The complementary functions of multiple immune evasion genes of murine cytomegalovirus

by

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AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen Presenting Cell
BAC	Bacterial Artificial Chromosome
BFA	Brefeldin A
CID	Cytomegalic Inclusion Disease
CMV	Cytomegalovirus
CPE	Cytopathic Effect
Crt	Calreticulin
EBV	Epstein- Barr Virus
EM	Electron Microscopy
Endo H	Endoglycosidase H
ER	Endoplasmic Reticulum
ERGIC	Endoplasmic Reticulum-Golgi Intermediate Compartment
GCR	G-Coupled Receptor
Glc	Glucose
GT	Glucosyltransferase
HC	Heavy chain
HCMV	Human Cytomegalovirus
HSV	Herpes Simplex Virus
HHV	Human Herpes Virus (designated by number)
HIV	Human Immunodeficiency Virus
IEF	Isoelectric Focusing
IP	Immunoprecipitation
i.p.	Intraperitoneal
KSHV	Kaposi's Sarcoma related Herpes Virus
MCMV	Murine Cytomegalovirus
MHV 68	Mouse Herpesvirus 68
MHC	Major Histocompatibilty Complex
MOI	Multiplicity of Infection
PAA	Phosphonoacetic Acid
PAGE	Polyacrylamide Gel Electrophoresis
p.f.u	Plaque Forming Unit
QC	Quality Control
SDS	Sodium Dodecyl Sulfate
Tpn	Tapasin
UDP	Uridine 5'-diphosphate
VV	Vaccinia Virus
VZV	Varicella Zoster Virus

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As part of a separate project, Marielle Gold, a graduate student in our lab, isolated the T cell clones used in Chapter 2, and contributed CTL data shown in Chapter 2, Figures 4 and 5, and parts of Figure 3.

The new mutant viruses described in Chapter 2 were constructed in the laboratory of Dr. Ulrich Koszinowski, Max von Pettenkofer Institut, Munich, Germany, by Markus Wagner, a graduate student in his lab.

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The immunoprecipitations shown in Chapter 4, Figures 2 and 3, were performed by Jennifer E. Robertson, an undergraduate from Reed College, working under my supervision as part of her undergraduate thesis.

Abstract

Murine cytomegalovirus (MCMV) is a member of the β subfamily of the herpes family of large, enveloped, double-stranded DNA viruses. Like other herpes viruses, cytomegaloviruses cause lifelong infections with little pathology in normal host animals. Both human cytomegalovirus (HCMV) and MCMV encode multiple genes which interfere with antigen presentation by major histocompatability complex (MHC) class I, and thus protect infected targets from lysis by virus-specific cytotoxic T lymphocytes (CTLs). HCMV has been shown to encode four such genes, and MCMV to encode two. MCMV *m152* causes the retention of class I in a pre-Golgi compartment by an unknown mechanism. MCMV *m6* encodes a glycoprotein that binds to class I and redirects it to the lysosome for degradation The reason for the multiplicity of immune evasion genes is unknown. Over the course of the project described in this dissertation, we have investigated the interacting functions of multiple immune evasion genes of MCMV.

MCMV *m4* was previously known to encode a glycoprotein, m4/gp34, which binds to class I and forms a complex that is expressed at the cell surface. Here we here show that *m4* is a CTL-evasion gene that, unlike previously described immune-evasion genes, inhibits CTLs without blocking class I surface expression. The immune evasive function of *m4* is biochemically and functionally complementary to that of *m152*. Although the immune evasive mechanism of *m4* is unknown, we also show that m4/gp34 engages class I molecules in biochemically distinct complexes at the cell surface and in a pre-Golgi compartment.

Finally, we show that the class I-specific chaperone tapasin has altered function in MCMV-infected fibroblasts. We show that *m152* prolongs the association between class I and tapasin, and that some m4/gp34 is also tapasin associated in MCMV infected cells.

Chapter 1

Background and Introduction

Murine cytomegalovirus (MCMV) is a β-herpesvirus which uses a number of mechanisms to evade the immune system. In particular, it encodes a variety of genes that alter the normal assembly of major histocompatibility complex (MHC) class I molecules, which present antigenic peptide to CD8+ cytolytic T lymphocytes (CTLs). This dissertation describes a novel CTL-evasion function of an MCMV gene, and proposes a model for the interaction of multiple MCMV immune-evasion genes. This chapter will provide background information needed to understand various aspects of our experimental system.

Murine and Human Cytomegaloviruses

Classification and phylogeny

The herpesvirus family is made up of large, enveloped, double-stranded DNA viruses. Herpesviruses are divided into three subfamilies: alphaherpesviruses, including herpes simplex viruses 1 and 2 (HSV 1 and 2) , varicella zoster virus (VZV), and related viruses; betaherpesviruses, including the cytomegaloviruses (CMVs) and human herpesviruses 6 and 7 (HHV 6 and 7); and gammaherpesviruses, including Epstein-Barr virus (EBV), Kaposi's Sarcoma herpesvirus (KSHV), and related viruses. These divisions were originally based on virological observation-- host range, replication rate, tissue tropism, etc. Fortunately, DNA sequencing shows that these subfamilies are phylogenetically valid (McGeoch et al., 2000), although a few viruses have had to be re-

assigned based on sequence (Buckmaster et al., 1988; Davison, 1992; Gompels et al., 1995; Telford et al., 1993).

McGeoch *et al.* have constructed the most comprehensive herpesvirus phylogeny to date (McGeoch et al., 2000). Of particular interest is the conclusion that β1 herpesviruses (cytomegaloviruses) show cospeciation with respective hosts. Thus MCMV has apparently co-evolved with mice over the entire course of their evolution. By contrast, data are not consistent with cospeciation for many γ herpesviruses, such as mouse herpesvirus 68 (MHV 68).

History

The history of the study of CMV is summarized in (Ho, 1991). The first clinical description of what we now recognize as CMV disease was based on observations made by Ribbert in 1881 and reported in 1904 (Ribbert, 1904). He reported seeing "protozoan-like" cells in the kidney of a stillborn fetus. Subsequent similar observations led Smith and Wiedman to name the supposed infectious agent *Entamoeba mortinatalium* (Smith and Wiedman, 1920). In 1921 Goodpasture and Talbot introduced the term "cytomegalia" to describe the enlarged cells, which they concluded were not protozoan, found in the lung, kidney, and liver of an infant (Goodpasture and Talbot, 1921); and the associated disease came to be called cytomegalic inclusion disease (CID). [Rather than "cytomegalia", we today generally use the term "cytopathic effect" (CPE).] Cytomegalic cells were observed in autopsy tissue from many organs, especially salivary glands. Eventually, a similarity was noted between the cytopathlogy of CID and that seen in herpetic lesions (Lipschutz, 1921).

The development of tissue culture also made possible the eventual propagation of isolated virus *in vitro*. In 1954, Margaret Smith isolated CMV from the salivary glands of mice (Smith, 1954) and of humans (Smith, 1956). At about the same time the Davis strain of HCMV was isolated by Weller, who trying to propagate toxoplasma (Weller et al., 1957), and who eventually proposed the name "cytomegalovirus" (Weller et al., 1960). Concurrently, the still commonly used AD169 strain was isolated by researchers trying to grow adenovirus (Rowe et al., 1956). CMVs came to be distinguished from other herpesviruses by slow growth in culture, salivary gland tropism, and species specificity. As discussed above, this distinction turned out to be phylogenetically sound.

The CMV virion

A general overview of CMV biology is provided by (Mocarski, 1996). The CMV virion is 150 to 200 nm in diameter, and consists of a 100 nm icosahedral capsid surrounded by a tegument which is encased in a lipid bilayer envelope. Each virion contains a DNA core consisting of a single, linear, double-stranded DNA genome. Virions are sensitive to low pH, lipid solvents, and temperature, with a half-life at 37° of one hour. HCMV-infected cells also produce two kinds of noninfectious enveloped particles: dense bodies, consisting largely of tegument protein pp65, and lacking detectable capsid and DNA; and noninfectious enveloped particles, which are like normal capsids except that they lack a DNA core. MCMV does not produce dense bodies; however, it does produce multicapsid virions (Hudson et al., 1976).

CMV genomes

Strains of both HCMV and MCMV have been completely sequenced. HCMV has a 230 kbp genome. It is the only cytomegalovirus known to have an isomerising genome (i.e. one containing segments which invert relative to each other). The HCMV genome is divided into a long (L) segment of approximately 180 kb, and a short (S) segment of approximately 50 kb. Each segment is composed of a unique (U) central region capped at either end with terminal or internal repeated segments (TR or IR). Repeat sequences mediate inversion of U or L segments, permitting the existence of four different isomers of each HCMV strain.

The sequence of the AD169 strain is predicted to encode 208 ORFs of greater than 100 residues (Chee et al., 1990). ORFs are numbered sequentially according to position within one of the six sections. There are several gene families defined by internal homology and which are most likely the product of gene duplication.

The sequence of MCMV Smith is 230,278 bp (Rawlinson et al., 1996). Although it contains short terminal repeats and some internal repeats, the MCMV genome does not isomerise. Based on the criteria of minimal length of 300 bp and maximal 60% overlap with adjacent ORFs, Smith is predicted to encode 170 ORFs. ORFs are numbered consecutively *m1* to *m170*, in the 3' to 5' direction of the primary coding (C) strand of the genome. One hundred fifteen ORFs are transcribed from the C strand and 55 are transcribed from the antiparallel strand. There is no nucleotide homology between MCMV and HCMV, but 78 ORFs have highly conserved amino acid homology with HCMV and many of these are essential genes shared with other herpesviruses. Based on coding regions, the MCMV genome is collinear with that of HCMV. Genes with

demonstrable homology to HCMV genes are designated with a capital M and have the same numerical designation as the HCMV gene (thus MCMV *M45* is the homolog of HCMV *UL45*), while non-homologous genes are designated with a small m.

MCMV also has six gene families defined by internal homology, and four of these are homologous to HCMV gene families. Of particular interest to us are the two families, named for the index ORFs *m2* and *m145* respectively, which do not have homologs in HCMV. Each family contain genes which have been shown to have immune evasive function, while no function has been shown for the other members of the family. With the exception of rat CMV (RCMV) homologs to *m145* (Vink et al., 2000), no member of either family has significant homology to any known gene outside the family. However, at equivalent positions within the HCMV genome are found HCMV genes with analogous immune-evasive functions. As detailed below, HCMV and MCMV seem to use distinct biochemical mechanisms to alter the assembly of class I MHC, so it is interesting to speculate as to why these non-homologous genes have come to occupy homologous positions in the collinear genomes. Perhaps the ancestral CMV had primitive immune evasion genes at these loci.

There are several CMV genes with known homology to cellular genes; interestingly, most seem to be related to the immune system. HCMV encodes three or four homologs of G-coupled receptors (GCRs)--*US27*, *US28*, *UL33*, and *UL78*. US28 is a C-C chemokine receptor. US27, US28 and US33 have been found in viral particles [(Margulies et al., 1996) and reported as personal communication in (Mocarski, 1996)]; and *US28* is implicated in the etiology of CMV-related vascular disease (Streblow et al., 1999). HCMV UL18 is a homolog of HLA class I (Beck and Barrell, 1988). HCMV

UL20 is homologous to the T cell receptor γ chain (Beck and Barrell, 1991); the function of this gene is unknown. HCMV UL111a is a homolog of human IL-10 (Kotenko et al., 2000). MCMV encodes *M33* and *M78*, homologs of the HCMV genes (*UL33* and *UL78*), as well as *m144*, a homolog of murine MHC class I, to be discussed below (Rawlinson et al., 1996). The significance of some of these genes is discussed in the Viral Immune Evasion section below.

Gene regulation

There is no evidence for a CMV RNA polymerase, and transcription of all viral genes is thought to be carried out by host RNA polymerase II. During productive infection of fibroblasts *in vitro*, CMV gene expression follows a strictly ordered cascade of regulatory events, and genes can be classified according to where they fall within the cascade. The first genes to be expressed upon infection are the immediate early (IE) class. These genes are expressed from the powerful CMV-IE promoter/enhancer which is activated by resident cellular factors. IE gene products are mostly regulatory factors which trans-activate the next class of viral genes, the early (E) genes. E genes provide a number of essential and non-essential functions. In particular, they encode the machinery of viral DNA synthesis, which is required for late (L) gene transcription. L genes almost all encode structural components of the virion.

Requirements for transcription:					
Viral Protein synthesis	-	+			+
Viral DNA synthesis	-	-			+
Nomenclature:					
Greek	α	β		γ	
		β_1	β_2	γ_1*	γ_2
English	Immediate early **(IE)****	Early **(E)**** (also called delayed early)			Late **(L)****

* γ_1 genes show low levels expression in the absence of DNA replication
** designations used in this dissertation

Table 1: designation of classes of CMV genes according to regulation of expression.

The different classes are defined functionally by sensitivity to inhibitors of protein synthesis or DNA synthesis (Table 1). Thus IE genes are those which are transcribed after infection in the presence of an inhibitor of protein synthesis such as cyclohexamide. E genes are those which cannot be transcribed in the presence of cyclohexamide, but can be transcribed in the presence of inhibitors of viral DNA synthesis such as phosphonoacetic acid (PAA). L genes are only transcribed in the absence of PAA. (In order to permit the exclusive expression of IE proteins in the absence of E gene translation, infected cells are incubated in the presence cyclohexamide, so that large amounts of IE transcript accumulate. Actinomycin D is then added, which prevents transcription of any new mRNA. Finally, cyclohexamide is removed, which now permits the translation of IE transcripts.) The actual regulation of viral protein expression, especially E products, is much more complicated than what is suggested by this scheme.

For example, different E genes are transcribed at different times after infection, and protein expression is under complex post-transcriptional control. Also expression of some IE genes is repressed during the E phase and restimulated during the L phase.

An interesting difference between CMVs and many other viruses is that both HCMV and MCMV stimulate host cell metabolism, including synthesis of DNA, RNA, and protein (Gonczol et al., 1978; Knight et al., 1999), although expression of some host genes is selectively suppressed (Cebulla et al., 1999; Heise et al., 1998; Phillips et al., 1998; Sedmak et al., 1994). Thus CMVs have no known analog to the HSV virion host shutoff function. Gene chip assays indicate that HCMV infection has nearly identical stimulatory effects on many cell types cells as does type 1 IFN; infection is not required to produce this effect, which is entirely mediated by HCMV gB engagement of an unknown cellular receptor (Simmen et al., 2001). Of particular interest to the present study is the fact that CMV infection upregulates transcription and translation of MHC class I; aside from gB-mediated effect described above, expression of HCMV IE1 and IE2 is sufficient to drive reporter gene expression from minimal elements of the HLA A2 promoter (Burns et al., 1993). We find that MCMV infection of fibroblasts leads to an induction of class I expression comparable to that caused by IFN-γ (unpublished data). However, there is no global modulation of murine transcription analogous to that of HCMV gB (Klaus Früh, personal communication).

Viral replication

CMVs are highly host specific. Also, many host cell types are non-permissive under standard culture conditions (non-permissive cells are defined as those which do not support the production of infectious virus particles). Cell-type restriction of productive

infection is determined by post-penetration barriers to viral gene expression (DeMarchi, 1983; Lafemina and Hayward, 1988; Nelson et al., 1990).

The cellular receptor is not known for any CMV, but it must be some ubiquitous protein(s) because CMVs can attach to and penetrate a wide variety of cells, across species barriers (DeMarchi, 1983; Lafemina and Hayward, 1988). MCMV attachment to mouse embryo fibroblats (MEFs) is reversible: the number of plaques formed on a monolayer is proportional to the concentration and not to the number of infectious particles in an inoculum (Hodgkin et al., 1988). Penetration occurs within 5 minutes of exposure of target cells to HCMV, and is pH independent, indicating a non-endocytic mechanism. In fact, viral entry via endocytosis does not result in productive infection by MCMV in monocyte macrophages (Ann Campbell, personal communication). Post-penetration, viral nucleocapsid is delivered to the nucleus and viral transcription occurs as soon as 20 minutes after exposure to inoculum. MCMV and HCMV genomes circularize within four hours of infection. DNA replication is proposed to be a relatively slow process which takes place in cycles during the E phase and occurs via the rolling circle mechanism.

Once late gene expression occurs, nucleocapsids accumulate in the nucleus and subsequently acquire DNA. The exact mechanism by which capsids acquire an envelope remains unclear. It is assumed that envelopment occurs as nucleocapsids cross the membrane into or out of some membranous compartment, and a prime candidate is the inner nuclear membrane. In addition, evidence from electron microcopy (EM) studies suggests that nucleocapsids may go through stages of de-envelopment and re-envelopment before release from the cell. The process of virion export is brefeldin A

(BFA)-sensitive, demonstrating a requirement for the Golgi apparatus, and the large cytoplasmic inclusion seen in CPE is a Golgi-derived compartment.

Infection and pathogenesis

Clinical aspects of HCMV infection are reviewed in (Britt and Alford, 1996). HCMV is relatively labile and is spread only by close personal contact. Post-natal infection occurs via oral and respiratory routes, and HCMV can be found in most body fluids. The majority of humans are infected with HCMV in the first few years of life, although rates of infection vary with socioeconomic status, probably as a function of population density. Infection of seronegative women during pregnancy can lead to intra-uterine infection, which is a major cause of congenital birth defects.

In immunocompetent patients, acute HCMV infection causes little pathology, although primary infection is sometimes associated with mononucleosis or encephalitis. Patients are infected for life and HCMV remains latent in macrophages, endothelial cells, and salivary glands. Occasional reactivation leads to shedding of virus and spread to new hosts. HCMV is suspected to be a cofactor in some chronic human diseases such as atheroclerosis (De La Melena et al., 2001; Hosenpud, 1999; Streblow et al., 1999)

In recent decades HCMV has become a serious health concern due to the rapidly growing number of patients who are immunocompromised due to AIDS or immunosuppressive chemotherapy associated with tissue transplants. In these patients, HCMV can cause life-threatening damage to many organs systems, including the gut, liver, lungs, and the CNS. HCMV infection is associated with failure of organ transplantation, due both to pro-inflammatory effects in the graft and to direct tissue

damage by lytic replication. A significant percentage of late-stage AIDS patients become blind due to HCMV retinitis.

MCMV infection offers a number of experimental parallels to HCMV. MCMV also causes lifelong latent infection which reactivates after immunosuppression (Duan and Atherton, 1996; Jordan et al., 1978; Mayo et al., 1977). MCMV is latent in macrophages, endothelial cells, and salivary gland (Koffron et al., 1998; Pollock et al., 1997), and sites of latency are widely disseminated (Collins et al., 1993). There are MCMV models for CMV-associated diseases such as retinitis (Dix et al., 1994), pneumonia (Reddehase et al., 1985; Shanley, 1991), and atherosclerosis (Berencsi et al., 1998). It should be noted that experimental MCMV infection almost always uses a route of infection which is unnatural and a dose size far larger than what probably occurs in nature. Furthermore experimental outcomes are highly dependent on the strain of mouse used. Thus the goal of MCMV research is not to describe the natural course of infection, but to ask specific questions and test limited hypotheses. The most common route of infection used for experimental MCMV infection is intraperitoneal (i.p.). Morbidity and mortality after i.p. infection are dose-dependent and vary with the strain of mouse; in normal mice death is probably a result of hepatitis (Shanley et al., 1993), although as with HCMV a broad range of tissues can be infected during acute disease (Stoddart et al., 1994). In mice immunocompromised by irradiation, death from MCMV may be due to a failure of hematopoesis (Mutter et al., 1988).

Besides being a site of latency, macrophages are believed to be an important vehicle for dissemination of MCMV throughout the body (Stoddart et al., 1994). Hanson *et al.* (Hanson et al., 1999) found that a mutant virus strain which was specifically

defective in the ability to replicate in macrophages was avirulent. However, depletion of macrophages led to an increase in tissue titers of both the mutant and wild type strains of virus, indicating that macrophages play counteracting roles by helping both to disseminate and control the virus.

MCMV pathogenesis is suppressed by innate immunity and by adaptive humoral and cellular immune responses. These are dealt with in a separate section at the end of this chapter.

Expression and assembly of MHC class I molecules

MHC class I molecules present short antigenic peptides to $CD8^+$ T cells. For non-professional antigen presenting cells (APCs), these peptides are derived from endogenous proteins degraded in the cytoplasm. The polygenic murine MHC contains up to three classical class I loci-- K, D, and L-- although the C57BL/6 strain (B6 strain) has no L locus. Each locus is polymorphic and has a large number of alleles, each of which is indicated by a respective superscript letter. Thus B6 mice express two class I molecules, K^b and D^b. The process by which class I molecules are assembled has been extensively reviewed (Cresswell et al., 1999; Pamer and Cresswell, 1998; Solheim, 1999). The following is a detailed review of assembly and peptide loading of class I in the ER.

Basic paradigm

Class I HC is a type1 transmembrane glycoprotein of approximately 45 kDa. Class I molecules are expressed on the cell surface as a trimolecular complex of heavy chain (HC), beta-2 microglobulin (β2m), and antigenic peptide. The basic structure of

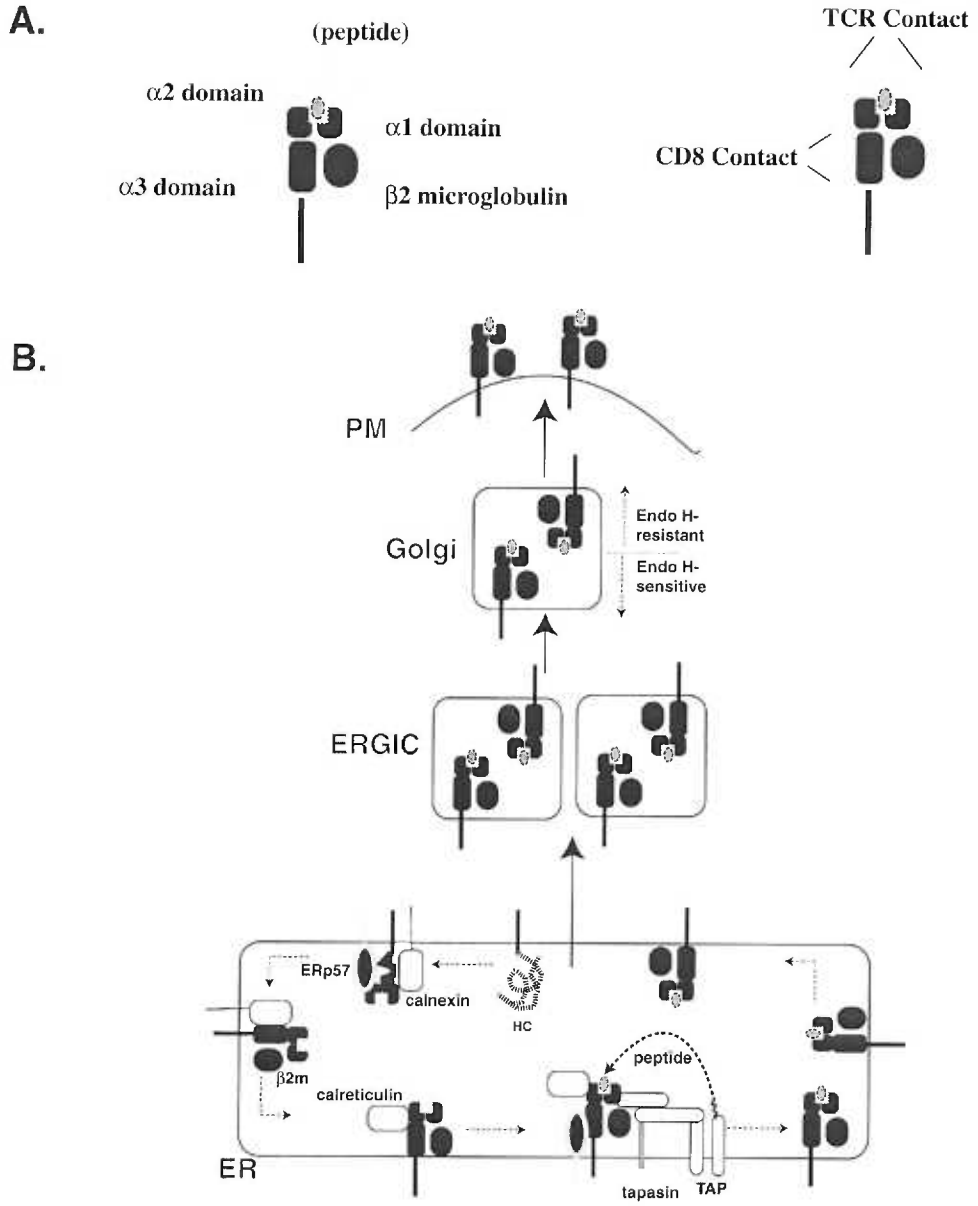

Figure 1. A. Structure of class I molecules, showing contact surfaces for TCR and CD8. **B.** Assembly and export of class I from the ER to the plasma membrane. Nascent heavy chain (HC) is cotranslationally translocated into the ER, where it is glycosylated and associates with a calnexin and ERp57. HC then associates with the light chain, beta-2-microglobulin (β2m). This bimolecular complex next joins the peptide loading complex, which includes the soluble chaperone calreticulin, ERp57, tapasin, and the peptide transporter TAP. Here class I is loaded with antigenic peptide transported from the cytoplasm. The mature trimolecular complex is now released by ER chaperones and exits the ER to be transported through the Golgi and to the cell surface. In the passage through the cis-Golgi, glycan moieties on the HC become modified such that they are resistant to digestion *in vitro* with endoglycosidase H (Endo H). ERGIC, ER-Golgi Intermidiate Compartment; PM, plasma membrane.

this complex is shown in Figure 1A. The expression of peptide-loaded class I molecules on the cell surface is the result of a highly order process, summarized in Figure 1B. Briefly, newly synthesized HC is cotranslationally translocated into the ER, in the process of which it is glycosylated and associates with the chaperones calnexin and ERp57. Next it associates with β2m, forming a heterodimer which can associate with the peptide-loading complex, a multimolecular structure including calreticulin, tapasin, ERp57, and TAP. At this point class I is loaded with peptide and undergoes a poorly-defined process of peptide-optimization, after which it may leave the ER. The trimolecular complex next passes through the ERGIC and cis-Golgi to reach the medial Golgi, where glycosyl residues are modified such that they become resistant to digestion with Endo H *in vitro*; thus Endo H digestion becomes a method of distinguishing "immature" and "mature" class I complexes. Finally the complex traverses the trans-Golgi network and reaches the plasma membrane.

Quality control in class I assembly

In order for the secretory pathway to function efficiently, eukaryotes have evolved complex systems of quality control (QC) [reviewed in (Ellgaard et al., 1999)]. These QC systems can be divided into two types: primary, or general, systems used by the majority of glycoproteins; and secondary, or specialized, systems used only by specific proteins. Example of primary systems include the lectin-like chaperones, protein disulfide isomerase, and BiP; these systems are largely conserved between yeast and mammals. Examples of secondary systems include the collagen-specific chaperone HSP47, expressed only in collagen producing cells; and invariant chain, an MHC-specific chaperone preferentially expressed in professional APCs.

The process of class I assembly involves both primary and secondary QC mechanisms. The first stage of QC uses primary systems to fold nascent HC into a β2m-receptive state; this process requires between 5 and 20 minutes post-synthesis (Farmery et al., 2000). The second stage involves a complex secondary QC system whereby HC associates with β2m and peptide, and then may go through an even more stringent QC process by which the affinity of bound peptide is assured. This stage may take an hour or more.

Glycosylation

Class I is glycosylated exclusively at asparagine (N) residues. Murine class I is glycosylated at two or three sites (N86, N176, and sometimes N256), each within a separate domain of the HC. Human HC is glycosylated only at N86. The significance of glycosylation to class I assembly and function is not entirely clear. Recombinant class I synthesized in bacteria, and thus lacking any glycosylation, is still capable of folding *in vitro*, binding peptide, and stimulating T cells (Boniface et al., 1998). However, glycosylation is required for association with chaperones such as calreticulin and ERp57 (Morrice and Powis, 1998), and contributes to association with calnexin. If glycosylation is blocked, class I expression is inhibited to varying degrees according to allele and locus (Neefjes and Ploegh, 1988). Thus glycosylation may serve to facilitate chaperone and quality-control functions in the ER; in addition, glycosylation does play a role in the interaction with some class I receptors other than the TCR (Parham, 1996).

Calnexin

Calnexin is a type 1 transmembrane protein which is ER-resident by virtue of a dilysine motif at the (cytoplasmic) C-terminus. Calnexin is often described as a "lectin-like" chaperone because it shows glycosylation-dependent substrate binding. As a consequence of cotranslational glycosylation in the ER, calnexin can bind to nascent glycoproteins before they exit the translocon (Chen et al., 1995).

Calnexin facilitates HC folding and assembly with β2m. However, it does not appear to be necessary for class I assembly. No calnexin-knockout mouse cell lines exist, but assembly of human class I is not compromised in a calnexin-negative cell line (Scott and Dawson, 1995). Furthermore, heterologous expression of mouse class I in human cells leads to equivalent surface expression in the presence and absence of calnexin (Prasad et al., 1998). In experiments using synchronized translation of HC in semipermiablized cells, calnexin preferentially bound class I molecules with incomplete disulfide bonds, while calreticulin preferentially bound HC with correct internal disulfide bonds (Farmery et al., 2000). Overall, results suggest that calnexin's role in class I assembly is confined to primary, or general, QC; an exact description of its function is complicated by redundancy in the primary QC functions of calnexin, calreticulin, and BiP.

Disulfide bonds

The next step in HC folding is disulfide bond formation. Class I has one such bond in the α2 domain and one in the α3 domain. When disulfide bond formation is blocked using DTT treatment of live cells or using cysteine to serine point mutations in

recombinant HC, the ability of HC to bind β2m is inhibited (Smith et al., 1995). It is possible that one or more ER chaperones may facilitate disulfide bond formation in class I. A likely candidate is ERp57, a member of the PDI superfamily which has been shown to function as a thiol reductase in the ER (Molinari and Helenius, 1999). Support for a specific role for ERp57 in class I synthesis comes from the fact that it, but not the homologous disulfide isomerase PDI, specifically associates with class I. The function of ERp57 in class I assembly remains to be demonstrated, and understanding the role of this protein is hampered by the lack of ERp57-deficient cells.

Lindquist *et al.* reported an association of ERp57 with nascent HC in the absence of β2m (Lindquist et al., 1998). Also, in a kinetic study using HLA-B27, Farmery *et al.* found that ERp57 associated with free HC very early in synthesis, consistent with a role in initial folding; of particular note is the fact that ERp57 and calnexin, but not calreticulin, associated with partially reduced forms of HC which migrated with a diffuse pattern on non-reducing gels (Farmery et al., 2000). In contrast to these reports, in a study of steady-state conditions using L^d, Harris *et al.* found ERp57 bound exclusively β2m-associated HC in a tapasin-dependent manner (Harris et al., 2001), inconsistent with a role in the initial stages of domain folding. These apparently contradictory results could be incorporated into a biphasic model of ERp57 association, where ERp57 first plays a role in the rapid folding of nascent free HC, and then plays a separate role in the slow association of class I with optimal peptide. It is easy to imagine that ERp57 serves a basic chaperone function at the early stage. What role it may play in peptide binding is less clear. This protein will be discussed again in the context of the peptide-loading complex, below.

β2m

After disulfide bond formation, calnexin-associated HC next associates with the light chain, β2m. β2m is an unglycosylated soluble protein of 12.5 kDa. β2m association is generally required for the subsequent assembly steps; in cells lacking β2m, the subsequent entry of class I into the peptide-loading complex is reduced to < 5% of normal (Cresswell et al., 1999; Solheim et al., 1997). β2m is not absolutely required for class I assembly however; although $\beta 2m^{-/-}$ mice are immune-deficient, they still develop some class I-restricted CD8+ T cells (Cook et al., 1995).

The crystal structure of class I seems to suggest that the purpose of β2m is structural, in that there is no obvious point of contact between β2m and the TCR or CD8. β2m is required for class I to stably achieve a peptide-receptive conformation (Wang et al., 1996). In transfected cell lines, mouse class I is more efficiently stabilized by human β2m than by mouse β2m, resulting in peptide-independent surface expression (Wei and Cresswell, 1992). Thus it seems that mouse β2m is tuned to provide an intermediate degree of stability to folded HC in preparation for peptide loading.

Solheim *et al.* (Solheim et al., 1995) constructed a recombinant form of β2m carrying the KDEL ER-retention motif, and expressed it in $\beta 2m^{-/-}$ cells, which normally have no folded class I on the cell surface. Interestingly, although recombinant β2m was fully retained in the ER, it was still capable of promoting expression of conformationally mature, peptide-loaded HC on the cell surface, as assayed by antibody staining. This implies that the role of β2m is more to facilitate proper folding of class I in the ER than it is to maintain the structural integrity of mature complexes.

The peptide-loading complex

After β2m associates with HC, the resulting bimolecular complex enters into a multiprotein complex containing a number of specialized ER-resident proteins which variously serve to transport peptides and to assure optimal peptide loading, and possibly contribute to peptide trimming. Besides calreticulin, HC, and β2m, this complex includes TAP, tapasin, ERp57, and perhaps other proteins. Depending on your point of view, TAP (Solheim et al., 1997), tapasin (Ellgaard et al., 1999), and even β2m [(Solheim et al., 1995)--see above] can all be considered chaperones. Indeed, the peptide-loading complex seems to function as a large, complex chaperone of class I assembly. Calnexin seems to participate in the pre-assembly of the complex, but disassociates before class I and calreticulin enter (Diedrich et al., 2001).

HC, β2m, TAP, and tapasin are all generally considered essential components of the complex. However, some independent interactions do occur between individual components. Tapasin binds independently to class I and TAP (Sadasivan et al., 1996). Conversely, small amounts of class I are associated with TAP in the tapasin-defective 721.220 cell line (Peh et al., 1998). Solheim and coworkers reported that β2m associated with TAP in the "class I negative" cell line 721.221 (Carreno et al., 1995); in fact, LCL 721.221 does express HLA-C (Grandea et al., 1995) as well as class Ib molecules, including HLA-E (Tzeng et al., 1996), which associates with TAP (Braud et al., 1998). However, the same group found β2m associated with TAP in these cells even after removal of class Ib molecules using conformation-dependent antibody W6/32 (Solheim et al., 1997). It remains to be proved that this association is not due to residual class I not precipitable with W6/32. Also, some class I association with TAP can be detected in the

absence of β2m (Bangia et al., 1999; Cresswell et al., 1999; Lindquist et al., 1998; Solheim et al., 1997). As discussed below, class I alleles vary in their dependence on chaperone expression. Nevertheless, the peptide-loading complex serves an essential role in the development of a functional class I-restricted immune response.

TAP

TAP (Transporter associated with Antigen Processing) is a heterodimer of two subunits-- TAP.1 and TAP.2-- both of which contain eight potential membrane-spanning sections. The membrane topology of these subunits is still unknown. TAP translocates peptides from the cytoplasm to the ER by an ATP-dependent mechanism and is the source of the vast majority of antigenic peptides presented at the cell surface in the context of class I. In the absence of one or both subunits, peptide is not translocated and class I surface expression is vastly reduced compared to surface expression in wild type cells.

TAP binds to cytosolic peptides in an ATP-independent manner. Human TAP is more promiscuous than mouse TAP, although both are somewhat selective when tested *in vitro* with short artificial peptides. The advantage to the immune system of such TAP selectivity is not immediately obvious, and in fact may not be significant in terms of limiting the pool of available peptides. For example, when HLA-A3 was transfected into a mouse cell line, it primarily bound to peptides with C-terminal lysine residues, which are not favored by mouse TAP. It is possible that there is a specific mechanism for delivery of peptides from the proteasome to TAP (perhaps involving peptide-binding heat-shock proteins such as HSP70-- see below) which renders the TAP selectivity

irrelevant. In addition, TAP can transport quite long peptides (up to 40-mers) which may be subsequently trimmed and loaded onto class I.

Tapasin

In 1996 Peter Cresswell and colleagues identified a novel spot, representing a protein which they named tapasin, on 2D electrophoretic gels of the peptide-loading complex from human cells (Sadasivan et al., 1996). Tapasin was found to associate with class I in the absence of TAP, and with TAP, but not class I, in the absence of β2m, suggesting a role for tapasin as a "bridge" between TAP and class I. Indeed, they were unable to detect tapasin expression in cell line 721.220, which has defective class I-TAP association. [It was eventually shown that 721.220 cells express a truncation mutant of tapasin which still interacts with TAP (Copeman et al., 1998).] The same group (Ortmann et al., 1997) and colleagues (Grandea et al., 1997) subsequently cloned the tapasin cDNA and demonstrated that transfection of 721.220 cells with the tapasin gene was sufficient to restore peptide loading and antigen presentation. The mouse tapasin was cloned soon afterward (Grandea et al., 1998; Li et al., 1999).

Tapasin is a 48 kDa type 1 glycoprotein which carries a C-terminal dilysine ER retention motif. Tapasin is necessary in mice and men for strong association of class I with TAP. A single TAP complex can simultaneously associate with four tapasin molecules, each of which can associate with a single class I molecule (Ortmann et al., 1997). Tapasin associates primarily with β2m-associated HC (Bangia et al., 1999; Sadasivan et al., 1996; Solheim et al., 1997). Thus the bridging function of tapasin brings four peptide-empty class I molecules into close proximity of each TAP molecule.

A number of studies have begun to elucidate the structural basis of the TAP-tapasin-class I bridge. The association of TAP with the rest of the peptide-loading complex occurs via TAP.1 (Li et al., 1999). The 50 N-terminal residues of tapasin mediate binding to class I, while the C-terminal mediates binding to TAP (Bangia et al., 1999). On class I, convergent results suggest a role for the residues in the region of E222 in the α3 domain (Carreno et al., 1995; Kulig et al., 1998; Suh et al., 1999), although other studies implicate the α2 domain (Lewis et al., 1996; Neisig et al., 1996; Peace-Brewer et al., 1996). It has been suggested that apparent involvement of the α2 domain may actually reflect altered association with calreticulin; however, recent results, discussed below, suggest that calreticulin is not required for the association of class I with tapasin.

Accumulating evidence demonstrates that the function of tapasin is more complex than that of a simple bridge between TAP and class I. Tapasin association with TAP is sufficient to significantly increase the stability of the TAP heterodimer (Bangia et al., 1999; Lehner et al., 1998) and thus increase the rate of peptide transport, although tapasin does not increase the rate of transport by individual TAP heterodimers. More intriguingly, tapasin association with class I facilitates peptide loading: recombinant soluble human tapasin, which cannot bind to TAP, still restores class I expression when transfected into the tapasin-defective 721.220 cell line (Lehner et al., 1998). [However, preliminary evidence (A. G. Grandea, personal communication) suggests that expression of recombinant soluble *mouse* tapasin leads to rapid degradation of class I, in a manner reminiscent of degradation via HCMV US2 or US11 (these genes are described below)] In addition Peh *et al.* (Peh et al., 2000) showed that levels of mouse tapasin expression

sufficient to bridge HLA B*4402 to TAP were insufficient to permit HLA B*4402 surface expression, but that overexpression of mouse tapasin restored HLA B*4402 expression. This implies that tapasin provides some necessary function in addition to increased bridging of class I to TAP.

Our understanding of tapasin function has been facilitated by convergent approaches using both "add-in" and "take-away" strategies. In the add-in approach, portions of the class I assembly pathway were constructed *de novo* by expression of human (Lauvau et al., 1999) or murine (Schoenhals et al., 1999) components in insect cells. Using this approach, Schoenhals *et al.* found that murine tapasin was necessary to retain peptide-empty class I molecules in the ER and thus ensure adequate peptide loading to permit stable surface expression of class I (Schoenhals et al., 1999).

Pursuing the take-away approach, two groups (Garbi et al., 2000; Grandea et al., 2000) constructed tapasin-knockout ($Tpn^{-/-}$) mice, and reported mutually consistent results. $Tpn^{-/-}$ mice showed a general defect in $CD8^+$ T cell selection (≈20% of normal $CD8^+$ numbers) less severe than $TAP^{-/-}$ mice (≈5% of normal $CD8^+$ numbers). Likewise hematopoetic cells from $Tpn^{-/-}$ mice showed a decrease to 10-15% of normal class I surface expression, compared to about 5% of normal for cells from $TAP^{-/-}$ mice. Lack of tapasin led to a defect in TAP activity which was much more pronounced in fibroblasts than in hematopoetic cells (con A splenocyte blasts) (Grandea et al., 2000).

Most strikingly, data from both groups were consistent with a general loss in the quality control of peptide loading. Class I in $Tpn^{-/-}$ cells exited the ER at a similar rate to that seen in wildtype cells; however these molecules were unstable and rapidly degraded. This degradation could be prevented by addition of exogenous high-affinity peptide,

consistent with the proposition that the molecules had either been empty or loaded with suboptimal peptide (Grandea et al., 2000). However, egress from the ER in $Tpn^{-/-}$ cells could be blocked by the proteasome inhibitor lactacystin, indicating that class I in these cells was initially loaded with suboptimal peptide which later fell off, eventually leading to class I instability (Garbi et al., 2000). This hypothesis has also been supported by subsequent investigations showing: (i) that class I in tapasin-deficient cells could be stabilized by external peptide (Barnden et al., 2000); (ii) that mutant D^d with defective tapasin association was stabilized by exogenous peptide, to an intermediate extent between empty D^d and wildtype D^d (Suh et al., 1999); and (iii) that HLA-B27 was differentially stained with peptide-specific antibodies in Tpn^- and Tpn^+ cells (Peh et al., 1998).

In summary, current evidence suggests that tapasin serves multiple functions in class I assembly, including bridging class I to TAP, enhancement of TAP activity, retention of empty class I molecules, and optimization of peptide loading.

Calreticulin

Calreticulin (crt) is another lectin-like chaperone with generally similar properties to calnexin, except that calreticulin lacks a transmembrane domain and is soluble in the ER lumen, where it is retained by a KDEL motif. Calreticulin and calnexin have some overlapping functions; however, they are not redundant with respect to class I assembly. Calreticulin is consistently found in association with TAP and tapasin, while calnexin is not (Gao et al., submitted; Solheim et al., 1997). Calreticulin binds only β2m-associated class I (Sadasivan et al., 1996). Also, calreticulin discriminates between open and peptide-loaded class I conformations, binding preferentially to open conformations

(Harris et al., 1998; Sadasivan et al., 1996). Calnexin shows no such discrimination (Carreno et al., 1995). Calnexin and calreticulin also use different glycosylation sites to associate with HC (Harris et al., 1998). Importantly, calreticulin was shown to require tapasin to associate with class I in mouse cells (Grandea et al., 2000): in heterozygous littermates but not $Tpn^{-/-}$ mice, HC was found in anti-calreticulin immunoprecipitates from splenocytes. A similar finding has been reported for human cells (Hughes and Cresswell, 1998; Lewis and Elliott, 1998). These findings are strong evidence that calreticulin specifically plays a role in the peptide loading complex.

New insight into the role of calreticulin comes from the work of Tim Elliott and coworkers (Gao et al., submitted). They report that class I assembly in $Crt^{-/-}$ murine cells is defective in ways very similar those previously found for $Tpn^{-/-}$ cells. Specifically, compared to wildtype cells, class I from these cells left the ER more rapidly, had a shorter half-life at the cell surface, and was more easily stabilized by exogenous peptide. No defect was detected in the steady-state association of TAP with class I HC, tapasin, or ERp57. This result suggests that calreticulin serves a necessary peptide-optimization function. This function may be connected to the ability of calreticulin to act as a peptide sink by binding and retaining TAP-transported peptides in the ER (Nair et al., 1999; Spee and Neefjes, 1997), which has also been proposed to contribute to the delivery of exogenous peptides for cross-presentation by dendritic cells (Basu and Srivastava, 1999).

These data are consistent with the possibility that tapasin exerts its quality-control function on peptide loading entirely by promoting the association of calreticulin with class I. However, results from our lab indicate that the absence of tapasin or calreticulin respectively leads to different effects on antigen presentation to T cell clones (Marielle

Gold, unpublished data). Thus these two chaperones seem to have parallel but non-redundant functions with regard to peptide loading.

ERp57

As discussed above, ERp57 has been found in association with nascent, incompletely reduced HC, consistent with its putative thiol reductase function. In addition, many reports indicate that ERp57 is associated with HC in the peptide loading complex (Harris et al., 2001; Hughes and Cresswell, 1998; Lindquist et al., 1998; Morrice and Powis, 1998). This association is dependent on tapasin (Harris et al., 2001; Hughes and Cresswell, 1998), but not calreticulin(Gao et al., submitted). What ERp57 is doing in this complex is a matter of speculation. It has been suggested that its thiol-reductase activity could contribute to peptide optimization via the hypothetical opening and closing of the peptide groove (Cresswell et al., 1999). In addition, ERp57 has been reported to act as a cysteine protease (Urade and Kito, 1992; Urade et al., 1997), and thus could potentially contribute to peptide trimming. Finally, based on unpublished data (Lindquist et al., 1998), ERp57 joins the growing ranks of chaperones reported to bind TAP-transported peptides in the ER, possibly providing a peptide sink for direct class I loading or cross-priming of DCs.

Allelic differences in class I assembly

The simplest model of class I genetic variation suggests that the primary advantage to class I diversity is the ability to bind a diversity of peptides. In fact, differences among class I molecules are concentrated in the peptide binding region (Parham et al., 1988; Watkins et al., 1990). However, class I molecules exhibit a variety

of complex differences in their assembly patterns which suggests a broader evolutionary significance than simple diversity of peptide binding.

Neefjes and Ploegh (Neefjes and Ploegh, 1988) found different rates of association with β2m of different HLA alleles, as well as different dependence on glycosylation as determined by treatment with tunicamycin, which inhibits glycosylation. Hill and coworkers (Hill et al., 1993) also found differences in the rate of export of different class I molecules. By comparing a number of natural variants, they found a region of the α2 domain which correlated with differing rates of assembly between HLA-B35 and -B51. These differences in the overall rate of class I assembly could be explained by many factors, such as the availability of different peptides, overall differences in affinity for peptide or β2m, or differential association with chaperones.

Neisig *et al.* pursued this line of inquiry by comparing the association of different HLA molecules with TAP (an association which we now know to be mostly mediated by tapasin). By running isoelectric focusing (IEF) gels of αTAP immunoprecipitates, they were able to simultaneously visualize the association with TAP of multiple class I molecules (Neisig et al., 1996). They found that most HLA-A and -C alleles studied had a strong association with TAP, while -B alleles did not. Nevertheless, all molecules had comparable kinetics of TAP association and dependence on TAP-transported peptides, and HLA-B alleles were not defective in surface expression. These results suggest that some class I molecules acquire peptides without participating in the conventional peptide-loading complex.

These results were brought into focus by the work of Peh *et al.* (Peh et al., 1998), who demonstrated different degrees of tapasin dependence for different class I molecules.

Surface expression of HLA-B*4402 and -B8 was greatly increased by tapasin. The effect on expression of HLA-B*2705, however was negligible in human cells and slight in murine cells [about 2X--this is actually concordant with the reported "strong" effect of tapasin on HLA-B27 expression in insect cells (Lauvau et al., 1999), which was about 1.7X], andB*2705 achieved high levels of surface expression in the absence of tapasin. B*2705 was found to require tapasin to associate with TAP, demonstrating that HLA-B*2705 can achieve high levels of surface expression without TAP association. The biochemical results were consistent with functional data comparing the tapasin dependence of presentation to CTL clones restricted by different class I molecules. Peh *et al.* speculate that this phenotype may be related to the strong association of HLA-B*27 with autoimmune diseases (Khan, 1995). In addition, they conclude that the tapasin-independence of HLA-B*2705 may represent an evolutionary response to interference with tapasin function by a hypothetical pathogen. Indeed, as discussed below, Bennet *et al.* demonstrated that a human adenovirus protein interferes with the association of murine class I and tapasin (Bennett et al., 1999). Results presented in this dissertation provide the first evidence for altered tapasin function due to infection with a herpes virus.

Generation of antigenic peptides

The 26S proteasome is an essential structure, found in the cytoplasm and nucleus of all cells, which contains a core proteolytic element called the 20S proteasome [reviewed in (Rock and Goldberg, 1999)]. The 20S proteasome is a cylindrical structure made of four connected rings surrounding a hollow core through which a protein passes during proteolysis. The two central rings are each made up of seven different β subunits and the two outer rings each contain seven different α subunits. α subunits are all

homologous to each other and β subunits are all homologous to each other. In addition, there are specialized catalytic and regulatory components which comprise the "immunoproteasome", a specialized form of the proteasome found in lymphoid cells and in other cells after IFN-γ induction [reviewed in (Fruh and Yang, 1999)]. The immunoproteasome has increased and slightly altered activity compared to the 20S proteasome.

The exact mechanisms by which antigenic peptides of the correct size for class I binding are generated is incompletely understood. The vast majority of peptides bound to class I in normal and virus-infected cells are generated in the cytoplasm by the proteasome. Thus various drugs which specifically inhibit the proteasome can cut off the supply of peptides and largely prevent class I maturation, although there is allelic variation in the degree of proteasome dependence (Benham et al., 1998).

The proteasome is not the only protease involved in antigen processing, however. Although the average size of proteasome degradation products is about 8 residues, peptides are produced in a very broad range of sizes. In general, the C-terminal of proteasomal peptides is unaltered after TAP transport and presentation in the context of class I. The N-terminal, however, can undergo extensive additional proteolysis. Evidence exists for both cytoplasmic (Beninga et al., 1998; Roelse et al., 1994), and ER-resident (Paz et al., 1999; Roelse et al., 1994) aminopeptidase activities which may contribute to generating correct N-termini for optimal class I loading. In the case of ER-resident activity, trimming may occur after the C-terminal is bound into the class I groove; this proposition is supported by the fact that it is extremely difficult to isolate trimming intermediates in the absence of appropriate class I molecules (Paz et al., 1999).

Thus this ER-resident aminopeptidase activity may be an important component of peptide optimization, in co-operation with tapasin and/ or ERp57.

Free peptides have an extremely short half-life both in the ER and the cytoplasm. It is believed that free peptides in the ER are retrotranslocated into the cytoplasm, where they join other free peptides in being rapidly destroyed by undefined endo- and exo-peptidases, although some may be recycled through TAP and eventually bind to class I (Roelse et al., 1994). It is reasonable to propose that specific mechanisms exist to convey peptides from the proteasome to TAP, and from TAP to class I. Candidates for such peptide sinks include chaperones-- such as HSP70 in the cytoplasm, and gp96 or calreticulin in the ER (Spee and Neefjes, 1997)-- which have been shown to sequester peptides which can later become exogenous antigen for cross-priming by professional APCs (Srivastava et al., 1998).

Regulated export from the ER

In spite of much recent progress in understanding the cell biology of vesicular transport, factors controlling the export of proteins from one cellular compartment to the next are still only partly understood. A recent paper from Michael Edidin's group (Spiliotis et al., 2000) suggests that selective export from the ER of correctly assembled class I complexes is mediated by specific association with ER-to-Golgi cargo protein(s). Spiliotis *et al.* showed that peptide-loaded class I molecules, but not TAP, colocalized with p137, a marker for ER exit sites. Furthermore, they showed that L^d associated with BAP31, a cargo protein which is proposed to mediate specific anterograde transport of some proteins from the ER (Annaert et al., 1997). However, due to incomplete controls Spiliotis *et al.* did not demonstrate that the association with BAP31 was specific for

class I or selective for peptide loaded molecules. Nevertheless, these results provide hope that we will soon have a better understanding of sorting mechanisms which permit specific export of fully conformed class I molecules. Obviously, this will be of particular interest to researchers who study viral interference with class I export.

Viral immune evasion: an overview

The term "immune evasion" can be applied to any process which specifically helps pathogens to survive in the face of an active immune response. Thus I define immune evasion genes to be those which have evolved for the specific purpose of downregulating particular aspects of the host immune system. Since viruses have many very complex interactions with host biology, defining an evolutionary "purpose" for viral genes can be difficult, and many people have suggested that the term "immune evasion" is overused. To definitively prove the immune-evasive function of a gene, it should ideally meet the following criteria: i) deletion of the gene should attenuate the virulence of the pathogen, and ii) mutant and wildtype viruses should have equivalent virulence in animals with targeted lesions in the immune system. Obviously, these criteria cannot be tested for obligate human pathogens, and so far they have been successfully applied only to three genes: MCMV *m152* and *m144*, and the gene encoding HSV ICP47 (see below). The importance of a conservative interpretation is demonstrated by the finding that mutant MCMV lacking an Fc receptor gene was attenuated even in B cell deficient mice (Crnkovic-Mertens et al., 1998), thus failing to fulfill criterion (ii), above. The MCMV Fc receptor is probably involved in some non-immunological aspect of pathogenesis.

Nevertheless, there are many viral genes for which there is good circumstantial evidence of an immune evasive function, and many others for which an immune evasive function is merely speculative. I will briefly review some of these genes here; for a more exhaustive review see (Tortorella et al., 2000). A separate section below is dedicated to evasion of CTLs by MCMV.

Evasion of humoral responses

A number of viruses encode receptors for the Fc portion of immunoglobulin (FcR). Since Fc mediates a number of effector functions such as opsonization, complement fixation, and antibody dependent cellular cytotoxicity, viral FcRs could protect infected cells or viral particles by blocking any of these functions. The best demonstration of such a function so far was applied to the HSV Fc receptor formed by the gE-gI protein complex (Nagashunmugam et al., 1998). In this case, targeted mutations in gE specifically prevented the Fc function of the complex. Passive transfer of antibody was able to reduce disease in mice infected with mutant but not wildtype virus. This is a strong indication that the gE-gI Fc receptor may actually function as an antibody-evasion factor in natural infections of humans.

Most antibody responses require T cell help, and a likely way for viruses to alter the humoral response would be to target the $CD4^+$ T cell compartment. Thus evasion of humoral responses may be a secondary function of interference with class II antigen processing (see below).

Interference with cytokines and chemokines

This is the category of putative immune evasion genes with by far the largest number of members. Tortorella *et al.* list fifty one different viral genes which are proposed to modulate host cytokine functions. Given this large number of genes, it is almost certain that some of them will be genuine immune evasion factors, and some of them will play roles in other aspects of the viral infectious cycle, such as cell to cell spread, and modulation of apoptosis. The criteria of immune evasion proposed above will have to be carefully addressed for each individual gene. For example, the myxoma virus T2 protein is a soluble TNF receptor homologue which has been demonstrated to be a virulence factor in rabbits. In addition to being a cytokine receptor, however, T2 serves an unrelated anti-apoptotic function in infected $CD4^+$ T cells. Determining which role of T2 is relevant to virulence will require careful experiments using targeted depletion of cytokines in infected animals.

Evasion of NK cells

CMVs

A few years ago, it was understood that CMVs downregulated class I expression, that NK cells kill targets with missing self, and that both MCMV and HCMV encode homologues of class I (*m144* and *UL18*, respectively). This suggested a relatively simple model in which the class I homologs were viral countermeasures to the missing self response. Accumulating results, however, demonstrate more complicated roles for these viral genes. With regard to NK evasion, there is better evidence for a role for *m144* (Cretney et al., 1999; Farrell et al., 1997; Kubota et al., 1999) than for *UL18*. In fact,

under some conditions, UL18 expression actually enhanced NK activity (Leong et al., 1998).

Experiments with a recombinant soluble form of UL18 led to the discovery of a whole new family of human leukocyte receptors, the leukocyte immunoglobulin-like receptors (LIRs) (Cosman et al., 1997). The family contains eight genes, and shares sequence homology with the mouse paired immunoglobulin-like receptors (PIRs); however, no functional homology between the families has been demonstrated. Different LIRs are expressed on different leukocyte populations, and some LIRs are inhibitory while others are stimulatory. UL18 is a ligand for LIR-1, which is expressed on DCs, monocytes, T cells, B cells, and NK cells (Fanger et al., 1999). Thus UL18 may serve to inhibit any of a broad range of leukocytes which contribute to innate and adaptive defense. It has been suggested that a central purpose of LIR-1 is to activate tissue DCs in response to the loss of surface class I in local cells and thus initiate an immune response against viruses or tumors that downregulate class I. By binding to LIR, UL18 may inhibit DC activation and retard CTL priming.

In the footsteps of the discovery of LIRs, the same group recently made another major discovery by staining cells with a soluble recombinant trimer of an HCMV protein. In this case the HCMV protein was UL16, and the discovery was the ULBP ("UL16-binding protein") family of ligands for the natural killer receptor NKG2D (Cosman et al., 2001). ULBPs are GPI linked proteins which are expressed by a variety of somatic cells and activate NK cells via NKG2D. By binding to ULBP, recombinant soluble UL16 interferes with NK activation by targets expressing ULBP. Thus unlike UL18, the ligand for UL16 is on the target cell, not on the effector lymphocyte. Cosman *et al.* hypothesize

that UL16 expressed on an infected cell may bind to ULBP at the cell surface and thus block NK activation.

With regard to *m144*, the most convincing demonstration of a specific role in NK evasion made use of a knockout virus lacking this gene. This virus was attenuated in an NK-dependent manner (Farrell et al., 1997). Additional evidence comes from the observation that *m144* can protect transfected tumor lines from NK lysis *in vitro* (Kubota et al., 1999) and *in vivo* (Cretney et al., 1999). However, direct *in vitro* NK activity against any MCMV infected cells in the presence or absence of *m144* has yet to be demonstrated.

Other viruses

In addition to those of the CMVs, an MHC class I homolog, for which no function has yet been demonstrated, is encoded by the poxvirus molluscum contagiosum (Moss et al., 2000; Senkevich and Moss, 1998). Also, as described below, a number of other viruses encode genes which downregulate class I, thus hypothetically exposing infected cells to attack by NKs. These viruses seem to have adapted to prevent such a response. Both adenovirus E19 and HIV Nef preferentially downregulate HLA-A and -B gene products, which are of greatest importance in the CTL response, while leaving HLA-C, -E, and -G gene products, which are of greatest importance in the NK response, unaffected. It seems that similar selectivity is shown by HCMV US2 [unpublished data cited in (Tortorella et al., 2000)]. The KSHV K5 protein has similarly selective effects (see below).

Evasion of CD4+ T cells

Antigens presented in the context of MHC class II stimulate $CD4^+$ T cells, which can exert a variety of anti-viral functions, such as secreting cytokines and providing help to CTLs, NK cells, and B cells. In contrast to the class I pathway, however, the potential benefit to a virus of interference with the class II pathway in infected cells is less clear. This is because $CD4^+$ cells generally exert their effect by releasing cytokines when they encounter antigen presented by uninfected professional APCs. Based on this understanding, we would predict that $CD4^+$-evasion factors should be secreted proteins which can affect uninfected APCs. A possible example of this was recently described for the eukaryotic parasite *Brugia malayi*, which has been shown to secrete a cathepsin inhibitor that blocks class II antigen processing *in vitro* (Manoury et al., 2001). To date, however, no such viral protein has been identified. On the other hand, some rare CD4+ T cells can lyse targets directly in a class II restricted manner, and this population may be the functional target of viral class II downregulation.

Although no known soluble viral protein downregulates class II, MCMV has been shown to induce class II downregulation *in vivo* and *in vitro* via induction of IL-10 by infected cells (Redpath et al., 1999). This mechanism provides the sort of action-at-a-distance which we would expect to be a necessary component of generalized $CD4^+$-evasion. This function has not been attributed to a particular gene.

In addition, a number of other reports have described class II downregulation *in vitro* by direct infection of APCs. Both MCMV (Heise et al., 1998) and HCMV (Miller et al., 1998) downregulate class II by modulating transcription in infected cells. In addition, HCMV US2 has been shown to downregulate class II by causing its degradation

via retrograde translocation from the ER to the cytoplasm (Tomazin et al., 1999). This is in addition to US2's previously known ability to downregulate class I (see below). In the absence of defined genes in MCMV, and an animal model of HCMV infection, the biological significance of these intriguing observations remains an open question.

Finally, an extreme example of $CD4^+$ evasion is the progressive and nearly complete destruction of the $CD4^+$ compartment by HIV. The loss of $CD4^+$ cells in AIDS leads to a weakening of antiviral CTL and antibody responses, and thus permits the uncontrolled replication of the virus. This extreme mode of immune evasion eventually leads to the death of the host, which may ultimately be disadvantageous for the virus. HIV is an emerging pathogen, and over time it may evolve into a less virulent human pathogen with less devastating effects on the $CD4^+$ compartment.

Evasion of CTLs

Adenovirus

The first putative CTL-evasion gene to be discovered was the adenovirus E3/19K (E19) protein. This protein binds to the $\alpha 1/\alpha 2$ regions of class I molecules in an allele-specific manner and causes retention in the ER, by virtue of a dilysine motif in the cytoplasmic tail, thus blocking CTL activation (Burgert and Kvist, 1985; Burgert et al., 1987; Paabo et al., 1987). In addition, mutant E19 lacking the ER retention signal can retard, but not block, class I export. This delay is associated with a block in the association of class I with tapasin (Bennett et al., 1999). Thus adenovirus may interfere with the peptide loading of those class I molecules to which it cannot directly bind.

Human adenovirus does not infect mice, and expression of E19 by vaccinia virus has no effect on CTL response (Cox et al., 1994) or pathogenesis (Grunhaus et al., 1994) *in vivo*.

Lentiviruses

The lentivirus gene products Nef and Vpu have both been reported to cause downregulation of surface class I on infected cells (Collins and Baltimore, 1999; Piguet et al., 1999). David Baltimore and coworkers (Collins et al., 1998) have convincingly shown that *nef* in particular has a significant CTL-evasive effect *in vitro*, and that CTL evasion can be attributed to loss of surface class I rather than other reported *nef* effects such as downregulation of Fas. Interestingly, *nef* specifically downregulates HLA-A and -B alleles, which are more important for CTL, and not -C alleles, which are more important for inhibiting NKs (Cohen et al., 1999).

SIV *nef* is close to satisfying our criteria for immune evasion genes. *nef* deletant SIV is greatly attenuated (Ruprecht, 1999). *In vivo* depletion of $CD8^+$ cells in SIV-infected animals leads to a rapid reactivation of latent Δnef SIV, demonstrating that this virus is controlled by CTL (Metzner et al., 2000). However, it remains to be shown that $CD8^+$ depletion abrogates the attenuation of Δnef SIV compared to parent-strain SIV. Given the multiplicity of functions attributed to *nef* (Carl et al., 2001; Janvier et al., 2001; Mahlknecht et al., 2000; Rasola et al., 2001), it remains a possibility that some or all of the attenuation of Δnef SIV is due to factors other than CTL control-- so for now *nef* must remain a putative immune evasion gene.

α- and γ-Herpesviruses

Examples of genes with specific CTL-evasion properties *in vitro* have been identified in every subfamily of *Herpesviridae;* those used by β-herpesviruses will be

described in more detail below. The first herpes CTL evasion gene to be discovered was ICP47 (Fruh et al., 1995; Hill et al., 1995; York et al., 1994). ICP47 is the product of an HSV IE gene. It is a short protein with no discernable secondary structure in aqueous solution (Beinert et al., 1997). It competes with antigenic peptide for binding to the cytosolic side of TAP, and is much more effective against human TAP than murine TAP (Ahn et al., 1996 ; Tomazin et al., 1998). ICP47 shuts off the supply of TAP-dependent peptides and abrogates CTL activity against HSV infected cells. In spite of the relative ineffectiveness of ICP47 against murine TAP, ICP47-knockout HSV is less neurovirulent in mice than wildtype HSV, and this attenuation is dependent on $CD8^+$ T cells (Goldsmith et al., 1998). Thus ICP47 is a bona fide immune evasion gene.

Recently, a second α-herpesvirus, VZV, has been shown to downregulate class I expression on infected cells (Abendroth et al., 2001). Class I in infected cells is not retained in the ER but appears to accumulate in the Golgi compartment. Transfection with VZV *ORF66* was sufficient to downregulate class I. It remains to be demonstrated whether *ORF66* itself causes Golgi accumulation of class I or has a functional effect on CTL activity.

The γ-herpesviruses KSHV (Coscoy and Ganem, 2000; Ishido et al., 2000) and MHV68 (Stevenson et al., 2000) carry homologous putative immune evasion genes which cause downregulation of class I from the cell surface by promoting its internalization. Transfection with these genes causes class I to accumulate in a post-Golgi compartment. KSHV *K5* also inhibits CTL and NK activity by downregulation of costimulatory (B7-2) and adhesion (ICAM) factors (Coscoy and Ganem, 2001; Ishido et al., 2000). Also, the γ-herpesvirus EBV encodes a *cis*-acting inhibitor of the proteasome

attached to the major latent antigen EBNA-1, which decreases the supply of EBNA-1-derived peptides to TAP and inhibits EBNA-1-specific CTL activity (Levitskaya et al., 1995).

Virus	ORF	Expression	Mechanism	Homologous ORFs
alphaherpesviruses				
HSV	***ICP47***	in virion	TAP cytoplasmic blockade	
VZV	***ORF66***	E	retention in Golgi?	
betaherpesviruses				
HCMV	***US2***	E	retrograde transport to cytoplasm	HCMV ***US2, US3, US6-US11***
	US3	IE	retention in ER	
	US6	E	TAP lumenal blockade	
	US11	E	retrograde transport to cytoplasm	
MCMV	***m4***	E	unknown	the *m2* family: MCMV *m2-m16*
	m6	E	redirection to lysosome	
	m152	E	retention in ER	the *m145* family: 10 MCMV genes and 15 rat CMV genes
gammaherpesviruses				
KSHV	***K3***		retention in trans Golgi network	KSHV ***K5***, ***K3*** MMHV68 ***K3***
	K5		retention in trans Golgi network and downregulation of costimulation	
MHV68	***K3***		retention in trans Golgi network	

Table 2. Herpesviruses genes that alter class I assembly

HCMV

Currently there are four HCMV genes which have been shown to alter the assembly and export of class I-- more than for any other virus. The mechanisms by which these genes affect class I are reviewed in (Loenen et al., 2001; Tortorella et al., 2000), and will be briefly described here. Of particular interest are the facts that (i) the functions of HCMV immune evasion genes are apparently analogous to those of MCMV,

yet the mechanisms used are quite different, and (ii) both viruses encode a number of genes whose functions at first appear redundant.

The first HCMV gene shown to alter class I export was *US11* (Jones et al., 1995), which was found to be sufficient to cause degradation of class I molecules from the ER. Wiertz *et al.* went on to demonstrate the biochemical mechanism of this degradation: retrograde transport of class I out of the ER and into the cytosol, where it is rapidly degraded by the proteasome (Wiertz et al., 1996). Soon afterward, *US2* was shown to degrade class I by the same mechanism (Jones and Sun, 1997). This virological discovery was the first demonstration of a type of ER-associated degradation which turned out to be important in the cell biology of all eukaryotic species. However, there is no evidence that this mechanism is relevant to immune evasion by MCMV. As noted above, recent work (Tomazin et al., 1999) has shown that in transfected cell lines, *US2* can also cause the destruction of MHC class II.

A recent report (Gewurz et al., 2001) described the crystal structure of US2 complexed to HLA-A2; this is the first crystalographic description of any herpes immune-evasion protein complexed to class I. An interesting finding was that US2 contains an unexpected Ig-like fold which was not predicted on the basis of primary sequence, and which is the point of contact between US2 and class I. This domain is shared among the family of US genes which includes *US3*, *US6*, and *US11*, leading to the speculation that this domain serves a similar function for all these proteins, and perhaps for the remaining uncharacterized members of this gene family. The point of contact for US2 is on the same side of HLA-A2 as that predicted for calnexin and calreticulin, opposite that of tapasin. Since there is no homology between the CTL-evasion genes of

HCMV and MCMV, the significance of this structure with regard to *m152* is uncertain. For now, we can only hope that someone will eventually determine crystal structures for complexes of class I with MCMV immune evasion proteins.

Around the same time as the discovery of *US2* and *US11*, *US6* was identified as sufficient to inhibit TAP-dependent transport of peptide into the ER (Ahn et al., 1997; Hengel et al., 1997; Lehner et al., 1997). This was clearly an analogous function to that served by the previously described HSV ICP47. However, US6 and ICP47 work by different mechanisms. US6 attacks TAP from the lumenal side (Ahn et al., 1997) and ICP47 blocks peptide binding on the cytoplasmic side. As with retrograde translocation of class I, TAP blockade is an HCMV immune-evasion strategy for which there is no evidence of an MCMV analog.

HCMV uses another mechanism to reduce the supply of antigenic peptides CTLs (Gilbert et al., 1996). The 72 kDa immediate early protein is phosphorylated in *trans* by the tegument component pp65, which makes the IE protein a poor substrate for the proteasome (Gilbert et al., 1996). This limits the generation and processing of IE peptides. So far, no other virus has been found to use a *trans* mechanism of proteosomal inhibition.

Also around the same time, two different groups (Ahn et al., 1996; Jones et al., 1996) demonstrated that the IE gene *US3* causes the retention of class I protein in the ER. Thus US3 is the only known IE CMV gene which interferes with antigen presentation. This led to the description of a "sequential multistep process" whereby *US3* first blocks export of class I from the ER, and *US2* and *US11* then cause its subsequent destruction. Of all the putative HCMV immune evasion genes, *US3* is the one with the closest

MCMV analog, in that both *US3* and *m152* inhibit class I maturation via poorly-understood transient interactions in a pre-Golgi compartment. These genes are compared in Table 3, below. The function of MCVM immune evasion genes is described in detail in the following section. First we will review general aspects of the immune response to MCMV.

Immunity to MCMV: host response and viral evasion

The NK response to MCMV

The first line of defense against many viruses is the innate response mediated by NK cells (Biron et al., 1999), which especially help to control virus in the first few days of infection before the development of specific CTLs. NKs are particularly important in the case of herpesviruses, as was demonstrated by the example of a human patient with defective NK function who presented with a succession of severe herpesviral infections, including HCMV (Biron et al., 1989). The importance of $CD3^-$ NK cells in MCMV infection has also been demonstrated in mice, using mice which are genetically deficient in (Shellam et al., 1985) or experimentally depleted of (Welsh et al., 1991; Welsh et al., 1994) NK cells. In addition, BALB/c mice are more susceptible to MCMV infection than are B6 mice. Genetically this susceptibility maps to a locus called *Cmv-1*, near the Ly49 NK receptors on chromosome 6 (Forbes et al., 1997; Scalzo et al., 1995). *Cmv-1* controls viral titer in the spleen, where control is perforin-dependent, to a greater extent than in the liver, where it is IFN-γ-dependent. In spite of much investigation, the sequence and exact position of *Cmv-1* remains to be identified.

NK cells can be activated both by stimulation of activating receptors and disinhibition of class I-specific inhibitory receptors. The latter mode of activation is responsible for the "missing self" response, by which targets lacking class I are recognized. That some anti-MCMV NK activity is independent of class I expression is demonstrated by the observation that NK cells help to control MCMV in mice lacking β2m (Polic et al., 1996).

A recent report (Daniels et al., 2001) has identified a subset of NK cells which are especially important in controlling MCMV. The subset was defined as recognized by an antibody to Ly49H, C, and I, and not recognized by antibody to C and I; this subset is thus provisionally defined as Ly49H$^+$, although it may include other unknown Ly49 subsets. Ly49H is an activating NK receptor which signals through DAP12. This is the first report assigning a particular role in controlling viral infection to a subset of NK cells. Daniels *et al.* found that control of MCMV, but not of MHV-68, VV, or LCMV, was dependent on the Ly49H$^+$ NK cells. It is possible that *Ly49H* is identical to *Cmv-1*, but such identity has yet to be demonstrated.

Adaptive immune control of MCMV

No single comprehensive study has defined the roles of distinct immune compartments in MCMV infection. However, a series of papers from Ulrich Koszinowski and coworkers has outlined distinct roles for different compartments in controlling MCMV. These papers reported the following results. (i) In BALB/c mice, adoptive transfer studies showed that CD8$^+$, and not CD4$^+$, T cells from MCMV-primed mice could control viral replication in lung, spleen, and adrenal glands of infected recipient mice which were first irradiated to reactivate MCMV (Reddehase et al., 1988;

Reddehase et al., 1987; Reddehase et al., 1985). (ii) In BALB/c mice, *in vivo* depletion studies showed that $CD4^+$ T cells were not necessary to control virus in organs other than the salivary glands of infected mice. However, $CD4^+$ cells were required to control MCMV replication in salivary glands , while control of dissemination from salivary gland to other tissues was mediated by $CD8^+$ T cells (Jonjic et al., 1989). (iii) Also in BALB/c mice, if donor mice were depleted of $CD8^+$ cells and infected with MCMV, splenocytes from these donors could control MCMV replication in various organs of recipient mice also lacking $CD8^+$ cells; this protective effect required the transfer of both $CD4^+$ T cells and some non-T cell splenocytes (Jonjic et al., 1990), and required IFN-γ (Lucin et al., 1992). Thus $CD4^+$ cells in cooperation with some other splenocytes were sufficient to control MCMV replication. (iv) In B6 mice, $CD4^+$ cells controlled virus in the salivary gland even in mice entirely lacking B cells. Lack of antibody had no effect on primary MCMV infection, but permitted increased viral replication after reactivation (Jonjic et al., 1994). (v) B6 mice lacking β2m were able to control viral replication in various organs when infected with tissue-culture passaged MCMV. This control required both $CD4^+$ and NK cell populations. However, $\beta 2m^{-/-}$ mice were more susceptible than wildtype mice when subjected to lethal challenge with virulent salivary gland (animal-passaged) virus stocks (Polic et al., 1996).

These somewhat confusing results can be interpreted as follows. B cells probably do not contribute to primary control of MCMV. $CD4^+$, $CD8^+$ and NK cells all contribute to control and provide partially redundant protection. $CD8^+$ cells are of primary importance in protecting animals against lethal disease in most major organs, but $CD8^+$ cells do not control virus in salivary glands. In this organ, $CD4^+$ cells are necessary but

not sufficient for protection, and probably cooperate with NK cells to control viral replication.

Overall, these results justify a continuing emphasis on understanding how $CD8^+$ cells control MCMV and how the virus modulates this response. The fact that MCMV carries so many CTL-evasion genes emphasizes the potential importance of this subset in control of MCMV replication. The following sections summarize what is known about the function of anti-MCMV CTLs.

CTL evasion by MCMV

An important first step in the investigation of anti-viral CTLs was the identification of a major viral antigen. By manipulating gene expression using techniques described above (see *CMV gene regulation*), it was determined that in BALB/c mice a significant proportion of anti-MCMV CTLs were specific for an IE antigen (Reddehase et al., 1984; Reddehase and Koszinowski, 1984). This antigen turned out to be a nonameric peptide derived from the IE protein pp89 and presented in the context of L^d (Del Val et al., 1991; Koszinowski et al., 1987; Volkmer et al., 1987). CTLs specific for this antigen were shown to protect against viral challenge (Jonjic et al., 1988). This pp89-derived peptide is still the best-defined class I-restricted antigen of MCMV and has served as the basis for much subsequent investigation of antigen presentation in MCMV.

In addition to pp89, however, other MCMV antigens, some of which are expressed during the E phase, are recognized by CTLs from MCMV-infected mice. The first E antigens to be identified were recently described in the BALB/c system by Holtappels *et al.* (Holtappels et al., 2001; Holtappels et al., 2000; Holtappels et al., 2000),

who identified antigenic peptides derived from the E genes *M83* and *M84*, and another derived from *m4* and presented in the context of D^d. There is no reason to suspect any functional connection between the antigenic properties of this peptide and the immunoevasive effects of *m4* described below, although its antigenicity may be related to the extreme abundance of m4/gp34 during the E stages of infection. In addition, our laboratory has recently identified a nine- or ten-mer antigen derived from *M45*, and recognized in the context of D^b (Marielle Gold, manuscript in preparation), making it the fourth E gene antigen to be described, and the first MCMV antigen in the B6 system.

MCMV E genes inhibit CTL activity

Using a CTL clone specific for pp89, Reddehase *et al.* (Reddehase et al., 1986) demonstrated that infected fibroblasts had a greatly diminished capacity to present pp89 antigen during the E phase of gene expression as compared to the IE and L phases. This led to a remarkable discovery: Del Val and coworkers (Del Val et al., 1989) demonstrated that E gene expression almost completely abrogated presentation of IE antigen to T cell clones without blocking the presentation of (uncharacterized) E antigens. Initially Del Val *et al.* interpreted these results to represent a pp89-specific block in antigen presentation (Del Val et al., 1989). As discussed below, it eventually became clear that the effects of multiple MCMV early genes in fact led to a general inhibition of class I maturation which is not specific for pp89-derived peptide. The intriguing observation that some E antigens still escape these multiple effects and are effectively presented to E-specific CTL clones remains unexplained, and is the subject of current investigation.

So far, three MCMV genes, *m152*, *m6* and *m4*, have been found to alter the assembly and function of class I molecules. A summary of what we currently know about these genes follows.

m152 Retains Class I in a Pre-Golgi Compartment

The first step in the biochemical description of CTL evasion by MCMV was the observation that L^d molecules synthesized during E gene expression fail to become Endo H resistant, indicating that class I is retained in a pre-medial Golgi compartment. In contrast to what is observed in HCMV infections, the retained class I molecules are not rapidly degraded, but in fact have a relatively long half-life and accumulate (del Val et al., 1992). Koszinowski and coworkers were able to replicate this effect by micro-injecting fibroblasts with defined fragments of the MCMV genome (Thale et al., 1995), and, using a close analysis of restriction sites, identified *m152* as being sufficient to cause class I retention (Ziegler et al., 1997). *m152* encodes a 40 kDa type 1 transmembrane glycoprotein, called "gp40", which is expressed during the E phase of infection with maximal expression between 3 and 6 hours post-infection. Expression of *m152* via transfection caused a marked reduction in surface expression of several classical class I molecules. Expression of *m152* via recombinant vaccinia virus (m152-Vac) was sufficient to protect targets from lysis by vaccinia-specific CTL clones. Recently, Krmpotic *et al.* (Krmpotic et al., 1999) have published results obtained for mutant viruses specifically knocked-out for *m152* (Δm152 MCMV) as well as control revertant viruses in which the knocked-out gene was restored. Using polyclonal CTL restimulated with pp89 antigenic peptide, Krmpotic *et al.* found that during the E stage of infection, targets infected with Δm152 were highly susceptible to lysis, while targets infected with either

wildtype virus or the revertant were completely protected. Thus *m152* serves to protect targets from class I-restricted lysis in the contexts of both MCMV infection and heterologous expression.

The effect of *m152* is specific for mouse class I molecules, since neither murine CD44 (an irrelevant cellular glycoprotein) nor human class I proteins are retained by m152-Vac (Ziegler et al., 1997). During infection with both MCMV and m152-Vac, retained class I molecules were not degraded but remained Endo H sensitive. Confocal microscopy demonstrated that retained class I molecules co-localize not with the ER marker BiP, but with p58, a marker for the ER-Golgi intermediate compartment (ERGIC) (Ziegler et al., 1997). The lumenal domain of m152/gp40 is sufficient to cause retention of class I in the ER (Ziegler et al., 2000).

In order to determine whether retained class I molecules were peptide loaded, Del Val *et al.* extracted processed peptides from MCMV-infected cells maintained under IE or E conditions. Using a pp89-specific T cell clone, they demonstrated that equal quantities of pp89 peptide were extractable under E and IE conditions. As a control, they showed that the antigenic epitope from pp89 could only be isolated from infected cells that expressed L^d and not from control cells lacking L^d (del Val et al., 1992). Del Val *et al.* concluded that retained L^d in MCMV-infected cells is peptide-loaded.

In spite of clear evidence for a specific effect of *m152* on class I transport, no direct biochemical interaction between m152/gp40 and class I has ever been found. In fact, it appears that m152/gp40 has a much shorter half life than the retained class I molecules, and that while class I is retained in a pre-Golgi compartment, m152/gp40 is transported through the Golgi and degraded in the lysosome (Ziegler et al., 2000; Ziegler

et al., 1997). Continuous expression of *m152* is not required for class I retention: even if *m152* expression is transient, class I remains in the ERGIC after all of the m152/gp40 has been degraded. Thus m152/gp40 appears to exert its effect on class I by some transient or indirect interaction in the ER or ERGIC. An unexplained observation of Ziegler *et al.* (Ziegler et al., 2000) is that the half-life of the association of HC with β2m is much longer for L^q retained by the effects of *m152* than for is for normal L^q. Thus the altered disposition of class I may be associated with an increased affinity for β2m.

	Protein	
	MCMV m152/gp40	**HCMV US3**
lumenal domain sufficient for class I retention	yes	yes
viral protein degraded in lysosome	yes	yes
coprecipitated with class I	no	yes
interaction with class I	hypothetical, transient	transient, recycled
continuous viral gene expression required for class I retention	no	yes
localization of retained class I	ERGIC	ER

Table 3. Functional comparison of *m152* and *US3*. Results are compiled from (Ziegler et al., 2000; Ziegler et al., 1997) and (Gruhler et al., 2001; Lee et al., 2000).

m152 Alters the CTL Response *in vivo*.

Using knockout and revertant viruses, Krmpotic *et al.* have demonstrated that *m152* has an effect on CTL control of virus *in vivo* (Krmpotic et al., 1999). In newborn BALB/c mice infected with 100 plaque forming units (PFU) of virus, mortality was 25% with Δm152 virus, 50% with wildtype virus, and 75% with revertant. Virus titers in both the spleen and the lung of animals infected with Δm152 were reduced tenfold compared

to animals infected with wildtype and revertant MCMV, and this reduction was dependent on CD8+ T cells. Finally, either MCMV-primed or naive CTLs were transferred to irradiated BALB/c mice which had been infected with Δm152 or revertant. Both CTL populations controlled replication of Δm152 better than revertant in the lung and liver (except that primed CTLs completely controlled both viruses in the liver). Thus *m152* is the only herpesvirus immune evasion gene for which an *in vivo* phenotype has been demonstrated in the natural host.

m6 Redirects Class I to Degradation in the Lysosome

Even after the identification of *m152*, it was clear from surface staining using a mutant virus that the effects of *m152* could not account for all of the loss of class I expression in MCMV-infected cells (Thale et al., 1995). Reusch *et al.* (Reusch et al., 1999) therefore used an innovative approach to look for viral gene products affecting class I expression. Reasoning that some important viral factor(s) could be found in tight complexes with class I molecules, Reusch and coworkers immunized BALB/c mice with class I molecules isolated from MCMV-infected BALB 3T3 fibroblasts. Since the mice were necessarily tolerant to H-2^d molecules, the resulting humoral immune response was especially directed against foreign proteins associated with class I. When a panel of B cell hybridomas was screened for activity against MCMV gene products, two hybridomas were found which specifically interacted with the product of *m6*.

m6 is another type 1 transmembrane glycoprotein, of molecular weight 48 kDa. It is an E gene, with maximal expression at 3-6 hours post-infection. m6/gp48 carries a functional dileucine motif in the cytoplasmic tail which targets the protein for destruction in the lysosome. In the ER, m6/gp48 binds tightly to β2m-associated class I molecules

and eventually redirects their transport away from the plasma membrane and into the lysosome where both proteins are destroyed; and this destruction is sensitive to specific inhibitors of lysosomal proteases. If the terminal dileucine motif of m6/gp48 is removed, class I complexes appear on the surface of transfected cells with an increased half-life relative to that seen with wildtype m6/gp48. As with *m152*, heterologous expression of *m6* is sufficient to protect target cells from lysis by class I-restricted CTLs. Thus MCMV encodes at least two different proteins which protect infected targets from class I-restricted CTLs by means of a general downregulation of class I.

m4/gp34 Forms Complexes With Class I Which Are Expressed On the Cell Surface

Shortly before the description of *m6* Kleijnen *et al.* identified another class I-associated MCMV protein using a similar but more direct strategy (Kleijnen et al., 1997). In this case, class I molecules were precipitated from metabolically labeled MCMV-infected fibroblasts and directly analyzed by SDS-PAGE. By comparing infected and uninfected cells, it was found that infected cells contained a glycoprotein of 34 kDa apparent molecular weight which was in a detergent-stable complex with class I molecules. Using mutant viruses and a serum generated against the predicted peptide sequence of *m4*, Kleijnen *et al.* demonstrated that gp34 is the product of *m4*. Like m152/gp40 and m6/gp48, m4/gp34 is a type I glycoprotein expressed as an E gene in MCMV infection. Since it is a major focus of this dissertation, I will review the original description of m4/gp34 (Kleijnen et al., 1997) in considerable detail.

All of the immunoprecipitations done by Kleijnen *et al.* were performed under similar conditions: MEFs were metabolically labeled (or, in one case, iodinated) and lysed in 0.5% NP40 lysis buffer (NLB); lysates were then subjected to precipitation with

specific antibodies, and precipitates were washed four times with a wash buffer containing 0.5% NP40 and 0.1% SDS. It is worth noting that the isolated complexes survived washing under these fairly stringent conditions. Biochemical analysis by Kleijnen *et al.* was limited to lysates of murine fibroblasts.

In the process of identifying gp34 as the product of *m4*, Kleijnen produced the serum R123 by immunizing a rabbit against the sixteen C-terminal residues of the predicted amino acid sequence of *m4*. This epitope from the cytoplasmic tail proved to be a good choice, since R123 recognizes both native and denatured m4/gp34. [The peptide sequence reported in the Methods section of (Kleijnen et al., 1997) is incorrect and does not match the published sequence of *m4* (Rawlinson et al., 1996) or the sequence of Fig. 7 of (Kleijnen et al., 1997). The actual peptide used to produce serum R123 was derived from the correct sequence and not the sequence listed in the Methods section (Ann Hill, personal communication)]. Serum R123 specifically recognizes m4/gp34: when Kleijnen *et al.* precipitated class I, boiled the precipitate, and reprecipitated with R123, they specifically recovered m4/gp34. However, they did not observe reciprocal co-precipitation: IP with serum R123 failed to coprecipitate class I. As shown in the Results section below, we have since defined conditions under which reciprocal coprecipitations are possible.

Kleijnen *et al.* tested the ability of m4/gp34 to form complexes with class I in wildtype and mutant H-2^b MEFs. As shown in Table 4, the ability of m4/gp34 to associate with class I was entirely β2m-dependent, and mostly TAP-dependent. Expression of human β2m, which has a higher affinity for mouse HC than does mouse β2m (Wei and Cresswell, 1992), reduced the requirement for TAP. These results suggest

that these m4/gp34-HC complexes are stabilized by a conformation of HC which is β2m-associated, although they could also indicate that β2m bridges the association between HC and m4/gp34 (see Chapter 3). The question of whether class I within these complexes is peptide-loaded remains unanswered.

Genotype	degree of m4/gp34 association with K^b, D^b
Parental (B6)	++++
$\beta2m^{-/-}$	-
$TAP^{-/-}$	+
$TAP^{-/-}$ $\beta2m^{-/-}$	-
human $\beta2m^{+}$	+++
$TAP^{-/-}$ human $\beta2m^{+}$	++

Table 4. Association of m4/gp34 with K^b and D^b in MEFs of different genotypes. Results are summarized from Fig. 2 of (Kleijnen et al., 1997).

Next, Kleijnen *et al.* investigated the cellular localization of m4/gp34. They found that all of the m4/gp34 which they recovered with R123 was Endo H sensitive, and concluded that class I-unassociated m4/gp34 was entirely ER-resident. In contrast, m4/gp34 which co-precipitated with class I was Endo H-resistant and capable of being labeled by extracellular ^{125}I-lactoperoxidase. An unusual feature of Endo H resistant m4/gp34 was that it only acquired partial Endo H resistance: using incremental Endo H digestion, they demonstrated that three of the four putative sites for N-linked glycosylation were used, and that of these three, only one became Endo H-resistant during the process of export. This is why exported m4/gp34, after Endo H-digestion, has a molecular weight intermediate between the undigested protein and the Endo H-digested, retained protein.

One function proposed for *m4* (Kleijnen et al., 1997) is that it could serve to counteract the "missing self" reaction by which some NK cells are activated when target

cells fail to express class I at the cell surface. The main impetus behind this proposal was the observation that cells infected with a mutant virus-- lacking genes *m01-m17*-- seemed to express less class I at the surface than cells infected with wildtype virus. To date there is no functional evidence for or against a role for *m4* in NK evasion. This is in sharp contrast to the case of the class I homolog *m144*, for which there is ample evidence of an effect on NK function, as described above.

Functional interaction of MCMV immune-evasion genes

Most of the above analyses of immune evasion functions were undertaken in transformed cells transfected with unique viral genes. Results from these important studies form the foundation of our understanding of viral immune evasion. However, transfection of transformed cells cannot provide a complete picture of viral gene functions for a variety of reasons. First, transformed cells often behave quite differently from primary cell lines and *ex vivo* tissue cultures. Second, the amount of viral protein expressed in transfected cells may be much more or less than what is expressed during viral infection. Finally, the natural expression of viral genes is regulated in a complex manner in the context of viral and cellular gene products. The alteration by multiple immune evasion genes of the presentation of multiple viral antigens cannot be replicated in tranfected cells. For these reasons, we undertook the present project using primary cells infected with MCMV as our main experimental model. Results presented in the following chapters describe the functional interaction of multiple viral gene products in MCMV infected cells.

Chapter 2

The Multiple Immune-evasion Genes of Murine Cytomegalovirus Are Not Redundant: *m4* and *m152* Inhibit Antigen Presentation In a Complementary and Cooperative Fashion.

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Abstract

Both human (HCMV) and murine (MCMV) cytomegaloviruses encode multiple genes which interfere with antigen presentation by major histocompatability complex (MHC) class I, and thus protect infected targets from lysis by virus-specific cytotoxic T lymphocytes (CTLs). HCMV has been shown to encode four such genes, and MCMV to encode two. MCMV *m152* blocks the export of class I from a pre-Golgi compartment, and MCMV *m6* directs class I to the lysosome for degradation. A third MCMV gene, *m4*, encodes a glycoprotein which is expressed at the cell surface in association with class I. Here we here show that *m4* is a CTL-evasion gene which, unlike previously described immune-evasion genes, inhibited CTLs without blocking class I surface expression. *m152* was necessary to block antigen presentation to both K^b- and D^b-restricted CTL clones, while *m4* was necessary to block presentation only to K^b-restricted clones. *m152* caused complete retention of D^b, but only partial retention of K^b, in a pre-Golgi compartment. Thus while *m152* effectively inhibited D^b-restricted CTLs, *m4* was required to completely inhibit K^b-restricted CTLs. We propose that cytomegaloviruses encode multiple immune-evasion genes in order to cope with the diversity of class I molecules in outbred host populations.

Introduction

Cytomegaloviruses (CMVs), including human CMV (HCMV) and murine CMV (MCMV) belong to the β subfamily of the *Herpesviridae*, a family of large, double-stranded DNA viruses. CMVs cause little pathology in normal host animals, but cause severe disease when the immune system is compromised. CMVs have developed intimate relationships with the host immune systems which permit the viruses to establish latency and reactivate in the face of primed immune responses. A number of mechanisms have been described by which CMVs modulate host immune responses; these include chemokine receptor homologues and viral gene products which interfere with the normal functions of T cells and natural killer (NK) cells (Alcami and Koszinowski, 2000). In particular, both HCMV and MCMV encode a number of gene products which specifically interfere with the ability of infected cells to present antigen to $CD8^+$ cytotoxic T lymphocytes (CTLs) (Hengel et al., 1998; Kavanagh and Hill, 2001).

$CD8^+$ T cells recognize a trimolecular complex, consisting of class I heavy chain, beta-2- microglobulin (β2m) and a short antigenic peptide, and which is assembled in the ER. In cells infected with HCMV, at least four different viral gene products interfere with this assembly (Jones et al., 1995): US6 blocks the peptide transporter TAP (Ahn et al., 1997; Hengel et al., 1997; Lehner et al., 1997); US3 prevents export to the Golgi (Ahn et al., 1996; Jones et al., 1996); and both US2 and US11 cause the destruction of class I molecules by retrograde transport into the cytosol (Wiertz et al., 1996; Wiertz et al., 1996). Similarly, at least three MCMV gene products also alter class I assembly. m152/gp40 blocks transport of class I molecules from the ER to the Golgi (del Val et al., 1992; Ziegler et al., 2000; Ziegler et al., 1997); m6/gp48 binds to class I molecules and

redirects their transport into the lysosome for destruction (Reusch et al., 1999); and m4/gp34 binds class I in the ER, forming a complex which is transported to the cell surface (Kleijnen et al., 1997). Only *m152* and *m6* have been previously shown to affect CTL function. There is no sequence homology between these MCMV genes and any mammalian or HCMV gene (Rawlinson et al., 1996).

Although HCMV *US3* and MCMV *m152* both cause class I retention, in HCMV-infected cells the dominant effect on class I is rapid degradation due to the actions of *US2* and *US11* (Beersma et al., 1993; Warren et al., 1994). In contrast, in MCMV-infected cells, class I is not degraded in the ER, but in fact accumulates in a pre-Golgi compartment (del Val et al., 1992), is degraded in the lysosome (Reusch et al., 1999), or reaches the cell surface, sometimes in association with m4/gp34 (Kleijnen et al., 1997). Similarly, TAP function is impaired by HCMV *US6* , whereas TAP function is apparently normal in MCMV-infected cells (unpublished observation). Finally, no molecule analogous to m4/gp34 has been observed to co-precipitate with class I in HCMV-infected cells. Thus, although there is no sequence homology between HCMV and MCMV genes that alter class I assembly, both viruses still effectively inhibit class I antigen presentation through the use of multiple genes.

It seems likely that interference with CTL recognition and the use of multiple genes to do so are both important features of the CMV-host relationship. It is not clear why both these viruses should carry a multiplicity of class I-modulating genes, but a number of possible explanations have been proposed. It has been suggested that individual genes may augment the function of others, or that viruses may require multiple

different genes in order to interfere with the function of diverse class I proteins in natural outbred host populations. This report provides evidence in support the latter hypothesis.

We have previously described the MCMV protein m4/gp34, which binds to MHC class I but whose function was not known. m4/gp34 is a 34 kD type 1 transmembrane glycoprotein, the product of the *m4* gene (Kleijnen et al., 1997). m4/gp34 is expressed abundantly during the early phase of viral gene expression, and accumulates in the ER, where it binds to class I molecules and forms a detergent-stable complex which is exported through the Golgi and to the cell surface. We previously speculated that *m4* might serve to oppose the action of *m152* by rescuing some class I molecules from retention, thus protecting infected cells from NK cells which might otherwise be activated by the loss of surface class I (Kleijnen et al., 1997); on the basis of this hypothesis, m4/gp34 has been referred to as an "NK decoy". However, until now there has been no evidence for an effect of *m4* on any immune function.

In this report we show that *m4* cooperates with *m152* to prevent recognition of virus-infected cells by $CD8^+$ T cells. *m4* is thus the third MCMV gene demonstrated to interfere with the class I pathway of antigen presentation. We show that *m152* has a differential effect on different class I molecules, efficiently retaining D^b in a pre-Golgi compartment but only partially retaining K^b. To completely prevent recognition of virus-infected cells by three K^b-restricted CTL clones, both *m4* and *m152* were necessary. In contrast, *m4* was not necessary to prevent recognition of infected cells by two D^b-restricted CTL clones. Thus *m4* and *m152* have complementary effects on different class I molecules.

Materials and Methods

Generation of mutant MCMVs

Generation and characterization of recombinants ΔMS94.5 (with a deletion of ORFs *m150* to *165*), ΔMC96.24 (with a deletion of ORF *m152*) and rMC96.27 (revertant for ΔMC96.24) were described previously (Krmpotic et al., 1999; Thale et al., 1995).

The recombinant Δm4-MC95.33, with an insertion of the *lacZ* gene in place of the *m4* ORF, was generated by insertional mutagenesis in eukaryotic cells as described previously (Crnkovic-Mertens et al., 1998), using the plasmid construct pm4. The homologous recombining region of pm4 was produced by flanking the *lacZ* gene with MCMV genomic sequences adjacent to the 5' (nt 2739 to 3250 left flank) and 3' (nt 4041 to 4737 right flank) ends of the ORF. Plasmid DNA (pHindIIIA) (Ebeling et al., 1983) serving as MCMV genomic template and primer pairs for the left flanking sequence [sense (5'-AA**CTCGAG**CATCACGGTGAACGATACCA), anti sense (5'-TT**GGATCC**TGGAACAACGAATGAGACAGA)] and right flanking sequence [sense (5'-AT**GCGGCCG**CTCGAACTTCAAACCGCTTAAGAG), anti sense (5'-AA**CCGCGG**ACTTATCGACGTACAATCCTGT)] were used in separate PCR reactions to produce fragments with convenient restriction sites to ligate to the *lacZ* gene (XhoI, BamHI and NotI and SacII, respectively in bold). These fragments were inserted into corresponding sites within the plasmid pIC4, which contains the *lacZ* gene under control of the Rous sarcoma virus (RSV) promoter, SV40 poly(A), and flanking loxP sites (Crnkovic-Mertens et al., 1998). Thirty fmol of linearized pm4 plasmid DNA was cotransfected with wt MCMV DNA (1.5 μg) into NIH3T3 fibroblasts by calcium phosphate precipitation to generate the recombinant virus Δm4-MC95.33. Recombinant

virus was isolated and plaque-purified as described previously (Crnkovic-Mertens et al., 1998). Correct recombinatorial mutagenesis within the genome of Δm4-MC95.33 was confirmed by restriction enzyme analysis (data not shown).

We have recently cloned the MCMV genome as an infectious bacterial artificial chromososme (BAC) in *E. coli* (Messerle et al., 1997). The MCMV-BAC plasmid pSM3fr contains the complete MCMV genome and was transfected into permissive eukaryotic cells to reconstitute the virus MW97.01 (wt) (Wagner et al., 1999). MW97.01 (wt), which contains the complete MCMV genome without any BAC sequence, has wildtype properties both *in vitro* and *in vivo*, indicating that the MCMV genome can be passaged in *E. coli* without altering the properties of the reconstituted viruses.

Recombinant MCMVs Δm4-MW99.03, Δm152-MW99.05, and Δm4+m152-MW99.04 were generated by transfection of the MCMV BAC plasmids pΔm4, pΔm152, and pΔm4+m152, respectively, into primary MEFs by calcium phosphate precipitation technique as described previously (Messerle et al., 1997). The MCMV BAC plasmid pΔm4, which encodes an exact deletion of the *m4* ORF (nt 3270 to 4067) by insertion of the prokaryotic kanamycin resistance marker (kan^r), was constructed using contiguous m4-kan sequence primer pairs: sense (5'-TAATGATCTAGACGGCAATTTCTGTCTCATTCGTTGTTCCAGAGCGACGGATGGTACAAG) and antisense (5'-TACTCAGAACACCGGAAAATGGTTTACTCAAGGGGATTTTTATTTAGGGGGTTAGTTACT). The plasmid pACYC177 (New England Biolabs) served as template for the kanamycin resistance marker. A linear DNA fragment containing flanking homologies of 55 bp to the *m4* gene (nt 3215 to 3269 and nt 4068 to 4123 in the MCMV

genome) and the kan^r was generated by PCR amplification. This fragment was inserted into the wt MCMV BAC plasmid pSM3fr (Wagner et al., 1999) by homologous recombination in *E. coli* to generate the MCMV BAC plasmid pΔm4. The MCMV BAC plasmid pΔm4+m152 was generated using contiguous *m152*-zeocin primer pair PCR amplification. The fragment containing flanking homologies of 60 bp to the *m152* gene (nt 210184 to 210243 and nt 210378 to 210437) and the zeocin resistance gene was generated using sense (5'-GCTCGAGCGAGAGCACCCGACGATCTGACATTGTCCAGTGTGCCGGTCGCAC GAACATCAGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCAACGTTTACA ATTTCGCCTGATGCG) and antisense (5'-TCACAAGCCGTGTCACCGCTCCACGTTTCACCGTCGTCGGTCTCCCGATCGCT AGCCTGAACAGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCTGAAGTTTT AGCACGTGTCAGTCCT) primer pairs and the plasmid pZero1 (Invitrogen) as template. This fragment was inserted into the MCMV BAC plasmid pΔm4 by homologous recombination in *E. coli,* generating plasmid pΔm4+m152. Plasmid pΔm4+m152 thus carries exact deletions of the *m4* and *m152* ORFs and insertions of the kanamycin resistance marker (in the case of *m4*) and the zeocin resistance marker (in the case of *m152*) instead. Plasmid pΔm152 was generated by homologous recombination between pSM3fr and the *m152*-zeocin fragment. Correct mutagenesis was confirmed by restriction enzyme and southern blot analysis (data not shown).

Recombinant MCMVs m4-Tn3514, m4Tn3516 and m4TnP (with Tn1721 transposon insertions within the *m4* gene or putative promoter, at nt 3514, nt 3516 and nt 3099 respectively) were reconstituted from recombinant MCMV-BAC plasmids

generated by direct transposon mutagenesis as described previously (Brune et al., 1999; Hobom et al., 2000). The site of mutagenesis was confirmed by restriction enzyme analysis and sequencing (data not shown).

The genomic organization of all MCMV mutants is shown schematically in Figure 1A-C. Loss of m4/gp34 expression in the BAC-derived recombinants was confirmed by Western blot analysis of cell lysates from infected NIH3T3 cells with the antiserum m04-3 that detects m4/gp34 (see Figure 1D).

Experimental Animals.

B6 mice were purchased from Simenson, and B10.A5R, and B10.A2R from Jackson Labs. $D^{b-/-}$ mice (Hoglund et al., 1998) were a gift from Francois Lemmonnier.

Virus Stocks and Cell Culture

MEFs were grown form trypsin-digested day 12-14 mouse embryos, and used between passage 3 and 6. Adult mouse fibroblast lines were generated from ears of $D^{b-/-}$ mice and from B6 x129 backcrossed mice, and used between passages 3 and 6. NIH 3T3s (CRL-1658) and Balb3T3s (CCL-163) were obtained from ATCC. MEFs and 3T3s were maintained in DMEM supplemented with 10% fetal (for MEFs, adult fibroblast lines and NIH3T3s) or newborn (for Balb3T3s) calf serum. Virus stocks were generated by infecting subconfluent MEFs with low-passage seed stock at an MOI of 0.001. Cells were then switched to DMEM + 10% normal calf serum until the monolayer became 100% infected. Stocks were harvested by scraping and sonication of cells. Titer of plaque forming units (PFU) was determined by serial dilution and agarose overlay on Balb-3T3s.

T Cell Line and Clones

B6 mice were infected intraperitoneally with 5 x 10^4 PFU MCMV-Smith, ΔMS94.5, or ΔMC96.24. Between 8 and 40 weeks later, spleens were harvested. 10% of splenocytes were infected with MCMV (of the same strain with which mice were infected) and returned to culture with the remaining splenocytes. For polyclonal effectors, cultures were used 5 days later in Cr release assays. To derive CTL clones, the cultures were cloned by limiting dilution on day 3 in the presence of irradiated mixed allogeneic feeder splenocytes and 2μg/ml concanavalin A (con A; Sigma). Clones were maintained in cloning medium [RPMI medium with 10% FCS, 5 X 10^{-5} M 2-mercaptoethanol (Sigma), 1% conditioned medium from IL-2-secreting cell line X63.653 (Karasuyama and Melchers, 1988), and 10% conditioned medium from conA-stimulated rat splenocytes], and restimulated with conA and irradiated mixed allogeneic feeders each 10 days. Clones have been maintained in culture for more than 12 months. Clones were screened for anti-viral function based on their ability to specifically kill IFNγ-boosted ΔMS94.5-infected MEFs compared to uninfected MEFs. Clones 3, 11 and 5 are from ΔMS94.5-infected mice (clone 5 is from a different mouse than clones 3 and 11); clone 96 is from a Smith-infected mouse; and clone 55 is from a ΔMC96.24-infected mouse. Clones 3, 11 and 96 recognize different HPLC fractions of peptides extracted from infected cells (data not shown); clones 5 and 55 have not been tested against HPLC-fractionated extracts.

Cytolytic T cell assays

MEF target cells were plated into 96-well plates at 5,000 cells/well and treated with recombinant mouse IFNγ (50 U/ml, Sigma) for 24 hours, infected with MCMV (at an MOI of 30 for Figures 3D and 4, and an MOI of 10 for all other figures) unless otherwise indicated, and labeled with ^{51}Cr (NEN) overnight, in

the presence of 0.3 mg/ml phosphonoacetic acid (PAA, Sigma) to prevent expression of viral late genes. CTL clones described here did not kill MEF targets without IFNγ pretreatement (data not shown). T cells were added at the indicated effector-to-target ratios for six hours, after which supernatants were harvested and assayed for γ-irradiation with a Topcount scintillation counter (Packard). Background Cr-release was determined by incubating targets with medium alone, and total Cr release was achieved by lysing targets with medium containing 2% Triton X-100. Percent specific lysis was calculated as (experimental cpm-background cpm)/(total cpm-background cpm). Each data point represents the mean of triplicate wells.

Antibodies

Serum 8010 (anti-p8) was generated by immunizing rabbits with synthetic peptide corresponding to exon 8 of K^b. Sera 8142 and 8139 (anti-m4/gp34) were both generated as follows. Serum R123 against the cytoplasmic tail of m4/gp34 (Kleijnen et al., 1997) was used to precipitate m4/gp34 from MCMV (Smith)-infected MEFs. After washing, the immune complex was suspended in complete Freund's adjuvant (Sigma) and used to immunize rabbits subcutaneously. Rabbits were boosted first with immune complex suspended in incomplete Freund's adjuvant (IFA, Sigma), and then by infection with recombinant vaccinia virus expressing m4/gp34 (generated by recombination between modified psc11 plasmid expressing the *m4* gene and WR strain vaccinia virus), and finally with recombinant m4/gp34 protein purified from baculovirus, (the kind gift of Pamela Bjorkman) in IFA. Serum m04-3 used for western blot analysis of m4/gp34 expression was generated by immunizing rabbits with synthetic peptide corresponding to amino acids 34 to 48 of m4/gp34 (peptide sequence

KEYKEKMKYRHSLGC). Monoclonal antibody 28.14.8S (ATCC HB-27) was purified from hybridoma supernatant.

Metabolic labeling and immunoprecipitations

B6 MEFs or adult ear fibroblasts were pretreated with recombinant mouse IFNγ at 50 U/ ml for 24-48 hours before infection. Without IFNγ, uninfected MEFs do not express detectable amounts of class I. Although infected cells express class I in the absence of IFNγ, they were also treated with IFNγ for the sake of consistency. Cells were maintained in the presence of 0.3 mg/ml PAA after infection or mock infection. 1 hour before the addition of metabolic label, cells were washed in PBS and placed in cysteine/ methionine-free DMEM (Gibco) supplemented with antibiotics, and 5% FCS. Cells were then labeled with [^{35}S]cysteine/methionine (NEN, ~0.2 mCi/ml for long labeling periods and ~0.5 mCi/ml for pulse labels) for the time periods indicated. For pulse-chase experiments, cells were washed with chase medium [DMEM supplemented with antibiotics, glutamate, 10% FCS, and 1mM L-cysteine and L-methionine (Sigma)] at the end of the labeling period. All lysis and precipitation procedures were carried out at 4°C. Cells were washed in the plates with PBS and lysed in NP-40 lysis buffer (0.5% NP-40, 50 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$). Just before use, lysis buffer was supplemented with protease inhibitor— either 1 mM PMSF (Sigma) or Complete EDTA-free Protease-Inhibitor Cocktail according to the manufacturer's directions (Boehringer Mannheim). Lysates were precleared by incubation with at least 20 μl of normal rabbit serum (NRS) and 500 μl of 10% suspension of fixed *Staphylococcus aureus* for 2 hours, and centrifuged for 5 min at 15,000 g. Precleared lysates were then subjected to specific immunoprecipitation as indicated in the figures. Unless otherwise indicated, each aliquot

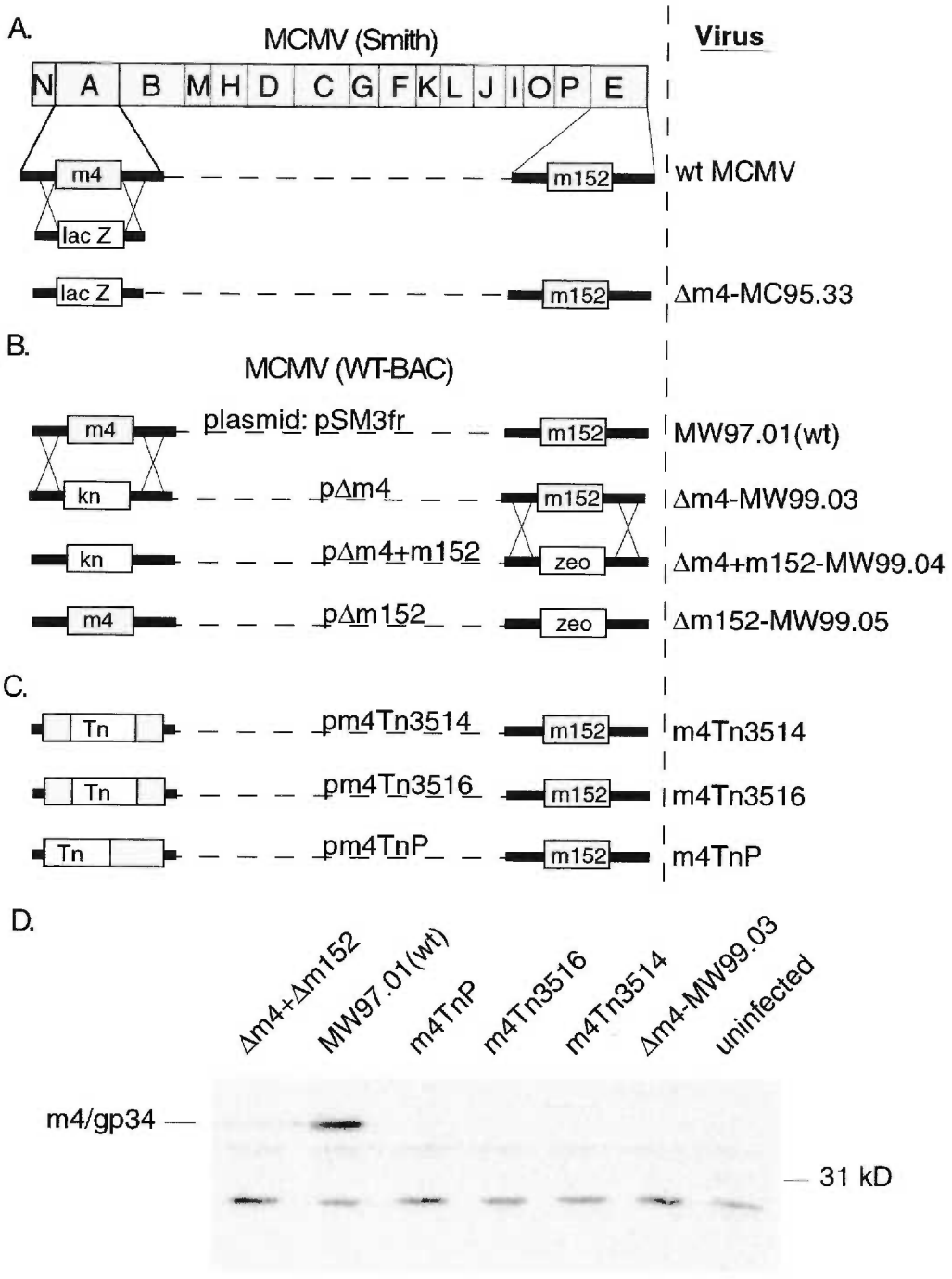
of lysate received ~10 μg of antibody plus 150 μl of 5% protein A agarose suspension (Sigma). Immunoprecipitates were washed four times in NET buffer (150mM NaCl, 50mM Tris pH 7.5, 5mM EDTA and 0.05% NP40) containing 0.1% SDS. Samples were digested with Endo H_f (NEB) according to manufacturer's protocol, resuspended in reducing sample buffer and separated by SDS-PAGE on a 12.5% gel. Quantitation of labeled protein was performed using a Molecular Dynamics phosphorimager.

Results

Construction of MCMV mutants lacking m4/gp34 expression.

MCMV mutants lacking m152/gp40 expression have already been described (Krmpotic et al., 1999). To compare the functions of *m4* and *m152*, we constructed mutant MCMVs with targeted deletions of *m4* and/or *m152*. The process of generating mutant viruses may lead to accidental mutations elsewhere in the genome, so in order to clearly attribute a phenotype to the deleted gene, we have constructed five separate mutant viruses lacking *m4*, using three different technologies (see Materials and Methods, Figure 1, and Table 1). Δm4-MC95.33 was made by homologous recombination between the viral genome and plasmid in transfected cells (Figure 1A). Other mutants were reconstituted from bacterial artificial chromosomes (BACs) constructed by homologous recombination (Figure 1B) or transposon insertion (Figure 1C). The correct genomic structure of all BACs was confirmed by restriction analysis and Southern blot (not shown). Lack of m4/gp34 expression by all Δm4 mutants was confirmed by Western blot (Figure 1D).

Figure 1. Construction of mutant viruses. A. Schematic representation of the 230kb linear MCMV genome. Hind III digestion generates 16 fragments, designated A-P by size, organized in the genome as shown (not to scale). Sequencing of the complete genome (17) revealed 170 potential open reading frames, numbered from the left to the right hand end of the genome, *1* to *170*. ORFs with homology to HCMV genes are given a capitalized *M* (e.g. *M84*), and MCMV genes without recognized homology are denoted by a lower case *m* (e.g. *m4*). Also shown are the positions of *m4* and *m152* within the genome, and the strategy for generation of the *m4* deletion mutant Δm4-MC95.33 by insertion of the *lacZ* gene. **B.** Generation of BAC mutants by insertional mutagenesis. Kanamycin and zeocin resistance genes were inserted into the pSM3fr BAC plasmid as shown to replace the *m4* and *m152* ORFs respectively. Transfection into permissive cells generated the mutant viruses Δm4-MW99.03, Δm4+m152-MW99.04, and Δm152-MW99.05. **C.** Generation of BAC mutants by transposon mutagenesis. **D.** Western blot using anti-m4/gp34 serum m4-03 of lysates from cells infected with the viruses shown. All viruses were used at the same MOI and all infected cells showed comparable cytopathic effect.


A.
MCMV (Smith)
Virus
N A B M H D C G F K L J I O P E
m4
m152
wt MCMV
lac Z
lac Z
m152
Δm4-MC95.33
B.
MCMV (WT-BAC)
m4
plasmid: pSM3fr
m152
MW97.01(wt)
kn
pΔm4
m152
Δm4-MW99.03
kn
pΔm4+m152
zeo
Δm4+m152-MW99.04
m4
pΔm152
zeo
Δm152-MW99.05
C.
Tn
pm4Tn3514
m152
m4Tn3514
Tn
pm4Tn3516
m152
m4Tn3516
Tn
pm4TnP
m152
m4TnP
D.
Δm4+Δm152
MW97.01(wt)
m4TnP
m4Tn3516
m4Tn3514
Δm4-MW99.03
uninfected
m4/gp34
31 kD

VIRUS:	METHOD	GENOTYPE	INSERTION
Smith strain	natural isolate	wildtype	none
MW97.01	BAC derived	wildtype	none
ΔMS94.5	mutagenesis in cells[1]	ΔORFs 150-165	lacZ
ΔMC96.24	mutagenesis in cells	Δm152	none
rMC96.27	mutagenesis in cells	wildtype, revertant of ΔMC96.24	none
ΔMS94.7	spontaneous mutant	ΔORFs 1-17	none
Δm4-MC95.33	mutagenesis in cells	Δm4 (nt 3250-4041)	lacZ
Δm4-MW99.03	BAC/recombination[2]	Δm4 (nt 3270-4067)	kan^r
m4Tn3514	BAC/transposon[3]	*m4* disrupted	Tn1721 into *m4* at nt 3514
m4Tn3516	BAC/transposon	*m4* disrupted	Tn1721 into *m4* at nt 3516
m4TnP	BAC/transposon	putative *m4* promoter disrupted	Tn1721 into putative *m4* promoter at nt 3099
Δm4+m152-MW99.04	BAC/recombination	Δm4+m152 (nt 3270-4067 and 210244-211377)	kan^r/$zeocin^r$
Δm152-MW99.05	BAC/recombination	Δm152 (nt 210244-211377)	$zeocin^r$

Table 1. Viruses used in this paper. The methods used to create mutant MCMVs are described in Materials and Methods.

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[1] recombination between plasmid and wildtype MCMV in transfected cells.

[2] homologous recombination in *E. coli* between MCMV bacterial artificial chromosome (BAC) and insert.

[3] transposon-mediated mutagenesis of MCMV BAC in *E. coli* .

m4 does not affect the export of K^b over a ninety minute chase.

NK cells can lyse target cells with low cell surface class I; thus viral functions that reduce cell surface class I in order to protect against CTL recognition might render the infected cells vulnerable to NK attack. We previously hypothesized that m4/gp34 might serve to inhibit NK activity by rescuing some class I molecules from *m152*-induced retention, thus increasing class I expression on the cell surface (Kleijnen et al., 1997). In order to test this hypothesis, we infected mouse embryo fibroblast (MEF) cells with either wildtype virus or the mutant virus Δm4-MC95.33, at a range of multiplicities of infection (MOIs), and measured the degree of K^b export as indicated by the acquisition of Endoglycosidase H (Endo H) resistance over a 90 minute chase period. Figure 2 shows that at any given MOI, infection with either Δm4-MC95.33 or wildtype virus caused comparable degrees of K^b retention. In addition, at a fixed MOI of 5, we found no significant difference between the amount of K^b that was exported in wildtype or Δm4-MC95.33-infected cells at a range of timepoints after infection (data not shown). We conclude that, for at least ninety minutes after synthesis, *m4* does not affect the extent of K^b export in MCMV-infected fibroblasts.

m4/gp34 expression inhibits CTL activity

We next investigated whether m4/gp34 expression could affect recognition of targets by class I-restricted $CD8^+$ CTL. The first experiments used polyclonal CTL from spleens of MCMV-infected C57BL/6 (B6) mice restimulated *in vitro* with virus. Figure 3A shows one such experiment. There was minimal specific lysis of targets infected with wild-type MCMV, but strong CTL activity against targets infected with ΔMS94.5, which

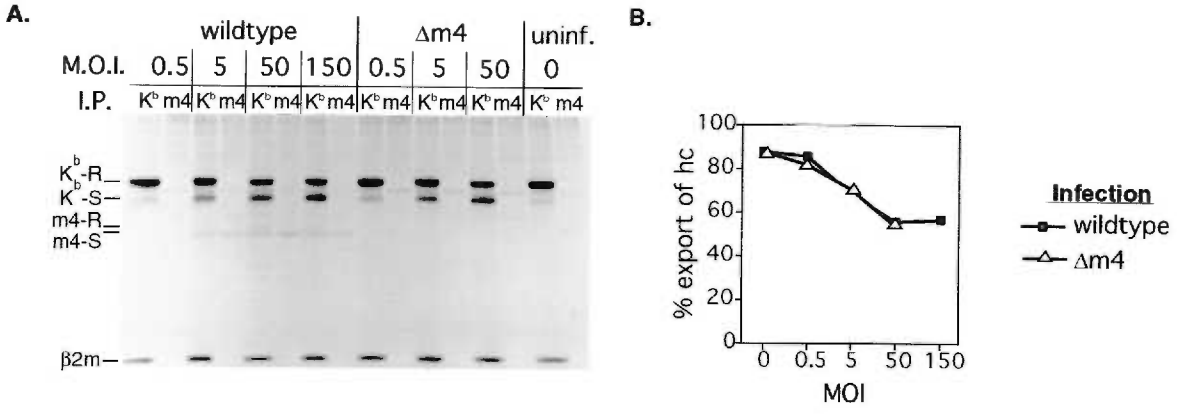

Figure 2. *m4* does not counteract the effects of *m152* on K^b. A. Immunoprecipitation of K^b and m4/gp34 from MEFs infected with increasing doses of wildtype (Smith) or Δm4-MC95.33 MCMV. Cells were infected at the indicated MOI for 5 hours, labeled for 30 min. with [^{35}S]methionine and chased for 90 min. with unlabeled methionine. Cells were lysed in NP-40 lysis buffer. K^b was immunoprecipitated with anti-p8 and m4/gp34 with serum 8142. All samples were treated with Endo H. The positions of bands corresponding to Endo H-resistant (R) and -sensitive (S) molecules are indicated. **B.** Quantitation of K^b export. The amount of Endo H-sensitive (retained) and Endo H-resistant (exported) K^b was determined with phosphorimage analysis of the gel shown in A. The degree of export is calculated as % export=(resistant)/ (resistant + sensitive).

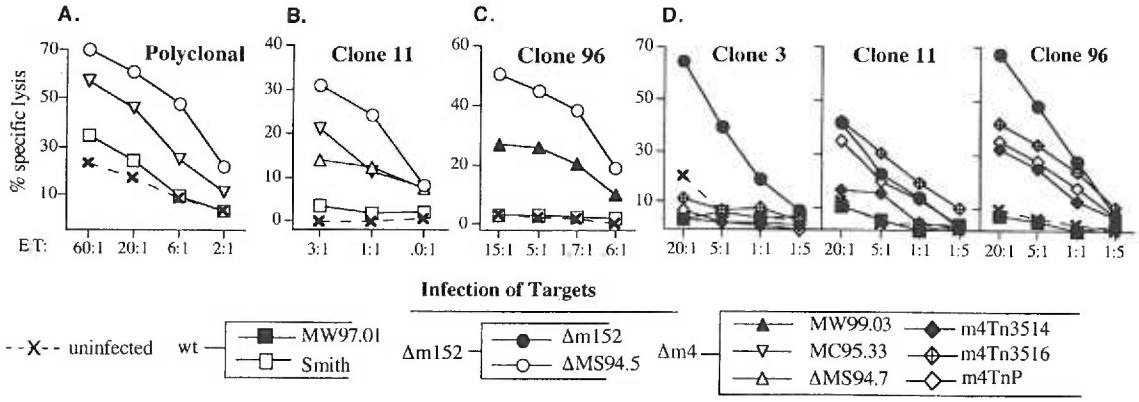

Figure 3. *m4* is an immune-evasion gene. Polyclonal CTL or CTL clones were tested for their ability to lyse B6 MEFs infected with the viruses shown. Targets were pretreated with IFNγ and infected overnight in the presence of PAA. **A.** Polyclonal CTL were tested against ΔMS94.5 (lacking ORFs 150-165), Δm4-MC95.33 and wildtype MCMV (Smith). **B-D:** MCMV-specific CTL clones were tested against Smith or the wildtype BAC virus MW97.01 and various *m4* deletion mutants, as follows. B: Δm4-MC95.33, ΔMS94.7 (lacking ORFs 1-17), ΔMS94.5 and Smith. C: Δm4-MW99.03, ΔMS94.5, and Smith. D: Δm152-99.05, MW97.01 (wt) and three *m4* deletion viruses: m4Tn3514, m4Tn3516 and m4TnP.

lacks *m152*. Thus in wild-type infection the combined effects of the immune-evasion genes were able to completely abrogate recognition; however, a virus lacking *m152* was readily detected. There was also significant killing of targets infected with Δm4-MC95.33, demonstrating that *m4*, in addition to *m152*, contributes to immune evasion from polyclonal CTL.

To investigate this phenomenon further, we generated a panel of MCMV-specific CTL clones from mice infected with either wildtype MCMV or two mutant MCMV viruses lacking *m152*. The antigens recognized by these clones have not yet been identified, but they are all expressed in the early phase of MCMV gene expression (data not shown). Remarkably, none of these clones were able to lyse cells infected with wildtype virus; this included clone 96 which was generated from a mouse infected with wildtype virus. However, all the clones recognized targets infected with viruses lacking *m152* (Figure 3 B-D), confirming the importance of this immune-evasion gene. We next tested whether the clones could recognize viruses lacking *m4* but expressing *m152*. Figure 3B shows an experiment using Δm4-MC95.33, and Figure 3C shows an experiment using Δm4-MW99.03. Both *m4* deletion mutants, which were independently constructed using different techniques, were recognized, whereas the wildtype virus was not. These results were extended in the assay shown in Figure 3D, in which the three *m4* deletion mutants generated by transposon insertion, m4Tn3514, m4Tn3516 and m4TnP, were tested for recognition by three different clones. All three mutants were recognized by clones 11 and 96, consistent with the previous results. However, we noted that none of the three mutants was recognized by clone 3.

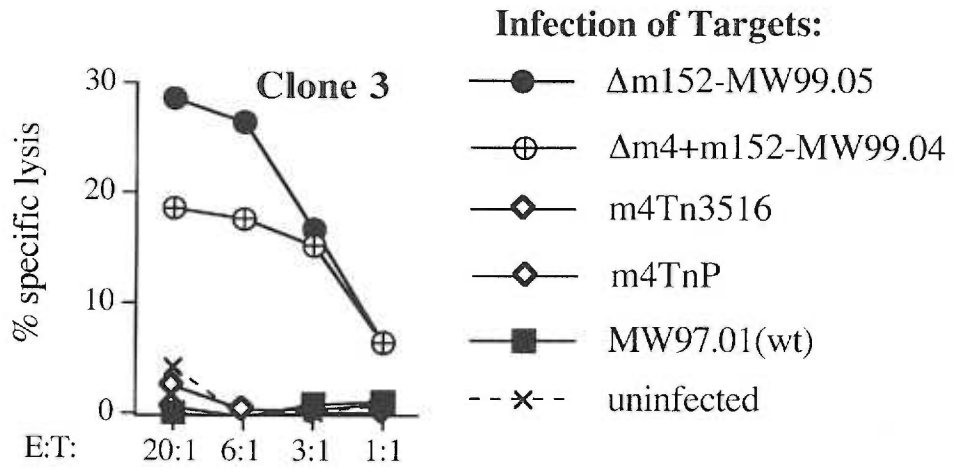

Figure 4. The antigen recognized by clone 3 is not m4/gp34. B6 MEF targets were infected with MCMV MW97.01 (wt), m4Tn3516, m4TnP, Δm4+m152-MW99.04, or Δm152-MW99.05. The difference in degree of lysis of targets infected with Δm152 and Δm4+m152 is not reproducible.

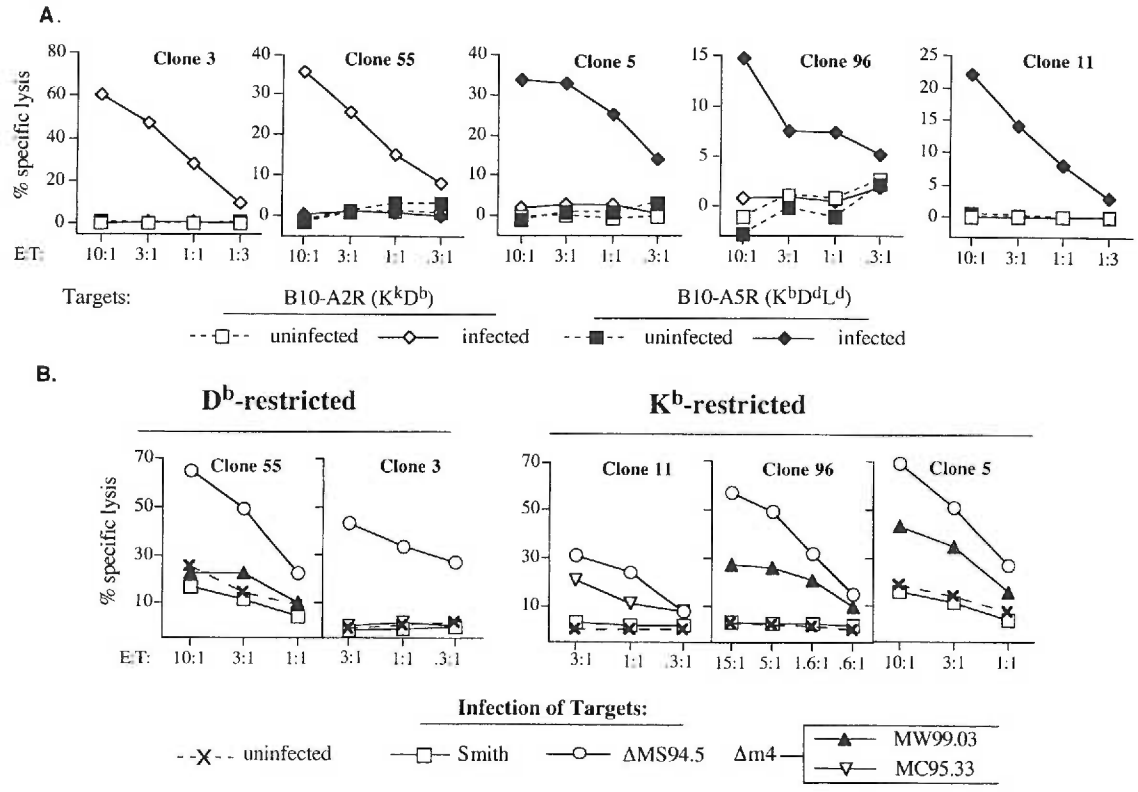

Figure 5. *m4* is necessary for evasion from K^b- but not D^b-restricted CTL clones. A. Two MCMV-specific clones are D^b-restricted and three are K^b-restricted. The class I restriction elements used by five CTL clones, derived from MCMV-infected B6 mice, were determined by testing the ability of each clone to lyse fibroblasts from B10A.2R (K^k D^b) or B10A.5R (K^b L^dD^d) mice. Fibroblast targets were either uninfected or infected with MCMV ΔMS94.5. **B.** K^b- but not D^b-restricted clones respond to MCMV lacking *m4*. The five CTL clones were tested for ability to lyse of B6 MEFs infected with the indicated viruses.

The results seen with five independent *m4* deletion mutants led us to conclude that the observed phenotype is indeed due to the functional deletion of the *m4* gene. These results demonstrate for the first time that *m4*, like *m152* and *m6,* acts as a viral immune-evasion gene. However, the results seen with clone 3 demonstrate that deletion of *m4* is not by itself sufficient to permit MCMV recognition by some CTL clones.

Clone 3 does not recognize an epitope within m4/gp34

We wondered why only some CTL clones could recognize cells infected with *m4* deletion mutants. m4/gp34 provides an epitope recognized by MCMV-specific CTL from Balb/C mice (Holtappels et al., 2000). One possible explanation for inability of clone 3 to recognize *m4* deletion viruses was that the epitope recognized by clone 3 could be derived from m4/gp34 itself. Since clone 3 can respond to viruses lacking *m152*, we constructed a new virus (Δm4+m152-MW99.04) lacking both *m152* and *m4*. Figure 4 shows that this virus was readily detected by clone 3, indicating that the epitope recognized by clone 3 is not contained within m4/gp34.

***m4* is necessary for evasion from K^b- but not D^b-restricted CTL clones.**

To further analyze why some clones were able to recognize *m4* deletion mutants and others not, we determined the restriction element used by five MCMV-specific CTL clones. MEFs from B10A.2R (K^kD^b) or B10A.5R ($K^bL^dD^d$) mice were infected with MCMV-ΔMS94.5 and used as targets in CTL assays. Figure 5A shows that clones 3 and 55 are restricted by D^b, and clones 5, 11, and 96 are restricted by K^b. These five clones were next tested for their ability to lyse targets infected with *m4* deletion mutants. The results are shown in Figure 5B. All three K^b-restricted clones were able to lyse targets

infected with *m4* deletion mutants, indicating that *m4* expression was necessary for complete immune evasion from these clones. In contrast, the two D^b-restricted clones did not recognize the *m4* deletion mutants, indicating that the other immune-evasion genes were sufficient to prevent MCMV-specific killing by these clones.

MCMV differentially inhibits maturation of different class I molecules.

The difference between K^b- and D^b-restricted CTL clones suggested that K^b and D^b might show different sensitivities to the effects of the immunomodulatory genes. The three K^b-restricted clones can lyse targets infected with virus containing a single deletion of either *m152* or *m4*, indicating that *m152* is necessary to prevent antigen presentation by K^b but is not sufficient for this task in the absence of *m4*. In contrast, the two D^b-restricted clones can lyse infected cells if *m152* is deleted, but are unable to detect virus in which *m4* is deleted while *m152* remains. This suggested that D^b might be more susceptible to the activity of *m152* than K^b. *m152* inhibits antigen presentation by retaining class I molecules in the ERGIC. We therefore performed a pulse-chase experiment comparing the relative rates of export of K^b and D^b in MCMV-infected cells. B6 MEFs were pulsed with [^{35}S]methionine for fifteen minutes and chased for one, two, or four hours. K^b and D^b were immunoprecipitated from the same lysates and the respective degree of maturation was determined by Endo H digestion. Figure 6A shows that in uninfected cells, both K^b and D^b become Endo H-resistant over the chase period, although the maturation of D^b was slower than maturation of K^b. In contrast, in infected cells almost all D^b was retained in an Endo H-sensitive form over the entire four hours, while approximately 50% of the K^b protein was exported and matured within two hours. We also noted that the m4/gp34 co-precipitating with the class I molecules displayed a

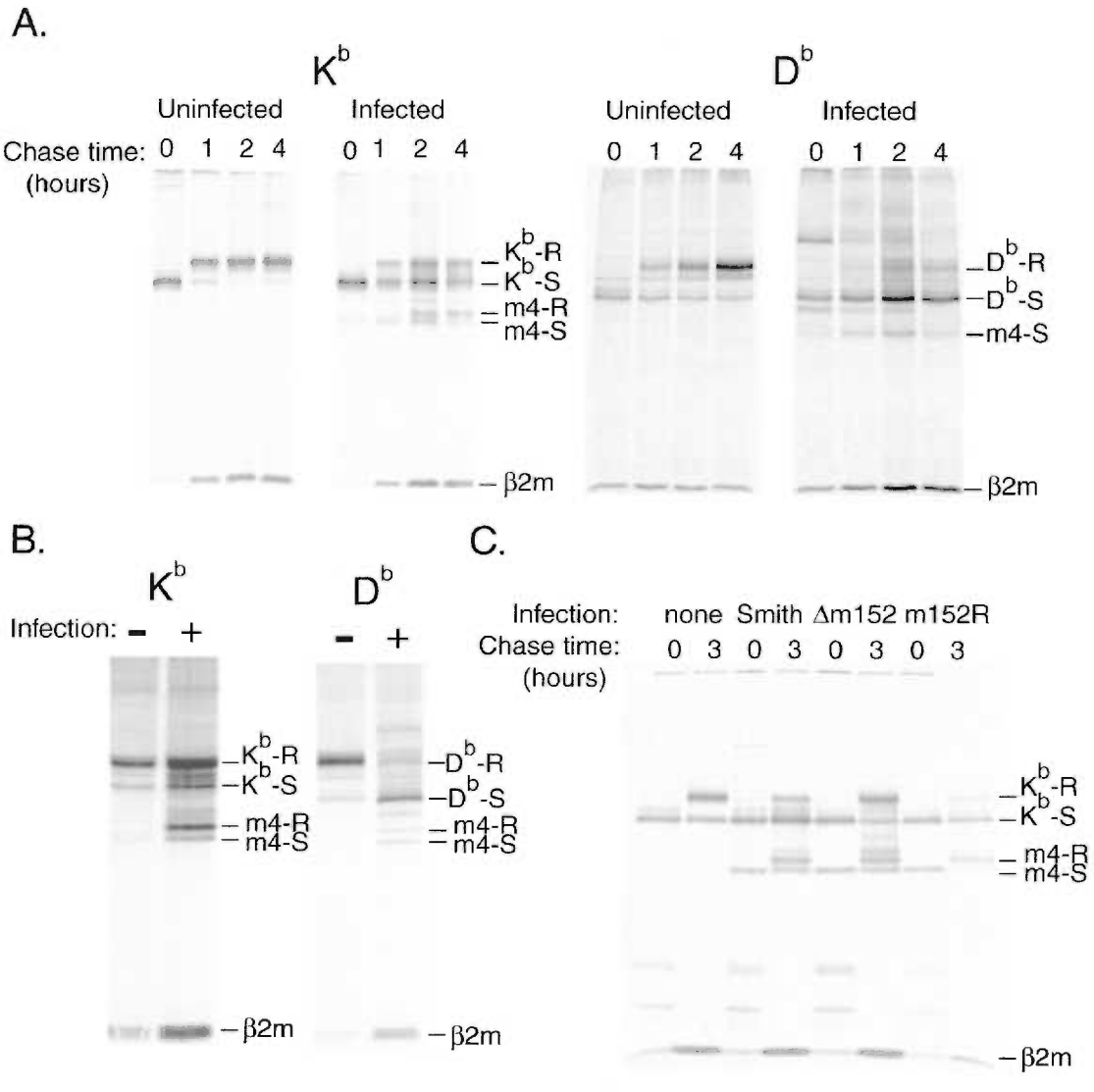

Figure 6. Differential effects of MCMV infection on maturation of different class I proteins. A. In a four-hour pulse chase, K^b partially escapes from MCMV-mediated retention, but D^b does not. B6 MEFs were infected with MCMV (Smtih) for 5 hours, pulsed with [^{35}S]methionine/ cysteine for 15 minutes, and chased for the indicated time period. K^b and D^b were sequentially immunoprecipitated from the same lysates. **B.** The differential effect is also apparent over a fourteen hour labeling period. Fibroblast lines from adult H-2^b (B6 X 129) mice were infected with MCMV(Smith). Cells were continuously labeled with [^{35}S]methionine/cysteine from 2 to 16 hours after infection. K^b and D^b were sequentially immunoprecipitated from the same lysates. **C.** Escape of K^b from the effects of *m152* is not due to competition between K^b and D^b. Fibroblasts from $D^{b-/-}$ mice were infected with the indicated virus, pulsed for 30 minutes, and lysed immediately or chased for 3 hours. The positions of bands corresponding to Endo H-resistant (R) and -sensitive (S) molecules are indicated.

parallel pattern: there was little m4/gp34 associated with D^b, and all D^b-associated m4/gp34 was Endo H-sensitive; in contrast, there was a significant amount of K^b-associated m4/gp34, which also became 50% Endo H-resistant by two hours of chase, and nearly 100% Endo H-resistant by four hours.

Because of the slower rate of maturation of D^b in uninfected cells, we wondered whether the effect of *m152* might simply be a general retardation of the maturation of both molecules with no eventual effect on the relative steady-state degree of export. To address this we labeled cells continuously from two to sixteen hours after infection, and sequentially immunoprecipitated first K^b and then D^b from the same lysates. Figure 6B shows that whereas all the D^b from infected cells remained Endo H-sensitive, the majority of K^b acquired Endo H resistance. Again, a parallel maturation pattern of class I associated m4/gp34 was observed. The results shown in Figures 6A and B are typical of a series of similar experiments in which D^b retention was always nearly complete, while K^b retention, although variable, was always less. Thus in infected fibroblasts relatively little D^b is available to reach the cell surface, but a large portion of K^b, some of which is m4/gp34-associated, eventually passes through the Golgi to the cell surface.

The observed differential effects of MCMV infection on K^b and D^b indicate that these molecules are differently affected by *m152*. Although a sustained interaction between m152/gp40 and class I has not been demonstrated, we reasoned that K^b might be able to escape retention because of competition by D^b (which is fully retained) for a limiting amount of m152/gp40. To test this possibility we determined the extent of export of K^b molecules in infected fibroblasts from mice with a targeted deletion of D^b. If competition for m152/gp40 were the cause of the differential retention of K^b and D^b, then

in the absence of D^b, K^b should be fully retained during the 3 hour chase period. However, as shown in Figure 6C, even in the absence of D^b, a significant amount of K^b escaped *m152*-mediated retention. We conclude that the difference in susceptibility to *m152* is intrinsic to the individual class I proteins and not due to intermolecular competition.

Discussion

In this paper we have provided the first evidence of a function for the MCMV gene *m4*. Previous discussion of the function of *m4* has been limited to speculation, based on its biochemical association with MHC class I molecules. We have demonstrated here that expression of *m4* by MCMV-infected target cells protects those targets from killing by some class I-restricted CTL clones. Thus *m4* joins *m152* and *m6* as the third MCMV CTL-evasion gene. Furthermore, the mechanism of action of *m4* is likely to be entirely novel, since all previously described viral CTL-evasion genes have had the effect of reducing class I surface expression— by inhibition of class I transport, removal of class I from the cell surface, or TAP blockade. In contrast, we show here that while *m4* does not inhibit K^b export from the ER, (Figure 2), it significantly inhibits killing of MCMV-infected targets by K^b-restricted clones. This inhibition is demonstrated in Figure 3, where we show that MEF targets infected with wild-type MCMV were not recognized by CTLs, while targets infected with mutant viruses lacking *m4* were recognized to a significant extent. Thus *m4* inhibits CTL recognition of infected targets even though they express significant amounts of mature K^b [Figures 2 and 6, and (Kleijnen et al., 1997)].

The mechanism by which *m4* inhibits CTL recognition is not yet known. We have found that between 50% and 70% of mature K^b synthesized over the course of MCMV infection coprecipitates with m4/gp34 in the presence of 0.5% NP40. In addition, immature K^b forms complexes with m4/gp34 which are observed in lysates made with the weaker detergent digitonin (manuscript submitted). Thus we imagine two mechanisms by which *m4* may inhibit CTL activity, either or both of which may be operative: ER-localized m4/gp34 may alter peptide-loading of K^b, and/ or surface-exposed m4/gp34 may alter class I recognition by the TCR or CD8. We are currently in the process of identifying peptide epitopes recognized by MCMV-specific CTL, which will facilitate the investigation of these possibilities.

In addition to demonstrating the immune-evasive function of *m4*, our results describe, for the first time, the functional interaction of multiple immune-evasion genes in cells infected with a herpesvirus. It has been a longstanding puzzle why CMVs should encode multiple genes (at least four in HCMV and at least three in MCMV) which all have the general effect of reducing class I-restricted antigen presentation. Multiple genes could interact in any of several ways, ranging from complete redundancy to cooperation or synergy. Many previous papers describing viral immune-evasion genes have relied on transfected cells over-expressing single viral genes, and thus can shed no light on this question; however, some possibilities have been discussed in the case of HCMV. Ahn *et al.* raised the hypothesis of synergy (Ahn et al., 1996): they observed that the HCMV gene *US3* is expressed earlier in the viral cycle than *US2* and *US11*, and thus might augment the function of the latter genes by retaining class I. Machold *et al.* proposed another reason for HCMV to encode both *US2* and *US11*, which both have the effect of

targeting class I for degradation by the proteasome: they suggested that these genes might preferentially target different class I molecules (Machold et al., 1997). Using cell lines transfected with either *US2* or *US11*, and infected with vaccinia viruses encoding various alleles of murine class I genes, they noted that *US2* degraded only a subset of the class I molecules that were degraded by *US11*. However, since no functional assays were done, and only murine class I was tested (while HCMV infects only humans), the biological relevance of the finding was unclear.

Here we have employed a biologically relevant system, using MCMV-infected primary cells to assess the effect of *m4* and *m152* on antigen presentation to MCMV-specific CTLs. The first clear conclusion from the results reported here is that the genes are not redundant: deletion of either *m152* or *m4* allows detection of infected cells by K^b-restricted CTL clones. Thus a contribution from both of these genes (and perhaps also from *m6* which was not tested here), is necessary for complete abrogation of antigen presentation in this experimental system. At present we have no data to indicate whether the effects of *m4* and *m152* are synergistic or merely additive. We also report a differential effect of the immune-evasion genes on antigen presentation by two different class I molecules, K^b and D^b. We found that while expression of both *m152* and *m4* was necessary for complete abrogation of antigen presentation to three K^b-restricted clones, expression of *m152*, but not of *m4*, was required to completely block antigen presentation to two D^b-restricted clones (Figure 5).

These observations, using a limited number of CTL clones, suggested that D^b would be more affected by *m152* than K^b. This prediction was confirmed by our biochemical analysis of class I assembly in MCMV-infected fibroblasts. Figure 6

demonstrates that the combined effects of *m152* and *m6* were insufficient to completely prevent maturation of K^b: during a 120 minute chase, approximately 50% of newly-synthesized K^b molecules became mature (i.e. were exported past the medial Golgi). In contrast, almost no D^b became Endo H-resistant over a four-hour chase. Furthermore, we note that the mature (Endo-H-resistant) K^b molecules had significant amounts of m4/gp34 associated with them, while there was relatively little m4/gp34 associated with D^b. Thus, the class I molecule which escapes from the effects of *m152* and *m6*—K^b— is preferentially targeted by *m4*. The difference in retention of K^b and D^b is even more strikingly evident over the course of a sixteen hour labeling period, as shown in Figure 6B. The CTL assays monitored antigen presentation by a small subset of total class I- that which was loaded with cognate peptides. The biochemical experiments, on the other hand, monitor the potential for antigen presentation of all the class I synthesized during infection. The almost complete retention of D^b due to *m152* contrasts with the significant export of K^b. This fully supports the prediction, based on the CTL assays, that K^b would need *m4* as a "backup" mechanism for *m152* in order to fully inhibit antigen presentation, whereas D^b may not. We conclude that *m4* complements the function of other MCMV immune-evasion genes.

These observations raise some interesting questions regarding the co-evolution of viruses and the immune system. Class Ia loci are both polygenic and highly polymorphic, and it is generally accepted that this diversity reflects evolutionary selection for the ability to present a broad array of different peptides. In addition to differences in peptide binding, however, different class Ia molecules also assemble at different intrinsic rates [Figure 6 and (Emerson et al., 1980; Hill et al., 1993; Neefjes and Ploegh, 1988)]

and with different dependence on various chaperones (Neisig et al., 1996; Peh et al., 1998; Peh et al., 2000) the evolutionary implications of these differences are less clear. We have now shown that K^b and D^b have differential susceptibility to the effects of MCMV *m152*, and that the virus requires a "backup gene" — *m4*— in order to achieve complete protection against CTL lysis *in vitro*. This raises the possibility that intrinsic differences in the assembly behavior of K^b and D^b may reflect evolutionary pressure to avoid the effects of viral genes such as *m152*. Such a tit-for-tat evolutionary model is already widely accepted in the case of NK cells, in which the "missing self" response is believed to have evolved to counteract virally-induced class I downregulation; in turn, CMVs encode genes [the signal sequence of HCMV *UL40* (Tomasec et al., 2000; Ulbrecht et al., 2000), MCMV *m144* (Farrell et al., 1997) and perhaps HCMV *UL18* (Reyburn et al., 1997)] which inhibit NK activity.

We have provided evidence suggesting that one function of the multiplicity of immune-evasion genes of MCMV is to provide more effective coverage of the diverse class I molecules present in natural outbred host populations. This does not preclude the possibility that some of the other hypothetical advantages discussed above may also be operative. It is interesting to note that the CTL-evasion genes of both MCMV and HCMV are encoded within families of related membrane glycoproteins which are not essential for virus replication *in vitro*, and which contain many genes whose functions have not yet been identified. There is much still to be learned about the ways that CMVs manipulate the cellular immune response, and the ways that the multiple genes interact to provide selective advantage for the virus.

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Chapter 3

The murine cytomegalovirus immune evasion protein m4/gp34 forms biochemically distinct complexes with class I MHC at the cell surface and in a pre-Golgi compartment.

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Abstract

We have recently demonstrated that the murine cytomegalovirus (MCMV) gene *m4* is an immune evasion gene which protects MCMV-infected targets from some virus-specific cytotoxic T lymphocyte (CTL) clones. *m4* encodes m4/gp34, a 34 kDa glycoprotein which binds to major histocompatibility complex class I in the ER and forms a detergent-stable complex which is exported to the surface of the cell. In order to investigate how m4/gp34 promotes CTL evasion, we analyzed the assembly and export of m4/gp34-K^b complexes. We found that 50-70% of K^b exported over the course of MCMV infection was m4/gp34-associated. Since these complexes are present at the cell surface, it is possible that *m4* mediates CTL evasion by interfering with contact between class I and receptors on the T cell. In addition, we found that K^b retained by the MCMV immune-evasion gene *m152* formed a novel type of complex with Endo H-sensitive m4/gp34; these complexes are distinguished from the exported complexes by being stable in 1% digitonin and unstable in 1% NP40. Since this association occurs in a pre-Golgi compartment, m4/gp34 might also interfere with antigen presentation by affecting some aspect of class I assembly, such as peptide loading, in a pre-Golgi compartment. While m4/gp34 requires β2m to bind class I, there was no significant binding of m4/gp34 to β2m in the absence of class I heavy chain, demonstrating that m4/gp34 forms NP40-stable complexes specifically with folded conformations of class I. We conclude that m4/gp34 promotes immune evasion by a novel mechanism involving altered assembly and/or T cell recognition of class I molecules.

Introduction

Cytomegaloviruses, including mouse cytomegalovirus (MCMV) and human cytomegalovirus (HCMV), are herpesviruses which cause lifelong infections, but are rarely associated with acute disease in immunocompetent hosts. As with other herpesviruses, cytolytic T cells (CTLs) play a vital role in controlling infections, and CMVs in turn encode multiple immune-evasion genes which alter the normal assembly of major histocompatibility complex (MHC) class I, and protect infected cells from CTL activity [reviewed in (Hengel et al., 1998; Kavanagh and Hill, 2001; Loenen et al., 2001; Tortorella et al., 2000)]. MCMV (Rawlinson et al., 1996) and HCMV (Bankier et al., 1991; Chee et al., 1990) have been completely sequenced, and have collinear genomes. MCMV contains 170 ORFs of predicted length greater than three hundred base pairs. MCMV ORFs are numbered sequentially from one end of the linear genome: *m1-m170*. ORFs with recognized homology to HCMV genes are labeled with a capital M.

During normal assembly, murine class I heavy chain (HC) is co-translationally translocated into the ER, where it associates first with the chaperone calnexin and binds to the light chain, β2m. The HC-β2m heterodimer associates with an assembly complex including the chaperones calreticulin, tapasin, ERp57, and the peptide transporter TAP [for recent reviews see (Cresswell et al., 1999; Solheim, 1999)]. TAP transports short peptides, generated by proteasomal degradation of cytosolic proteins, into the ER, where they are loaded onto empty class I molecules. The trimolecular complex of HC, β2m, and peptide dissociates from the assembly complex, leaves the ER and travels through the Golgi and out to the cell surface.

Three MCMV genes have been shown to alter this process in infected cells. *m152* encodes a glycoprotein (m152/gp40) which, by an unknown mechanism, prevents normal export of class I and causes immature class I molecules to accumulate in the ER-Golgi

intermediate compartment (ERGIC) (del Val et al., 1992; Ziegler et al., 1997). Because normal amounts of antigenic peptide can be extracted from MCMV-infected cells that are not recognized by cognate CTL, it is believed that these retained molecules are loaded with peptide (del Val et al., 1992; Ziegler et al., 1997). It has not been possible to detect a direct interaction between m152/gp40 and class I, and the lumenal domain of m152/gp40 is sufficient to cause retention of MHC class I (Ziegler et al., 2000). The immune evasion gene *m6* encodes a glycoprotein (m6/gp48) which binds to class I in the ER and redirects it to the lysosome for degradation (Reusch et al., 1999). Expression of either *m152* or *m6* via recombinant vaccinia is sufficient to protect targets from CTL lysis *in vitro* (Reusch et al., 1999; Ziegler et al., 1997). The third CTL-evasion gene is *m4*.

m4 encodes a 34 kDa type 1 glycoprotein (m4/gp34) which is abundantly expressed in the ER. m4/gp34 remains ER-resident until it binds to class I, forming tight complexes which are readily observed by immunoprecipitation of class I from NP40 lysates of infected cells. These complexes exit the ER and are expressed at the cell surface where they can be labeled by surface iodination (Kleijnen et al., 1997). Based on our initial observations, we hypothesized that these complexes might serve as decoy signals to prevent NK activation due to loss of class I expression in infected cells. To date, however, there is no functional evidence for an effect of *m4* on NK activity. A recent report (Holtappels et al., 2000) demonstrates that peptides derived from m4/gp34 are recognized by some CTLs in the context of the class I molecule D^d. This antigenicity has no obvious connection to the function *m4* and is probably a consequence of the abundance of m4/gp34 in the ER of infected cells.

We have recently demonstrated that *m4* does inhibit CTL activity: mutant MCMV strains lacking *m4* are recognized by some MCMV-specific CD8+ CTLs, while wildtype MCMV is not. In addition, the functions of *m4* and *m152* are complementary with respect to different class I molecules: in H-2^b fibroblasts, *m152* retards the export of D^b more than K^b. On the other hand we observed that *m4* was required for maximal immune

evasion from three K^b- but not two D^b-restricted CTL clones. Since the mechanism by which *m4* inhibits CTLs is unknown, we undertook the biochemical analysis described here to gain a clearer understanding of how and when m4/gp34 affects the MHC class I antigen presentation pathway. In this paper we demonstrate that at least 50% of mature (Endo H-resistant) K^b in infected fibroblasts was found in NP40-stable complexes with m4/gp34. This is consistent with a mechanism for *m4* inhibiting CTLs by direct interference with contact between class I and the TCR or CD8. However, we could not demonstrate m4/gp34 association for a significant minority of these mature K^b molecules, which, along with the fact that the vast majority of m4/gp34 is resident in a pre-Golgi compartment, prompted us to look for other effects of *m4* on K^b. We found that K^b accumulated, in an *m152*-dependent manner, with m4/gp34 in a novel type of complex which is unstable in NP40 lysate but stable in 1% digitonin. These complexes are largely confined to a pre-Golgi compartment. This suggests a second possible mechanism by which *m4* may interfere with antigen presentation: by altering aspects of class I assembly such as peptide loading. In addition, we investigated factors required for NP40-stable binding of class I by m4/gp34. Kleijnen *et al.* showed that m4/gp34 and class I do not form complexes in the absence of β2m (Kleijnen et al., 1997); here we show that m4/gp34 did not bind significantly to β2m in the absence of classical class I molecules, demonstrating that the requirement for β2m is due to a specific association of m4/gp34 with a folded conformation of class I.

Materials and Methods

MCMV strains. The recombinant Δm4-MC95.33, with an insertion of the *lacZ* gene in place of the *m4* ORF, was generated using the plasmid construct pm4 and performing insertional mutagenesis in eukaryotic cells as described previously (Krmpotic et al., 1999). pm4 was constructed as follows. The homologous recombining region was produced by flanking the *lacZ* gene with MCMV genomic sequences adjacent to the 5'

(nt 2739 to 3250 left flank) and 3' (nt 4041 to 4737 right flank) ends of the *m4* ORF. Plasmid DNA (pHindIIIA) (Ebeling et al., 1983) serving as MCMV genomic template and primer pairs for the left flanking sequence [sense (5´-AA**CTCGAG**CATCACGGTGAACGATACCA-3`), anti sense (5`-TT**GGATCC**TGGAACAACGAATGAGACAGA-3`)] and right flanking sequence [sense (5-`AT**GCGGCCG**CTCGAACTTCAAACCGCTTAAGAG), anti sense (5`-AA**CCGCGG**ACTTATCGACGTACAATCCTGT)] were used in separate PCR reactions to produce fragments with convenient restriction sites to ligate to the *lacZ* gene (XhoI, BamHI and NotI and SacII, respectively—restriction sites denoted in bold). These fragments were inserted into corresponding sites within the plasmid pIC4, which contains the *lacZ* gene under control of the Rous sarcoma virus (RSV) promoter, SV40 poly(A) and flanking loxP sites (Crnkovic-Mertens et al., 1998). Thirty fmol of linearized pm4 plasmid DNA was cotransfected with wt MCMV DNA (1.5 μg) into NIH3T3 fibroblasts by calcium phosphate precipitation to generated the recombinant virus Δm4-MC95.33. Recombinant virus was isolated and plaque-purified as described previously (Crnkovic-Mertens et al., 1998). Correct recombinatorial mutagenesis within the genome of Δm4-MC95.33 was confirmed by restriction enzyme analysis (data not shown).

All other mutant MCMVs used in this report have been previously described. ΔMC96.24 (*m152* knockout) and ΔMC96.27 (revertant) are described in (Krmpotic et al., 1999) and ΔMS94.5 (lacking ORFs *m151-165*) in (Thale et al., 1995).

Experimental Animals. C57BL/6 (B6) mice were purchased from Simonsen. $D^{b\,-/-}$ and $K^{b\,-/-}$/ $D^{b\,-/-}$ mice (Hoglund et al., 1998) were a gift from Francois Lemmonnier. C57BL/6 $\beta2m^{-/-}$ mice were purchased from Jackson Labs.

Virus Stocks and Cell Culture. Mouse embryo fibroblasts (MEFs) from B6 and $\beta2m^{-/-}$ mice were grown from trypsin-digested day 12-14 mouse embryos, and used between passage 3 and 6. MEFs from $D^{b-/-}$ and ($K^{b\,-/-}$, $D^{b\,-/-}$) mice were the gift of David Raulet.

NIH 3T3s (CRL-1658) and Balb3T3s (CCL-163) were obtained from ATCC. MEFs and 3T3s were maintained in DMEM supplemented with 10% fetal (for MEFs, adult fibroblast lines and NIH3T3s) or newborn (for Balb 3T3s) calf serum. Virus stocks were generated by infecting subconfluent MEFs with low-passage seed stock at an MOI of 0.001. Cells were then switched to DMEM + 10% normal calf serum until the monolayer became 100% infected. Stocks were harvested by scraping and sonication of cells. Titer of plaque forming units (PFU) was determined by serial dilution and agarose overlay on Balb-3T3s.

Antibodies. Serum 8010 (anti-p8) was generated by immunizing rabbits with synthetic peptide corresponding to exon 8 of K^b. Sera 8142 and 8139 (anti-m4/gp34) were both generated as follows. Serum R123 against the cytoplasmic tail of m4/gp34 (Kleijnen et al., 1997) was used to precipitate m4/gp34 from MCMV (Smith)-infected MEFs. After washing, the immune complex was suspended in complete Freund's adjuvant (Sigma) and used to immunize rabbits *s.c.* Rabbits were boosted first with immune complex suspended in incomplete Freund's adjuvant (IFA; Sigma), and then by infection with recombinant vaccinia virus expressing m4/gp34, and finally with recombinant soluble m4/gp34 protein purified from baculovirus (the kind gift of Pamela Bjorkman) in IFA. Anti-D^b monoclonal antibody 28.14.8S (ATCC HB-27) and anti-β2m monoclonal antibody Lym11 (a gift from A. Simmons) were purified from hybridoma supernatants. Anti-transferrin receptor/CD71 antibody (rat IgG2a) was purchased from Leinco Technologies Inc, Ballwin, MO.

Antibody-binding reagents. Rabbit antibodies were precipitated using either fixed *Staphylococcus aureus* or 5% w/v protein A-agarose (Sigma). Rat IgG2a antibody was precipitated using 5% w/v protein G agarose (Sigma). Just before use antibody-binding reagents were washed three times in the appropriate lysis buffer.

Metabolic labeling and immunoprecipitations. All immunoprecipitations used adherent MEFs which were pretreated with recombinant mouse IFN-γ at 50 U/ ml for 48

hours before metabolic labeling. Unless otherwise indicated, virus infections used an MOI of 10 and infected cells were continuously grown in the presence of 0.3 mg/ml PAA (phosphonoacetic acid, Sigma). 1 hour before the addition of metabolic label, cells were washed in PBS and placed in labeling medium [cysteine/ methionine-free DMEM (Gibco) supplemented with antibiotics, and 5% FCS]; at the end of 1 hour cells were labeled with ^{35}S-cysteine/methionine (NEN) for the time periods indicated in the figures. For pulse-chase experiments, cells were washed with chase medium [DMEM supplemented with antibiotics, glutamate, 10% FCS, and saturating concentrations of TC-grade cysteine and methionine (Sigma)] at the end of the labeling period, after which they were cultured in chase medium for the chase period indicated in the figures. All lysis and precipitation procedures were carried out at 4°. At the time of lysis, TC plates were placed on ice, washed in cold PBS, lysed in the plate with lysis buffer, scraped, and transferred to tubes. Lysis buffer was either NP-40 lysis buffer (0.5% NP-40, 50 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$) or digitonin lysis buffer [1% high purity digitonin (Calbiochem) in PBS]. Just before use, lysis buffer was supplemented with protease inhibitor-- either 1 mM PMSF (Sigma) or 1X inhibitor cocktail [Complete EDTA-free Protease-Inhibitor Cocktail (Boehringer Mannheim)]. To remove non-specific antibody-binding proteins, lysates were precleared with normal rabbit serum (NRS): each ml of lysate received at least 20 µl of NRS and 500 µl of staph A, after which lysates were mixed by slow rotation for 2 hours, and centrifuged for 5 min at 15,000 g to remove non-specific proteins and cellular membranes. Precleared lysates were then subjected to specific immunoprecipitation as indicated in the figures. Unless otherwise indicated, each aliquot of lysate received 10 µl of antibody plus 150 µl of protein A or G suspension. Lysates were mixed by slow rotation for 2 hours and then centrifuged for 1 min at 12,000 g. Lysate supernatant was removed and stored at -80° for further analysis. Immune complex pellets were washed four times in 1 X NET buffer (150mM NaCl, 50mM Tris pH 7.5, 5mM EDTA and 0.05% NP40) +0.1% SDS. Samples were digested

with Endo H_f (NEB) according to manufacturer's protocol and separated by SDS-PAGE on a 12.5% gel. Quantitation of labeled protein was performed using a Molecular Dynamics phosphorimager.

Results

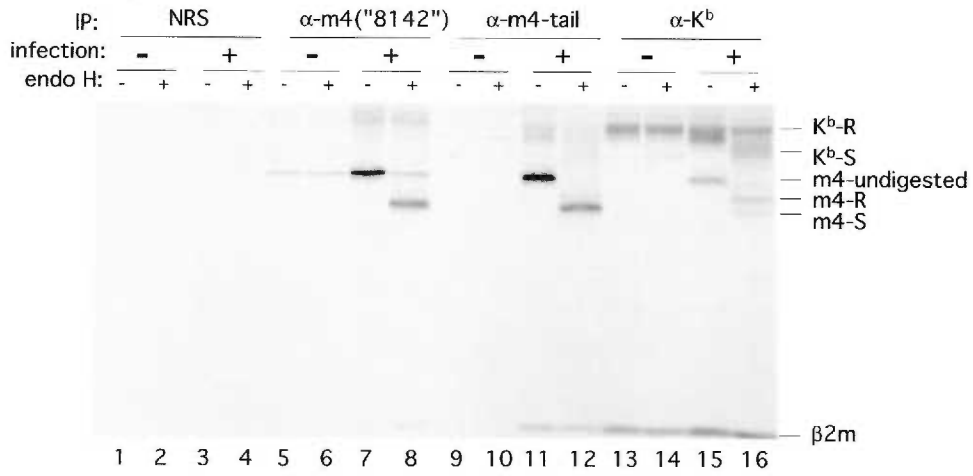
Most of the K^b reaching the surface of infected MEFs is associated with m4/gp34 in NP40-stable complexes.

m4 is required to protect MCMV-infected B6 mouse embryo fibroblasts (MEFs) from lysis by K^b-restricted T cell clones, which demonstrates that *m4* expression interferes with antigen presentation by K^b. It is possible that this interference occurs at the level of TCR-K^b contact, since, unlike any other immune-evasion protein, m4/gp34 forms complexes with class I which are expressed on the surface of the cell (Kleijnen et al., 1997). However, it was previously not known what percentage of K^b reaching the cell surface of MCMV-infected MEFs was m4/gp34-associated. We were unable to estimate this quantity because we did not precipitate significant amounts of K^b with our anti-m4/gp34 serum R123, generated against a peptide in the cytoplasmic tail (Kleijnen et al., 1997); we did not know if this failure was due to a steric blockage of the R123 epitope or to a stoichiometric excess of free m4/gp34 over m4/gp34-class I complexes. Therefore we generated two new sera, 8139 and 8142, by immunizing rabbits against the entire m4/gp34 protein (see materials and methods). As shown below, we have defined conditions under which it is possible to co-precipitate some class I using any of our antibodies to m4/gp34. We observed the CTL-evasion function of *m4* using MCMV-infected MEFs which were pretreated with interferon-γ to increase class I expression, and treated with the viral DNA synthesis inhibitor phosphonoacetic acid (PAA) to limit viral cytopathic effect. We therefore performed all of our biochemical analyses using the same cell type and infection conditions.

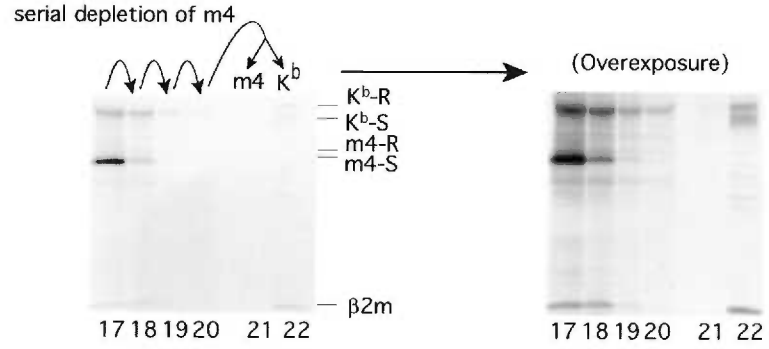
Figure 1. Over 50% of Endo H-resistant K^b is m4/gp34-associated in NP40-stable complexes. B6 MEFs were infected with MCMV (Smith) or mock-infected, labeled 16 hours with ^{35}S-methionine, and lysed in NP40 lysis buffer. **A.** Primary immunoprecipitation using 10μl each of normal rabbit serum (NRS, lanes 1-4), anti-m4/gp34 serum 8142 (lanes 5-8), anti-m4/gp34 cytoplasmic tail serum R123 (lanes 9-12), or anti-K^b (lanes 13-16). Samples were divided in half and digested with Endo H or mock digested. **B.** Supernatant from the precipitation shown in 5A, lanes 7-8, was subjected to serial depletion of m4/gp34 protein. For each of lanes 17-20, 40 μl of serum 8142 was used to precipitate m4/gp34, and the supernatant from that precipitation was subjected to the next round of immunoprecipitation with another 40 μl of 8142. Finally, the supernatant from the immunoprecipitation shown in lane 20 was cleared of residual antibodies with staph A, divided in half, and subjected to immunoprecipitation with anti-m4/gp34 serum R123 (lane 21), or anti-K^b (lane 22). All samples were treated with Endo H before analysis by SDS-PAGE. The immunoprecipitation shown in lane 22 represents an equal amount of the original lysate as that shown in 1A, lane 16. Overexposure of the gel in 1B demonstrates that there is no K^b-associated m4/gp34 detectable in lane 22. Bands of a similar weight to K^b in lanes in lanes 19 and 20 probably represent an unidentified/ nonspecific protein, which sometimes co-precipitates with anti-m4/gp34 sera. **C.** Cells were infected and labeled as above, and subjected to immunoprecipitation with NRS (lanes 1 and 8), anti-m4/gp34 (serum 8139, lanes 2 and 9), or anti-K^b (lanes 3 and 10). The lysates from which m4/gp34 had been precipitated were then completely depleted of m4/gp34 by four rounds of immunoprecipitation. A parallel "mock" depletion was performed with NRS. After depletion, these lysates were then subjected to immunoprecipitation with NRS or anti-K^b. Lanes 5 and 12 show the amount of K^b remaining after mock depletion with NRS; lanes 7 and 14 show the amount remaining after specific depletion of m4/gp34 with serum 8139. Each lane of Fig 1C shows proteins precipitated from an equal volume of the original lysate. All samples

were treated with Endo H. Phosphorimage data from this gel are shown in Table 1. Relevant bands are labeled -R and -S to designate Endo H-resistant and -sensitive proteins, respectively.

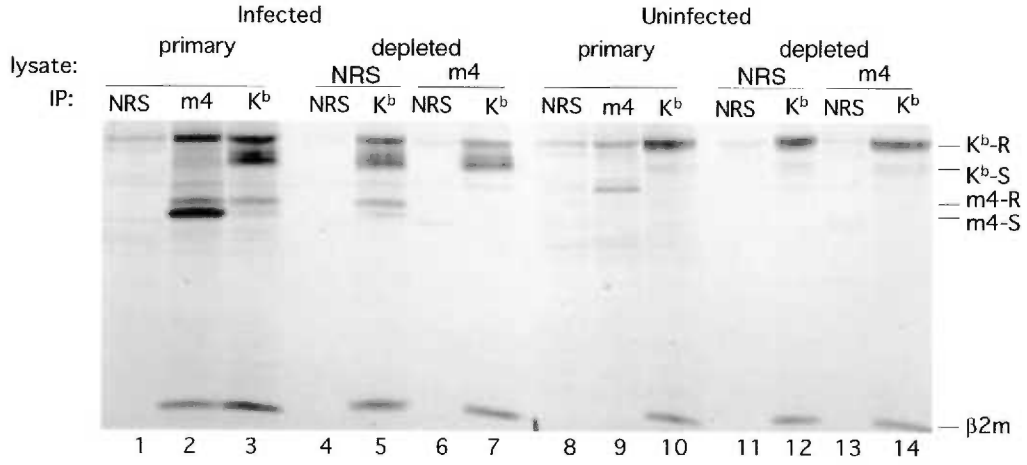
A.


IP:
NRS
α-m4("8142")
α-m4-tail
α-Kb
infection:
endo H:
Kb-R
Kb-S
m4-undigested
m4-R
m4-S
β2m
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

B.


serial depletion of m4
m4 Kb
(Overexposure)
Kb-R
Kb-S
m4-R
m4-S
β2m
17 18 19 20 21 22
17 18 19 20 21 22

C.


Infected
Uninfected
lysate:
primary
depleted
NRS
m4
IP:
NRS m4 Kb NRS Kb NRS Kb NRS m4 Kb NRS Kb NRS Kb
Kb-R
Kb-S
m4-R
m4-S
β2m
1 2 3 4 5 6 7 8 9 10 11 12 13 14

		Amount of K^b (arbitrary units)		
Lysate	Endo H sensitivity of K^b	Initial IP	After mock depletion (% remaining)	After anti-m4/gp3 depletion (% remaining)
Infected	resistant	140	93 (67%)	42 (30%)
	sensitive	296	209 (71%)	174 (59%)
Uninfected	resistant	133	115(86%)	141 (106%)
	sensitive	n/a	-	-

Table 1. Over half of the exported K^b, but less than half of the retained K^b, is in NP40-stable complexes with m4/gp34 in infected cells. The amount of radio-labeled K^b in Figure 1C was determined by phosphorimager analysis; measurements are expressed in arbitrary volume units. We determined the initial amount of exported (Endo H-resistant) and retained (Endo H-sensitive) K^b for infected (Fig 1C, lane 3), and uninfected (Fig 1C, lane 10) cells; uninfected cells did not have a measurable amount of retained K^b. Lysates were subjected to serial depletion as explained in the caption to Figure 1C. The amount of K^b remaining after mock (Fig 1C, lanes 5 and 12) or anti-m4/gp34 (Fig 1C, lanes 7 and 14) depletion is shown. For each depletion, we calculated the percent of K^b remaining as (depleted IP / initial IP). Thus 70% of exported K^b was removed from infected lysates by anti-m4/gp34 serum, indicating that 70% of these molecules were m4/gp34 associated. The fact that a mock preclear with normal serum yields between 14% and 33% depletion of K^b indicates the degree of error in the system.

In order to estimate what percentage of K^b synthesized during the course of MCMV infection is exported in complexes with m4/gp34, we undertook the following experiments, shown in Figure 1. NP40 lysates of metabolically labeled, MCMV-infected cells were subjected to serial depletion with anti-m4/gp34 serum to remove all of the m4/gp34 protein from the lysates; we then compared the amount of K^b present before or after total m4/gp34 depletion. This comparison allowed us to estimate the minimal percentage of total labeled K^b which was in NP40-stable complexes with m4/gp34. Because the only class I molecules on infected MEFs which are likely to be relevant to the anti-viral CTL response are those synthesized after viral infection, we chose to begin the metabolic labeling after infection of MEFs with MCMV.

Figure 1A shows a typical initial immunoprecipitation of m4/gp34-K^b complexes from lysates of infected cells; as a control, we performed parallel immunoprecipitations from lysates of uninfected cells. B6 MEFs were infected with MCMV and metabolically labeled for sixteen hours, lysed in 0.5% NP40 buffer, and subjected to immunoprecipitation as shown. As previously demonstrated (Kleijnen et al., 1997), m4/gp34 co-precipitates with K^b (lanes 15-16). This co-precipitated m4/gp34 runs in two positions after Endo H digestion: a lower band (m4-S), representing ER-resident m4/gp34, has lost all three N-linked glycans; an upper band (m4-R), representing m4/gp34 which has traversed the medial Golgi, has retained one of its three glycans. We have previously demonstrated (Kleijnen et al., 1997) that m4/gp34 is an unusual protein in that only one of its three N-linked glycans becomes Endo H-resistant as it passes through the Golgi; therefore Endo H-resistant m4/gp34 (lane 16) is of a lower molecular weight than undigested m4/gp34 (lane 15). Immunoprecipitation with either of two anti-m4/gp34 sera yields an Endo H-sensitive m4/gp34 band (lanes 7-8 and 11-12). At this exposure, there is no Endo H-resistant m4/gp34 visible. However, both

immunoprecipitates do contain faint bands that co-migrate with class I HC and β2m and that are absent in precipitates from uninfected cells (lanes 5-6 and 9-10). In this experiment, co-precipitation of class I was much more apparent with serum R123 (lanes 11 and 12) than 8142 (lanes 7-8). The amount of co-precipitating class I seen with different sera is somewhat variable between experiments, due largely to the vast stoichiometric excess of m4/gp34 over class I in these lysates (see below). However, these complexes can be shown to contain K^b by re-precipitation with specific antiserum (data not shown). Thus these sera (R123 and 8142) can initially precipitate some small number of class I-m4/gp34 complexes.

In order to remove all available m4/gp34 from the lysates, we took the supernatant lysate from the immunoprecipitation shown in Figure 1A, lanes 7-8, and subjected it to serial depletion with 40 µl aliquots of serum 8142. Figure 1B shows that 2 rounds of serial depletion (lanes 17-18), using a total of 80 µl of antibody, removed all remaining m4/gp34 from the lysate, since there is no further m4/gp34 band visible in lane 19, even on overexposure of the gel. The immunoprecipitates shown in lanes 17-18 also contain bands of molecular weights corresponding to K^b, β2m, and Endo H-resistant m4/gp34. Furthermore, when K^b was immunoprecipitated from the fully depleted supernatant, we found no more m4/gp34 associated with this remaining pool of K^b (lane 22), even after extensive overexposure of the gel, as shown. Thus serial immunoprecipitation with serum 8142 was able to remove all K^b-associated and -unassociated m4/gp34 from the lysate. We conclude that our previous failure to co-precipitate class I with m4/gp34 was largely due to a stoichiometric excess of free m4/gp34 over the K^b-m4/gp34 complexes in NP40 lysates. It is also possible that prolonged exposure to anti-m4/gp34 antibodies has the effect of dissociating m4/gp34-class I complexes; for this reason we kept the total time of incubation of lysates with antibodies to a minimum for both experiments shown in Figure 1. Based on a comparison of the amount of K^b precipitated from the original lysate (Figure 1A, lane

16), with the amount precipitated from the same volume of lysate after total depletion of m4/gp34 (Figure 1B, lane 22), we concluded that a substantial proportion of the total labeled K^b in the lysate was m4/gp34-associated.

In order to estimate this percentage more accurately, we repeated the procedure using additional quantitative controls (Figure 1C). In this case, NP40 lysates from infected and uninfected cells were totally depleted of m4/gp34 using serum 8139, or mock depleted with normal rabbit serum (NRS). The amount of labeled K^b precipitated before and after depletion was determined by phosphorimager analysis. These quantities are shown in Table 1. Mock depletion with NRS serves as a control for non-specific loss of class I due to serial immunoprecipitations. The fact that no class I was lost from the lysates of uninfected cells depleted with serum 8139 shows that this serum is not cross-reactive with class I. After total depletion of m4/gp34, 33% of the original Endo H-resistant K^b remained in the lysate. There is of course a large error inherent in sequential immunoprecipitations, the range of which is indicated by the fact that 0% of class I was removed by depletion of m4/gp34 from lysates of uninfected cells, while between 14% and 33% of class I was nonspecifically depleted by NRS from lysates of infected cells. Taking this into account, we conclude that between 50% and 70% of Endo H-resistant, metabolically labeled K^b was associated with m4/gp34. We note that the depletion of Endo H-resistant K^b is much greater than that of Endo H-sensitive K^b. This is consistent with our previous observation that NP40-stable complexes of K^b with m4/gp34 are exported to the cell surface.

It is possible that, of the remaining mature K^b not co-precipitated with m4/gp34, some may have been m4/gp34-associated at the time of lysis but disassociated during the immunoprecipitation procedure. If this were the case, up to 100% of the surface-exposed labeled K^b could be m4/gp34 associated. However, the present data suggest that at least 30%, and up to 50% of K^b which matures over the course of MCMV infection may be m4/gp34-unassociated. Given this possibility, we wanted to look for other potential

mechanisms for *m4*-mediated immune evasion besides direct interference with contact between class I and the receptors on the T cell.

K^b molecules whose export is blocked by MCMV infection are found in NP40-unstable complexes with m4/gp34

Comparing the transport of different MCMV immune-evasion proteins provides some interesting contrasts. While *m152* causes class I to be retained in the ERGIC, m152/gp40 itself is rapidly exported from the ER to the lysosome for degradation. m6/gp48 is also targeted to the lysosome, where it is destroyed, along with any associated class I proteins. Although *m4* was originally discovered because m4/gp34 forms complexes with class I found on the cell surface, the vast majority of m4/gp34 in infected cells remains in a pre-Golgi compartment (see Figure 1). This suggested that m4/gp34 might have a separate function in a pre-Golgi compartment, and we therefore undertook a closer examination of m4/gp34 in these compartments.

MCMV infection has a mixed effect on maturation of K^b. Some molecules are retained in a pre-Golgi compartment by the effects of *m152*, while others are exported to the cell surface. As demonstrated above, the majority of K^b on the cell surface is m4/gp34-associated, but we also wanted to know more about the disposition of K^b which is retained in the ERGIC. We therefore used a more sensitive assay for protein-protein interactions: lysis of infected cells in the weak detergent digitonin and identification of co-precipitating bands in immunoprecipitations. We chose digitonin because weak interactions such as those between class I, TAP, and tapasin are preserved in 1% digitonin but disrupted by NP40. This led to a discovery (Figure 2): m4/gp34 and K^b were found in a novel type of complex which, unlike the complexes described above and previously (Kleijnen et al., 1997), were unstable in 1 % NP40 lysis buffer, but stable in 1% digitonin lysis buffer. Furthermore, these NP40-unstable complexes were almost

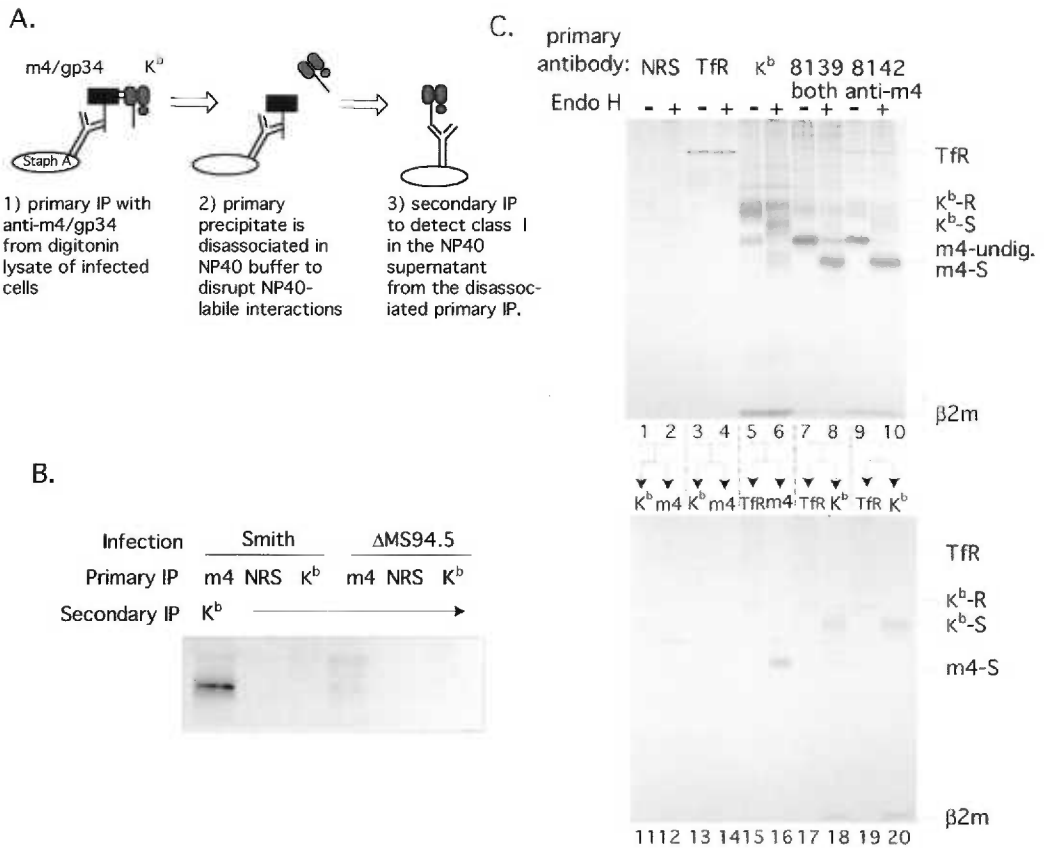


Figure 2. Specific association of Endo H-sensitive m4/gp34 and Endo H-sensitive K^b in NP40-unstable complexes. **A**. Method for distinguishing NP40-stable and -unstable complexes. Metabolically labeled cells are lysed in 1% digitonin in PBS, and the primary immunoprecipitation is performed in this buffer. The primary complex is then disrupted in 1% NP40 buffer and subjected to secondary IP as shown. **B**. K^b is found in NP40-unstable complexes with m4/gp34 in cells infected with MCMV. B6 MEFs were infected with wildtype (Smith) MCMV or mutant ΔMS94.5 (lacking ORFs 151-165). MEFs were infected for five hours, metabolically labeled for four hours, and lysed in 1% digitonin. Lysates were then subjected to immunoprecipitation as shown for part A, and digested with Endo H. **C**. NP40-unstable complexes represent a specific interaction between m4/gp34 and K^b. MEFs were infected with MCMV and labeled as above. Primary lysates were subjected to immunoprecipitation with NRS (lanes 1-2), mAb against mouse transferrin receptor (lanes 3-4), anti-K^b (lanes 5-6), anti-m4/gp34 8142 (lanes 7-8) or 8139 (lanes 9-10). Each lane represents precipitation with roughly 10 μg of antibody. Precipitates were resuspended in NP40 lysis buffer to disrupt NP40-unstable complexes, after which they were re-centrifuged to precipitate the NP40-stable complexes shown in lanes 1-10. Supernatant remaining from these IPs was divided in half and subjected to sequential precipitation with two different antibodies as shown (lanes 11-20). All samples in B and C were digested with Endo H.

entirely composed of Endo H-sensitive proteins, as opposed to the previously described NP40-stable complexes, which were mostly Endo H resistant.

Figure 2A is a schematic representation of the method used to distinguish NP40-stable and -unstable complexes. Infected cells are metabolically labeled and lysed in 1% digitonin buffer, and lysates are subjected to immunoprecipitation with the primary serum (e.g. anti-m4/gp34). Precipitated complexes are then resuspended in 1% NP40 buffer so that any NP40-unstable associations will be disrupted. Precipitated complexes are re-centrifuged. NP40-stable complexes remain with the pellet, while proteins from NP40-unstable complexes are now found in the supernatant, from which they can be specifically re-immunoprecipitated.

The results shown in Figure 2B demonstrate that K^b and m4/gp34 are found together in NP40-unstable complexes. Cells were metabolically labeled starting five hours post infection with MCMV, and four hours later were lysed in 1% digitonin buffer. K^b from NP40-unstable complexes was isolated as described above. The results show that if the primary antiserum is anti-K^b, no K^b is recovered from the NP40 supernatant. This is because the antibodies still bind strongly to K^b in this buffer, and all of K^b remains in the primary pellet. Likewise, NRS does not precipitate any K^b in NP40-unstable complexes. If anti-m4/gp34 serum is used for the primary precipitation, however, then K^b is found in the NP40 supernatant, indicating that some m4/gp34 and K^b are in NP40-unstable complexes. In cells infected with wildtype MCMV, this K^b is almost entirely Endo H-sensitive, indicating that these complexes reside in a pre-Golgi compartment. In cells infected with MCMV ΔMS94.5, a mutant lacking ORFs 151-165 (Thale et al., 1995), the overall amount of K^b in these complexes is greatly reduced, and of the amount remaining a greater percentage is Endo H-resistant. This suggests a role for some gene from the deleted region in retaining and stabilizing NP40-unstable complexes. This observation is pursued in Figure 3, below.

We wanted to confirm that these NP40-unstable complexes represented a specific interaction between K^b and m4/gp34, and were not merely a side effect of the abundance of m4/gp34 in these lysates. We therefore compared the protein content of NP40-stable and unstable complexes with K^b, m4/gp34, and the irrelevant control protein transferrin receptor (TfR-- Figure 2C). Primary IPs from digitonin lysate of wildtype MCMV-infected cells were performed using the following sera or antibodies: NRS, anti-TfR, anti-K^b, or either of two anti-m4/gp34 sera (8139 and 8142). These pellets were dissociated, and the resulting NP40 supernatants subjected to secondary IP as described above. NP40-stable pellets are shown in lanes 1-10; IPs from NP40 supernatants are shown in lanes 11-20. As seen previously in Figure 1, NP40-stable pellets from IP with anti-K^b serum contain m4/gp34 (lanes 5-6). In addition, the NP40 supernatant from this IP also contains m4/gp34 (lane 16), but not TfR (lane 15). Thus the NP40-unstable interaction between K^b and m4/gp34 is specific to these two proteins and does not include an irrelevant protein. The reciprocal precipitations with anti-m4/gp34 sera for the primary IP (lanes 7-10) also demonstrate a specific interaction with K^b (lanes 18 and 20) but not TfR (lanes 17 and 19). Primary IP with NRS (lanes 1-2) or anti-TfR (lanes 3-4) yields no specific protein in the NP40 supernatant (lanes 11-14), confirming the specificity of these interactions.

We are confident that NP40-stable and -unstable complexes are distinct entities because they reside in different cellular compartments. All of the m4/gp34 and K^b from NP40-stable complexes eventually becomes Endo H-resistant (lane 6 and Figure 3, below), while all of the m4/gp34 (lane 16) and K^b (lanes 18 and 20) from NP40-unstable complexes remains Endo H-sensitive, and is thus confined to some pre-Golgi compartment.

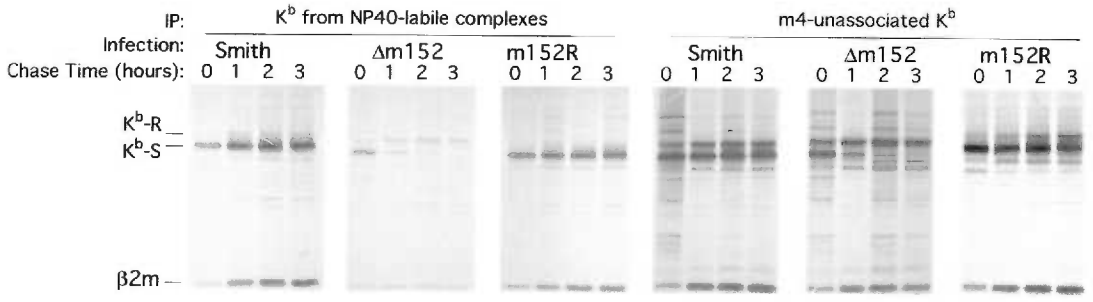


Figure 3. Pulse-chase analysis shows that K^b accumulates in NP40-unstable complexes with m4/gp34 in an *m152*-dependent manner. MEFs were infected as above, metabolically labeled for one hour, and chased with excess cysteine/methionine for the indicated time period. K^b from NP40-unstable complexes with m4/gp34 was isolated as for Figure 2 above. As a control to demonstrate the efficacy of the chase protocol, the total remaining (m4/gp34-unassociated) K^b was isolated from the remaining supernatants. All samples were digested with Endo H.

Cooperative effects of *m152* and *m4* on retained, immature class I.

Given the discovery of m4/gp34 and K^b in novel NP40-unstable complexes, we wanted to know to what degree the specific association of m4/gp34 with Endo H-sensitive class I in these complexes was an independent function of *m4*, or a cooperative function of *m4* with other MCMV genes. Figure 2B demonstrates a decrease of these complexes in the absence of ORFs *m151-165*, and this led us to suspect that *m152* might play a role. Figure 3 shows a pulse-chase analysis of K^b from NP40-unstable complexes with m4/gp34, isolated from cells infected with wildtype, Δm152, or revertant (m152R) MCMV. Five hours after infection, B6 MEFs were pulsed with ^{35}S-methionine/ cysteine for one hour, and chased with medium containing excess unlabeled methionine/ cysteine for one, two, or three hours. K^b from NP-40 unstable complexes with m4/gp34 was isolated as described in Figure 2, and K^b which was not associated with m4/gp34 was subsequently isolated from the supernatant that remained after depletion of m4/gp34. In cells infected with wildtype or revertant MCMV, K^b accumulated in NP40-unstable complexes with m4/gp34 over the course of the chase period. By contrast, in cells infected with Δm152, K^b initially entered into NP40-unstable complexes with m4/gp34 (chase time 0) and then rapidly left the complexes, presumably to be exported to the cell surface.

Class I retained by the effects of *m152* is thought to accumulate in the ER-Golgi intermediate compartment (ERGIC), forming a distinct population from newly-synthesized, ER-localized class I molecules (Ziegler et al., 2000). The most striking result from Figure 3 is that in wildtype MCMV-infected cells, nascent K^b is gradually recruited into NP40-unstable complexes with m4/gp34 over a period of hours. This accumulation is dependent on the expression of *m152*, although the initial formation of the complexes is not. This implies that m4/gp34 molecules are co-localized and

specifically interacting with some portion of the *m152*-retained K^b. Whether this interaction represents a significant function of *m4* (for example, related to peptide loading), or an adventitious interaction with class I already on a dead-end pathway, remains to be determined.

m4/gp34 does not associate significantly with β2m in the absence of class I heavy chain

It is known that m4/gp34 specifically associates with class I HC which is bound to β2m rather than with free HC; this is demonstrated by the observation that NP40-stable m4/gp34-HC complexes were absent in fibroblasts lacking β2m [(Kleijnen et al., 1997) and this report, Figure 4 (lanes 13-16)], and reduced in fibroblasts lacking TAP (Kleijnen et al., 1997). This led to the tentative conclusion that m4/gp34 associates specifically with peptide-loaded, fully conformed MHC class I (14). However, the data were also compatible with the possibility that m4/gp34 might bind directly to β2m and that β2m might be the primary bridge between m4/gp34 and HC. The availability of primary fibroblasts from mice lacking both K^b and D^b made it possible for us to test whether m4/gp34 could associate directly with β2m in the absence of classical class I HC (Figure 4). Immunoprecipitation of β2m from NP40 lysates of wildtype or $D^{b-/-}$ cells co-precipitated m4/gp34 (lanes 4 and 8). However, very little or no m4/gp34 co-precipitated with β2m from lysates of $K^{b-/-}D^{b-/-}$ cells, despite a comparable level of β2m expression in these cells (lane12). Likewise, there is a prominent β2m band in the anti-m4/gp4 precipitations from wildtype or $D^{b-/-}$ cells (lanes 1 and 5), but little or no β2m apparent in precipitations from $K^{b-/-}D^{b-/-}$ cells (lane 9). Thus the association of β2m with m4/gp34 is almost entirely dependent on class Ia expression. We conclude that m4/gp34 does not form significant NP40 stable associations with either free HC or free β2m but only with a specific conformation of class I which is β2m-associated. Whether or not the class I in these complexes is peptide-loaded remains to be demonstrated.

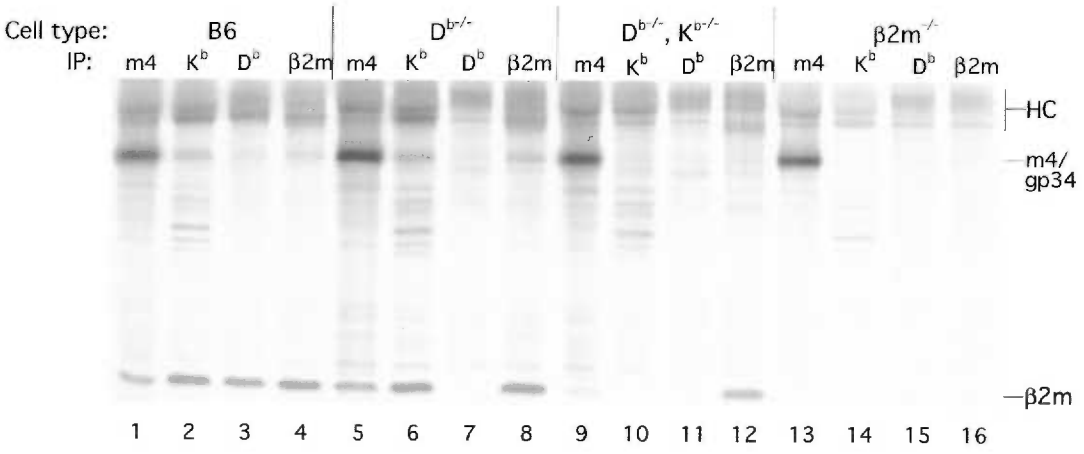


Figure 4. m4/gp34 does not significantly associate with β2m in the absence class Ia expression. MEFs from wildtype H-2^b mice (B6, expressing both K^b and D^b), or knockout mice as shown, were infected with MCMV (Smith) and metabolically labeled overnight. Cells were lysed in NP40 lysis buffer and lysates subjected to IP with serum against m4/gp34 (8139) or K^b (8010), or purified hybridoma supernatant against D^b (28.14.8S) or β2m (Lym11). Immunoprecipitates were not Endo H digested. Molecular weights of class I heavy chain (HC), m4/gp34, and β2m are indicated.

We also note that if m4/gp34 formed abundant complexes with non-classical class I molecules such as Qa-1 or the viral class I homologue m144, we should still have seen a prominent β2m band in lane 9. Since the amount of β2m in lane 9 is extremely small, we conclude that such complexes, at least in fibroblasts, are few or absent.

Discussion

CMVs share an extensive evolutionary history with their mammalian hosts, over the course of which the viruses have developed intimate and complex relationships with the host immune systems. The altered assembly of class I MHC in MCMV-infected cells is a prime example of this complexity. Two MCMV genes have been identified in published reports as directly interfering with CTL recognition of fibroblasts: *m152* (Krmpotic et al., 1999; Ziegler et al., 1997), which causes retention of nascent class I molecules in the ERGIC, and *m6* (Reusch et al., 1999), which redirects class I to the lysosome where it is degraded. In addition, we have recently demonstrated that a third gene, *m4*, cooperates with *m152* and *m6* to modulate immune responses restricted by K^b. We know that *m4* is necessary for evasion of K^b-restricted, MCMV-specific CTL clones, because fibroblasts infected with Δm4 MCMV were readily recognized by these clones while those infected with wildtype virus were not. However, the mechanism by which m4/gp34 interferes with antigen presentation is not known.

We undertook the experiments described here to define more carefully the nature of the interaction of m4/gp34 with class I. Since m4/gp34 was originally identified on the basis of its association with class I in NP40-stable complexes expressed on the cell surface (Kleijnen et al., 1997), we considered it possible that *m4* might block antigen recognition by directly interfering with contact between class I and the TCR or CD8. However, previous work was unable to demonstrate whether a significant percentage of K^b at the cell surface was associated with m4/gp34. We now show (Figure 1) that at least

half of K^b synthesized over the course of infection and exported past the medial Golgi is associated with m4/gp34 in NP40-stable complexes. This finding seems consistent with a mechanism by which m4/gp34 directly interferes with contact between K^b and the TCR. Such inhibition could involve steric hindrance of TCR contact with MHC-peptide, although the ability of antibodies, such as Y3 and B22.249, which recognize the α1 and α2 domains of class I molecules, to co-precipitate m4/gp34 (Kleijnen et al., 1997) argues against this idea. Alternatively, m4/gp34 might prevent CD8 coreceptor contact with class I, exclude class I from the immune synapse, alter the association of class I with the cytoskeleton, or prevent class I dimerization. Distinguishing these possibilities would be greatly assisted by knowing the peptide content of m4/gp34-class I complexes. However, none of the MCMV antigens recognized by K^b-restricted CTLs are currently known, which makes determining the peptide content of these complexes difficult. The fact that m4/gp34 associates specifically with folded conformations of class I (Figure 4) and that these associations are promoted by TAP expression (Kleijnen et al., 1997), suggests that peptide loading is required at least for the initial formation of these complexes.

In spite of the above findings, we were unable to demonstrate, over the course of several experiments, complete association of mature K^b with m4/gp34: a significant percentage (at least 30%) always failed to co-precipitate with antisera to m4/gp34. This might be due to anti-m4/gp34 sera causing dissociation of m4/gp34-class I complexes, or to a genuine population of m4/gp34-unassociated ("free") K^b at the surface of infected cells. The latter possibility, along with the fact the vast majority of m4/gp34 resides in a pre-Golgi compartment, prompted us to look for additional interactions between m4/gp34 and K^b aside from those at the cell surface.

We therefore undertook a more sensitive assay for protein-protein interactions using the weak detergent digitonin. Figure 2 demonstrates that K^b and m4/gp34 engage in a novel type of complex in a pre-Golgi compartment. These complexes are defined by their stability in digitonin lysis buffer and their instability in NP40 lysis buffer. These

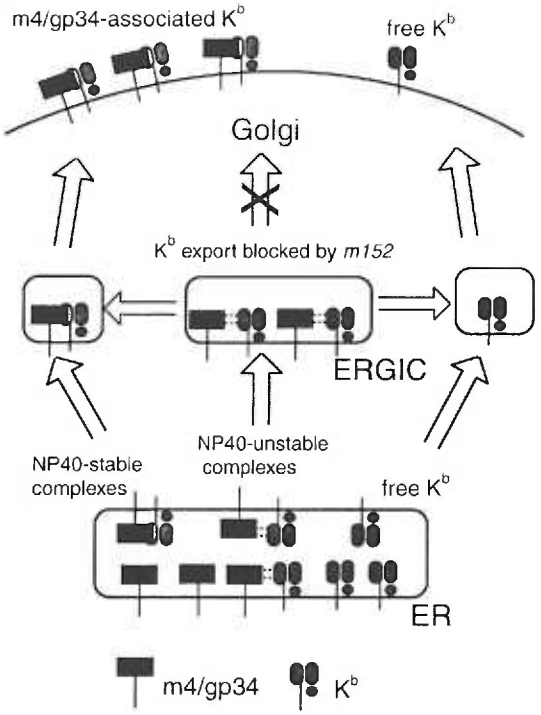


Figure 5. Hypothetical relationships among m4/gp34-containing complexes. β2m-associated K^b forms NP40-unstable associations with m4/gp34 in the ER or ERGIC. The transport of K^b from these complexes to the Golgi is retarded by *m152*. It may be that K^b in this compartment undergoes peptide exchange or some other alteration, which makes the molecules non-immunogenic. Eventually a significant amount of K^b escapes the effects of *m152* and is transported to the cell surface. Over half of these molecules are associated with m4/gp34 in NP40-stable complexes; up to 30% may be unassociated with m4/gp34, but *m4* nevertheless renders the target incapable of stimulating a CTL clone. Thus, *m4* might prevent antigen presentation by altering peptide loading in a pre-Golgi compartment and/or by interfering at the cell surface with contact between K^b and receptors on the T cell. Arrows indicate multiple hypothetical pathways of traffic between compartments.

complexes are specific, because neither protein is found in association with an irrelevant control protein (transferrin receptor) or with immunoprecipitates of normal rabbit serum. They are distinct from the NP40 stable complexes, because they accumulate in a pre-Golgi compartment over time, while the latter are exported and become Endo H-resistant. The accumulation of K^b in these complexes is dependent on *m152*. The biochemical basis of the difference in detergent stability of the two types of complex, and the kinetic relationship between then, is unknown. The existence of these novel, NP40-unstable complexes suggests that m4/gp34 may be altering K^b assembly and function at either or both of two different points: in a pre-Golgi compartment at the level of class I assembly, and/or at the cell surface, at the level of contact between class I and receptors on the MCMV-specific T cell.

K^b that is retained by the effects of *m152* accumulates in NP40-unstable complexes with m4/gp34 in a pre-Golgi compartment, most likely the ERGIC (Figure 3). Immature class I in the ER associates with a large assembly complex including tapasin, TAP, ERp57, and calreticulin (Cresswell et al., 1999), and it is possible that NP40-unstable complexes between m4/gp34 and K^b represent an indirect interaction mediated by one or more ER chaperones. Current models of class I assembly suggest that class I in the ER repeatedly exchanges peptides in a process which favors the eventual presentation of immunodominant peptides. By associating with ER- or ERGIC-resident class I molecules, m4/gp34 might alter this process. We have found that MCMV infection also prolongs the association of K^b with tapasin (unpublished observation). Taken together, these results provide circumstantial evidence for an effect of m4/gp34 on an early aspect of K^b assembly and export, such as peptide loading.

We have shown that m4/gp34 associates with K^b in distinct complexes in different cellular compartments. One or both of these interactions is presumably responsible for *m4*'s immune-evasive effects. A general model of interactions between K^b and m4/gp34 is shown in Figure 5. As shown, *m152* causes the partial retention of K^b in a pre-Golgi

compartment, where it accumulates in NP40-unstable complexes with m4/gp34. Eventually, a significant amount of K^b escapes the effects of *m152* and progresses to the cell surface, where much of it is found in NP40-stable complexes with m4/gp34. It is possible that the effects of m4/gp34 in the ER have a direct impact on the function of complexes at the cell surface. For example, if all of the NP40-unstable complexes eventually become stable complexes, interference with peptide loading in the ER would result in a different spectrum of peptides being found on free and m4/gp34-associated class I at the cell surface. A number of other scenarios can be imagined, and it should be possible to test these hypotheses after we have identified the MCMV epitopes recognized by K^b-restricted CTL.

In any case, it is clear that m4/gp34 inhibits CTL activity by some novel mechanism, since the biochemical relationships described here are unlike those seen for any other immune-evasion protein. Results shown in this paper provide insight into the complex and dynamic biochemical relationship between a class I molecule and viral immune-evasion proteins.

Acknowledgements

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Chapter 4

The murine cytomegalovirus immune evasion gene *m152* prolongs the association of major histocompatibility complex class I with tapasin

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Abstract

Murine cytomegalovirus encodes multiple immune evasion proteins which interfere with the activation of cytolytic T cells (CTLs). These include m6/gp48, which causes class I to be degraded in the lysosome; m152/gp40 , which blocks class I export in a pre-Golgi compartment; and m4/gp34, which forms distinct complexes with class I in the ER and at the cell surface, and inhibits CTL activity by an unknown mechanism. We have previously found that retention of K^b by m152/gp40 is incomplete and that m4/gp34 complements the immune-evasive capacity of m152/gp40. The class I-specific ER chaperone tapasin serves as a bridge between class I and the peptide transporter TAP, and also promotes optimal peptide loading. For this report, we investigated the interaction of the class I molecule K^b with tapasin in MCMV infected cells. We found that infection of fibroblasts with MCMV caused a prolonged association between K^b and tapasin, and that this effect required viral expression of m152/gp40. In addition, we found that m4/gp34 associates with tapasin in a class I-dependent manner. These results are consistent a model in which m152/gp40 and m4/gp34 cooperate to block CTL activation by interfering with optimal peptide loading.

Introduction

In order to strike a delicate balance with the host immune system, many viruses have evolved complex mechanisms to modulate the cellular immune response (Brodsky et al., 1999; Fruh et al., 1999). Cytomegaloviruses (CMVs) in particular encode multiple genes which alter the normal assembly of MHC class I (Loenen et al., 2001; Reddehase, 2000). Human cytomegalovirus (HCMV) encodes proteins which block class I export from the ER, block the peptide transporter TAP, and cause degradation of class I via retrograde translocation from the ER to the cytosol (Wiertz et al., 1997). Murine cytomegalovirus (MCMV) has at least three genes (*m6*, *m152*, and *m4*) which alter class I assembly (Hengel et al., 1999; Kavanagh and Hill, 2001). m6/gp48 causes degradation of class I in the lysosome; m152/gp40 blocks class I export from the ER; and m4/gp34 binds to class I and inhibits CTLs by an unknown mechanism. The class I molecule K^b is incompletely retained by *m152*, and *m4* complements this defect to provide complete protection of infected cells from K^b-restricted CTLs (Chapter 2, manuscript submitted).

During normal assembly, newly synthesized class I heavy chain first associates with light chain (β2m) and then enters into a peptide-loading complex containing the chaperones tapasin and calreticulin, and the peptide transporter TAP. After a transient association the duration of which varies for different class I alleles, class I is released from association with the peptide loading complex and is exported through the Golgi and out to the cell surface [reviewed in (Cresswell et al., 1999; Solheim, 1999)]. Tapasin plays at least four important roles in this process: it provides a bridge between class I and TAP (Sadasivan et al., 1996, Grandea, 1997 #1611), it promotes optimal peptide loading

of class I (Barnden et al., 2000; Lehner et al., 1998), it stabilizes expression of TAP heterodimers (Grandea et al., 2000; Peh et al., 2000), and it retains incorrectly loaded class I molecules in the ER (Schoenhals et al., 1999).

The mechanism or mechanisms by which *m152* inhibits class I presentation are incompletely understood. *m152* expression causes retention of class I molecules in the ER-Golgi intermediate compartment (ERGIC) (Ziegler et al., 1997), but no biochemical association between m152/gp40 and class I has ever been demonstrated. m152/gp40 itself is only transiently localized in the ER/ERGIC, before it is transported to the lysosome and degraded (Ziegler et al., 2000; Ziegler et al., 1997). The lumenal domain of m152/gp40 alone is sufficient to cause class I retention, and *m152* does not need to be continuously expressed to cause prolonged retention of class I (Ziegler et al., 2000).

We have recently demonstrated that *m4* is able to inhibit K^b-restricted CTL clones (Chapter 2; manuscript submitted). The mechanism of inhibition is unknown. m4/gp34 engages class I in two biochemically distinct types of complexes. One type of complex is stable in 1% NP40 buffer and is expressed at the cell surface (Kleijnen et al., 1997). A second type of complex is unstable in 1% NP40 buffer, but stable in 1% digitonin, and is retained in a pre-Golgi compartment (Chapter 3; manuscript submitted). We have hypothesized that m4/gp34 might block CTL activation by interfering with TCR contact at the cell surface and/ or interfering with peptide loading in the ER.

We investigated the association of class I with tapasin and m4/gp34 in MCMV infected cells. We report that MCMV infection caused a prolonged association of class I with tapasin in murine fibroblasts. *m152* was necessary to cause this prolonged association. Furthermore m4/gp34 also associates with tapasin, in class I-dependent

manner. We discuss possible implications of these results with regard to the mechanisms of immune evasion of *m4* and *m152*.

Materials and Methods

Virus Strains. MCMV (Smith) is from ATCC. Mutant strain Δm152 (ΔMC96.24) is described in (Krmpotic et al., 1999). ΔMS94.5, lacking ORFs *m151-m165* is described in (Thale et al., 1995). Δm4 (m4Tn3514) is described in Chapter 2 (manuscript submitted).

Cell culture. B6 mouse embryo fibroblasts (MEFs) from were grown from trypsin-digested day 12-14 mouse embryos, and used between passage 3 and 6. MEFs from ($K^{b/-}$ $D^{b-/-}$) mice were the gift of David Raulet. MEFs were maintained in DMEM supplemented with 10% fetal calf serum. Virus stocks were generated and titred as described previously (Chapter 3).

Antibodies. Antiserum 8010 against the cytoplasmic tail of K^b (anti-p8) was generated by immunizing a rabbit with a synthetic peptide derived from the sequence of exon 8, in the cytoplasmic tail of K^b. Antiserum 8139 was generated by immunizing a rabbit with whole m4/gp34, as previously described (Chapter 2; manuscript submitted). Antiserum 14950 was generated by immunizing a rabbit with a synthetic peptide (SKEKATAASLTIPRNSKKSQ-OH) derived from the cytoplasmic tail of tapasin. Serum 45668, the kind gift of Klaus Früh, was generated by an identical method. The anti-tapasin serum used in Figure 1 is 45668, and in the remaining figures it is serum 14950.

Immunoprecipitations. MEFs were pretreated with recombinant mouse IFN-γ at 50 U/ml for 48 hours before metabolic labeling. MEFs were infected with MCMV at an MOI. of 10 and infected or mock infected cells were continuously grown in the presence of 0.3 mg/ml PAA (phosphonoacetic acid, Sigma). 1 hour before the addition of metabolic label, cells were washed in PBS and placed in labeling medium [cysteine/ methionine-free DMEM (Gibco) supplemented with antibiotics, and 5% FCS]; at the end of 1 hour cells were labeled with ^{35}S-cysteine/methionine (NEN) for the time periods indicated in the figures. For pulse-chase experiments, cells were washed with chase medium [DMEM supplemented with antibiotics, glutamate, 10% FCS, and saturating concentrations of TC-grade cysteine and methionine (Sigma)] at the end of the labeling period, after which they were cultured in chase medium for the chase period indicated in the figures.

All lysis and precipitation procedures were carried out at 4°. At the time of lysis, TC plates were placed on ice, washed in cold PBS, lysed in the plate with lysis buffer, scraped, and transferred to tubes. Cells were lysed in digitonin lysis buffer [1% high purity digitonin (Calbiochem) in PBS]. Just before use, lysis buffer was supplemented with 1X Complete EDTA-free Protease-Inhibitor Cocktail (Boehringer Mannheim). To remove non-specific antibody-binding proteins, lysates were precleared with normal rabbit serum (NRS): each ml of lysate received at least 20 μl of NRS and 500 μl of staph A, after which lysates were mixed by slow rotation for 2 hours, and centrifuged for 5 min at 15,000 g to remove non-specific proteins and cellular membranes. Precleared lysates were then subjected to specific immunoprecipitation as indicated in the figures.

For each primary precipitation, lysates were subjected to IP with anti-tapasin antibody or NRS, plus fixed Staph A. Primary IP pellets were washed once in digitonin lysis buffer. For Figure 1, primary pellets were resuspended in 1 ml 1% NP40 in PBS plus 1X protease inhibitor cocktail. For other figures, primary pellets were first resuspended in 100 μl 1% SDS in PBS and boiled for 10 minutes, and then diluted in 1 ml 1% NP40 plus 0.1% BSA.

Secondary precipitations were carried out as described in the text, using the indicated antibody plus a suspension of protein A-agarose (Sigma). Immune complex pellets were washed four times in 1 X NET buffer (150mM NaCl, 50mM Tris pH 7.5, 5mM EDTA and 0.05% NP40) +0.1% SDS. Samples were digested with Endo Hf (NEB) according to manufacturer's protocol and separated by SDS-PAGE on a 12.5% gel. Quantitation of labeled protein was performed using a Molecular Dynamics phosphorimager.

Results

The association between K^b and tapasin is prolonged by MCMV infection.

In order to examine kinetic relationships among m4/gp34, K^b, and tapasin in the ER, we took advantage of the fact that all of these proteins form associations which are stable in 1% digitonin but unstable in 1% NP40 (Figure 1). Infected or uninfected MEFs were metabolically labeled for 15 minutes and then chased with excess methionine for 0, 45, or 145 minutes, after which cells were lysed in 1% digitonin buffer. Primary IPs to isolate digitonin-stable complexes were performed with antisera to m4/gp4, tapasin, or K^b. Primary precipitates were washed once in digitonin and resuspended in 1% NP40 buffer, which disrupts complexes between tapasin and class I as well as some complexes between K^b and m4/gp34 (manuscript submitted). 1% NP40 supernatant was removed from the primary pellet and subjected to secondary precipitations against either m4/gp34 or K^b as shown. Thus bands in Figure 1 represent only proteins which were stably associated with primary IPs in 1% digitonin and which subsequently disassociated in 1%

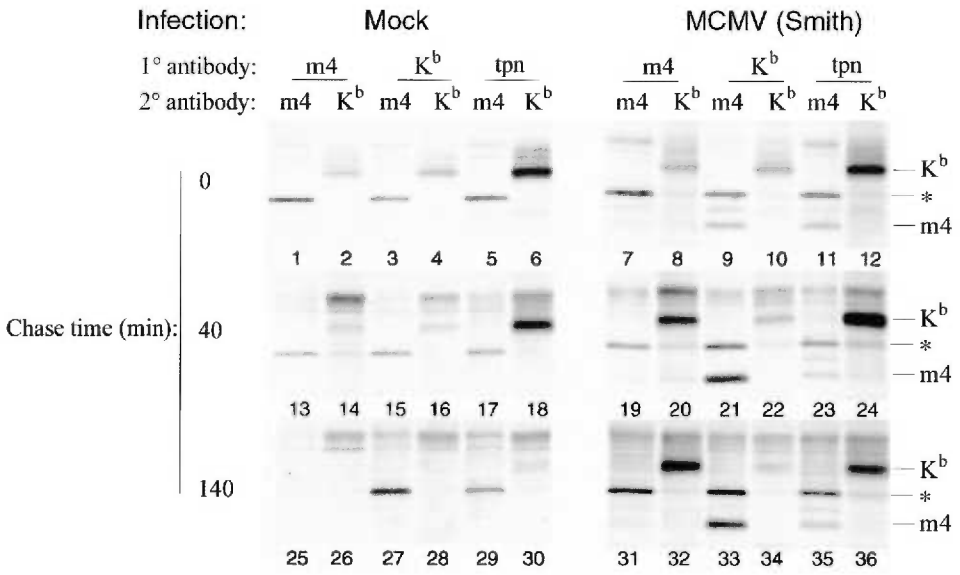


Figure 1. Analysis of tapasin-containing complexes in infected and uninfected cells. B6 MEFs were mock infected or infected with MCMV (Smith). 4.5 hours later, cells were pulse-labeled with ^{35}S-methionine for 15 minutes, and then chased with unlabeled methionine for the indicated time period. Cells were lysed in 1% digtionin buffer and lysates subjected to primary immunoprecipitation with the indicated sera. Pellets from primary IP were washed once in 1% digitonin and then resuspended in 1% NP40 buffer. Immune complexes were again precipitated, and the remaining NP40 supernatants were subjected to secondary IP with the indicated sera. All samples were treated with Endo H and separated by SDS-PAGE. * Indicates an irrelevant background band precipitated by anti-m4/gp34 serum.

NP40. Figure 1 demonstrates several features of protein complexes in the pre-Golgi compartments of MCMV-infected cells.

First, as previously reported, Endo H-sensitive m4/gp34 and K^b are associated in NP40-labile complexes. This is demonstrated by the fact that K^b coprecipitates with m4/gp34 (lanes 8, 20 , and 32), and that m4/gp34 coprecipitates with K^b (lanes 11, 23, and 35). Also, as previously shown, K^b accumulates in these complexes over time. In addition, we show for the first time that m4/gp34 associates with tapasin (lanes 9, 21, and 33). The nature of this association is addressed by further experiments, described below.

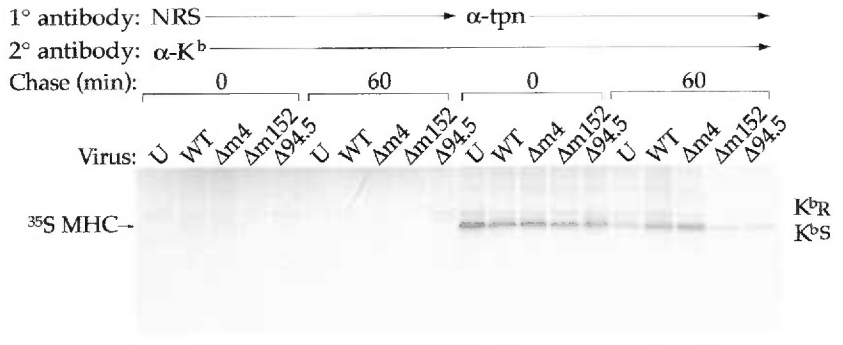
Second, results in Figure 1 allow us to compare the kinetic association of tapasin and K^b in infected and uninfected MEFs. Under normal conditions, K^b should associate with tapasin soon after synthesis, after which tapasin mediates a poorly-understood process of peptide loading and optimization, and K^b is released. This transient interaction is seen in the uninfected cells: K^b associates with tapasin early after synthesis (lanes 6 and18) and then departs before 145 minutes (lane 30). In infected cells, however there is a highly prolonged association of K^b with tapasin, even out to 145 minutes (lane 36).

The prolonged association of K^b with tapasin requires *m152.*

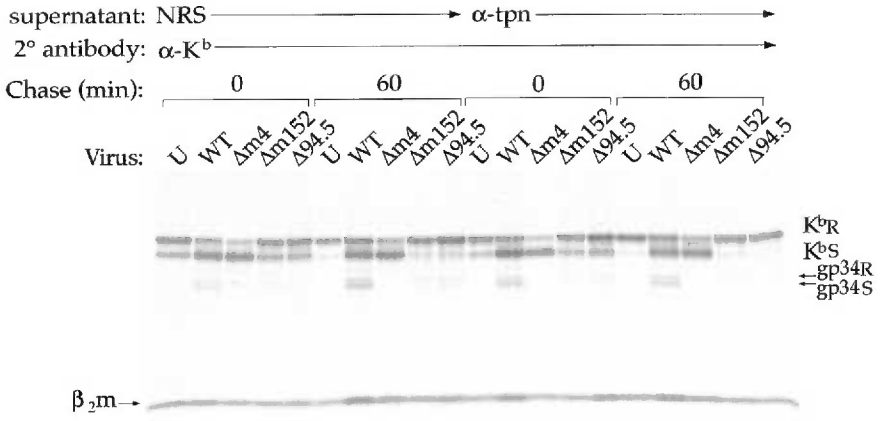
The fact that MCMV infection led to a prolonged association between K^b and tapasin could have been a general side effect of MCMV infection or the specific effect of a particular MCMV gene. m4/gp34 and m152/gp40 are both known to interact with class I pre-Golgi compartments, and both alter class I assembly via poorly defined mechanisms, so we decided to test the effects of each gene by infecting cells with wildtype MCMV, Δm4, Δm152, or ΔMS94.5, which lacks ORFs *m150-m 165* (Figure 2).

Figure 2. *m152* is necessary and *m4* is unnecessary for prolonged association of tapasin with K^b. B6 MEFs were mock infected or infected with indicated MCMV strain. ΔMS94.5 is a mutant MCMV lacking ORFs *m150-m165*. 3.5 hours later, cells were labeled with ^{35}S-methionine for a period of 2 hours, and then chased with unlabeled methionine for the indicated time period. **A.** Isolation of tapasin-associated K^b. Cells were lysed and subjected to primary IP as for Figure 1, using either NRS or serum against tapasin. Primary precipitates were washed once in 1% digitonin, boiled in 1% SDS for 10 minutes, resuspended in 1% NP40 lysis buffer, and subjected to secondary IP against K^b. All samples in were treated with EndoH and separated by SDS-PAGE. **B.** Isolation of tapasin-unassociated K^b. Digitonin supernatant remaining from primary precipitation shown in Figure 2A was subjected to futher immunopreciptation with antserum to K^b. All samples were treated with EndoH and separated by SDS-PAGE. **C.** Relative effects of infection with different viral strains on association of tapasin with K^b. The amount of K^b for each lane in Figures 2A and 2B was quanitfied using a phosphorimager. For each timepoint, the percent tapasin association was calculated as (amount of K^b associated with tapasin)/(total K^b). The total K^b is taken to be amount remaining in digitonin lysate after primary IP with NRS.

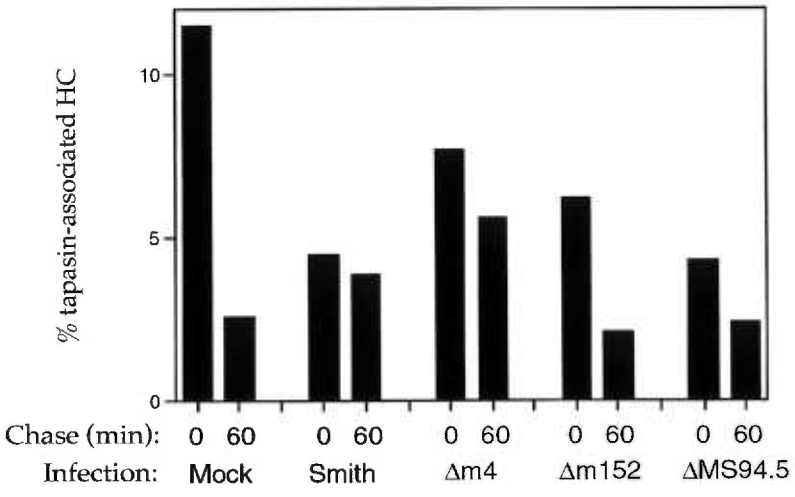
A.


1° antibody: NRS
α-tpn
2° antibody: α-K^b
Chase (min): 0 60 0 60
Virus: U WT Δm4 Δm152 Δ94.5
35S MHC
K^bR
K^bS

B.


supernatant: NRS
α-tpn
2° antibody: α-K^b
Chase (min): 0 60 0 60
Virus: U WT Δm4 Δm152 Δ94.5
K^bR
K^bS
gp34R
gp34S
β2m

C.


% tapasin-associated HC
10
5
0
Chase (min): 0 60 0 60 0 60 0 60 0 60
Infection: Mock Smith Δm4 Δm152 ΔMS94.5

MEFs were infected with the indicated virus or mock infected. Three and a half hours later they were labeled with ^{35}S-methionine for a period of two hours, after which they were lysed immediately or chased with unlabeled methionine for 1 hour. In order to measure the degree of tapasin association at each time point, we performed primary precipitations from digitonin lysates using either normal rabbit serum (NRS) or specific antiserum to tapasin. K^b associated with primary precipitates was isolated as described for Figure 1, except that instead of directly resuspending the primary pellets in NP40 buffer, they were first boiled in 1% SDS to completely disrupt all protein-protein associations, and then resuspended in 1% NP40 buffer (Figure 2A). In addition, we isolated all of the K^b remaining in the digitonin lysates after the primary precipitation (Figure 2B). The specificity of all re-IPs is demonstrated by the fact that no K^b is recovered from a primary IP with NRS.

For each virus, the rate of release of K^b from tapasin is indicated by the difference between the pulse and chase time points in the amount of K^b recovered from association with tapasin. We quantified the amount of labeled K^b in each precipitate using a phosphorimager, and expressed this amount as a percent of total labeled K^b recovered from the lysate (Figure 2C). As seen in Figure 2C, there is a large reduction in tapasin-associated K^b in uninfected cells, consistent with the rapid release which is expected under normal conditions. In both wildtype- and Δm4-infected cells, there is no release of K^b from tapasin over the sixty minute chase period. This both confirms the results from Figure 1, and demonstrates that *m4* is not required to prolong the association of tapasin with K^b. In contrast, both mutant viruses lacking *m152* also lack the prolonged

association, and show a rate of release of K^b from tapasin equivalent to that seen in uninfected ells. This indicates that *m152* is necessary for the prolonged association.

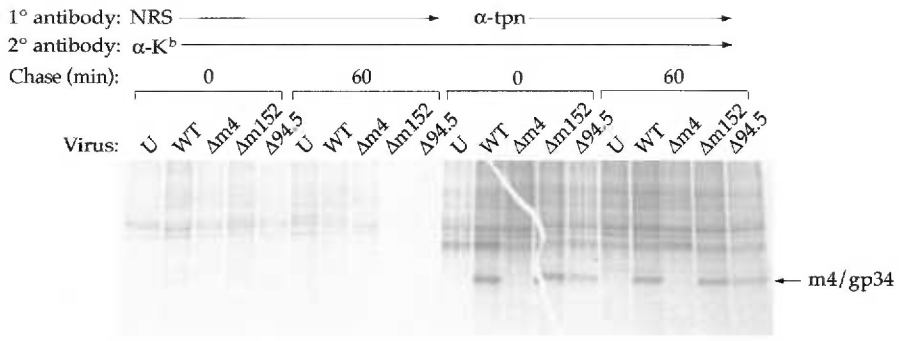
m4/gp34 associates with tapasin indirectly, via class I HC.

Our previous results (Chapters 2 and 3, manuscripts submitted) demonstrated that K^b forms both NP40-stable and -unstable associations withm4/gp34 a pre-Golgi compartment, although only the NP40-stable complexes are exported to the cell surface. Results shown in Figure 1 suggested that m4/gp34 was associated with tapasin in infected cells, although results in Figure 2 demonstrated that *m4* is not responsible for the prolonged association of K^b with tapasin. Because we have hypothesized that m4/gp34 may alter peptide loading in infected cells (Chapter 3), we wanted to confirm the observation (Figure 1, above) that m4/gp34 associates with tapasin. We looked for the association using the additional controls of a primary IP with NRS and infection with Δm4 MCMV.

1% NP40 supernatant from immunoprecipitation of K^b from tapasin complexes (Figure 2A) was subjected to re-immunoprecipitation with anti-m4/gp34 serum 8139 (Figure 3A). Figure 3A shows that m4/gp34 associates with tapasin in cells infected with Smith, Δm152, and Δ94.5 MCMV. The specificity of the interaction is demonstrated by the fact that no m4/gp34 is recovered from primary immunoprecipitation with NRS, and the identity of the m4/gp34 band is confirmed by the fact that it is absent from lysates infected with Δm4

m4/gp34 could associate with tapasin in three ways: by being in NP40-stable complexes with K^b which was tapasin-associated, by being in NP40-unstable complexes with K^b which was tapasin-associated, and/ or by being in direct complexes with tapasin

A.



B.

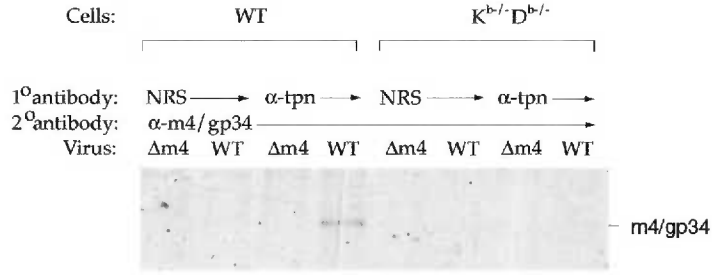


Figure 3. Class Ia-dependent association of m4/gp34 with tapasin. A. Specific association of m4/gp34 with tapasin. NP40 supernatant remaining from from the IP shown in Figure 2A was subjected to further immunoprecipitation with antiserum against m4/gp34. **B.** m4/gp34 does not associate with tapasin in the absence of classical class I molecules. B6 or $K^{b-/-}D^{b-/-}$ MEFs were infected with Smith or Δm4 MCMV as shown. 3.5 hours later, cells were labeled with ^{35}S-methionine for a period of 2 hours and lysed in 1% digitonin lysis buffer. Digitonin lysates were subjected to primary IP against tapasin. Primary pellets were washed once in 1% digitonin, boiled in 1% SDS for 10 minutes, resuspended in 1% NP40 lysis buffer., and subjected to secondary IP against m4/gp34. All samples in A and B were treated with Endo H and separated by SDS-PAGE.

independent of class I. Figure 1 demonstrates that m4/gp34 is both in NP40-stable complexes with tapasin-associated K^b (lanes 9, 21, and 33), and in NP40 unstable complexes containing tapasin (11, 23, and 35). However, results in Figures 1 and 2 do not show whether any of the associations between m4/gp34 and tapasin are direct or whether they are entirely mediated by class I.

In order to distinguish these possibilities, we used a fibroblast cell line derived from mice lacking classical class I HC (Figure 3B). If m4/gp34 were capable of binding directly to tapasin, then it should still be found in complexes with tapasin in the absence of class Ia. B6 or $K^{b-/-}D^{b-/-}$ fibroblasts were infected with wildtype or Δm4 MCMV. Three and a half hours later, cells were labeled with ^{35}S-methionine for a period of two hours, after which they were lysed in 1% digitonin buffer. Lysates were subjected to primary IP with anti-tapasin serum or with NRS, after which pellets were boiled in 1% SDS, resuspended in 1% NP40, and subjected to re-IP with anti-m4/gp34 serum. Figure 3B shows that a significant amount of m4/gp34 was recovered only from tapasin complexes from cells expressing class Ia HC. Thus, m4/gp34 did not associate directly with tapasin, but rather formed this association via class I HC.

Discussion

Tapasin serves multiple roles in the assembly of class I molecules. It binds independently to both class I and TAP, thus allowing it to serve as a bridging molecule. It also serves a separate quality-control function, by binding and retaining class I until it is loaded with optimal peptide. Given the importance of this molecule in antigen

presentation, it seems to present a likely target for viral interference with class I assembly.

Viruses interfere with class I expression by a wide variety of mechanisms, including TAP blockade, destruction of HC, and sequestration of class I in post-Golgi compartments. In addition, there are three viral proteins which are known to specifically prevent the export of class I molecules to the Golgi compartment. The first of these is the adenovirus E3 19K protein (E19), which binds to some class I molecules and retains them in the ER, by a "grab and hold" mechanism, by virtue of a dilysine retention motif in the cytoplasmic tail (Cox et al., 1991). The second is the HCMV IE protein US3, which transiently associates with human class I and prevents export from the ER by an unknown mechanism (Gruhler et al., 2001; Jones et al., 1996). The third protein is MCMV m152/gp40, which has not been shown to associate with class I, but which blocks export of murine class I from the ERGIC. Continuous expression of m152/gp40 is not required for continuous retention of H-2 L^q. Thus *m152* has been proposed to induce a long-lived change in class I which prevents it from reaching the Golgi (Ziegler et al., 2000). An alternative possibility is that *m152* disrupts some aspect of the cellular export machinery which is required for normal maturation of class I.

Results presented here show that in addition to preventing export of K^b to the Golgi compartment, *m152* also causes a prolonged association between K^b and tapasin. It is thus possible that tapasin mediates retention by *m152*, perhaps by a mechanism similar to that of E19, except that the retention signal is in tapasin rather than in the viral protein. Even if such a mechanism were demonstrated, the nature of the changes induced by *m152* would remain an open question. If m152/gp40 attacks class I, hypothetical possibilities

would include alterations in glycosylation or folding of the class I protein which increase the association of class I with tapasin. If, on the other hand, m152/gp40 attacks tapasin, *m152* might inhibit its ability to release properly loaded class I.

Adenovirus E19 causes long-term retention of class I proteins by a grab and hold mechanism which relies on the ER retention motif in the cytoplasmic tail. However, Bennett *et al.* (Bennett et al., 1999) showed that mutant E19 lacking the ER retention motif is also able to delay (but not block) the export of class I molecules by a second mechanism. They showed that E19 inhibits the association of TAP with class I but not with tapasin; thus E19 somehow blocks the ability of tapasin to bridge the connection between TAP and class I. Bennett *et al.* hypothesized that this interference leads to inefficient peptide loading and thus delayed export of class I from the ER. The ability of E19 to bind class I molecules varies with class I allele (Beier et al., 1994). Bennet *et al.* suggested that by blocking the interaction of class I with tapasin, E19 could delay peptide loading of a broad array of class I alleles, including those to which it could not directly bind.

We find an interesting parallel to E19 in the case of *m152,* which shows a differential ability to retain different class I molecules. Specifically, *m152* causes a profound retention of D^b molecules, but only partial retention of K^b molecules, and this differential retention is reflected in the degree of inhibition of D^b- or K^b- restricted CTL clones (Chapter 2; manuscript submitted). It is possible that the differential effects of *m152* on D^b and K^b reflect two different mechanisms used by this protein to alter class I assembly. The complete retention of D^b may reflect a permanent biochemical

modification of this molecule by m152/gp40 (Ziegler et al., 2000), while the incomplete retention of K^b may reflect a delay in peptide-loading of an otherwise unaltered molecule.

In contrast to the findings of Bennet *et al.* with regard to E19, we find that *m152* causes prolonged rather than reduced association between tapasin and class I. However, the two viruses might actually use analogous mechanisms if E19 prevents association of tapasin with class I, and *m152* prevents association of tapasin with TAP. In either case, the predicted result would be failure of class I to associate with TAP, and a delay in class I export during a prolonged period of peptide loading. The prolonged association of tapasin with K^b in MCMV-infected cells would thus reflect a period of inefficient peptide loading of TAP-unassociated K^b. This line of reasoning suggests that *m152* might not alter class I directly but may in fact attack TAP or tapasin, rendering them incapable of binding each other. If this hypothesis is correct, then K^b in MCMV-infected cells should show a decreased association with TAP.

As proposed by Bennet *et al.* for E19, altered tapasin function may be a secondary mechanism used by *m152* to delay the export of class I molecules which it is not capable of directly retaining. Thus complete retention of D^b may reflect the primary escape mechanism of *m152* and may be tapasin-independent; delayed export of K^b may reflect a secondary escape mechanism of *m152* and may be tapasin-dependent.

In addition to the altered relationship between tapasin and class I in infected cells, we demonstrate that m4/gp34 also associates with tapasin, in a class I-dependent manner (Figure 3). This result is intriguing since we have previously hypothesized that *m4* may inhibit CTL activity by altering class I peptide loading. The most likely explanation for the class I dependence of the tapasin-m4/gp34 association is that class I molecules

themselves bridge the association between m4/gp34 and tapasin. By associating with K^b simultaneously with tapasin, m4/gp34 could hypothetically inhibit the quality control function of tapasin, leading to suboptimal peptide loading, and accounting for *m4*-mediated inhibition of K^b-restricted CTLs. According to this model, delayed export of K^b, and partial inhibition of K^b-restricted CTL by *m152*, would be due to a blockage in the tapasin-TAP association. Complementary inhibition of K^b-restricted CTL by *m4* would be due to loss of peptide quality control by tapasin. Finally, m4/gp34 might be expressed at the cell surface to stabilize K^b loaded with suboptimal peptide and thus inhibit NK activation.

All of these models suggest hypotheses which can be tested in infected cells, by measuring the degree of TAP-tapasin association, by determining the quality of peptide loading, and by determining the tapasin dependence of *m152*-mediated immune evasion. Ongoing work should continue to unravel the complex alterations in class I assembly induced by the immune evasion functions of MCMV.

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Chapter 5

Discussion and Conclusions

Summary of results

In the preceding chapters we have introduced the relationship between MCMV and the host immune system, and described our experimental results examining the traffic of class I in MCMV-infected cells. In this final chapter I will broadly outline some models which integrate these results, and propose an experimental program which could be used to test these models. Implications of specific results are discussed in preceding chapters, but I will briefly summarize them here.

1) *m4* is a CTL evasion gene *in vitro*. This is demonstrated in Chapter 2, Figure 3. Infection with Δm4 sensitizes MEF targets for lysis by MCMV-specific CTL to an intermediate extent between that seen for wildtype virus and for Δm152. Preliminary data using several experimental systems suggests that *m4* has an even greater effect in DCs and macrophages. In these cell types, Δm4 virus is often recognized as well or better than Δm152. However, the fact that *m4* serves as CTL evasion gene for some clones *in vitro* does not prove that this a major function of the gene in the viral lifecycle. The problem of demonstrating function *in vivo* will be discussed below.

2) *m4* complements the immune-evasive function of *m152* in MEFs. This is demonstrated by the following facts (Chapter 2): (i) *m4* is reqired for complete evasion of K^b- but not D^b-restricted CTL clones. (ii) *m152* is sufficient for complete evasion of D^b- but not K^b- restricted clones. (iii) the differential effects of *m152* on antigen presentation by D^b and K^b are corroborated by differential rates of export as determined biochemically. Results shown are limited to three K^b- and two D^b-restricted CTL clones.

In addition, we have extended these results to another CTL clone which I isolated from the spleen of a mouse infected with Δm4 MCMV (not shown). This clone is D^b-restricted and, consistent with results in Chapter 2, is also entirely inhibited by *m152* in the absence of *m4*. We hope to strengthen our conclusions further by testing more CTL clones as they become available.

3) Over half of K^b at the surface of infected MEFs is m4/gp34-associated. This is demonstated in Chapter 3, Figure 1. We found that at least half of the mature K^b in infected cells is m4/gp34-associated, but were unable to demonstrate such an association for up to 30% of K^b. Given the fact that m4/gp34-K^b complexes on live intact cells can be labeled with ^{125}I (Kleijnen et al., 1997), and biotin (D.G.K. unpublished observation), we conclude that the majority of K^b on the surface of infected cells is m4/gp34-associated. The fact that a significant percentage of surface-labeled K^b did not coprecipitate with polyclonal serum against m4/gp34 may be an artifact of the serum (which could tend to dissociate some complexes) or may indicate a genuine "free" population of K^b. To address this, we are trying to develop monoclonal antibodies against m4/gp34. Alternatively, there may be geniune population of free K^b which will have to be accounted for in any model of immune evasion by *m4*.

4) K^b which is retained by the effects of *m152* acccumulates in NP40-unstable complexes with m4/gp34. These complexes are described in Chapter 3. m4/gp34 is expressed in vast excess of class I, where it accumulates in a pre-Golgi compartment, and as class I is retained by the effects of *m152*, it accumulates in NP40-unstable complexes

with m4/gp34. Experiments described below are intended to distinguish the function of NP40-stable and -unstable complexes.

5) m4/gp34 is also in complexes containing tapasin in a pre-Golgi compartment. This result is consistent with the hypothesis that *m4* promotes immune evasion by somehow altering peptide loading. (Chapter 4)

6) *m152* causes prolonged association between retained K^b and tapasin. Discovering the mechanism by which *m152* retains class I was not the goal of this thesis project. However, in the course of studying class I assembly in infected cells, we made this interesting observation. (Chapter 4)

Unanswered Questions

In this section I will propose a variety of experimental approaches to understanding the evasion of cellular immunity by MCMV, with an emphasis on the role of *m4*, which has been to focus of most of my work. The following are the major outstanding questions which I believe remain to be addressed.

Is m4/gp34 a genuine immune evasion gene? Given the strong *in vitro* evidence summarized above, this seems very likely. However, in Chapter 1 I made a case for applying stringent *in vivo* standards to the identification of immune evasion genes, and these have yet to be fulfilled for *m4*. Therefore I will propose *in vivo* experiments to address this question. A closely related question is: what immune cell type is the major target of *m4*? Again, results so far show an *in vitro* effect on CTLs. However, it is certainly possible that *m4* has a more important effect on another cell type such as NKs. I

propose several *in vivo* and *in vitro* experiments which will help to determine the true relevance of *m4* to evasion of cellular immunity.

How does MCMV evade CTLs *in vitro*? We have presented circumstantial evidence for several new mechanisms of CTL evasion, including steric hindrance of TCR contact, altered peptide loading, and altered tapasin function. Which of these effects is relevant, and how do they work together to promote complete protection from CTLs? The experiments described below are intended to determine the individual importance of the biochemical effects we have described in the preceding chapters, and to determine how they contribute to the overall immune escape strategy of the virus. Finally I will present a speculative model of cooperation between *m152* and *m4*.

Choosing a relevant cell type for *in vitro* experiments

All of the data presented here have come from *in vitro* experiments using infected fibroblasts. This is not because anyone thinks that fibroblasts are a particularly important cell type with regard to MCMV pathogenesis or immunity, but because they have been the only primary cell type which we could easily propagate and infect in tissue culture. However, primary macrophages are permissive for MCMV *in vitro* (Brautigam et al., 1979), and experimental antigen-presenation sytems using these cells have recently been developed (Heise et al., 1998; Hengel et al., 2000).

Diane LoPiccolo in our lab has recently adapted the necessary techniques to study antigen presentation in B6 macrophages. She has demonstrated that infection with MCMV sensitizes B6 macrophages for lysis by MCMV-specific CTL clones, and that immune-evasion genes have a profound effect on CTL activity (unpublished

observations). Of particular relevance to *m4* is her observation that the specific lysis by K^b-restricted CTL clones of macrophages infected with Δm4 is often even greater than that of macrophages infected with Δm152. This implies that *m4* may even be the dominant immune-evasive gene in this cell type with respect to K^b-restricted CTL. Given the apparent importance of macrophages in latency and pathogenesis of MCMV infection, it seems appropriate to pursue the majority of *in vitro* studies in this cell type rather than fibroblasts. For all of the *in vitro* experiments proposed below, I suggest that primary macrophages should be the cell type of choice.

Given the stronger effect of *m4* on specific lysis of macrophages compared to fibroblasts, one possible benefit of moving to work in macrophages is that this may help to distinguish the relative importance of NP40-stable versus -unstable complexes. If, for example, in macrophages the majority of surface K^b is m4/gp34 -associated, but NP40-unstable complexes are not detectable, this will strongly suggest that NP40 stable complexes are more relevant to CTL evasion than NP40-labile complexes.

In addition to macrophages, other specific cell types, such as DCs or salivary gland epithelium are appropriate for some specific experiments, as described below.

Can *m4* affect NK activity *in vitro*?

There is a great deal of confusion in the field regarding the role of *m4* in the NK response. Based on speculation in the original report of Kleijnen *et al.*, (Kleijnen et al., 1997), m4/gp34 is sometimes described as an "NK decoy". Although we have now shown that *m4* is in fact a CTL evasion gene, we have not disproved the hypothesis that it

is NK-evasion gene as well. Definitively addressing this question would yield useful results and would be a service to the field in general.

The original basis for proposing that m4/gp34 may be an NK decoy was the suggestion that it might serve to rescue class I from the effects of *m152* and increase its rate of transport to the cell surface. We attempted to address this possibility in the experiments shown in Chapter 2, Figure 2 and described as unpublished findings in the accompanying text. These experiments used a brief pulse followed by a ninety minute chase to analyse the rate of export of K^b in wildtype- or Δm4-infected MEFs. We concluded that there was no difference in the rate of export between the two infections. In retrospect we recognize that it would have been more useful to do these experiments over a much longer chase period, with multiple time points, and I suggest that such experiments would still be informative. These kinetic analyses would more definitively address the question of whether *m4* affects the rate of export of K^b. Whatever the results, however, they would only provide circumstantial evidence for or against an effect of m4/gp34 on NK activity.

At the very beginning of my Ph.D. research, I attempted to examine the effect of *m4* on NK activity. I was able to induce populations of NK cells by injecting mice with poly-IC or by infection with MCMV (data not shown). These populations showed strong NK activity against cell lines of hematopoetic origin, but not against any infected or uninfected fibroblast targets. In fact, to my knowledge, NK killing of MCMV-infected fibroblasts has never been demonstrated. MCMV-infected primary macrophages may be better NK targets, which could permit a true functional determination of the effect of *m4* on NK activity. NK activity could be measured by ^{51}Cr-release and by intracellular

cytokine staining. It would be useful to compare the effects of infection with wildtype or mutant viruses. Genes which down regulate class I would hypothetically tend to increase NK activity, while *m4* may decrease NK activity. The relationship of m4/gp34 to NK function should also be demonstrated by *in vivo* depletion experiments, described below.

The hypothetical effects of *m4* on NK activity are not irrelevant to the issue of *m4*'s role as a CTL-evasion gene. If *m4* is found not to affect NK function, this will strengthen the conclusion that CTL evasion is the primary role of *m4* in the viral life cycle. If *m4* does promote NK evasion, this would favor certain hypotheses regarding the mode of CTL evasion. For example, anti-MCMV CTL in B6 mice express a variety of so called "NK" receptors which are known to regulate the activity of NK cells (Marielle Gold, unpublished data). One possible mechanism for immune evasion by *m4* is that m4/gp34 stimulates inhibitory NK receptors or blocks the stimulation of activating receptors. This is a mechanism which could potentially promote escape from both NKs and CTLs. Alternatively, if *m4* is shown to inhibit NKs as well as CTLs, this could explain the discovery of biochemically distinct types of complexes between m4/gp34 and K^b (Chapter 3); it is possible that m4/gp34 in pre-Golgi compartments may inhibit proper peptide loading (thus interfering with CTL), and that m4/gp34 at the cell surface may stabilize improperly loaded K^b (thus preventing a missing self response). This possibility is included in the model of immune evasion presented below (Figure 1).

What lymphocyte compartments are affected by *m4 in vivo*?

One major advantage of studying a mouse virus as opposed to a human virus is that definitive *in vivo* experiments can be used to determine the effects of an immune

evasion gene on specific immune compartments. These techniques have already been used to demonstrate *in vivo* that loss of *m152* decreases viral titer in a CD8-dependent manner (Krmpotic et al., 1999), and that loss of *m144* decreases viral titer in an NK-dependent manner (Farrell et al., 1997). By comparing *in vivo* titers of Δm4 and revertant virus in mice specifically depleted of various lymphocyte subsets, we can identify the primary immune compartment target of *m4 in vivo*. Favored targets for *in vivo* depletion would be Ly49H, NK1.1, and CD8.

Our new appreciation of the overlap between supposed "NK" and "CTL" compartments requires that depletion experiments be interpreted carefully. It also provides new opportunities to pinpoint the functional target of *m4*. For example, depleting antibody to NKG2A could potentially identify an effect of *m4* on subpopulations of NK and $CD8^+$ lymphocytes. Results from *in vivo* studies will provide direction regarding which of the biochemical studies described below are most likely to be informative.

What is the structure of m4/gp34 -K^b complexes

Obviously, determining the crystal structure of m4/gp34-K^b complexes could potentially provide a great deal of information regarding the mechanism of CTL evasion, especially in determining whether m4/gp34 hinders TCR contact withK^b. Attempts are under way in collaborating labs to crystalize these complexes. Given that X-ray crystalography of membrane proteins is a difficult and uncertain undertaking, I will describe a number of ways to infer the structure of these complexes using existing techniques and reagents.

Does m4/gp34 act by a steric blocking mechanism?

This is perhaps the simplest possible mechanism for *m4*-mediated immune evasion, and one which could explain the paradox that K^b is expressed on the surface of infected cells but is unable to stimulate MCMV-specific CTL.

Access of TCR to class I-peptide

The observation that monoclonal antibody Y3, recognizing a K^b epitope near the peptide binding groove, is able to coprecipitate m4/gp34 (Kleijnen et al., 1997), is inconsistent with m4/gp34 blocking access to the entire $\alpha1/\alpha2$ region. Nevertheles, m4/gp34 might block TCR contact by covering a relatively small area of the TCR contact surface. Also, the ability of Y3 to precipitate m4/gp34-K^b complexes in NP40 lysate does not prove that the relevant epitope would have been seen on intact cells.

Steric blockage of TCR contact could be measured in several ways. Non-permiablized cells infected with Smith or Δm4 MCMV can be stained with a panel of anti-K^b mAbs, and the relative brightness of staining by each antibody can be measured. If m4/gp34 does not block any epitopes on K^b, then the relative degree of staining with each antibody should be equivalent for both Smith or Δm4 infections. A relative loss of staining between Smith and Δm4 for certain mAbs would indicate that m4/gp34 blocks the relevant epitopes.

In addition to staining of intact cells, the panel of antibodies may be tested another way. In this case metabolically labeled cells can be lysed in NP40 buffer and subjected to IP with the panel of anti-K^b antibodies. The degree of m4/gp34

coprecipitation can be seen by SDS PAGE. Antibodies which precipitate K^b but not m4/gp34 probably recognize epitopes which are obscured by m4/gp34.

Another way to probe the accessibility of K^b-peptide to TCR is to stain intact cells with reagents specific for K^b presenting unique peptides. Examples are mAb 25-D1.16, specific for K^b-OVA, and the soluble TCR 2C TCR-2Ig, specific for K^b-SIY. Both of these reagents have been used to stain peptide-loaded complexes on intact cells (Spiliotis et al., 2000). If both of these reagents do stain m4/gp34-associated K^b, this will be strong evidence that m4/gp34 does not block TCR access to antigenic peptide. If, on the other hand, these reagents do not stain m4/gp34-associated K^b, the result will be inconclusive unless it can also be demonstrated that these K^b molecules are in fact peptide-loaded. One way to do this is to load cells with radioactive synthetic peptide and measure the degree of radioactivity coprecipitating with m4/gp34-containing complexes.

Any of the analyses above may identify epitopes on K^b which are sterically obscured by m4/gp34. These epitopes may overlap the surface of K^b-TCR interface, which would be strong evidence in favor of steric hindance with TCR contact contributing to immune evasion. On the other hand, antibody analysis may indicate that m4/gp34 obscures epitopes on the $\alpha 3$ domain, which would favor interference with CD8 contact as the most likely explanation for immune evasion (see below). Finally, staining may indicate that m4/gp34 does not block any important epitopes on K^b. This result would suggest that steric interference is not siginificant to *m4*-mediated evasion.

A complementary approach to the above methods can also be used. Even if m4/gp34 is shown to block binding of K^b by soluble TCR, this will not unambiguously prove that this is the primary mechanism of immune evasion by *m4*. This is because it at

least some free K^b is expressed at the surface of infected cells, which provokes the question of whether m4/gp34 can block T cell stimulation by simply titrating out a majority of peptide-loaded K^b through steric hindrance. To answer this question we can perform the following experiment.

Macrophages or DCs lacking K^b can be transfected with a recombinant gene encoding K^b, β2m, and OVA peptide as a single molecule. (Construction and use of this gene has recently been described. If macrophages and DCs are resisitant to transfection, $K^{b-/-}$ mice can be made transgenic for K^b-β2m-OVA, and macrophages from these mice can be used.) The ability of m4/gp34 to bind to and block presentation by K^b-β2m-OVA can be determined. If *m4* blocks presentation of K^b-β2m-OVA, this will show that altered peptide loading is not an essential component of immune evasion by *m4*. In this case, *m4* might block TCR access to peptide, or it might work through a different mechanism such as inhibitory receptors on CTLs. To determine whether m4/gp34 functions by reducing the exposure of available class I-peptide to TCR/CD3, we will titrate in an anti-class I blocking antibody (e.g. anti-K^b-OVA mAb 25-D1.16) and measure the anti-OVA cytolytic response. If m4/gp34 inhibits the CTL response by blocking access to class I, then it will take less antibody to inhibit the lysis of Smith-infected cells than Δm4-infected cells. If steric hindrance is not relevant to immune evasion by *m4*, then antibody will inhibit lysis of Δm4- and Smith-infected cells equally.

Access of CD8 to class I

Experiments described above may tend to rule out interference with TCR contact as an immune evasion mechanism. Another possible mechanism would be interference

with contact between α3 domain of K^b and the CD8 coreceptor. One way to test this hypothesis would be by blocking CTL stimulation using anti-CD8 antibody. The fact that K^b-restricted clones weakly recognize Smith-infected macrophages permits a controlled experiment. If m4/g34 blocks CD8 contact, then blocking CD8 with antibody should reduce CTL killing of Δm4-infected macrophages to the level of Smith-infected macrophages, while killing of Smith-infected macrophages should be relatively unaffected (see Table 1). Another way to test this hypothesis would be to generate CTL clones from CD8 knockout mice. If *m4* causes immune evasion by blocking CD8 function, *m4* should have no effect on CTL acitivty in these mice.

MCMV Infection of macrophage targets	**CTL Acitivity**	
	without anti-CD8	with anti-CD8
uninfected	-	-
Smith	+	+
Δm4	+++	+

Table 1. Predicted effect of anti-CD8 antibody on CTL activity, assuming that m4/gp34 blocks CD8 contact with class I.

Does *m4* alter class I peptide loading?

One possible explanation for why K^b expressed on the surface of MCMV-infected cells cannot activate CTL clones is that it is not loaded with antigenic peptide. As suggested by its association with K^b and tapasin in NP40-unstable complexes in a pre-Golgi compartment, m4/gp34 might cause some alteration in peptide loading. It is also possible that m4/gp34 may alter peptide loading via the NP40-stable interaction.

In order to determine whether *m4* alters peptide loading, we can acid-elute peptide from the surface of target cells infected with wildtype or Δm4 MCMV. Eluted peptide can be separated and purified by HPLC, and tested for antigenicity by exogenously loading targets and measuring specific lysis by CTL clones. The relative amount of antigenic peptide can be determined by titrating the antigenic HPLC fraction. By comparing the amount of antigenic peptide extracted from Smith or Δm4-infected cells, we can determine whether *m4* affects peptide-loading of K^b.

Using only infected H-2^b cells as the peptide source, it would be difficult to determine whether quantitative differences are due to the action of *m4* or to accidental differences in the efficiency of peptide recovery. Therefore we can use cells (MEFS or macrophages) derived from B6 x BALB/c F1 mice. These cells will express K^d, which does not form NP40-stable complexes with m4/gp34 (Kleijnen et al., 1997). Before peptide extraction, cells can be loaded with equal, sub-saturating concentrations of a defined, irrelevant K^d-binding peptide . The amount of K^d-binding peptide eluted from target cells will indicate the efficiency of peptide recovery from each cell population, and can be used to as a control when comparing the recovery of peptides recognized by K^b-restricted clones. If, using this controlled comparison, we find that Δm4-infected cells express significantly more antigenic peptide bound to K^b, we can conclude that *m4* prevents normal peptide loading of K^b.

Even if we demonstrate that *m4* alters the peptide loading of K^b, we will not know whether this is an effect of the NP40-stable or -unstable interaction with m4/gp34. I see three possibilities regarding the relationship of these two interactions to altered peptide loading. First, peptide loading may be altered by the NP40-unstable interaction, and all

the K^b from NP40-unstable complexes may end up in NP40-stable complexes. Second, peptide loading may be altered by the NP40-unstable interaction, and some or all of the K^b from these complexes may end up as free K^b. Third, peptide loading may be altered only by the NP40-stable interaction. Distinguishing among these possibilities poses a potentially difficult problem, but I will suggest a couple of ways to address it here.

We can try to distinguish the functions of NP40-stable and -unstable interactions by finding conditions under which one or the other does not occur. As mentioned above, *m4* has a strong effect on antigen presentation by macrophages, and m4/gp34-stable complexes have been found in these cells. If NP40-unstable complexes are not found in these cells, we will thus demonstrate that these complexes are not necessary to immune evasion. If in addition *m4* alters peptide loading in macrophages, then this will also most likely be a function of NP40-stable complexes.

Another potentially useful observation is that m4/gp34 does not form NP40-stable complexes with K^d (Kleijnen et al., 1997). It is not known whether m4/gp34 alters antigen presentation by this molecule or forms NP40-unstable complexes it. There is a defined peptide, derived from the MCMV E gene M84, which is presented by K^d (Holtappels et al., 2000), and it should be easy to determine whether *m4* can alter presentation of this peptide. Splenocytes from an MCMV-immune BALB/c mouse can be restimulated for five days in culture with defined K^d-binding peptide. After five days, effectors will be tested for ability to lyse Smith- or Δm4-infected targets. If Δm4 targets are better recognized, this will be strong evidence that the NP40-stable complexes are not necessary for CTL evasion. To extend this line of inquiry, it would be worthwhile to search for any other class I proteins which cannot bind m4/gp34 in NP40.

Another approach to the question of whether peptide loading may be altered by NP40-stable or -unstable interactions is to isolate m4/gp34-associated and unassociated K^b from NP40 lysates, and compare the peptide content in these populations to that in K^b from Δm4-infected cells. If peptide isolated from free K^b from Smith-infected cells is less antigenic than that isolated from free K^b from Δm4-infected cells, then we can conclude that peptide alteration is a function of NP40-unstable rather than NP40-stable interactions.

Does *m4* affect immune synapse formation?

As discussed in Chapter 1, the immune synapse (IS) is believed to be an important mechanism for information transfer between the APC and the T cell. Even if the above experiments fail to demonstrate altered contact between the TCR and K^b, m4/gp34 might still block T cell activation by interfering with proper IS formation. IS formation could be monitored by transfecting $K^{b-/-}$ targets with a K^b-GFP construct (Spiliotis et al., 2000), exposing Smith, Δm4, and infected cells to CTL clones, and using video microscopy to look for the characteristic ring of MHC which progessivey resolves into a central cluster. If in Δm4- but not Smith- infected cells, K^b-GFP forms normal synapses, we can conclude that *m4* interferes with IS formation. We could also determine whether m4/gp34 is preferentially excluded from the contact zones between T cells and APCs by contructing a YFP-m4/gp34 chimera and expressing it in MCMV infected cells.

Another way to to address this question would be to try to specifically capture proteins from the IS. To my knowledge this technique has not been used elsewhere, probably because of the technical difficulty, and should be seen as a high-risk project. If

successful, however, it could represent a significant advance in IS study. T cells will be treated with a heterobifunctional crosslinking reagent which can be linked on one end to lysine residues on T cell proteins, and which carries a photoactivatable functional group on the opposite end. T cells will then be exposed to APCs, and after a set period of time, photocrosslinked to APC surface proteins. T cell proteins can next be immuoprecitipated, and monitored for the presence of crosslinked APC proteins by Western blot. By performing a series of such crosslinkings at short intervals after T cell exposure, it should be possible to specifically identify APC proteins which enter and leave the maturing IS. Using this method, it should be possible to determine if and when m4/gp34 enters the IS. The reciprocal experiment-- labeling the APC and looking for crosslinked T cell proteins-- will enable us to compare of the degree of TCR contact between m4/gp34-associated and free K^b. It must be noted that defective IS formation is the predicted downstream effect of many mechanism which may interfere with T cell stimulation. Therefore a finding of defective IS formation will be most informative if other upstream events such as peptide loading and TCR contact are found to be normal.

Does m4/gp34 alter the mobility of K^b in the plasma membrane?

The importance to T cell stimulation of MHC mobility within the plasma membrane is incompletely understood. Class I oligomerization in the membrane could promote TCR cross-linking. High degrees of class I mobility could promote faster IS formation and thus speed T cell activation. On the other hand, in experiments using human cells, depletion of cholesterol led to tethering of MHC in the plasma membrane,

which caused increased stimulation of an allogeneic T cell clone[4]. Edidin and coworkers have developed elegant systems to measure the degree of mobility of class I molecules (Marguet et al., 1999). By measuring the rate of diffusion of m4/gp34-containing complexes, it would be possible to determine whether these complexes have significantly different mobility from free K^b, and whether they are tethered to the cytoskeleton. Incidently, it should also be possible to estimate the molecular weight of complexes based on the rate of diffusion, which would permit us to determine the stochiometry of m4/gp34-K^b complexes.

Are there receptors for m4/gp34 on lymphocytes?

Several remarkable discoveries in recent years have demonstrated that T cells and NK cells are regulated by a number of receptors with positive and negative signaling capacities. It is noteworthy that two such discoveries resulted from the search for ligands for the HCMV proteins UL18 (Cosman et al., 1997) and UL16 (Cosman et al., 2001).

To apply a similar strategy, we could produce recombinant soluble m4/gp34, and complexes of m4/gp34 with K^b. These could be expressed as Ig fusion proteins or as tetramers, and used to stain lymphocytes. This is clearly a high-risk project, since, aside from the difficulty of constructing soluble multimeric complexes, it will not work if the sole effect of m4/gp34 is to block TCR binding or prevent proper peptide loading. However, especially if other mechanisms are ruled out, this approach could provide very interesting results. Soluble m4/gp34 or m4/gp34 complexes may stain a specifc class of

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[4] M. Edidin, symposium presentation, Annual Meeting of the American Association of Immunologists,

lymphocytes (eg. NK or T cells), which may indicate the major immune target of *m4 in vivo*. In addition, identifying a ligand for m4/gp34 would strongly indicate a functional mechanism of *m4*. As with HCMV UL16 and UL18, this identification might even lead to the discovery of a novel lymphocyte receptor.

We can also look for lymphocyte receptors for m4/gp34 using known reagents, such as blocking antibodies against known receptors. For example, to determine whether immune evasion by *m4* involves any NKG2 receptors on T cells, mAb 20d5 (Vance et al., 1999) can be used to block all NKG2 receptors on CTL clones. If *m4* loses its effect in the presence of this anitbody (*i.e.* if Δm4 and wildtype MCMV are recognized equally), this would indicate that m4/gp34 functions by engaging one of these receptors.

Does MCMV induce covalent modification of K^b?

Aside from altering peptide loading, it is possible that the action of *m4* in the ER could biochemically alter class I in some other way to affect its function. As discussed in Chapter 1, there is currently no evidence that glycosylation of class I plays any role in TCR recognition of class I-peptide. In contrast, there is evidence for a role for glycosylation in recognition of class I by NK receptors (Parham, 1996). Although there is little evidence for any other covalent modification of murine class I molecules aside from N-linked glycosylation, it is possible that *m4* could affect the function of K^b via covalent modifications such as phosphorylation, palmitylation, or myristoylation.

There are two pieces of circumstantial evidence for MCMV-related covalent modification of class I in a pre-Golgi compartment. First, as discussed in Chapter 1, Ziegler *et al.* (Ziegler et al., 2000; Ziegler et al., 1997) demonstrated that prolonged contact with m152/gp40 is not required for prolonged retention of class I in the ERGIC. Ziegler *et al.* concluded that *m152* causes some uncharacterized alteration in class I which blocks its interaction with the cellular export machinery. There is currently no satisfactory hypothesis in the literature regarding the biochemical nature of this alteration.

The second piece of circumstantial evidence comes from our own work. After Endo H digestion, K^b from uninfected cells generally resolves into two distinct bands on SDS-PAGE. By contrast, K^b from MCMV-infected cells consistently runs as series of bands, forming a sort of ladder or schmear. This difference is apparent for example in Chapter 2, Figure 6. A possibly related observation is that as K^b accumulates in NP40-labile complexes with m4/gp34, a novel band appears in the anti-K^b precipitate, running slightly higher than the Endo H-sensitive band (Chapter 3, Figure 3). This band may represent some K^b-associated molecule which accumulates in these complexes. It is also possible that this band represents an altered form of K^b which has a slightly higher molecular weight.

In order to determine whether these bands represent modfied K^b molecules, or some other protein which coprecipitates with K^b in NP40 buffer, we can simply boil K^b precipitates in 1% SDS and reprecipitate with the conformation indepentdent serum 8010. Only K^b HC will be isolated in this reprecipitation. If precipitates from infected cells still contain differerent bands from those from uninfected cells, this will show that infection has induced some alteration in K^b.

In order to determine whether this alteration is in the glycosyl component of K^b, precipitates will be treated with N-Glyconase F which removes all Endo H-sensitive and -resistant glycans. If all of the various bands resolve themselves into a single band after this treatment, this will demonstrate that MCMV infection modifies the glycosylation of K^b.

What role does tapasin play in MCMV immune evasion?

In Chapter 4 we showed that in the context of MCMV infection *m152* causes a prolonged association of tapasin with K^b. This may be an incidental effect of *m152*'s interference with class I export, or it may be an essential component of K^b retention by *m152*. Some experiments proposed below using cells from $Tpn^{-/-}$ mice are designed to disitinguish theses possibilities.

There are a number of ways by which *m152* might affect class I by altering tapasin function. For example, since retention of some class I molecules is a normal function of tapasin (Schoenhals et al., 1999), MCMV could exploit this mechanism by altering tapasin or class I to increase the affinity of the interaction between them. In this case tapasin would replace the function of a viral protein such as adenovirus E19 by acting as an independent retention factor. Alternatively, *m152* could affect tapasin function in more subtle ways, such as altering its abilty to bridge the association between class I and TAP. Further experiments below are designed to address these possibilities.

Preliminary results from our lab suggest that class I assembly and antigen presentation is defective in $Tpn^{-/-}$ fibroblasts (data not shown). This is consistent with the report by Grandea *et al.* that TAP function is defective in $Tpn^{-/-}$ mice; this defect was

much more severe in MEFs than in con A splenocyte blasts (Grandea et al., 2000). Since I have found that both *m152* and *m4* inihibit antigen presentation by B6 DCs (data not shown), we should be able to measure the effects of these genes in DCs derived from $Tpn^{-/-}$ mice. For example, we can infect $Tpn^{-/-}$ and $Tpn^{+/-}$ DCs with wildtype or Δm152 MCMV and measure the CTL response. If the mechanism of *m152* is tapasin-dependent, antigen presentation by $Tpn^{+/-}$ DCs should be partially defective, but *m152*-resistant (see Table 2).

	CTL response	
Infection	$Tpn^{+/-}$ DCs	$Tpn^{-/-}$ DCs
uninfected	-	-
wildtype	+	++
Δm152	+++	++

Table 2. Predicted CTL activity assuming that the effects of *m152* are tapasin-dependent

As discussed in Chapter 4, Bennet *et al.* have demonstrated that the adenovirus E19 protein uses two different mechanisms to cause retention of class I: there is a grab-and-hold mechanism dependent on the dilysine retention motif in the cytoplasmic tail of E19, and there is a second mechanism independent of the dilysine motif and associated with loss of tapasin binding to class I. Similarly, if retention of class I by *m152* is found to be tapasin-dependent, such retention could be caused by a grab-and-hold mechanism or by some other functional failure of tapasin.

If *m152* causes tapasin to retain class I by a grab-and-hold mechanism, then *m152*-mediated retention should be dependent on the ER retention signals in tapasin.

Deletion of the dilysine motif in the cytoplasmic tail of tapasin should cause tapasin to be exported from the ER. $Tpn^{-/-}$ cells can be transfected with tapasin lacking the dilysine motif. The grab-and-hold model predicts that *m152* should have no effect in these cells. In contrast, if transfection of $Tpn^{-/-}$ cells with tapasin lacking the dilysine motif restores the effects of *m152*, this will demonstrate that *m152* interferes with some other function of tapasin, such as bridging the connection between class I and TAP.

As discussed in Chapter 4, dissociation of TAP and tapasin is a possible mechanism for retardation of K^b by *m152* that may help explain *m152*'s long-lasting effects. Ziegler *et al.* reported that continuous expression of *m152* is not required for prolonged retention of class I (Ziegler et al., 2000). When *m152*-transfectant cells were treated with cyclohexamide, all detectable m152/gp40 was gradually degraded, but class I molecules were not released to be exported from the ERGIC. Based on these results, Ziegler *et al.* suggested that via some transient but undetectable interaction with class I, m152/gp40 catalyzed a permanent change in class I which made it incapable of being exported. An alternative hypothesis consistent with the results of Ziegler *et al.* is that *m152* causes some long-lived change not in class I but in the cellular machinery required for class I export (e.g. tapasin and TAP). This mechanism would account for the failure of m152/gp40 to coprecipitate with class I.

The cyclohexamide experiment of Ziegler *et al.* does not distinguish between an attack by *m152* on class I and an attack on cellular machinery because it relies on a pulse-chase method in which cyclohexamide was added during the chase. Thus no labeled class I was synthesized after the degradation of m152/gp40. To help determine whether or not the biochemical target of *m152* is class I itself, it will be useful to regulate

expression of *m152* and class I separately. This can be accomplished by transfecting cells with *m152* under the control of Tet repressor. Thus expression of *m152* can be transiently turned on, and then shut off without altering expression of class I. Under these conditions, cells can be pulse labeled after all the m152/gp40 has degraded in the lysososme. The model of Ziegler *et al.* predicts that class I synthesized after the degradation of m152/gp40 will be fully export-competent. In contrast, if class I is still retained in the ERGIC after complete degradation of m152/gp40, this will prove that class I is not the biochemical target of *m152*. If this proves to be the case, we can determine whether *m152* alters the association of TAP with tapsin. We can also look for evidence of modification of putative ER-to Golgi cargo proteins which are proposed to mediate selective class I export from the ER (Spiliotis et al., 2000).

Immune evasion and the *in vivo* CTL response.

If one looks at the CTL response to infected MEFs (e.g. Chapter 2, Figure 3), it seems that the combined effects of MCMV immune evasion genes completely block all CTL activation. This raises at least two questions. If the virus blocks CTL activation, where do anti-MCMV CTL come from? What is the benefit for the host to develop a CTL response which is seemingly useless against infected cells?

Given their known importance in other systems, it is likely that DCs are major contributors to CTL priming in primary MCMV infection. DCs could potentially prime the CTL response by two mechanisms. First, DCs may be infected with MCMV and may prime CTLs directly. This is possible because, in contrast to MEFs, MCMV-infected DCs can detectably activate CTLs in spite of partial inhibition by immune evasion genes (D.G.K. unpublished observation). Second, uninfected DCs may cross-prime CTLs by

taking up antigens from dead infected cells. It should be possible to distinguish the relative importance of these two routes experimentally. For example, B6 mice can be primed with either B6 or $K^{b-/-}D^{b-/-}$ DCs infected with wildtype MCMV. To prevent viral infection of APCs *in vivo*, DCs and recipient animals can be treated with gangcyclovir for the course of the experiment. If cross presentation is the primary mechanism of CTL priming, there should be no difference in the magnitude of the CTL response between animals primed with B6 or $K^{b-/-}D^{b-/-}$ DCs. If direct presentation by infected cells is the primary method of priming, then B6 DCs should be more effective than $K^{b-/-}D^{b-/-}$ DCs at priming CTLs. In addition, the relative importance to CTL priming of various immune evasion genes can be tested in the same system using relevant knockout viruses. I would expect that using wildtype virus, cross-priming would be the primary mechanism; but that using Δm4/m6/m152 virus, direct priming would be the primary mechanism.

Is it possible that the virus has a mechanism to inhibit cross-priming? In theory, the virus could either interfere with uptake of antigen or with antigen processing by DCs. It may be possible to answer this question using a panel of transposon knockout MCMVs. Transposon technology should soon make available a panel of viruses with single-gene knockous at each non-essential ORF. This panel can be screened as follows: each virus can be used to infect a population of $K^{b-/-}D^{b-/-}$ DCs. These infected cells can be mixed with B6 DCs and exposed to CTLs. CTL activation can be measured by cytokine production. If any knockout causes a significant increase in CTL activation over wildtype virus, the relevant gene would be a good candidate cross-priming-interference gene. In the absence of a panel of knockouts, this problem could also be addressed by

transfecting the $K^{b-/-}D^{b-/-}$ DCs with a model antigen, such as ovalbumin, and infecting them with wildtype MCMV.

The fact that at least some CTL priming does occur should not be seen as a failure of viral reproductive strategy. In fact, CTL control is probably an integral element of the infectious cycle, since a failure of CTL control would probably lead to super-virulence [as seen in $\beta 2m^{-/-}$ mice (Polic et al., 1996)]and thus impaired viral transmission to new hosts.

As noted in the introduction, transfer of MCMV-primed $CD8^+$ T cells is sufficient to control virus in most tissues but not in salivary gland (SG) (Jonjic et al., 1989; Jonjic et al., 1990). This suggests that CTL evasion genes may be particularly important for allowing virus to reproduce to high titre in this tissue. Three predicitons can be derived from this hypothesis. (1) Mutant viruses lacking one or more immune evasion gene will be specifically compromised in SG replication *in vivo* (*i.e.* have greater reduction in tissue titre in SG than in other tissues). Krmpotic *et al.* did not find a difference in SG titre between wildtype and Δm152 MCMV in neonatal mice [data not shown in (Krmpotic et al., 1999)]. This does not disprove the hypothesis that there may be an effect in adult mice or in mutant viruses deleted for other genes. (2) Mutant viruses lacking immune evasion genes will be compromised, in a CD8-dependent manner, in the rate of vertical transmission from mother to pups. (3) The *in vitro* effect of MCMV infection on SG epithelium will be more like that which it has on fibroblasts (complete immune evasion) than that which it has on macrophages (incomplete immune evasion).

A biochemical model for the interaction of *m4* and *m152*

In the preceding sections I have presented an array of hypotheses to explain the individual effects of *m152* and *m4*. These will need to be tested experimentally. For the purposes of discussion, I have put together a model which includes all of our experimental results and gives them all equal weight. Clearly many other models can be imagined in which some phenomena, such as the NP40-unstable interaction of m4/gp34 with class I, are not important. For the sake of argument I have assumed that all of the phenomena we have described are important to viral immune evasion. This is not the simplest possible model but it should be a good platform for discussion.

The model is presented in Figure 1. The main components are as follows.

1) m152/gp40 uses different mechanisms to alter transport of K^b and D^b. This proposal is inspired by the finding of Bennett *et al.* (Bennett et al., 1999) that the adenovirus E19 protein retains some class I molecules by direct interaction and retards others by blocking the interaction with tapasin. In this case, I propose that m152/gp40 directly attacks D^b and induces the permanent biochemical change suggested by Ziegler *et al.* (Ziegler et al., 2000). In contrast, I propose that m152/gp40 does not affect K^b directly but retards K^b export via an attack on TAP or tapasin which inhibits the association between them-- a consequence of this inhibition is delayed peptide loading and prolonged tapasin association.

Proposing two separate mechanisms for the action of *m152* is not parsimonious. However, there is certainly precedent for a single viral protein performing multiple immune evasion functions (Bennett et al., 1999; Carl et al., 2001; Tomazin et al., 1999).

This model is consistent with the total inhibiton of D^b function and partial inhibition of K^b function described in Chapter 2.

2)m4/gp34 alters peptide loading in a pre-Golgi compartment. In this model, m4/gp34 associates with K^b during its prolonged association with tapasin in NP40-unstable complexes. By virtue of this interaction m4/gp34 interferes with the quality-control functions of tapasin and prevents optimal peptide loading. This proposition could explain why K^b-restricted CTLs respond to targets infected with Δm4 but not wildtype MCMV.

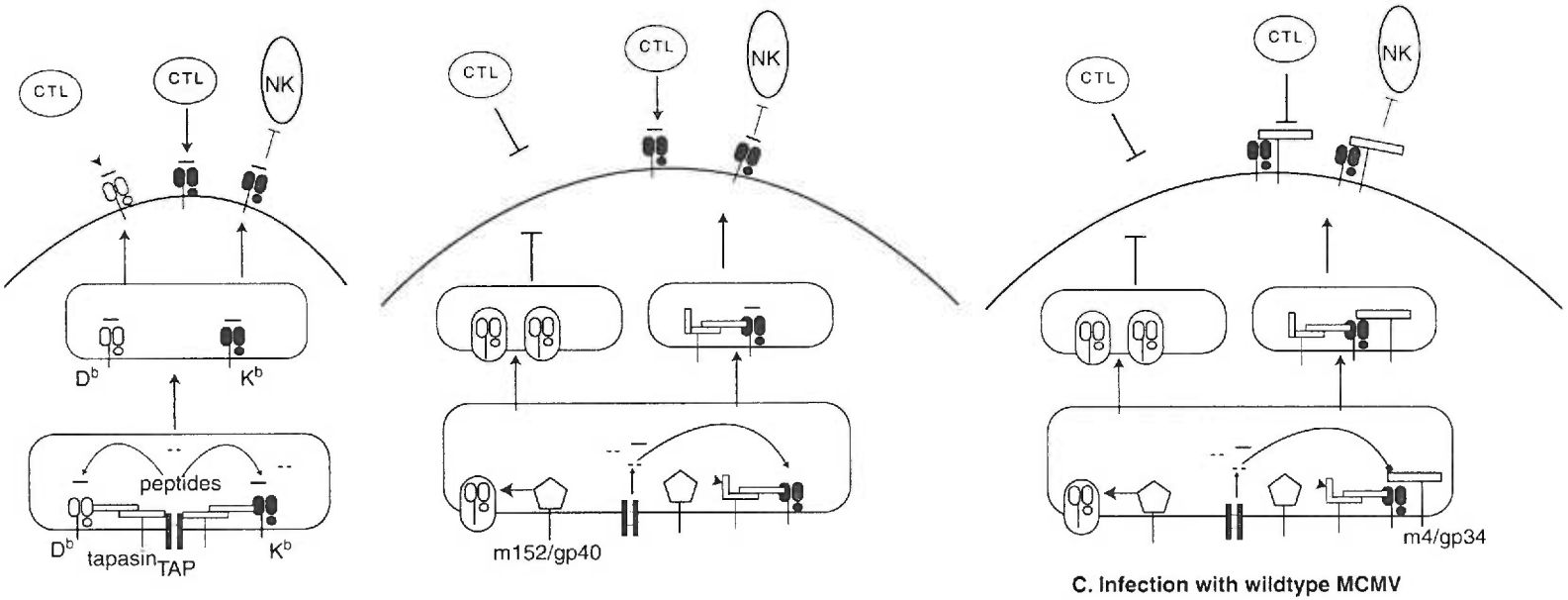
3) m4/gp34 stabilizes suboptimally loaded K^b and promotes its expression at the cell surface. As a result of suboptimal peptide loading, K^b would normally rapidly disappear from the cell surface, thus exposing infected cells to clearance by NKs. In the model shown, m4/gp34 prevents this by forming the NP40-stable interaction with K^b, which stabilizes K^b-β2m heterodimers and prevents their loss from the cell surface. Again, I have proposed that a single viral protein performs two different functions; in this case we have already demonstrated that the protein is involved in two biochemically distinct types of complexes (Chapter 3).

An interesting aspect of the model in Figure 1 is that under none of the three conditions shown are NK cells activated. This is because m4/gp34 destabilizes and "restabilizes" K^b in sequential steps. Therefore in order to test the hypothesis that m4/gp34 stabilizes suboptimally loaded K^b, it will be necessary to express *m4* in some cell line where K^b is already unstable, e.g. tapasin-deficient cells.

Conclusion

This dissertation describes a number of novel findings regarding the altered assembly and function of class I molecules in MCMV infected cells. In particular we

have advanced new evidence regarding the cooperative functions and biochemical mechanisms of different immune evasion genes. Our results are strengthened by the fact



A. Infection with Δm152/m4 MCMV

-both K^b and D^b are exported to the cell surface and activate CTLs.

-NK cells are inhibited by class I expression

B. Infection with Δm4 MCMV

-m152/gp40 alters D^b by transient interaction and causes its full retention in ERGIC.

-m152/gp40 does not interact with K^b but does atack tapasin and inhibit TAP-tapasin association, such that peptide loading and class I export are delayed.

-K^b eventually reaches the cell surface, where it is available to stimulate CTLs and inhibit NKs.

C. Infection with wildtype MCMV

-m152/gp40 alters D^b by transient interaction and causes its full retention in ERGIC.

-m152/gp40 does not interact with K^b but does alter tapasin.

-m4/gp34 forms NP40-unstableassociation with K^b and prevents loading of optimal peptide.

-Lack of optimal peptide would destabilize K^b and lead to NK activation, but m4/gp34 forms tight complexes with K^b which are expressed at the cell surface, K^b and thus inhibits NK activity.

Figure 1. Hypothetical cooperative mechanisms of immune evasion. The function and disposition of class I molecules are shown in the presence of (A) neither *m152* nor *m4*, (B) *m152* only, or (c) both *m152* and *m4*.

that we use primary, non-transformed cells, which are infected with MCMV and not transfected with individual genes. The experiments described in this chapter should advance our understanding of MCMV immune evasion another step.

Many broader questions, outside the scope of this project, remain. One is why MCMV and HCMV have developed divergent mechanisms to accomplish convergent functions. Why doesn't MCMV block TAP? Why doesn't HCMV degrade class I in the lysosome? Possible answers may lie in some intrinsic differences between mice and humans which make some molecules (e.g. TAP) more vulnerable to biochemical attack. Another possible reason could be the complex interaction of CTL and NK control, still poorly understood, which may make certain strategies, such as TAP blockade, more useful in humans than in mice.

Another question is whether all these interesting findings about immune evasion will lead to any new therapies against HCMV or other diseases. For example, developing antibody or peptide reagents which block UL16 might be clinically beneficial by preventing NK inhibition. We hope that a better understanding of antigen presentation can lead to improved therapeutic or prophylactic vaccines. With regard to altered class I assembly, however, the virus has put itself in a very good position by blocking antigen presentation at so many points; it is difficult to develop a vaccine to an antigen that isn't presented. Developing antiviral drugs always requires identifying biochemical targets essential for viruses and inessential for the host. CMV immune evasion proteins may present useful pharmaceutical targets: by blocking the function of US3 or US6, for example, a drug may be able to shift the balance between CTL control and viral escape. Standard pharmaceutical screening protocols may only identify target functions, such as

DNA polymerases, which are essential *in vitro*. It may be possible to design and screen new drugs for the ability to specifically limit viral replication in the presence of CTLs. Developing such drugs for MCMV immune evasion genes could provide both a useful model of clinical therapy and new experimental tools for basic research into viral immunity.

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