreement with the efficient presentation of allogeneic determinants by gro29, but in contrast to the HSV-1 presentation data, gro29 could efficiently process and present Influenza peptides. This was first demonstrated using gro29 cells infected with Influenza virus (Fig. 38, Panel A) and second with gro29 cells incubated with the K^k restricted NP 50-63 Influenza peptide (Fig. 38, Panel B). The lack of specific killing of the infected IT22.6-K^d allogeneic control targets (Fig. 38, Panels A, B) and the competitive inhibition of the killing by H-2^k specific antibodies (Fig. 38, Panel C) showed that the response was MHC class I restricted. The gro29 cell line was therefore specifically deficient in the processing and/or presentation of HSV-1 antigenic determinants.

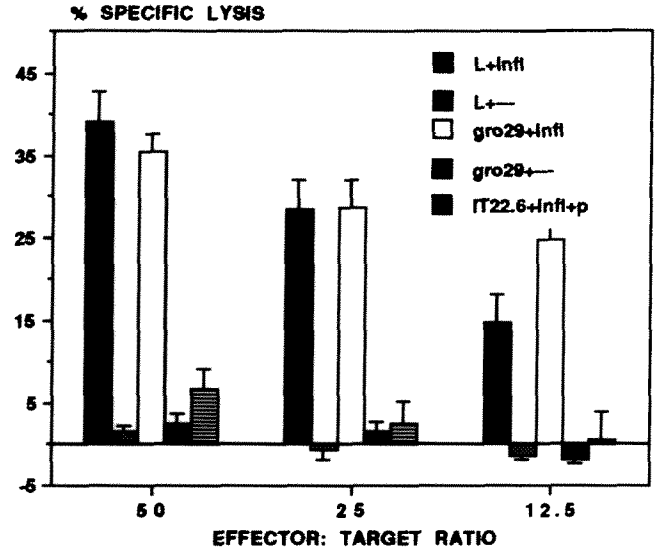
4.2.6- Proteasome component in gro29

The specific inability of gro29 to present HSV-1 peptides argued against a general defect in the processing and presentation pathway of the mutated cell line. It was nevertheless possible that some aspect of the MHC class I restricted pathway was specifically deficient in gro29. Given the proposed proteolytic function of the proteasome in antigen processing and the availability of a proteasome specific polyclonal antibody in the laboratory, the proteasome

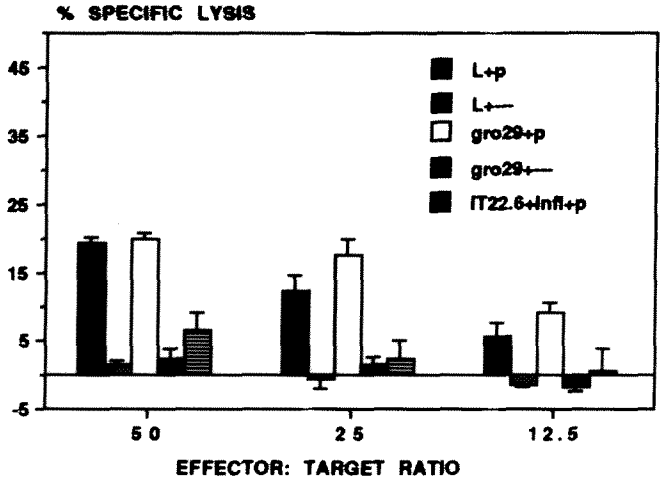
Fig. 38: Specificity of the deficiency of gro29 in presenting peptides to CTL

The presentation of Influenza to viral specific CTL was performed to evaluate the ability of gro29 cells to process and present other peptides. Therefore, target Ltk^- or gro29 cells were labeled with ^{51}Cr and simultaneously mock treated (+ ---) or infected with 400 HA units of Influenza (+ Infl; Panels A, C), as described in Materials and Methods. They were then assayed in a 3 hour ^{51}Cr release assay at three different E:T ratios. In panel C, H100.27.55 monoclonal antibodies were added to the assay to determine if they could inhibit recognition by the Influenza specific CTL (see Materials and Methods). Alternatively (Panel B), gro29 or Ltk^- target cells were mock treated (+ ---) or incubated with the Influenza peptide NP 50-63 (+ p) (see Materials and Methods) and assayed as above. The $H\text{-}2K^d$ transfected IT22.6 cell line was also used as an allogeneic control. In all cases, the spontaneous release never exceeded 15% of the maximum release. A statistical analysis indicated that the difference between gro29 and Ltk^- cells was not significant ($p>0.05$) at the E:T ratios of 50:1 and 25:1 (Panel A; infected cells) and 50:1 (Panel B; peptide treated cells).

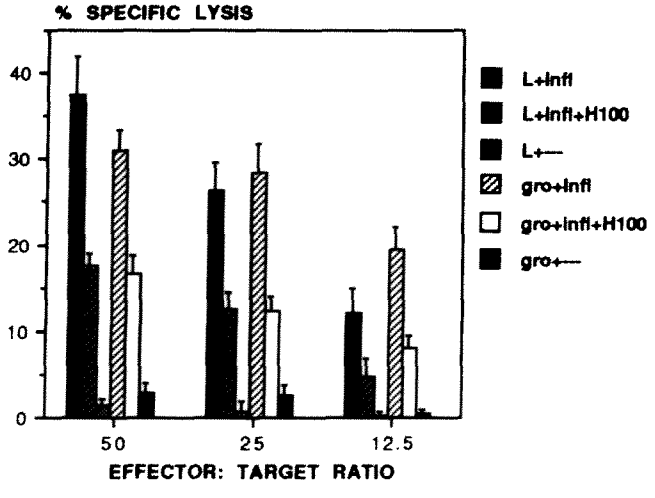
A L vs gro29 +/- Influenza



B L vs gro29 +/- p (NP 50-63)



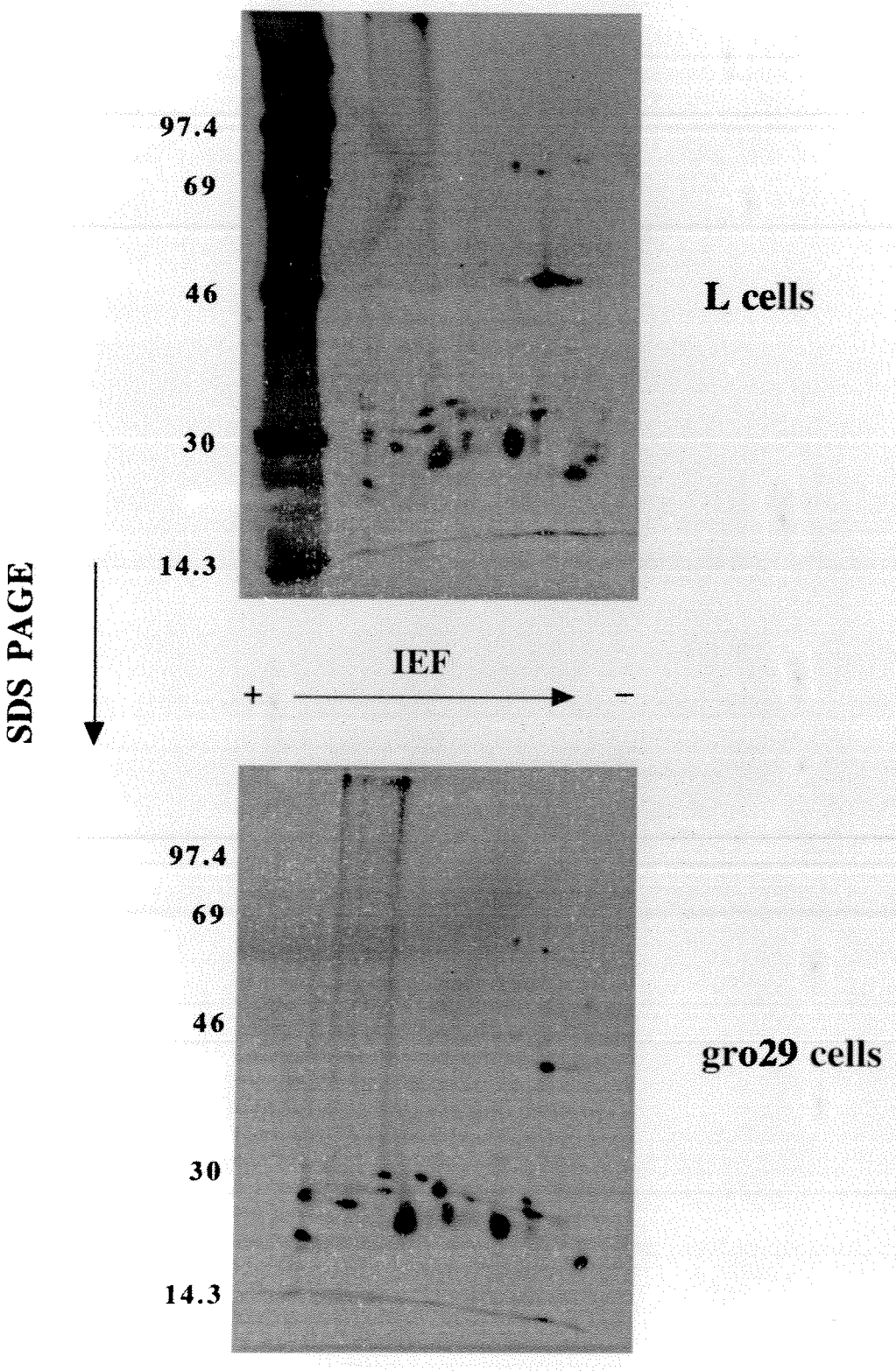
C L vs gro29 +/- Infuenza; Ab Inhibition



component of gro29 was examined. Two dimensional gels were performed after immunoprecipitation of the proteasome with the polyclonal antibody. Although minor differences could be detected between gro29 and Ltk$^-$ cells (Fig. 39), they could not be reproduced, thus indicating that the proteasome of gro29 was not significantly different from that of Ltk$^-$ cells.

Fig. 39: Analysis of the proteasome in gro29 cells

The proteasome from gro29 cells was compared to that from its parental Ltk$^-$ cell line to assess if it was affected by the mutation(s) contained in gro29. Ltk$^-$ and gro29 cells were therefore metabolically labeled with ^{35}S methionine/cysteine (Pro-Mix) for 2 hours following a 1 hour starvation in methionine, cysteine, and serum free DMEM. Equal TCA counts of the cell lysates were precleared and immunoprecipitated with a rabbit α rat proteasome polyclonal antibody, which cross reacts with the murine proteasome. The samples were first separated by IEF, further resolved on 10% SDS-PAGE gels, and finally analyzed by autoradiography. Although some spots differed in intensity and location between gro29 and Ltk$^-$, the differences were not reproducible. See Materials and Methods for details.


97.4
69
46
30
14.3
L cells
SDS PAGE
IEF
+
–
97.4
69
46
30
14.3
gro29 cells

4.3- DISCUSSION

4.3.1- Intracellular transport of proteins in gro29

The gro29 mutant cell line was investigated to determine if its deficiency to process proteins and virions also affected the MHC class I antigen processing and presentation pathway. Examination of the transport of MHC class I and TR proteins indicated that their egress through the secretory pathway was indeed reduced in the gro29 mutant (Fig. 34). Densitometry analysis further revealed that the transport rate of these glycoproteins in gro29 was approximately threefold slower than in the parental Ltk^- cell line. This was consistent with the reduced transport rates of VSV G (402), HSV-1 gB and gD (403), various PRV proteins (406), and non viral proteins (407). As for these latter proteins, the synthesis of the MHC class I and TR proteins was also unaltered by the mutation in gro29 (Fig. 34). Despite this generally slower transport rate, the steady state expressions of various host proteins, including MHC class I, TR, FNR, Thy-1, and PGP1, were normal on the surface of gro29 cells (Fig. 32, 33). This suggested that their reduced transport rate was inconsequential for their expression on the surface of the cells (Fig. 33). This contrasted with the expressions of the VSV and HSV-1 viral glycoproteins which are significantly reduced at the surface of the infected cells (402,403). Although it is unclear why such a difference exists between cellular and viral surface expression, the difference may be attributable to the constitutive expression of the host proteins and may depend on their half lives. Alternatively, or perhaps in addition, viral proteins may be synthesized in such large quantities that the protein transport and/or

processing machinery is overwhelmed during the infection of the gro29 cells, as suggested by Michaelis *et al.* (407) and Banfield (404).

4.3.2- Differential presentation of peptides

Given the slightly reduced transport rate of the MHC class I proteins in gro29 (Fig. 34), it was of interest to determine if this had any impact on the presentation of peptides. The presentation of allopeptides was unaltered, since gro29 cells were lysed by allogeneic CTL as efficiently as the Ltk^- cells (Fig. 35). The lack of killing of the NIH/3T3 H-2^d allogeneic targets indicated that this presentation was specific and MHC restricted. In addition, the presentation of other peptides, namely Influenza peptides, was also efficient (Fig. 38). As for the allogeneic response, the αInfluenza response was specific and MHC class I restricted, since mock treated gro29 or infected IT22.6-K^d allogeneic control cells were not lysed (Fig. 38). The specificity of the presentation was also confirmed by the incubation of gro29 cells with Influenza peptides (Fig. 38) known to be presented in the H-2^k context (135). This specificity was finally confirmed in competition assays using the αH-2^k H100.27.55 antibody (Fig. 38). In sharp contrast to the presentation of allogeneic and Influenza peptides, gro29 did not efficiently process and/or present HSV-1 peptides (Fig. 36). Preliminary data indicated that HSV-1 infected gro29 cells were not appreciably lysed by α viral CTL even at times as late as 24 hpi (G. Kolaitis, personal communication).

The above experiments were performed using bulk CTL populations (see Materials and Methods). This indicated that the entire polyclonal response against HSV-1 was affected by the gro29 defect. This was important, since the use of one or a few individual CTL clones

may not have reflected the wide impact of the deficiency in gro29. Furthermore, since no H-2^k restricted HSV-1 peptide has been mapped to date, the relative importance of individual T clones could not be assessed. Once such HSV-1 peptides are identified, individual CTL clones will be very useful. It will also be possible to examine if the deficiency in gro29 specifically affects the generation, the transport, and the binding of HSV-1 peptides to MHC class I molecules.

4.3.3- Modulation of MHC class I surface expression by HSV-1

Although gro29 expressed a normal complement of K^k and D^k proteins at its surface (Fig. 32), the surface expression of the H-2 molecules could have been modulated by the HSV-1 viruses during the infection. This did not appear to be the case, however, since there was no difference between the MHC class I surface expression in infected (4 and 8 hpi) and mock treated (0 hpi) gro29 cells (Fig. 37). Furthermore, HSV-1 infection did not result in any significant alteration of MHC class I surface expression on gro29 cells compared to the parental Ltk^- cell line (Fig. 37). It is noteworthy that the span of the assay (8 hpi) was equivalent to the duration of both the HSV-1 infection and CTL assay (9 hpi). Most importantly, HSV-1 infection had no significant impact on MHC class I synthesis, transport, or surface expression since HSV-1 infected Ltk^- cells efficiently presented HSV-1 peptides to CTL (Fig. 36). Thus, HSV-1 did not affect the expression of the MHC class I molecules at the surface of infected gro29 cells. These results contrasted with that obtained by Jennings *et al.* (412) and Hill *et al.* (413), which showed that both HSV-1 and HSV-2 interfere with MHC class I surface expression. Although it is unclear why their results differ from the ones

reported here, it may be that HSV modulation of MHC class I surface expression is specific to certain cell types or MHC alleles. Such a hypothesis would be consistent with the modulation of MHC class I proteins by Adenoviruses (Chapter 3). Hence, in contrast to our data which used H-2^k mouse fibroblasts, the study by Jennings *et al.* (412) was done with mouse fibroblasts expressing H-2^b molecules, whereas the study by Hill *et al.* (413) was done with human fibroblasts. Interestingly, Confer *et al.* (414) and Posavad and Rosenthal (415) showed that HSV-1 and -2 infections also result in the inactivation of NK, LAK, and CTL. However, the mechanism of this inactivation is as of yet unresolved and appears to require contact between the cytotoxic cells and HSV infected cells. It is unclear at the moment whether this phenomenon has any relevance for gro29, particularly since this cell line is deficient for the release of HSV-1 infectious particles.

4.3.4- Impact of the gro29 deficiency on MHC class I presentation

The ability of gro29 to efficiently present allogeneic and Influenza peptides (Fig. 35, 38) indicated that it did not have a gross defect in the processing of proteins into peptides. It also indicated that gro29 did not have a major defect in the transport of those peptides to the ER lumen. Finally, it indicated that the transport of the peptide loaded MHC class I molecules to the cell surface was sufficient to promote normal CTL recognition, despite the lower MHC class I transport rate (Fig. 34). The MHC class I molecules were therefore qualitatively and quantitatively functional. Thus, the reduced transport rate of MHC class I molecules in gro29 cells was not critical for antigen presentation and did not explain the eight fold reduction in the presentation of the HSV-1 peptides. The insignificance of a threefold reduction of the

MHC class I transport rate on the presentation of peptides to CTL can also be inferred from the comparison of the transport rates and the presentation efficiencies of individual MHC class I alleles (330,416,417). This therefore suggested a requirement for a minimal amount of MHC class I proteins present on the surface of cells. This also indicated that the gro29 mutant cell line met that minimum requirement. In support of this idea, it has been suggested that as few as 200 MHC class I-peptide complexes are required for presentation to CTL (38). Significantly, the surface expression of both K^k and D^k molecules was normal on gro29 cells (Fig. 32). It is therefore unlikely that the differential presentation of peptides could be explained on the basis that HSV-1 presentation required one of the H-2 molecules while the allogeneic and Influenza peptides required the other H-2 molecule. In any case, the mutation in gro29 clearly and specifically did affect the processing and presentation of HSV-1 peptides.

4.3.5- Differential generation of HSV-1 peptides

The difference between the allogeneic and Influenza presentation and the HSV-1 presentation strongly hinted at the processing or presentation of these antigens by two partially or totally distinct pathways. Alternatively, it suggested that the mutation in gro29 distinctly affected the HSV-1 antigens. Gro29 is deficient in the synthesis of chondroitin sulfate chains (404,407), the maturation of N- and O-linked sugars (402,403,406,407), and lacks ricin and modeccin binding molecules present in Ltk^- cells (407). This indicated that the HSV-1 antigens could indeed be improperly processed in gro29. In addition, given the slower transport through the secretory apparatus of gro29 cells (Fig. 34), proteins likely spent more time in some of the subcellular compartments en route to the cell surface. This implied that

the gro29 mutation could have directly affected the transport of proteins through the secretory pathway. Alternatively, gro29 may have harbored deficient enzymes needed for the above post translational modifications. It has indeed been suggested that an enzyme required for the metabolism of N-acetylgalactosamine, a common denominator for many of the above modifications and lectin binding specificities, is deficient in gro29 (404). It was therefore possible that the longer or inefficient processing of the HSV-1 proteins in gro29 resulted in proteins differently processed than those found in Ltk^- cells. It is also conceivable that this resulted in the generation of non immunogenic peptides. Such peptides could potentially outcompete the normal HSV-1 peptides and prevent their presentation to CTL. Significantly, the αHSV-1 CTL used in this study were elicited by the direct injection of HSV-1 virus in the foot pads and ears of mice. The immunization of mice with infected gro29 or Ltk^- cells instead of HSV-1 alone would allow us to examine if gro29 synthesized different peptides than Ltk^-. For instance, if different peptides were made in gro29, then CTL recognizing HSV-1 infected gro29 cells, but not HSV-1 infected Ltk^- cells, may be isolated. Similarly, if non immunogenic viral peptides were generated in gro29 cells, then no HSV specific CTL response would be detectable. An alternative and complementary approach to examine this issue would be the characterization of the peptides presented on the surface of gro29 cells. The recent technological advances in peptide elution, purification, and identification should allow such an analysis.

4.3.6- Differential recruitment of HSV-1 peptides

Although gro29 may generate distinct HSV-1 peptides, it is difficult to justify why this would be specific for this virus and not affect allogeneic or Influenza peptides. An alternative explanation for the inability of gro29 to present HSV-1 peptides is therefore its possible deficiency in recruiting HSV-1 peptides in the MHC class I pathway. The specificity of this deficiency for HSV-1, and not for allogeneic or Influenza peptides, may be explained by the particular mode of replication of HSV-1. For instance, HSV-1 virions are assembled in the nucleus using immature viral glycoproteins. HSV-1 then buds at nuclear membrane (418-420) and the immature glycoproteins are further processed to their mature form en route to the cell surface. To explain this phenomenon, it has been suggested that the assembled virions go through the secretory pathway and in particular through the Golgi apparatus where the immature carbohydrates would be processed (421-423). It is therefore possible that the recruitment of HSV-1 peptides was dependent on the egress of the virions through or beyond the Golgi apparatus. The severe block of virion egress in gro29 and the sequestering of immature virions in perinuclear vacuoles (402,403) might thus prevent gro29 from processing HSV-1 proteins into peptides that could be recruited for presentation to CTL. This is particularly relevant considering that, apart from the time when they were synthesized, the HSV-1 proteins possibly never encountered the proteasomes present in the cytosol. If, as indicated in Chapter 1, the generation of peptides is dependent on such encounter, then it is conceivable that no appropriate HSV-1 peptides were present in HSV-1 infected gro29 cells. In contrast, Influenza is assembled in the cytoplasm using mature glycoproteins and buds at the plasma membrane (424). Thus, as for allogeneic antigens, there is no sequestering of the

proteins from proteasomes and presumably the proteolysis of antigens into peptides was normal in gro29 cells. In support of this view, it is very interesting that the presentation by wt cells of the HSV-1 gC glycoprotein to H-2^k restricted HSV-1 specific CTL is dependent on the source of the viral protein. For instance, when expressed in its native environment, that is in HSV-1 infected cells, gC is efficiently presented to H-2^k restricted αHSV-1 CTL (425). However, it is not presented when encoded by an Adenovirus based vector or when transfected in Lta cells (426). This underlines the impact of the maturation pathway of HSV-1 virions on antigen presentation, as suggested above.

Examination of the ability of gro29 to present other viral peptides might clarify this issue. If the maturation pathway of the HSV-1 glycoproteins influences their presentation to CTL, one could expect a correlation between the route of maturation of virions and the presentation of viral peptides to CTL. Consistently, Whealy *et al.* (406) showed that infection of gro29 with PRV, another alphaherpesvirus, results in the inefficient production of infectious particles. The PRV phenotype is strikingly similar to that of HSV-1. It would be most interesting to see if the block of egress of PRV virions alters the ability of gro29 to present PRV peptides to virus specific CTL. This, however, has not been documented so far.

CHAPTER 5

GENERAL CONCLUSION

5.1- Adenoviruses

5.1.1- Structural and functional analysis of the Ad2 E3/6.7K protein

The clustering of genes in the E3 region of the adenoviral genome involved in the regulation of the immune system and the functional subdivision of the adenoviral genome suggests a role for the E3 region as a whole as an immunomodulating unit. As discussed in detail in Chapter 3, the E3/19K viral protein is a central component of this immunomodulation, since it blocks the surface expression of many MHC class I proteins. Given the cotranscriptional regulation of the E3/6.7K and E3/19K proteins, it was of interest to characterize the properties of the Ad2 E3/6.7K protein. The data yielded a significant wealth of information. First, such studies indicated that the E3/6.7K protein specifically interacted with E3/19K (Fig. 23, 24). Second, this interaction was complex as only the upper N-glycosylated E3/6.7K polypeptide was coimmunoprecipitated with the E3/19K protein (Fig. 23). Thus, this interaction appeared to either require the additional post translational modification of E3/6.7K or its interaction with a third molecule. Third, the coimmunoprecipitation of E3/6.7K and E3/19K from Ad2, but not Ad5 or rec700, infected cells (Fig. 24) suggested the need for a non E3 encoded viral protein. It is unclear whether this non E3 viral protein and the putative molecule interacting with the upper E3/6.7K

polypeptide are the same. Fourth, despite the binding of E3/19K to MHC class I proteins and the binding of E3/6.7K with E3/19K, an interaction between E3/6.7K and MHC class I molecules has yet to be found. It is unknown whether such complex exists or whether the interactions of E3/6.7K or MHC class I proteins with E3/19K are mutually exclusive. Although the data do not directly address this issue, they support the possibility that a binding of E3/6.7K and MHC class I molecules was transitory and could not be detected with the W6/32 antibody. Finally, the presence of both disulfide bonds (Fig. 22) and N-linked sugars suggested that E3/6.7K adopted a unique orientation in the ER membrane (Fig. 31).

We also originally questioned the role of the Ad2 E3/6.7K protein in the inhibition of MHC class I surface expression by Ad2. The data clearly showed that, unlike the E3/19K, E3/6.7K did not down regulate MHC class I surface expression (Table 4). Surprisingly, they underlined the need for an additional viral components or two to optimally block MHC class I surface expression. This view was supported by the residual block of MHC class I surface expression in the 621.13 transfected cell line, which expresses an E3/19K protein devoid of its ER retention signal (Fig. 25). Interestingly, the residual block of MHC class I plasma membrane expression in the 621.13 cell line (Fig. 25) was not as important as the residual block found for cells infected with E3/19K$^-$ viral mutants (Table 4). Although it is unclear why such a difference exists, it may be because of a different expression level of all the E3 proteins in the transfectant compared to the infected cells. This is particularly relevant, since the expression of the viral gene products is tightly regulated by the virus. The need for an additional component or two was also supported by the specific down regulation of MHC

class I surface expression in certain cell types (Table 3). The identity of this(these) additional molecule(s) is unknown at the moment.

Finally, we questioned the likelihood that Ad2 might block MHC class I phosphorylation as a mechanism to further reduce their plasma membrane expression. Hence, Ad2 did block MHC class I phosphorylation (Fig. 27) and E3/19K, but not E3/6.7K, was involved in this process (Fig. 29). Nevertheless, the data also indicated that this did not represent a mechanism used by Ad2 to further modulate MHC class I surface expression. They rather suggested that it was the consequence of their ER retention by E3/19K (Fig. 28, 30). Finally, these experiments also indicated that MHC class I proteins were phosphorylated in a post ER/cis Golgi compartment (Fig. 30).

Altogether, the current data have unveiled a complex system, where the block of MHC class I surface expression (Table 3) and phosphorylation (Fig. 29) was influenced by the cell type. In contrast, the interaction of E3/6.7K with E3/19K appeared to be dependent on the adenoviral serotype (Fig. 24), but not the cell type (Fig. 23, 24).

5.1.2- Possible role of the Ad2 E3/6.7K protein

The function of E3/6.7K remains a mystery! The current study only examined one aspect of the immune response, that is the participation of E3/6.7K in the down regulation of the MHC class I surface expression by Ad2. The failure to identify the function of E3/6.7K is thus likely the consequence of such a focused approach. So what is the function of the E3/6.7K protein? Its interaction with E3/19K (Fig. 23, 24) and their coregulated transcription suggests a complementary function, but it is unclear what this function might be.

Interestingly, E3/6.7K had no homology to any known molecules, but shared structural homology to certain molecules, including ion channels (Table 2). Preliminary experiments using transfected oocytes failed to detect any ion channel activity (Stea, personal communication). However, the lack of detectable ion channel activity at the surface of the oocytes did not rule out this possibility, because E3/6.7K has since been localized in the ER membrane. Experiments measuring intracellular ion channel activity would therefore be required. Alternatively, E3/6.7K may modulate or be essential to the function of other gene products that are believed to alter the immune response. Potential scenarios include the ability of the E3 region to down regulate the expression of the E1A antigenic determinants (367), to affect the surface expression of the epidermal growth factor receptor (261), or to mediate TNF resistance (261). The E3 mediated resistance against the TNF cytokine also raises the possibility that E3/6.7K may modulate the expression or function of other cytokines in infected hosts. Finally, the function of E3/6.7K might be a yet uncharacterized mechanism to modulate the immune system. Of course, it may also be that E3/6.7K plays a role unconnected with the immune system.

5.1.3- Potential regulation of other components of the MHC class I pathway

The current study reiterates the role of Ad2 E3/19K on the surface expression of MHC class I proteins. It is interesting to ponder whether Adenoviruses can also modulate the function of other components of the MHC class I processing and presentation pathway. The characterization of the proteasome, the TAP transporters, and the possible need for chaperones suggests numerous other potential sites of interaction by pathogens, and perhaps

Adenoviruses. Since the transport of MHC class I proteins out of the ER is dependent on the function of these accessory molecules, it is conceivable that Ad2 might modulate their function as a complementary mechanism to alter MHC class I surface expression. To this effect, it is particularly noteworthy that Adenoviruses can modulate the induction of interferons (261). This may be quite relevant since various genes in the MHC locus, including the MHC proteins, the TAP transporters and components of the proteasome, are inducible by γ interferon (192). Although the immunologically relevant E3 region of Ad2 encodes 9 proteins, the functions of many of those gene products, including the E3/6.7K protein, still remain to be established. It would therefore be interesting to examine if Ad2 modulates other components of the MHC class I surface expression.

5.1.4- Role of the E3 region *in vivo*

Although the E3 region, and in particular the E3/19K protein, clearly protects infected cells against CTL *in vitro* (328,332,344,350), its impact *in vivo* is elusive at the moment. Interestingly, the E3/19K protein may only be functional *in vivo* in the context of the Adenoviral genome, since it does not protect against Vaccinia virus when it is expressed in a Vaccinia vector (427). Surprisingly, some studies have indicated that the entire E3 region is not required for the effective replication of Adenoviruses in their hosts (291,292). This suggests that the replication and survival of the viruses may not be favored by evasion of the immune system, as one might intuitively think. This may nevertheless not be necessary since the viruses would have sufficient time to replicate before the onset of the cellular response of its host. Interestingly, the presence of the E3/19K protein appears to reduce the severity of

the pathogenesis caused by the infection (292,428), indicating that it does indeed play a role *in vivo*. This suggests that the function of E3/19K, and perhaps of the E3 region, is to minimize the impact of the immune response on the host itself rather than to evade the immune system. The recent generation of transgenic mice encoding the E3 region should be very useful (295). It may also clarify the mechanism by which Adenoviruses can become persistent or establish latent infections. The impact of the E3/6.7K protein *in vivo* has not yet been documented.

5.2- Gro29

5.2.1- Characterization of the gro29 mutant cell line

Given the inefficient processing of various proteins and virions in the gro29 mutant cell line, we wondered if these observations could be extended to MHC class I molecules. The analysis of the gro29 mutant revealed that the transport of MHC class I molecules was indeed slower in gro29 than in the parental Ltk^- cell line (Fig. 34). Not surprisingly, the approximately threefold reduction of the rate of maturation of proteins was not specific for the H-2 molecules and was also observed for the TR (Fig. 34). However, this slower transport rate appeared inconsequential for the steady state surface expression of these and other surface markers as their expression was normal on gro29 cells (Fig. 32, 33).

We also wondered whether the gro29 phenotype would perturb the processing and the presentation of peptides by MHC class I proteins. Interestingly, despite the slower transport of the MHC molecules, the presentation of allogeneic and Influenza peptides was unaffected by the gro29 lesion (Fig. 35, 38). Thus, the reduced transport rates of glycoproteins observed

in gro29 cells was not critical for antigen presentation. In contrast, it was found that the killing of HSV-1 infected gro29 cells by HSV-1 specific CTL was drastically reduced compared to infected Ltk$^-$ cells (Fig. 36). Importantly, this reduced killing was not caused by the modulation of MHC class I surface expression by HSV-1 (Fig. 37). The data implied that the HSV-1 peptides were processed by a partially or totally distinct pathway than the allogeneic or the Influenza peptides. As mentioned in Chapter 4, the particular mode of replication of alphaherpesviruses may have influenced the choice of pathway used to present the HSV-1 peptides. Hence, the route of maturation of HSV-1 and the block of egress of the virions in gro29 may have prevented the processing of the viral proteins into presentable peptides. This sequestering of proteins from recruitment into the MHC class I processing and presentation pathway did not occur for Influenza and allogeneic antigens, since they are readily available for degradation by the proteasome.

5.2.2- Molecular basis of the deficiency of gro29

As discussed in Chapter 1, the effective presentation of peptides to CTL requires the participation of a number of components. These include the generation of peptides by proteasomes, their transport to the ER lumen by the TAP transporters, their binding to newly synthesized MHC class I heavy and light chains, and the possible need for chaperones that may facilitate the assembly and transport of the MHC class I complex. The gro29 mutation may therefore affect one of these components. Not all of these components have been examined in gro29, primarily because they were more recently identified, but also because of the lack of reagents to analyze them at the time. However, as mentioned above, no gross

deficiency of the MHC class I antigen processing and presentation pathway is present in gro29, since it efficiently processed and presented allogeneic and Influenza peptides. This also underlined the fact that the MHC class I molecules themselves were functional in gro29 cells. Furthermore, the analysis of gro29 proteasomes indicated that there were no reproducible differences between gro29 and Ltk$^-$ cells (Fig. 39). Although unclear, it is possible that some of the variability observed from experiment to experiment was due to the presence of degradation products. It should be noted that this analysis only examined the expression level, the isoelectric point, and the molecular masses of the various components of the proteasome. Therefore, it did not exclude the possibility that one of the proteasome subunits had a point mutation and was functionally altered in some way. It would also be interesting to determine the expression level of the TAP proteins and their functionality in gro29. The current availability of TAP specific DNA probes (184) and the existence of TAP specific antibodies (190) should prove useful for these purposes. Finally, it is possible that the deficiency in gro29 affected the function of a chaperone molecule.

Importantly, it is conceivable that the gro29 mutation did not significantly alter the conformation of the mutated protein and hence could not be detected serologically. Given that peptide presentation results from the combined selection by proteasomes, the TAP molecules, and the MHC class I molecules, it may be that the specificity of one or some of these components was altered by the EMS mutagenesis. Hence, the presentation of HSV-1 peptides could specifically be altered. Therefore, to thoroughly analyze the gro29 mutation and its effect on antigen processing and presentation, the evaluation of the efficiency and the specificity of the proteasome, the TAP proteins, and MHC class I molecules will be required.

Functional *in vitro* studies using isolated proteasomes, microsomes, and purified MHC class I molecules should address this issue. Finally, analysis of the peptides that are presented by gro29 may be very informative and provide clues as to the nature of the mutation in this cell line.

The gro29 cell line was generated by EMS mutagenesis and thus contains one or more point mutations. Although the reversion rate for this cell line has not yet been determined, the rate of isolation of the mutant after EMS mutagenesis was 1 per 10^6 cells (402). This is consistent with a single hit by the EMS and would suggest that the gro29 cell line only contains one mutation. This is important, since this would suggest that a single mutation generally impairs protein and virion maturation, but specifically reduces HSV-1 presentation. The mutation in gro29 appears to have affected an enzyme involved in the metabolism of N-acetylgalactosamine. If, as indicated above, the EMS mutagenesis only resulted in a single hit, the question then remains how carbohydrate metabolism can affect MHC class I presentation. Although there are no obvious answers, one might speculate that molecules involved in the MHC class I pathway or proteins to be degraded into presentable peptides are directly affected. Since the three dimensional conformation of proteins is important for their function, a differential maturation of the carbohydrates of components of the MHC class I pathway could affect their activity or specificity. It is also conceivable that such differential glycosylation might alter the stability of substrates tagged for degradation into peptides. It may also alter the accessibility of enzymes to their respective sites on the substrates (ex: addition of ubiquitin, proteolytic degradation). It would be most interesting to see if addition of exogenous N-acetylgalactosamine would restore the presentation of HSV-1 by gro29 cells.

Unfortunately, this may not yield positive results, since this approach does not restore HSV-1 virion production, despite a restored synthesis of chondroitin sulfate (404).

5.3- Closing remarks

Two approaches were undertaken to study the MHC class I antigen processing and presentation pathway and proved to be very informative. The first approach (Chapter 3) unveiled a complex modulation of the surface expression of MHC class I molecules by Ad2. It also revealed the need of not only the Ad2 E3/19K protein but also that of other viral proteins to optimally block MHC class I surface expression. Although the identity and the mechanism of action of these additional molecules are unknown, this suggested that the regulation of the surface expression of MHC class I proteins in normal and healthy hosts is complex. This complexity was confirmed by the characterization of the gro29 mutant cell line, which proved to be a very useful antigen processing and presentation mutant. Most notably, its characterization unveiled the possibility of alternative MHC class I processing and presentation pathways.

These studies contribute to the better understanding of the MHC class I processing and presentation pathway. This improved knowledge should be beneficial for the treatment of infections and autoimmune diseases as well as for cancer research and vaccine development. For instance, a precise knowledge of the interactions between pathogens and the immune system may allow a specific intervention to alleviate the infections or attenuate their pathogenicity. Furthermore, a better understanding of the rules governing the selectivity of the diverse components of the MHC class I pathway may allow the specific alteration of the

presentation of relevant peptides. It may also lead to the therapeutic modulation of the immune response. Finally, the precise prediction of the peptides presented for each MHC allele may allow custom tailored vaccines specific for a given disease in a given individual.

NOMENCLATURE

2-ME	β_2 mercaptoethanol
αMEM	alpha minimal essential medium
ABC	ATP binding cassette
Ad2	Adenovirus serotype 2
Ad5	Adenovirus serotype 5
Ad12	Adenovirus serotype 12
APC	antigen presenting cell
araC	1-β-D-arabinofuranosyl cytosine
ATCC	American Tissue Culture Collection
β_2M	β_2 microglobulin (MHC class I light chain)
BSA	bovine serum albumin
CH	cycloheximide
cim	MHC class I modifier
CLIP	class II Ii associated invariant chain
CPE	cytopathic effect
CS	calf serum
CTL	cytotoxic (or cytolytic) T lymphocytes
DMEM	Dulbecco minimal essential medium
DTT	dithiothreitol
E:T	effector to target ratio
E3/3.6K	3.6 kDa protein of the early region 3 of Adenoviruses
E3/6.7K	6.7 kDa protein of the early region 3 of Adenoviruses
E3/7.5K	7.5 kDa protein of the early region 3 of Adenoviruses
E3/10.4K	10.4 kDa protein of the early region 3 of Adenoviruses
E3/11.6K	11.6 kDa protein of the early region 3 of Adenoviruses
E3/12.5K	12.5 kDa protein of the early region 3 of Adenoviruses
E3/14.5K	14.5 kDa protein of the early region 3 of Adenoviruses
E3/14.7K	14.7 kDa protein of the early region 3 of Adenoviruses
E3/19K	19 kDa protein of the early region 3 of Adenoviruses
EDTA	ethylenediaminetetraacetic acid
EMS	ethyl methanesulfonate
endo H	endoglycosidase H
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FNR	fibronectin receptor
GAM-FITC	goat α mouse IgG coupled to FITC
H-2	murine MHC
hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	histocompatibility locus antigen (human MHC)
hpi	hours post infection
HSV-1	Herpes simplex type 1

HSV-2	Herpes simplex type 2
IEF	isoelectrofocusing
Ii	invariant chain
kDa	kilodaltons
LAK	lymphocyte activated killer
LMP	low molecular weight protein
Max	maximum (^{51}Cr release assay)
MHC	major histocompatibility complex
MIIC	MHC class II containing compartment
Min	minimum (^{51}Cr release assay)
MOI	multiplicity of infection
n	sample size (independent experiments)
NFA	no first antibody
NK	natural killer
NP40	nonidet P40
ORF	open reading frame
PBS	phosphate buffered saline
PBS^{--}	phosphate buffered saline without calcium and magnesium
PBS-BSA	PBS^{--} supplemented with 20 mM hepes and 0.5% BSA
PGP1	precursor glycoprotein 1
PMSF	phenylmethylsulfonylfluoride
PRV	Pseudorabies virus
SDS-PAGE	sodium dodecyl sulfate polyacylamide gel electrophoresis
TAP	transporter associated with antigen processing
TCA	trichloroacetic acid
TCR	T cell receptor
$TCID_{50}$	tissue culture infectious dose
TNF	tumor necrosis factor
TR	transferrin receptor
VSV	Vesicular stomatitis virus
wt	wild type

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APPENDIX

A.1- Calculation of the percentage of specific CPE

In Table 3, the evaluation of the cpe caused by Ad2 is presented. Although these estimates were rather crude, they provided an insight as to the physiological relevance of the block of MHC class I surface expression by Ad2. Here is presented the rationale and details pertaining to those evaluations.

As a consequence of the infection with Ad2, cells rounded up, became detached from the dishes, and eventually were lysed by the virus. Therefore, at any given time, the total cpe consisted in the sum of the adherent cells exhibiting cpe and the cells already detached or lysed due to the viral infection. Cells were therefore examined at various times post infection and the confluence of the cells (% confluence) and the cpe (% cpe) estimated by light microscopy. The background cpe (mock treated dish) was subtracted to obtain the specific effect caused by the viral infection. The data are expressed as a percentage of the mock treated dish for comparison among experiments and time points.

In conclusion:

Total percentage cpe

= {specific % cells already floating or lysed} + {specific % adherent cells with cpe}
= A + B

where A and B are described below.

A.2- Specific % cells already floating or lysed

The cells were generally subconfluent at the start of each experiment. The cells found on the mock treated dish were therefore normalized to 100% for simplicity.

Hence:

$$A = \frac{\text{Confluence of mock dish - confluence of Ad2 dish}}{\text{Confluence of mock dish}} \times 100$$

A.3- Specific % adherent cells exhibiting cpe

Of the remaining (adherent) cells, some were infected with virus and exhibited cpe. For example, perhaps half of the remaining adherent cells were infected and displayed cpe at a particular time point. However, in the mock treated dishes, some cells sometimes displayed cpe like morphology (rounding of cells) due to random cell death or overgrown dishes. To obtain the Ad2 specific cpe, this cpe like factor was therefore subtracted.

Hence:

$$B = \frac{\text{(Cpe of Ad2 dish x confluence of Ad2 dish) - (cpe of mock dish x confluence of mock dish)}}{\text{Confluence of mock dish}} \times 100$$

A.4- Rules!

As the cells grew more confluent on the mock treated dishes, they exhibited cpe like morphology indistinguishable from viral cpe (rounding of cells, increased refraction, and eventual detachment from the dishes). This was minimal at low confluence, but sometimes constituted a significant factor at late times post infection due to the overcrowding of the plates. Therefore, in equation B, subtraction of the non specific mock cpe could result in a negative term. This, of course, was not representative of the true viral specific cpe. This was only a problem if the difference of confluence between the mock and infected dishes was significant. To alleviate this artifact, the rule below was used. These conditions were arbitrarily set based on personal observations and routine viral infections of cultured cells.

Rule:

If, at a given time point, the confluence of the mock treated dish was equal to or greater than 65% and if the mock treated dish was 20% more confluent than the infected dishes, then the cpe like factor for the mock treated dish was set to "0" in equation B (i.e. cpe of mock dish = 0).