Evasion of CD8+ and CD4+ T Cell Recognition by Herpesviruses

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ABSTRACT

Infections with herpesviruses are endemic throughout much of the world and represent a major cause of morbidity and mortality in newborns and immunodeficient patients. Herpesviruses establish lifelong latent infections in their hosts that are characterized by periodic bouts of reactivation and viral shedding. As a strategy for viral persistence, many of the herpesviruses have evolved elaborate mechanisms to evade or suppress host immunity. In particular, the recognition of infected cells by T lymphocytes is a major target for viral interference, as these cells typically play a vital role in the clearance of viral infections. This thesis describes two mechanisms by which herpesviruses escape detection by T lymphocytes.

Herpes simplex virus (HSV) encodes an immediate early protein, ICP47, that has been shown to inhibit the MHC class I presentation of viral antigens to $CD8^+$ T lymphocytes. Previous observations in our laboratory demonstrated that ICP47 bound to the transporter associated with antigen presentation (TAP) and was capable of blocking the translocation of peptides into the ER. Using an in vitro system consisting of insect microsomes containing human TAP and recombinant ICP47 produced in bacteria, I was able to characterize the molecular mechanism by which ICP47 inhibited TAP. I found that ICP47 blocked peptide binding to TAP and bound tightly and specifically to TAP with a much higher affinity (5.2×10^{-8} M) than did peptides. Competition experiments using a complex library of over 2000 peptides revealed that in order to abolish the

binding of ICP47 to TAP, a 1000-fold molar excess of peptide was required. Peptides were also found to behave as competitive inhibitors of ICP47 binding to TAP. Together, these results demonstrate that ICP47 binds to a site which includes or overlaps the peptide-binding domain of TAP and, by doing so, precludes the binding of peptides to TAP and their subsequent translocation into the ER.

Human cytomegalovirus (HCMV) infects a wide variety of cells in vivo, many of which are capable of expressing MHC class II proteins, such as monocytes/macrophages, and are likely to elicit responses from antiviral $CD4^+$ T lymphocytes. Monocytes/macrophages are thought to play an important role in the lifecycle of HCMV by providing a reservoir for latent virus and a means for viral dissemination. HCMV is known to express several inhibitors of the class I presentation pathway, and thus, by analogy, it seemed likely that the virus would also encode inhibitors of the class II pathway. Characterization of cells infected with wildtype HCMV revealed that expression of class II proteins was dramatically reduced. Experiments with deletion viruses and transfected cells established that this effect was due to the HCMV early protein, US2. US2 was found to mediate the degradation of class II DR-α and DM-α chains by the proteasome. Expression of US2 in cells reduced or abolished their ability to present exogenous antigen to $CD4^+$ T lymphocytes. Thus, by blocking the presentation of class II antigens, US2 may allow HCMV-infected macrophages to remain relatively invisible to $CD4^+$ T lymphocytes, a property that would be critical following reactivation.

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LIST OF ABBREVIATIONS

A	deoxyadenylate
Ad	adenovirus
ATP	adenosine triphosphate
Bac	baculovirus
BSA	bovine serum albumin
C	deoxycytidylate
Ca	calcium
CD	circular dichroism
Cl	chloride
CMV	cytomegalovirus
CNS	central nervous system
Con A	concanavalin A
CPE	cytopathic effect
CTL	cytotoxic T lymphocyte
DMEM	Dulbecco's modified minimal essential medium
DNA	deoxyribonucleic acid
DOC	deoxycholate
EDTA	ethylenediaminetetraacetate
ER	endoplasmic reticulum
FBS	fetal bovine serum

FcR	Fc receptor
G	deoxyguanylate
GST	glutathione-S-transferase
HCMV	human cytomegalovirus
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
hr	hour
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
^{125}I	radioisotope of iodine
IC_{50}	inhibitor concentration at 50% activity
ICP47	infected cell protein-47 (product of the immediate early gene US12)
IFN-γ	interferon gamma
IgG	immunoglobulin G
K	potassium
K_d	equilibrium dissociation constant
kbp	kilobase pairs
LATs	latency associated transcripts
μCi	microCuries
μg	microgram
μl	microlitre

μM	micromolar
M	molar
MAb	monoclonal antibody
MHC	major histocompatibility complex
min	minute
ml	millilitre
mM	millimolar
MOI	multiplicity of infection
mRNA	messenger RNA
N	asparagine
Na	sodium
NP40	Nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming unit
PMSF	phenylmethylsulfonyl fluoride
PNS	peripheral nervous system
rICP47	recombinant ICP47
RNA	ribonucleic acid
rpm	revolutions per minute

^{35}S	radioisotope of sulfur
SDS	sodium dodecyl sulphate
T	deoxythymidylate
TAP	transporter associated with antigen presentation
TGN	trans-Golgi network
Tris	tris(hydroxymethyl)aminomethane
TX-100	Triton X-100
U_L	unique long
U_S	unique short
VV	vaccinia virus
w/v	weight per unit volume
ZL_3VS	carbonylbenzyl-leucyl-leucyl-leucyl-vinylsulphone

PREFACE

The work presented in this dissertation was performed by the author under the supervision of Dr. David C. Johnson in the program of Molecular Microbiology and Immunology at Oregon Health Sciences University. The data in this thesis are presented in three chapters which have been published in peer-reviewed journals. Chapter 2 ("Stable binding of the herpes simplex virus protein ICP47 to the peptide binding site of TAP") was published in the EMBO Journal (15: 3256-3266, 1996). This paper describes the molecular mechanism by which ICP47 inhibits the TAP-mediated translocation of peptides into the ER. Chapter 3 ("Herpes simplex virus type 2 ICP47 inhibits human TAP but not mouse TAP") was published in The Journal of Virology (72: 2560-2563, 1998) and extends the previous observations by addressing the species specificity of ICP47 from both HSV serotypes 1 and 2 for TAP. Chapter 5 ("Cytomegalovirus US2 destroys two components of the MHC class II pathway, preventing recognition by $CD4^+$ T cells") was published in the journal Nature Medicine (5:1039-1043, 1999) and represents the first description of a viral inhibitor of the MHC class II presentation pathway. Chapters 2 and 3 are preceded by an Introduction which describes the interaction of herpes simplex virus with the host immune system. Chapter 5 is preceded by a separate Introduction which conveys similar material regarding human cytomegalovirus. Following the chapters is a Discussion which summarizes the salient results presented in the thesis and attempts to address several issues raised by my research.

CHAPTER 1

HERPES SIMPLEX VIRUS
INTRODUCTION

1.0 INTRODUCTION

Through millions of years of coexistence with their hosts, viruses have continually evolved to exploit various aspects of host biology. The survival of a virus depends on its successful transmission from one host to the next before it is destroyed by the host immune system. In response to this selective pressure, many viruses have evolved sophisticated mechanisms to evade or suppress host immunity. The primary goal of these evasion strategies is not to overwhelm the host, as this would be counterproductive, but instead, to simply offer the virus a brief window of opportunity to replicate and produce infectious progeny. In most cases, as the mature immune response intensifies, the host is able to control and limit viral replication.

One of the most successful families of viral pathogens is the *Herpesviridae.* Herpesviruses establish lifelong latent infections within their hosts and periodically, reactivate from the latent state to produce infectious progeny for transmission to new hosts. Reactivation takes place in the face of a fully primed immune response, and thus, it is not surprising that these viruses have evolved elaborate strategies to deal with host immunity. The aim of this thesis is to describe in detail two such immune evasion strategies and, hopefully, offer insight into the virus-host relationship. Chapters 2 and 3 will describe the inhibition of the major histocompatibility complex (MHC) class I presentation pathway by the herpes simplex virus (HSV) protein ICP47, while Chapter 5 will deal with the identification and characterization of the first viral inhibitor of the MHC class II presentation pathway, the human cytomegalovirus (HCMV) protein US2.

1.1 *Herpesviridae*

The *Herpesviridae* is comprised of an ancient family of phylogenetically related viruses that are found ubiquitously in nature and are responsible for significant disease in a variety of species ranging from fish to mammals (reviewed in Roizman, 1996). There are over 100 herpesviruses characterized to date, 8 of which are human pathogens: herpes simplex virus type 1 and type 2 (HSV-1, HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), and human herpesviruses 6,7 and 8 (HHV-6, HHV-7, HHV-8). Herpesvirus infections typically cause relatively benign or asymptomatic disease in healthy individuals, but can become life-threatening in newborns and immunodeficient patients.

All members of the *Herpesviridae* share certain structural and biological properties. Herpesviruses are large, lipid-enveloped viruses with a linear, double-stranded DNA genome that ranges in size from 125-250 kilobase pairs (kbp) in length (reviewed in Roizman, 1996). The virions of herpesviruses can often be distinguished from those of other viruses due to their characteristic architecture and appearance. All mature herpesvirions consist of a dense, fibrillar DNA core surrounded by a proteinaceous capsid shell that is icosahedron in shape and is composed of 162 interlocking capsomeres. This nucleocapsid structure is then encompassed by an amorphous, electron-dense matrix called the tegument which is in turn enveloped by a host cell-derived lipid bilayer containing several viral glycoprotein spikes on its surface. The diameter of the complete virion particle ranges from 120-300 nm (reviewed in Roizman, 1996). In addition to these structural similarities, the herpesviruses also share four significant biological

properties including: i) expression of a large array of DNA replication and modifying enzymes, ii) a common mode of replication and capsid assembly in the host cell nucleus, iii) irreversible destruction of the host cell upon release of progeny virus and iv) the ability to establish latency in their natural hosts (reviewed in Roizman, 1996).

The *Herpesviridae* family can be further subdivided into three distinct classes based on tissue tropism, reproductive lifecycle and site of latency. These subfamilies are designated the *alphaherpesvirinae*, the *betaherpesvirinae* and the *gammaherpesvirinae*. Members of the *alphaherpesvirinae* are characterized by a broad host range, a relatively short reproductive lifecycle and the ability to establish latent infections in sensory ganglia of the nervous system (reviewed in Roizman, 1996). Several of the most prevalent human viral pathogens are included in the *alphaherpesvirinae* such HSV-1, HSV-2 and VZV. Infections with HSV-1 or HSV-2 lead to oral and genital herpes, while VZV is the causative agent of chicken pox and shingles.

The *betaherpesvirinae* display a more restrictive host range, often limited to the genus or species of the natural host, and their reproductive lifecycle progresses rather slowly, on the order of days to weeks (reviewed in Roizman, 1996). Infection with betaherpesviruses often results in the enlargement of the infected cell, known as cytomegalia, characterized by the formation of dense DNA inclusion bodies in the nucleus and cytoplasm. Tissues that harbor latent betaherpesvirus infections include the spleen, kidneys, secretory glands and blood. HCMV, which is a life-threatening pathogen in AIDS patients and organ transplant recipients, HHV-6, the causative agent of the childhood disease roseola

infantum, and HHV-7 are members of the *betaherpesvirinae*.

The *gammaherpesvirinae* constitute a more diverse group of viruses with variable replication and pathogenic properties. Members of this subfamily share a limited host range and a tropism for B or T lymphocytes. Lymphoid tissue often represents the latent site of infection for these viruses (reviewed in Roizman, 1996). Members of the *gammaherpesvirinae* that infect humans include EBV, which causes infectious mononucleosis and Burkitt's lymphoma, and the more recently identified HHV-8, which has been implicated as the etiological agent of Kaposi's sarcoma (Cesarman et al., 1995; Chang et al., 1994).

1.2 Herpes Simplex Virus

HSV infections have plagued humans throughout natural history and have been recorded as early as ancient Greek times (Wildy, 1973). These early accounts of infection were merely descriptions of cutaneous lesions such as lip ulcers or blisters. The infectious nature of HSV was not discovered until the early 20th century, when two independent researchers, Lowenstien (1919) and Gruter (1920), demonstrated that rabbits could be infected with the vesicular fluid from human herpetic lesions. During the next four decades, with the isolation of HSV and the development of animal and cell culture systems to propagate the virus, major advances were made in characterizing fundamental properties of HSV such as host range, lifecycle and disease pathogenesis. More recently, the introduction of molecular biology to the study of HSV has enabled researchers to dissect in great detail the role of individual viral gene products in HSV replication and disease. Today, HSV remains one of the most intensively studied of all viruses and is recognized as the etiological agent of several diseases of the mouth, genitals,

eyes and skin (reviewed in Whitley, 1996).

1.2.1 Structure

Two serotypes of HSV have been identified, HSV-1 and HSV-2, which share considerable sequence homology (approximately 50% identity at the genomic level) and can be distinguished on the basis of restriction fragment length polymorphism (Buchman et al., 1980; Kieff et al., 1971). The HSV genome consists of a linear, double-stranded DNA molecule that is approximately 152 kbp in length and may code for 79 or more genes (reviewed in Ward and Roizman, 1994). The organization of the HSV genome is quite distinct among DNA viruses in that two unique stretches of DNA are flanked by inverted repeat sequences. The two segments of the genome are designated the unique long region (U_L), of approximately 108 kbp, and the unique short region (U_S), of approximately 13 kbp (reviewed in Roizman and Sears, 1996). During productive infection, these two segments of DNA have been shown to invert relative to one another, generating four possible isomeric forms of the complete DNA genome (Delius et al., 1976; Hayward et al., 1975). The HSV DNA replication machinery is thought to mediate this inversion process through the homologous recombination of genomic DNA at the inverted repeat sequences (Mocarski and Roizman, 1982; Weber et al., 1988).

1.2.2 Replication Cycle

A striking feature of HSV infection is the ability of the virus to rapidly and completely overrun host cell machinery for its own use. To initiate infection, HSV first attaches to host cells through interactions with heparin sulphate and cell surface receptors, such as the mannose-6-phosphate receptor (M6R) (Brunetti et

al., 1994) or the herpesvirus entry mediator (HVEM) (Montgomery et al., 1996), then fuses with the host cell plasma membrane in a pH-independent manner (Koyama and Uchida, 1987; Morgan et al., 1968). Immediately following entry of virus into susceptible host cells, virion structural components are released into the cytosol which drastically alter cellular function. The virion host shutoff protein (Vhs) nonspecifically degrades cellular mRNA, effectively blocking the synthesis of host proteins, thereby setting the stage for viral gene expression (Fenwick and Everett, 1990; Read and Frenkel, 1983; Smibert et al., 1992). Another structural component of the virion, VP16, is a potent transactivator and, shortly after its delivery into the nucleus, directs the transcription of the first wave of viral genes, the immediate early (IE) or α genes (Batterson and Roizman, 1983; Campbell et al., 1984; Honesss and Roizman, 1974). Viral gene expression during HSV infection is coordinately regulated in a temporal cascade. Several of the IE genes (ICP0, ICP4, ICP22 and ICP27) regulate the transcription of the next series of genes, known as the early (E) or β genes (Roizman and Sears, 1996). Although the majority of the E genes code for proteins or enzymes involved in nucleic acid metabolism and synthesis, a few envelope glycoproteins such as glycoprotein E (gE) and glycoprotein D (gD) are also expressed during this time (reviewed in Wagner, 1985). Unlike IE genes, E genes are not expressed in a synchronized fashion, but instead, display a continuum of induction extending throughout the entire E phase. Following the replication of genomic DNA, expression of the late (L) or γ genes is induced. The products of this final wave of genes are predominantly virion structural components such as envelope glycoproteins, capsid and tegument proteins, that will comprise the viral particle (Roizman and

Sears, 1996). The expression of these structural proteins results in the assembly of capsids in the nucleus, followed by the packaging of viral genomic DNA. These nucleocapsids then acquire a lipid envelope by budding through the inner nuclear membrane, which contains immature forms of the viral envelope glycoproteins (Morgan et al., 1959; Schwartz and Roizman, 1969; Torrisi et al., 1992). The maturation and subsequent egress of these newly enveloped particles remains one of the most controversial topics in the HSV replication field. Essentially, two models have been proposed to account for this process. The first ER/Golgi model suggests that enveloped virions traverse the perinuclear space and enter the default secretory pathway (Campadelli-Fiume et al., 1991; Johnson and Spear, 1982; Morgan et al., 1959; Schwartz and Roizman, 1969; Torrisi et al., 1992). As virions transit through the ER and Golgi compartments, immature glycoproteins acquired at the inner nuclear membrane become processed to mature forms (Johnson and Spear, 1982). Ultimately, mature virions are released from the cell by exocytosis. The second model proposes that following envelopment at the inner nuclear membrane, viral particles within the perinuclear space de-envelop by fusing with the outer nuclear membrane, thereby depositing naked nucleocapsids into the cytosol (Gershon et al., 1994; Jones and Grose, 1988; Stackpole, 1969). These nucleocapsids then acquire their final lipid envelope by budding into the Golgi apparatus or trans-Golgi network (TGN), where mature forms of the envelope glycoproteins have accumulated (Gershon et al, 1994; Jones and Grose, 1988). Vesicular traffic then delivers the mature virions to the plasma membrane for release from the cell. Regardless of the route of viral egress, production of infectious virus is quite efficient, often reaching 50,000 to

200,000 virions per infected cell (Corey and Spear, 1986). The entire infectious cycle typically extends from 24 to 48 hours, often resulting in the lysis of the infected cell following the release of progeny virus.

In contrast to the lytic phase, however, latent infections with HSV exhibit a severe reduction in the expression of viral gene products. Under appropriate conditions, HSV establishes latency in ganglionic neurons and maintains a non-infectious, quiescent state (Hill, 1981; Nesburn et al., 1972; Stevens and Cook, 1971). The HSV genome in latently infected neurons is thought to exist as a circular episome, similar in structure to eukaryotic chromatin, with approximately 10-1000 copies per cell (Deshmane et al., 1989; Ho, 1992; Mellerick ad Fraser, 1987). Viral gene expression during latency is limited to three overlapping, antisense transcripts which map to the ICP0 gene (Spivak and Fraser, 1987; Stevens et al., 1987; Wagner et al., 1988). These latency associated transcripts (LATs) are thought to play important roles in the induction and maintenance of the latent state, but the precise mechanisms by which these events occur remain poorly understood.

1.2.3 Pathogenesis

Infections with HSV are widespread and have been reported in both developed and underdeveloped countries alike. Current estimates for North America predict that by early adolescence 70-80% of the population will become infected with HSV-1, while for HSV-2, it is thought that approximately 30-40 million adults in North America are presently infected (Magder et al., 1989; Whitley, 1996). Animal vectors for HSV infections have not yet been identified and, therefore, humans remain the sole reservoir and host for the virus.

Transmission of HSV relies upon direct person to person contact. The most common vehicle of HSV transmission is vesicular fluid released from herpetic lesions, but oral and genital secretions that harbor virus as a result of asymptomatic shedding may also play a significant role in spread of infection (Whitley, 1996). Primary HSV-1 infections usually occur during the first decade of life, most likely as a result of casual contact, while primary HSV-2 infections are usually transmitted perinatally at birth or during adulthood through sexual contact (Liesegang, 1992; Whitley, 1996).

Initial exposure to HSV at mucosal membranes or cutaneous abrasions permits entry of virus and the initiation of replication in susceptible cells. Primary infections are often asymptomatic, but in some cases, sufficient replication of virus may occur in superficial tissues to cause the formation of vesicular lesions or "blisters" (reviewed in Corey and Spear, 1986; Whitley, 1996). After several rounds of productive replication at the mucocutaneous site, progeny virus spreads to neighboring cells, including the fibrillar projections of sensory nerves. Virus is then transported via retrograde axonal flow along innervating axons until sensory ganglia become infected (Cook and Stevens, 1973; Hill et al., 1972; Kristensson et al., 1971). Replication of virus may occur in the ganglia and contiguous neural tissue for a brief period, but usually within days of the initial inoculation, HSV establishes latency in neuronal cell bodies. During latent infection, the virus remains quiescent in ganglionic neurons and awaits the opportunity to initiate the next recrudescent infection. This latent state may continue for days, months or years, but periodically, in response to stress, ultraviolet (UV) light or other unknown factors, latent HSV reactivates, travelling

back through axons and reinfecting cells at the original site of entry (reviewed in Ho, 1992; Stevens, 1989). Recurrent infections are often less severe than the primary incident, but in either case, lesions usually resolve within 1-3 weeks (Corey and Spear, 1986; Whitley, 1996). The location of the initial exposure also defines the route of viral spread and the site of latency. Infections of the oropharynx or mouth lead to latent infection of the trigeminal ganglia, whereas genital infections often lead to latency in the dorsal root ganglia (Baringer and Swoveland, 1973; Liesegang, 1992). Both HSV-1 and HSV-2 show a degree of tissue tropism, with the most common sites of infection being the mouth and genitalia, respectively. However, this tropism is not exclusive, in that either virus can infect virtually any mucosal surface.

Although the majority of HSV infections do not pose a serious clinical threat, in some cases, HSV can cause severe disease, often resulting in blindness or death. Viral infections of the eye are typically caused by HSV-1 and are second only to trauma as the major cause of corneal blindness in the United States (Kaufman, 1978; Mader and Stulting, 1992). Exposure of the cornea to HSV can result in several manifestations of disease including self-limiting blepharitis, conjunctivitis or epithelial keratitis (reviewed in Mader and Stulting, 1992). Active replication of HSV within the anterior stroma of the cornea often leads to the migration of host inflammatory or immune mediators to the site of infection in an attempt to limit viral spread (Babu et al., 1996; Liu et al., 1996; Metcalf et al, 1979; Meyers-Elliot and Chitjian, 1981). A robust immune response, however, often results in permanent tissue damage to the cornea causing stromal keratitis or disciform edema (reviewed in Mader and Stulting, 1992). Progressive disease

often results in scarring, ulceration or opacification of the cornea leading to permanent visual loss and blindness.

Encephalitis is a life-threatening disease that is caused by spread of HSV to the central nervous system (CNS) and eventually the brain. Most infections resulting in encephalitis are mediated by HSV-1 rather than HSV-2 and in 70% of the cases the disease is fatal (Roizman and Kaplan, 1992). Early diagnosis of HSV encephalitis is critical, as intravenous treatment with acyclovir has been shown to significantly reduce the mortality and morbidity associated with the disease (Whitley et al., 1986). Two theories have been proposed to account for the spread of HSV to the brain. In the first model, HSV is thought to gain access to the CNS by transneuronal spread from the trigeminal system to the brainstem nuclei (raphe and locus ceruleus) with known connections to temporal regions (Margolis et al., 1989; Ugolini et al., 1987). Alternatively, the recent isolation of HSV DNA from the brains of autopsied adults suggests that neurotropic strains of HSV may establish latent infections within the CNS itself (Fraser et al. ,1981). In either case, reactivation of HSV leads to replication of virus in the temporal lobes of the brain resulting in massive neuronal and non-neuronal tissue damage, progressive loss of neurologic function, and ultimately, death (reviewed in Roizman and Kaplan, 1992).

Immunocompromised patients and neonates are particularly susceptible to HSV infection because they lack sufficient immunity to fend off disease. HSV infection of patients immunosuppressed for organ transplantation or with AIDS may result in progressive disease involving the CNS, the respiratory tract, the esophagus, or even the gastrointestinal (GI) system (reviewed in Whitley, 1996).

Recurrent HSV infections in these patients also occur more frequently, and often display enhanced severity and duration of disease. The developing immune system of the newborn is exceptionally vulnerable to HSV infection and disease in this case can have catastrophic results. Depending on the time of transmission, intrauterine, intrapartum or postpartum, presentation of neonatal infection may show several clinical manifestations. Most severe are the systemic intrauterine or intrapartum infections which may lead to congenital defects such as microcephaly or hydranecephaly, major organ dysfunction, neurological damage, encephalitis or death (reviewed in Corey and Spear, 1986; Whitley, 1996).

1.3 Immune Response to HSV Infection

The ability of HSV to establish and maintain a lifelong infection within the host is due in large part to the unique interaction of the virus with the host immune system. During the primary exposure to HSV, the virus encounters a naive immune response which offers minimal protection against infection. In most cases, viral replication ensues and is only adequately controlled when the adaptive or specific immune response is activated. Even under these circumstances, HSV is not completely cleared from the host but remains dormant in latently infected neurons. Periodically, latent HSV reactivates and goes on to produce an active secondary infection. A remarkable feature of reactivation is the ability of the virus to replicate in the presence of a fully primed immune response.

Studies of the immune response to HSV infection have largely used the mouse as a convenient and inexpensive animal model. Unfortunately, mice do not represent a natural host for HSV, and thus, several manifestations of the infection and the resulting immune response differ from that in humans. Although the

outcome of herpetic disease varies with the mouse strain, the dose of virus and the route of inoculation, mice infected with HSV often display an increased susceptibility to CNS infection and the development of encephalitis (reviewed in Kern, 1987; Knotts et al., 1974). In addition, following primary HSV infection in mice, spontaneous recurrent infections from the latent state rarely occur as they do in humans (Roizman and Sears, 1996). Certain parameters of the immune response to HSV infection also differ in mice and humans. The predominant HSV-specific cytotoxic T lymphocyte (CTL) population in mice is $CD8^+$ and restricted by MHC class I antigens (Martin and Rouse, 1990; reviewed in Mester and Rouse, 1991). In humans, the reverse seems to be true, with the majority of anti-HSV CTL being $CD4^+$ and restricted by MHC class II antigens (Schmid, 1988; reviewed in Schmid and Mawle, 1991; Yasukawa and Zarling, 1984a; Yasukawa and Zarling, 1984b). This disparity suggests that the relevant antigens recognized by murine and human T cells during the course of HSV infection may differ drastically. Due to the inherent incompatibilities between the host species, conclusions drawn from mouse studies must be interpreted with caution or misleading results could be obtained. Since the mouse animal model is flawed, several researchers have expanded their studies to include other animal models such as primates, pigs, rabbits and guinea pigs with varying degrees of success. In particular, both rabbits and guinea pigs represent more suitable models for the study of host immunity during recurrent HSV infections as both these animals are capable of reactivating virus from the latent state (Nesburn et al., 1967; Stanberry et al., 1982). Collectively, however, these various animal studies have begun to yield valuable insight into the immune response against HSV infection. Moreover,

when these observations are compared and correlated with the limited number of human studies, similarities begin to emerge which define the anti-HSV immune response.

1.3.1 Innate Immunity

The immune response to HSV infection involves all major branches of the host immune system including innate and adaptive, humoral and cellular responses. During the primary exposure to HSV, the innate or non-specific immune response represents the first line of defense against viral infection. The first barrier the virus encounters is the protective mucosal membrane that covers the surfaces of the mouth and genitalia. Mucosal membranes are bathed in secretions that offer both mechanical and chemical barriers to infection. Most notably, mucosal secretions contain non-specific, low-avidity antibodies and complement factors which function to neutralize invading pathogens (reviewed in Janeway and Travers, 1997; Roitt, 1994). These defense mechanisms, unfortunately, provide minimal protection against primary HSV infection and typically, the virus gains access to the underlying epithelium. Cells infected with HSV begin to produce and secrete various cytokines which sensitize the immune response to the ongoing viral infection. The best characterized HSV induced cytokines are the type 1 interferons (IFNs), IFN-α and IFN-β (Engler et al, 1981; Kirchner et al, 1983; Hendricks et al., 1991). Replication of viruses or the presence of double-stranded RNA in infected cells leads to the induction and secretion of the type 1 IFNs into the surrounding milieu. The initial infected cell and surrounding uninfected cells express receptors for the type 1 IFNs which bind the cytokine and initiate a cascade of antiviral effects. The most potent antiviral

mechanisms include the induction of ribonuclease L (RNAse L), which degrades viral RNA, PKR protein kinase, which blocks protein synthesis, and upregulation of antigen presentation machinery for effective T cell recognition (reviewed in De Maeyer and De Maeyer-Guignard, 1998). Several studies have demonstrated an important role for the type 1 IFNs in combatting HSV infection. Treatment of mice with neutralizing antibodies specific for IFN α/β markedly increased the severity of HSV infection in these animals (Gresser et al., 1976; Kunder et al., 1993; Wrzos et al., 1986). Similar results were also observed with knockout mice lacking the IFN-AR1 subunit of the type 1 IFN receptor. In this case, replication of HSV exceeded three orders of magnitude above control levels resulting in the death of the infected animals (Lieb et al., 1999; Muller et al., 1994). Depending on the cell type infected, additional cytokines such as macrophage chemoattractant and activating factor (MCAF or MCP-1), tumor necrosis factor-α (TNF-α) or interleukin-8 (IL-8) may also be secreted leading to the induction of inflammatory acute phase proteins and migration of cellular immune mediators to the site of infection.

Natural killer (NK) cells and macrophages, both components of the innate cellular response, have been shown to play an important role in the early containment of HSV infection. NK or lymphokine-activated killer (LAK) cells have the capacity to interfere with viral replication by releasing various cytokines with antiviral activity, the most prominent being IFN-γ (a type II IFN), or by directly lysing infected cells (reviewed in Karre, 1993; Frederick et al., 1997). NK and LAK cell populations exhibit very limited clonal diversity and therefore, do not express antigen specific receptors, but must recognize their infected targets

through other means. NK cell recognition is thought to be mediated by both inhibitory and stimulatory signals. Normal surface expression of MHC class I proteins delivers an inhibitory signal via the killer inhibitory receptor (KIR) (reviewed in Colonna, 1997; Moretta et al., 1996) and thus, aberrant or low expression of class I, as is the case with HSV infection, results in the destruction of the infected cell (Fitzgerald-Bocarsley et al., 1991; Kaufman et al., 1992). Immunodepletion of NK cells in mice using anti-NK1.1 antibodies makes these animals much more susceptible to HSV infection resulting in increased severity and duration of herpetic disease (Bukowski and Welsh, 1986; Habu et al., 1984). Moreover, two well documented cases exist in which patients lacked functional NK cells with no other apparent immune defects. Both patients harbored multiple persistent herpesvirus infections, including HCMV, VZV and HSV, with frequent and severe recurrences (Biron et al., 1989; Jawahar et al., 1996). Stimulated NK cells produce large amounts of IFN-γ and this cytokine in conjunction with other stimuli leads to the potent activation of macrophages. Macrophages are vigorous phagocytes with the ability to engulf and destroy virus particles and infected cells (Keller et al., 1994; reviewed in Mosser, 1994). Activated macrophages are relatively resistant to infection with HSV, and thus, the phagocytosis of infectious virus may limit viral replication by reducing the effective dose of virus (Hendrazk and Morahan, 1994). In mice, depletion or activation of macrophages leads to increased or decreased severity of primary HSV infection, respectively (Kodukula et al., 1999; Pinto et al., 1991; Zawatsky et al., 1982). However, adoptive transfer or depletion of specific lymphocyte populations indicate that NK cells, macrophages and other mechanisms of innate immunity are insufficient to control

HSV infections (Nash et al., 1981; Nash et al., 1987). The innate immune response, nonetheless, does serve two important roles in combatting HSV infection: 1) innate mechanisms sufficiently delay infection allowing the adaptive immune response to develop and 2) innate cellular mediators secrete cytokines and present viral antigens which help orchestrate the mounting immune response. Macrophages and dendritic cells belong to a subset of cells known as professional antigen presenting cells (APCs) and these cells can process and present antigen to both $CD8^+$ and $CD4^+$ T lymphocytes (reviewed in Janeway and Travers, 1997). The phagocytosis of virus particles or infected or apoptotic cells by these APC may play a prominent role in priming the adaptive immune response to specific HSV antigens.

1.3.2 Adaptive Immunity

The adaptive or specific immune response to HSV is mediated by both humoral and cellular components. HSV-specific antibodies and the classical complement cascade comprise the arms of humoral immunity. Production of HSV-specific antibodies by B cells in response to primary infection may take from 5-7 days to reach appreciable levels in the blood (Worthington et al., 1980; Zawatsky et al., 1981). By this time, HSV infection may be well established. The ability of HSV to spread directly from cell-to-cell also provides very little opportunity for antibody- or complement-mediated clearance of virus. During direct cell-to-cell spread of HSV, particles are not exposed to the extracellular environment, and thus, remain inaccessible to antibody and complement factors. The inability of humoral immunity to protect individuals from recurrent HSV infections or the lack of correlation between disease severity and neutralizing antibody titres may stem

largely from this fact (Kahlon and Whitley, 1988; Whitley et al., 1980; Whitley et al., 1988). During reactivation, spread of virus through axons and mucosal epithelium also occurs through the direct cell-to-cell mode. Indeed, reactivation of HSV occurs in the presence of high titres of neutralizing antibody (Kohl, 1992; Zweerink and Stanton, 1981). The main benefit of humoral immunity, then, may lie in the prevention of reinfection with other strains or serotypes of HSV. Most notably, prior infection with HSV-1 has been shown to provide a somewhat protective effect on the acquisition of HSV-2 (Corey et al., 1981; Kaufman et al, 1973).

The second component of the adaptive immune response is cellular immunity, and in both mice and humans, specific cell-mediated immunity is most important in clearing ongoing HSV infections (Corey and Spear, 1986). T lymphocytes are divided into two distinct populations based on the expression of CD8 or CD4 accessory molecules. $CD8^+$ T lymphocytes are cytotoxic in nature and destroy infected targets on contact. $CD4^+$ T lymphocytes display a more diverse range of effector functions, but generally, these cells either stimulate B cells to produce antibody, activate macrophages and NK cells or lyse infected targets (reviewed in Janeway and Travers, 1997; Roitt, 1994). Studies in mice and humans indicate that both $CD8^+$ and $CD4^+$ populations are critical for the clearance of HSV infection. In mice depleted of $CD8^+$ T cells and then infected with HSV, neural spread of virus was significantly increased, a high proportion of ganglionic neurons were killed and clearance of virus was severely impaired (Simmons and Tscharke, 1992). In other studies, adoptive transfer of draining lymph node cells enriched for $CD8^+$ T lymphocytes was shown to protect

syngeneic recipient mice against potentially lethal HSV infection (Larsen et al., 1983; Simmons et al., 1992). Equally convincing evidence also supports a role for $CD4^+$ T cells in the elimination of HSV infection. Mice immunodepleted of $CD4^+$ T lymphocytes cannot clear HSV infections from the skin (Nash et al., 1987) and $CD4^+$ T cell-deficient AIDS patients also fail to resolve cutaneous herpetic lesions (Mann et al., 1984; Whitley, 1996). In addition, adoptive transfer of HSV-immune $CD4^+$ T cells into naive mice has been shown to confer antiviral immunity upon challenge (Leung et al., 1984; Nagafuchi et al., 1982; Nash and Gell, 1983). In healthy individuals with normal $CD8^+$ and $CD4^+$ T cell responses, HSV lesions are typically cleared within 1-3 weeks, at the peak of the adaptive immune response (Corey and Spear, 1986; Whitley, 1996). Although there are no apparent signs or symptoms of the current infection, HSV persists in latently infected neurons. Latent infection of neurons goes largely unnoticed by the host immune response for two primary reasons. First, during latent infection with HSV, virtually all viral gene expression is turned off and thus, no detectable antigens are produced within the infected cell (Ho, 1992; Stevens, 1989). Second, the sensory ganglia and surrounding neural tissue often represent an immunoprivileged site, in which host immune responses are drastically limited. Neurons do not express appreciable levels of MHC class I proteins and immune effector cells are often restricted from entering these sites (Lampson et al., 1983; reviewed in Rall, 1998). Thus, during latency, HSV avoids most, if not all, contact with the host immune response. However, under the appropriate conditions, usually a mild depression in host health, HSV reactivates from the latent state, travelling back through axons and reinfecting cells at the original site of entry. This represents a rather perplexing

feature of the HSV lifecycle; even in the face of a fully primed immune response, complete with memory B and T cells poised for viral destruction, HSV has the ability to reactivate and establish an active recurrent infection. The capacity of HSV to replicate in this hostile environment is probably due in part to a number of immune evasion strategies employed by the virus.

1.4 Antigen Recognition by CD8$^+$ T Lymphocytes

Since the primary focus of my early research dealt with the inhibition of CD8$^+$ T cell recognition by HSV ICP47, the following sections will describe the activation of CD8$^+$ T cells and the presentation of antigens by the MHC class I pathway.

1.4.1 CTL Activation

Detection of infected cells by CD8$^+$ T lymphocytes is mediated through the recognition of viral peptides displayed on cell surface MHC class I molecules (reviewed in York and Rock, 1995). Expression of MHC class I proteins is ubiquitous, and thus, CTL can scan virtually every cell in the body for the presence of viral antigens. T cell receptors (TCRs) expressed on the surface of CTL have the capacity to bind to an almost infinite array of peptides presented in the context of class I molecules. The specific recognition of peptide/MHC complexes by the TCR provides the initial signal for the activation of naive CTL (reviewed in Janeway and Travers, 1997; Roitt, 1994). The second signal comes from the binding of co-stimulatory molecules, such as B7.1 or B7.2, on the surface of infected cells to CD28 on the CTL. Expression of co-stimulatory molecules is generally limited to APCs and is upregulated in these cells in response to bacterial or viral infection (reviewed in Greenfield et al., 1998). Simultaneous delivery of

both signals results in the potent activation of CTL and the induction of specific cytolytic effector functions. Most importantly, cytotoxins such as perforin and granzyme are released from secretory granules within the CTL resulting in the lysis of infected cells (reviewed in Janeway and Travers, 1997; Roitt, 1994). Activated CTL also secrete various antiviral cytokines such as IFN-γ, TNF-α and TNF-β that contribute to the mounting immune response. Recognition of antigen by CTL in the absence of co-stimulatory signals often leads to a state called anergy, in which the CTL can no longer become activated. Antigens recognized in this manner are typically self-antigens and thus, co-stimulation provides a means for protecting host tissues from autoimmune attack. Anergic T cells fail to proliferate and are eventually deleted from the CTL population.

1.4.2 MHC Class I Antigen Presentation Pathway

The MHC class I presentation pathway has evolved primarily as a host surveillance system to signal the presence or replication of pathogens within host cells. MHC class I proteins are expressed on virtually all nucleated cells within the body and thus, can provide an effective window into the intracellular environment. During productive infections, several proinflammatory cytokines, in particular the type I and II IFNs, can upregulate MHC class I expression to enhance antigen presentation (reviewed in De Maeyer and De Maeyer-Guignard, 1998). Peptide presentation via the class I pathway involves multiple steps and in recent years, several novel proteins have been identified that play important roles in this process. A current model of the MHC class I antigen presentation pathway, illustrated in Figure 1, is summarized below.

1.4.2.1 The Ubiquitin-Proteasome Pathway

Peptides presented by MHC class I molecules are typically derived from proteins which are endogenously synthesized within cells. The initial step in the generation of class I peptides is degradation of proteins in the cytosol. The major source of proteolytic activity in the cytosol is the ubiquitin-proteasome pathway and thus, this degradative system has been postulated to play a vital role in class I antigen processing (Goldberg and Rock, 1992; Peters, 1994; Rechsteiner et al., 1993). Proteins targeted for degradation via the ubiquitin-proteasome pathway are typically modified with the small polypeptide ubiquitin. A series of enzymes (E1, E2 and E3) mediate the covalent addition of multiple molecules of ubiquitin to the lysine residues of targeted proteins (Ciechanover, 1994; Hershko, 1988; Rechsteiner, 1987). E3, the final enzyme in this cascade, directly binds to potential protein substrates and is thought to play an important role in selection of proteins for degradation. Preferentially, denatured or misfolded proteins or those containing oxidized or abnormal amino acids are selected as substrates for ubiquitination (Ciechanover, 1994). Once proteins become polyubiquitinated, they are marked for rapid and efficient degradation by a large, multisubunit proteolytic complex known as the proteasome.

The proteasome is found in the cytosol and nucleus of cells and exists in one of two major forms, designated the 20S and 26S, depending on the incorporation of various regulatory subunits. The smaller of the two forms is composed of 13-15 distinct but related subunits of approximately 21-31 kDa each (Kanayama et al., 1992; Rechsteiner et al., 1993). These subunits share homology with components of the large proteolytic complex found in archaebacteria and

thus, based on this similarity, have been classified into two ancient families, α and β. The α subunits are characterized by 20 conserved residues at the amino terminus and highly charged carboxy terminal extensions, which are thought to play important roles in the assembly of the proteasome and the incorporation of regulatory proteins, respectively (Rechsteiner et al., 1993). β subunits are more heterogeneous in nature, and are proteolytically processed at their amino termini, possibly activating these catalytic subunits before their assembly into the proteasome (Rechsteiner et al., 1993). α and β subunits dimerize with one another, and 6-7 of these dimers associate to form large rings, four of which are stacked one upon the other to yield the characteristic barrel or cylindrical structure of the proteasome (Lowe et al., 1995). Based on the sedimentation coefficient, this protease complex is referred to as the 20S proteasome. Degradation mediated by the 20S proteasome is both ATP and ubiquitin independent and is largely restricted to fully unfolded proteins (Goldberg and Rock, 1992; Rechsteiner et al., 1993). Recent evidence suggests that β subunits are largely responsible for the proteolytic activity of the 20S proteasome (Seemuller et al., 1996), however, it remains unclear whether the association with α subunits influences substrate specificity. In vitro, multiple peptidase activities have been attributed to the 20S proteasome, but in vivo, the functions of the complex are still unclear.

The second form of the proteasome is a much larger complex which sediments with a coefficient of 26S and is capable of degrading ubiquitin conjugated proteins in an ATP-dependent manner (Goldberg and Rock, 1992; Rechsteiner et al., 1993). The 20S proteasome is thought to form the catalytic

core of the larger 26S particle (Peters, 1994; Rechsteiner et al., 1993). Surrounding this cylindrical structure on either end are several additional subunits which regulate proteasomal activity. In particular, two of these subunits, S4 and S5a, confer ATPase and ubiquitin binding activities to the 26S proteasome, respectively (Dubiel et al., 1992; Deveraux et al., 1994). ATP-dependent degradation of polyubiquitinated proteins by the 26S proteasome is mediated through the hydrolysis of peptide bonds at various cleavage sites resulting in the generation of oligopeptides which vary in length. A molecular ruler is thought to exist within the proteasome which governs the length of cleavage products (Wenzel et al., 1994). To date, studies have shown that the majority of products lie within the 7-12 amino acid range, but peptides from 3 to 22 amino acids are also produced (Kisselev et al., 1999).

Although it has been postulated that other proteases in the cytosol may be responsible for the generation of class I restricted peptides, several lines evidence implicate the ubiquitin-proteasome pathway as the predominant source. Cell lines that harbor temperature sensitive mutations in enzymes involved in ubiquitin conjugation are severely impaired in their ability to present class I restricted peptides at the non-permissive temperature (Michalek et al., 1993). Treatment of cells with specific aldehyde inhibitors of the proteasome also blocks the production of most antigenic peptides presented on class I proteins (Rock et al., 1994). More recently, two proteasomal subunits, LMP-2 and LMP-7, which are encoded in the MHC locus (Glynne et al., 1991) and are inducible by IFN-γ (Yang et al., 1992), have been shown to influence peptide production by the proteasome. In knockout mice lacking the LMP-7 subunit, surface expression of

MHC class I proteins was reduced when compared to control mice and presentation of the endogenous HY antigen was extremely inefficient (Fehling et al., 1994). Thus, the incorporation of the LMP-7 subunit into the mature 26S structure may serve to alter proteasome activity by favoring the generation of immunogenic peptides.

1.4.2.2 Peptide Stability and Delivery to the ER

Once generated in the cytosol, peptides must be delivered to the ER for transport across the membrane and into the ER lumen. Peptides released in the cytosol, however, are very unstable and are rapidly degraded to their constituent amino acids (Momburg et al., 1994; Schumacher et al., 1994). Thus, it has been postulated that some type of relay system must exist in order to stabilize peptides in the cytosol, and then transfer them safely to the ER. Potential candidates for this role include various members of the heat shock family of proteins (HSPs). Several HSP70 genes, for example, are encoded within the MHC locus, suggesting a potential link to antigen processing (Sargent et al., 1989). HSP70 has also been shown to bind tightly to peptides and the release of these peptides seems to require the hydrolysis of ATP (Flynn et al., 1989; Undo and Srivasta, 1993). Most convincing is the observation that purified HSP70 from malignant cells injected into mice is capable of inducing tumor-specific cell-mediated immunity (Undo and Srivasta, 1993). This immunization appears to be due to the antigenic peptides bound to HSP70, but the mechanism by which this occurs is not well understood and may be unrelated to normal class I antigen processing events. To date, there is no direct evidence supporting a role for HSPs in class I antigen processing.

It has also been postulated that the direct interaction of proteasomes with the ER membrane may negate the need for a peptide relay system altogether. Proteasomes are known to be associated with the ER membrane (Dusseljee et al., 1998; Palmer et al., 1996) and thus, after protein degradation, hydrolyzed peptides may be fed directly to the ER transport machinery. In this model, peptide stability is not an issue because exposure to the cytosol is very brief or does not occur at all. In addition, the localization of proteasomes to the ER may increase local concentrations of peptide thereby facilitating transport across the ER membrane. Definitive evidence for this hypothesis is also lacking and awaits further investigation. Currently, the mechanisms by which peptides are stabilized in the cytosol and shuttled to the ER remain unknown.

1.4.2.3 Peptide Translocation into the ER Lumen

Loading of peptides onto class I complexes occurs in the lumen of the ER and thus, peptides generated in the cytosol must pass across the ER membrane. Translocation of peptides into the ER is mediated by a dedicated peptide transporter which is known as the Transporter associated with Antigen Presentation (TAP). Evidence implicating TAP in class I antigen presentation first came from the characterization of several mutant cell lines that displayed a similar defect in class I assembly; class I complexes in these cells did not acquire peptide, and as a result, became extremely unstable and were retained in the ER (Schumacher et al., 1990; Townsend et al., 1989). Genetic analysis of these mutants demonstrated that several cell lines contained deletions or mutations in the genes encoding the TAP subunits. Subsequent transfection of these cells with DNA encoding functional TAP genes resulted in the complete restoration of class

I presentation, supporting a vital role for TAP in supplying peptides for class I loading in the ER (Monaco, 1992; Spies and DeMars, 1991).

TAP is a member of the ATP binding cassette (ABC) family of transporters and as such, mediates the translocation of peptides in an ATP-dependent fashion (reviewed in Higgins, 1992; Kelly et al., 1992). The members of this family typically contain 12 membrane-spanning regions, either as a single polypeptide or, as is the case for TAP, as a heterodimer of two proteins with 6 transmembrane domains each. The two subunits of TAP are approximately 69 (TAP1) and 74 kDa (TAP2) and associate non-covalently with one another to form a functional TAP complex (Kleijmeer, 1992; Russ et al., 1995). TAP is rapidly and efficiently assembled in cells and is localized to membranes of the ER or cis-Golgi. Both TAP1 and TAP2 contain consensus nucleotide binding sites, the so-called Walker A and Walker B motifs, and have been shown to bind ATP and other nucleotides in a Mg^{2+} dependent manner (Muller et al., 1994; Russ et al., 1995). The formation of the peptide binding site of TAP is thought to depend on the interaction between both TAP1 and TAP2, with each subunit contributing essential residues. The ATP and peptide binding domains of TAP are distinct and both lie on the cytosolic face of the complex (Russ et al., 1995; van Endert et al., 1994).

Transport of peptides across the ER membrane by TAP occurs through a sequential, multistep process. The initial step in peptide translocation is binding of peptides to the peptide-binding domain of TAP. In the absence of ATP, the peptide-binding site seems to adopt a conformation which shows a high affinity for peptides (van Endert et al., 1994). Thus, inactive TAP favors the binding of peptides and not ATP. Binding of ATP to TAP, however, results in a shift to a

second conformation with a lower affinity for peptide (Covitz et al., 1994; van Endert et al., 1994). This reduced affinity presumably allows for the removal of peptides from the peptide-binding site, so that they might be transferred across the ER membrane. To avoid loss of substrate, the binding of ATP to TAP must be immediately followed by its hydrolysis to ADP. ATP hydrolysis provides the energy required for the translocation step, resulting in the transfer of peptide across the ER membrane. After release of peptide into the ER lumen and ADP into the cytosol, TAP returns to its native conformation awaiting the binding of the next suitable substrate.

TAP functions as a promiscuous transporter with the capacity to bind and translocate a diverse array of peptides. This substrate diversity is absolutely essential for effective antigen presentation because it enables MHC class I proteins to display virtually any epitope expressed within the cell. Binding of peptides to class I molecules, however, does impose certain constraints on peptide structure, such as length and the identity of carboxy terminal residues, which seem to be reflected in TAP substrate specificity. TAP seems to preferentially transport peptides which are 8-12 amino acids in length, while those shorter or longer are translocated much less efficiently (Androlewicz and Cresswell, 1994; Momburg et al., 1994; van Endert et al., 1994). The carboxy terminal residues of peptides play a vital role in binding to class I, by anchoring the peptide in the F-pocket of the binding groove. The importance of this constraint is clearly apparent when the TAP transport selectivities from different species are compared. In humans, class I alleles favor peptides with either hydrophobic or basic residues at the C-terminus, and accordingly, the human TAP shows this

same preference (Momburg et al., 1994; Neefjes et al., 1995). Mouse class I proteins, however, preferentially bind peptides with hydrophobic C-terminal residues, and thus, the murine TAP has evolved to favor the transport of peptides with hydrophobic or aromatic amino acids at the C-terminus (Momburg et al., 1994; Schumacher et al., 1994). In the rat, two classes of TAP2 alleles, cim^a and cim^b, have been shown to convey specificities similar to either the human or murine TAP, respectively (Heemels et al., 1993: Momburg et al., 1994). In contrast to rats, polymorphisms in human or mouse TAP do not seem to significantly alter peptide selectivity (Obst et al., 1995).

1.4.2.4 Assembly of MHC Class I Complexes in the ER Lumen

The translocation of peptides into the ER is inextricably linked to the assembly of newly synthesized class I molecules. The mature MHC class I complex consists of a 44-49 kDa polymorphic heavy chain, a 12 kDa soluble light chain, β_2-microglobulin (β_2m), and a short peptide of 8-10 amino acids in length. The proper folding and assembly of class I molecules is an intricate process involving several components and accessory factors (refer to Figure 1; reviewed in Fourie and Yang, 1998; Pamer and Cresswell, 1998; York and Rock, 1995). First, heavy chains are cotranslationally translocated into the ER and remain anchored in the lipid bilayer via a single transmembrane domain. Nascent heavy chains are rapidly glycosylated and may interact briefly with a chaperone called BiP, but the precise role of this interaction is not well understood (Kahn-Perles et al., 1994). Glycosylation of heavy chains leads to the association with a second chaperone, known as calnexin, which binds to carbohydrate moieties through lectin domains and aids in the folding and stabilization of the heavy chain

(Jackson et al., 1994; Zhang et al., 1995). Interaction of nascent β_2m with the calnexin/heavy chain complex results in the dissociation of calnexin, and the binding of a third chaperone, calreticulin, which is similar to calnexin and probably mediates a similar chaperone function (Sadasivan et al., 1996; Van Leeuwen and Kearse, 1996). This trimolecular complex then interacts with another protein, tapasin, which aids in folding as well as in mediating the interaction between assembling MHC class I complexes and TAP (Sadasivan et al., 1996). Peptide, translocated from the cytosol to the ER, is then transferred from TAP to the peptide-empty complexes. Binding of peptide dramatically enhances the stability of the class I complex, resulting in the dissociation of calreticulin, tapasin and TAP. Recently, ERp57, a thiol reductase, has been shown to interact with assembling class I molecules and may assist in peptide loading (Hughes and Cresswell, 1998). Once class I molecules are loaded with antigenic peptide, they are then exported from the ER, via the secretory pathway, to the cell surface for presentation to $CD8^+$ T lymphocytes.

1.4.2.5 Diversity of MHC Class I Proteins

In humans, the class I heavy chain is extremely polymorphic and is encoded by three genes, HLA-A, -B and -C, with many alleles at each locus (see Figure 2; reviewed in Bodmer et al., 1991; Parham and Ohta, 1996). The HLA-B locus, for example, is comprised of approximately 281 different alleles. This diversity plays a significant role in peptide binding by class I proteins. Peptides bind to class I molecules in an elongated peptide-binding groove. The binding of peptides is stabilized at either end by contacts with the free amino and carboxy termini, and along the length of the peptide at various anchor residues which lie

deep in the groove (Bjorkman and Parham, 1990; Young et al., 1994). These anchor residues insert into pockets in the MHC molecule that are lined by highly polymorphic amino acids (Madden et al., 1992; Matsumura et al., 1992). Thus, different allelic variants of class I may preferentially bind different peptides. MHC class I alleles are expressed in a co-dominant manner, and thus, in any given individual, there may be as many as 6 different class I proteins expressed on the surface of cells. Expression of multiple class I alleles, then, will ensure that a broad spectrum of peptides are presented and that no pathogen escapes detection by CTL.

1.5 Immune Evasion Strategies of HSV

The capacity of HSV to establish and maintain a lifelong infection within the host is due in large part to the ability of the virus to remain dormant in latently infected neurons. During latent infection, HSV does not express detectable levels of viral proteins and thus, this allows the virus to remain relatively invisible to the host immune system. However, for transmission of virus to new hosts, HSV must reactivate from the latent state and go on to produce infectious progeny. Reactivation of latent HSV takes place in the face of a fully primed immune response and therefore, infectious virus must come in direct contact with all branches of the host immune response. Since HSV is able to replicate in this hostile environment, it is not surprising that the virus has evolved several elaborate strategies to evade host immunity. In addition to blocking $CD8^+$ T cell recognition, HSV is also capable of interfering with complement activation, Fc receptor binding and lysis by cytolytic effector cells.

Figure 1: The MHC class I presentation pathway. The primary role of the class I pathway is to display endogenous antigens to $CD8^+$ T lymphocytes. 1. The class I heavy chain (HC) is initially translocated into the ER where it remains anchored via a single transmembrane domain. 2. Following glycosylation, the heavy chain associates with the chaperone calnexin (CXN), which aids in the stabilization and folding of the protein. 3. As the heavy chain adopts a stable conformation, it binds nascent β_2-microglobulin (β_2m) and calnexin is replaced by the soluble chaperone calreticulin (CRT). 4. The order of assembly is unclear from this point, but ultimately, the assembling class I complex consisting of class I, calreticulin and ERp57 associates with TAP through an interaction with the adaptor protein tapasin. Tapasin is thought not only to function as a bridge between TAP and the multimeric class I complex, but may also play a role in peptide loading. 5. Peptides generated from the degradation of proteins in the cytosol by the proteasome are then transported across the ER membrane by TAP and loaded onto empty class I complexes. 6. Once peptide is bound, calreticulin, ERp57, tapasin and TAP dissociate from the loaded class I molecule, facilitating its egress from the ER. 7. The class I molecule then moves along the exocytic pathway through the Golgi apparatus, where immature carbohydrates are processed to their mature forms, and eventually arrives at the cell surface for presentation to $CD8^+$ T cells.

MHC Class I Presentation Pathway

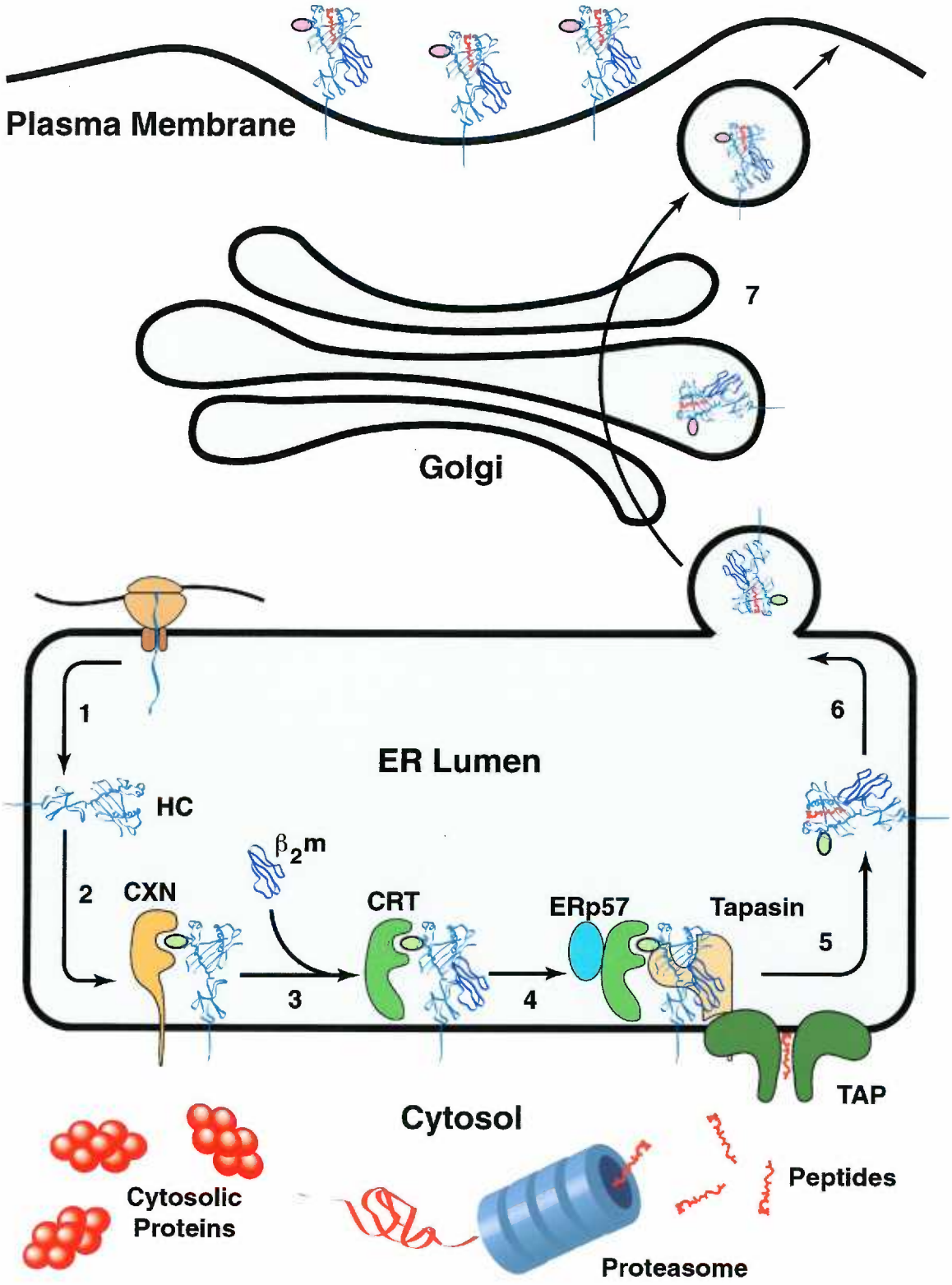
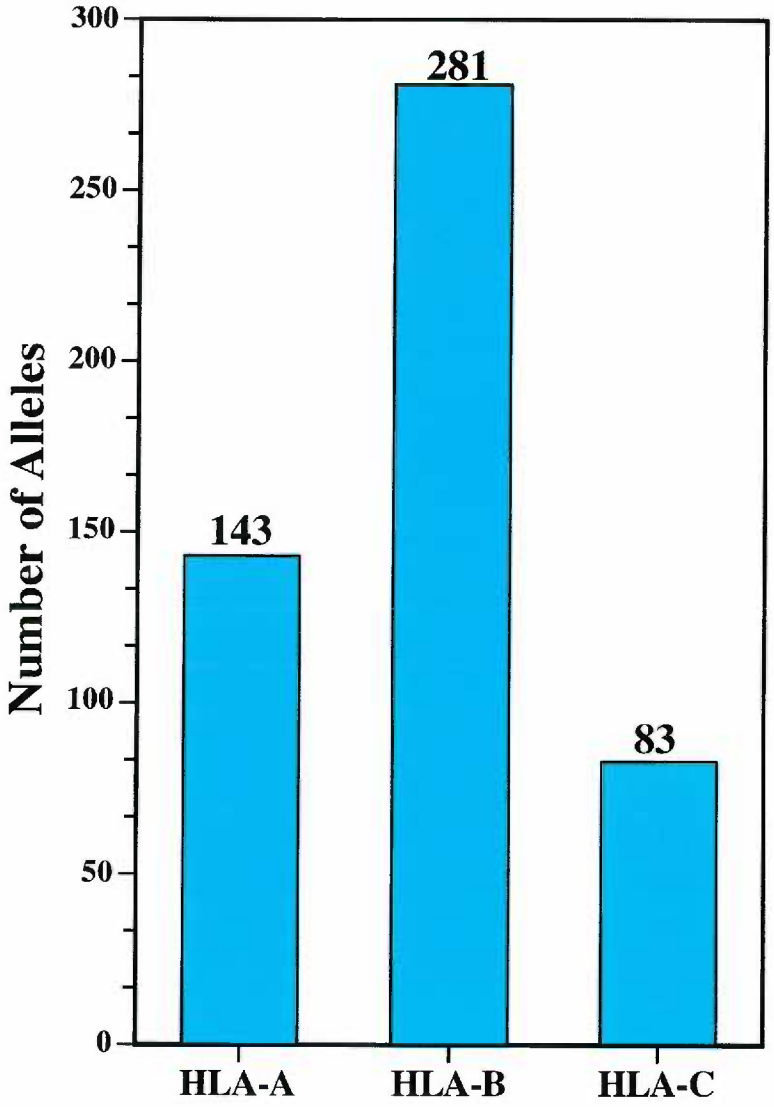


Figure 2: Polymorphism of MHC class I genes. Number of alleles currently assigned to the HLA-A, -B and -C loci based on DNA sequence analysis. Data obtained from the HLA Informatics Group (www.anthonynolan.com).

Polymorphism of MHC Class I Genes



1.5.1 Complement Inhibition

The complement system is a vital component of the humoral immune response and is comprised of a series of about 20 proteins which typically remain dormant in plasma, but once activated by the appropriate stimulus, produce a rapid, highly amplified response resulting in the lysis or neutralization of pathogens and infected cells (reviewed in Abbas et al., 1994; Janeway and Travers, 1997; Roitt, 1994). The complement system can be activated through two different pathways: classical and alternative. The classical pathway is activated by the binding of complement factor 1 (C1) to antigen-antibody complexes. The alternative pathway is initiated when spontaneously activated complement factors, usually C3, bind to the surface of a pathogen and are preferentially stabilized by properdin. The early events of both pathways lead to the formation of an enzymatic activity called a C3 convertase, which results in the specific cleavage of C3 to produce C3a and C3b. C3b plays a pivotal role in the regulation of complement activity. After cleavage, C3b acquires the ability to covalently bind to cell surface molecules and immune complexes. Complement receptors which bind to C3b are expressed on phagocytes, such as macrophages and neutrophils, and facilitate the opsonization and destruction of C3b-coated pathogens. Alternatively, C3b can recruit additional complement factors such as C5, allowing it to be cleaved by the C5 convertase and thus, initiating the formation of the membrane attack complex (MAC). Sequential attachment of C6, C7, C8 and C9 to the cleavage product of C5, C5b, results in the formation of a hydrophilic pore in the lipid bilayer which is fully permeable to electrolytes and water. This breach in the lipid bilayer leads to the disruption of ionic gradients

across the membrane, the penetration of lysozyme and other enzymes into the cell or pathogen and ultimately, the lysis of the target. Host cells are protected from accidental activation of complement by the expression of specific surface regulatory proteins such as decay accelerating protein (DAF), membrane co-factor protein (MCP) and protectin (CD59).

The ability of HSV to replicate in vivo in the presence of high titres of neutralizing antibodies and complement factors is in large part due to the ability of the virus to spread directly from cell-to-cell. However, during primary infections at mucosal surfaces, HSV is directly exposed to high concentrations of complement and yet, infection remains quite efficient. The failure of complement to lyse HSV particles during primary infection may stem from the fact that HSV encodes a receptor capable of blocking complement activation. Infection of cells with HSV-1 induces the expression of a virally encoded receptor that has the ability to bind complement factor C3b (Clines et al., 1982; Kubota et al., 1987; Smiley and Friedman, 1985). Several studies have established that the receptor responsible for this activity is glycoprotein C (gC), which is an envelope glycoprotein expressed on the surfaces of both infected cells and viral particles. Infection of cells with mutant HSV-1 viruses lacking gC do not bind C3b (Friedman et al., 1984) and conversely, cells transfected with gC acquire the ability to bind C3b (Seidel-Dugan et al., 1988). Further characterization of the complement binding activity of gC indicated that this glycoprotein was capable of binding another C3 fragment, called iC3b (Tal-Singer et al., 1991), which is thought to enhance opsonization of infectious virus by binding to phagocyte receptors. Both HSV-1 and HSV-2 express gC, designated gC-1 and gC-2,

respectively, but only cells infected with HSV-1 show complement binding activity (Friedman et al., 1984; Smiley and Friedman, 1985). This result was unexpected considering that gC-1 and gC-2 share a high degree of sequence homology and purified preparations of each protein can bind C3b and iC3b in vitro (Eisenberg et al., 1987; McNearney et al., 1987). Nevertheless, it remains unclear why cells infected with HSV-2 do not possess complement binding activity. Possible explanations for this difference may include inhibition of C3 fragment binding by other HSV-2 glycoproteins, a lower affinity of gC-2 than gC-1 for C3 fragments or differences in the cell surface expression of gC-2.

Several in vitro studies have demonstrated that both gC-1 and gC-2 regulate complement and provide protection against complement-mediated neutralization (Eisenberg et al., 1987; McNearney et al., 1987). Comparison of wildtype HSV-1 with a mutant virus unable to express gC-1 indicated that the mutant virus was much more susceptible to cytolysis by antibody and complement or complement alone (Harris et al., 1990; Hidaka et al., 1991). Similar studies have demonstrated that gC-1's protective activity seemed to be more pronounced in the absence of antibodies, suggesting that gC-1 may be more effective in blocking the alternative pathway (Fries et al., 1986; Hung S-L et al., 1994). Purified gC-1 was also shown to accelerate the decay of the alternative C3 convertase and inhibit the binding of properdin to C3 (Hung S-L et al., 1994; Kostavasili et al., 1997). Properdin plays a critical role in the alternative pathway by preferentially stabilizing the C3 convertase on the surfaces of pathogens, thereby providing sufficient time for the MAC to form. Unlike gC-1, gC-2 stabilizes the C3 convertase and does not inhibit binding of properdin (Hung S-L

et al., 1994; Kostavasili et al., 1997). The mechanism of complement inhibition by gC-2 has not yet been identified. It appears that gC-1, and to some extent gC-2, primarily influence the alternative complement pathway, suggesting that gC's effectiveness may be more apparent during primary HSV infections, when antibodies are not yet present.

In vivo, the evidence supporting a protective role for gC in HSV pathogenesis has been more difficult to obtain. Initial studies in mice showed no difference in severity or extent of infection between wildtype and gC^- viruses (Dix et al., 1983; Johnson et al., 1986; Sunstrum et al., 1988). These studies, however, were found to be flawed in that the intracerebral route of inoculation and the use of mouse strain DBA/2, which is deficient in C5, negated any contribution by complement in virus clearance. Moreover, gC-1 was found to bind very poorly to murine C3 fragments (Hidaka et al., 1991). Studies have since focused on the guinea pig as an animal model, and in this case, gC-1 was found to have a protective effect on HSV pathogenesis (Friedman et al., 1996). Viruses deleted of gC-1 were found to produce less severe vaginitis and grew to much lower titres than wildtype HSV-1. When these same experiments were conducted in guinea pigs lacking C3, both mutant and wildtype viruses showed identical disease.

1.5.2 Fc Receptor Inhibition

In addition to the complement system, humoral immunity is also comprised of antigen specific antibodies. Antibodies or immunoglobulins consist of two distinct functional domains: Fab fragments, which bind a diverse array of compounds through a highly variable antigen-binding site, and Fc fragments,

which interact with effector molecules and cells of the immune system. The Fc portion of immunoglobulins contains several conserved domains which define the isotype of the antibody (IgG, IgM, IgA, IgD or IgE) and confer specialized properties to each isotype (reviewed in Janeway and Travers , 1997: Roitt et al., 1994). Most importantly, the Fc portion of IgG is responsible for interactions with complement factor C1q and Fc receptors of immune effector cells such as macrophages, neutrophils and NK cells. Thus, IgG bound to virus particles or infected cells can induce their destruction via the classical complement cascade, opsonization by phagocytes or antibody dependent cellular cytotoxicity (ADCC) by NK cells.

Infection of fibroblast or epithelial cells with either serotype of HSV results in the expression of receptors able to bind the Fc region of IgG (Baucke and Spear, 1979; Para et al., 1980; Para et al., 1982). Based on studies using IgG affinity columns, this Fc receptor activity was originally attributed to a single HSV protein known as glycoprotein E (gE) (Baucke and Spear, 1979). However, subsequent studies revealed that the HSV Fc receptor was indeed a heterodimer comprised of two distinct polypeptides: gE and glycoprotein I (gI) (Johnson et al., 1988; Johnson and Feenstra, 1987). gE and gI are encoded by adjacent genes in the HSV genome, designated US8 and US7, respectively (McGeoch et al., 1985). During HSV infection, gE and gI are expressed in a coordinate fashion and this expression directly parallels the Fc binding activity of infected cells. Studies with mutant viruses deleted in gE or gI indicated that gE alone retained the ability to bind monomeric and complexed IgG, while gI did not (Dubin et al., 1990). However, subsequent studies demonstrated that gE alone bound much less of

both forms of IgG than did the gE/gI complex, supporting the notion that the functional form of the HSV Fc receptor is indeed the gE/gI heterodimer (Bell et al., 1990; Hanke et al., 1990; Johnson et al., 1988). Characterization of the domains required in the Fc portion of IgG for binding to gE/gI demonstrated that the CH_2 and CH_3 constant domains of IgG are essential for this interaction (Johansson et al., 1986). These same domains are required for Fc binding by Staphylococcus aureus protein A (PrA), and both gE/gI and PrA show the same binding specificity for subclasses of human IgG: IgG4 > IgG1 > IgG2 and not IgG3 (Johansson et al., 1989; Wiger and Michaelsen, 1985). However, the simultaneous binding of gE/gI and PrA to complexes of non-immune IgG (Johnson et al., 1988; Johnson and Feenstra, 1987) seems to suggest that these proteins interact differently with the CH_2 and CH_3 domains. Alternatively, since a single IgG molecule contains two heavy chains, it remains possible that both gE/gI and PrA bind to identical regions on opposite sides of IgG. In terms of species specificity, gE/gI binds IgG from human, cow, goat, sheep and rabbits, but does not bind horse, cat, rat or mouse IgG (Johansson et al., 1985).

In vitro, several lines of evidence suggest that the HSV Fc receptor may have a protective role during HSV infection. A double binding or bi-polar bridging hypothesis has been proposed to explain how gE/gI might counteract the destructive effects of complement, opsonization or ADCC mediated by IgG (Frank and Friedman, 1989; Lehner et al., 1975). The bi-polar bridging model refers to the binding of a single anti-HSV antibody to its antigenic target by its Fab domain and concomitantly to the HSV Fc receptor by its Fc domain. Binding of gE/gI to the Fc region of engaged anti-HSV antibodies would effectively

preclude the binding of complement factors or recognition by Fc receptors on immune effector cells. In support of this hypothesis, wildtype HSV-1 has been shown to be much more resistant to antibody and complement-mediated neutralization than mutant viruses lacking gE (Frank and Friedman, 1989). Subsequent characterization revealed that this protective effect was mediated through the inhibition of C1q binding by bi-polar bridging of antiviral IgG. Furthermore, additional studies have established a role for bi-polar bridging in the protection of HSV-infected cells from opsonization and ADCC (Dubin et al., 1991; Van Vliet et al., 1992).

Evidence supporting a protective role for the HSV-Fc receptor in vivo has been more elusive. A major barrier in this respect is interpretation of results obtained in animal studies using mutant viruses deleted for either gE or gI. In addition to Fc binding activity, gE/gI has been shown to play an important role in the cell-to-cell spread of virus across junctions (Dingwell et al., 1994). Therefore, any reduction in viral replication or severity of disease observed with gE^- or gI^- viruses cannot be automatically attributed to defects in immune evasion, but must also account for defects in viral spread. In addition, although it has been well established that gE/gI binds mouse IgG very poorly (Frank and Friedman, 1989; Johansson et al., 1985), most in vivo studies examining the role of the Fc receptor during HSV infection have been conducted in mice. Recently, Friedman and colleagues have made serious attempts to circumvent these problems (Nagashunmugam et al., 1998; Weeks et al., 1997). First, a mutant form of gE was constructed in which a small insertion of 4 amino acids was introduced into the putative Fc receptor binding site in the hopes that Fc binding activity would be

lost without affecting viral spread. Second, prior to infection with HSV, mice were passively immunized with human anti-HSV serum, providing a compatible source of IgG. Initial results seemed to support a protective role for the Fc receptor as infection of mice with a recombinant virus containing the mutant form of gE resulted in reduced viral titres and disease severity when compared to animals infected with wildtype virus. However, the interpretation of these results remains quite controversial, as viral spread in the mice infected with the recombinant gE virus was also reduced. Quite possibly, the 4 amino acid insertion introduced into gE may affect both Fc binding activity and cell-to-cell spread, suggesting that these two activities may be linked. Although there is little compelling evidence in vivo to support a role for the HSV-Fc receptor in immune evasion, in vitro studies clearly demonstrate the effectiveness of gE/gI in blocking complement fixation, opsonization and ADCC. Moreover, with the capacity of gE/gI to block the classical activation of complement, and gC to block the alternative pathway, HSV may drastically limit the destructive effects of complement during infection.

1.5.3 Inhibition of Cytolytic Effector Function

The antiviral effects of the humoral immune response during HSV infection may be largely negated by the capacity of HSV to spread directly from cell-to-cell in solid tissues. Thus, for the clearance of ongoing HSV infections, cellular immunity is thought to play the most important role. Essential in this respect are cytolytic effector cells such as CTL, NK or LAK cells which have the capacity to identify and lyse infected targets. An absolute requirement for the containment of HSV infection is the early destruction of infected cells which harbor progeny virus. Given that cytolytic cells play such an important role in combatting HSV

infection, the virus has also evolved several mechanisms to interfere with cytolytic activity.

For recognition and destruction of infected cells, cytolytic effector cells must come in close intimate contact with their potential targets. HSV exploits this transient cell-to-cell contact to disrupt the normal cytolytic functions of these cells. NK and LAK cells comprise a branch of the cellular innate response and can lyse a diverse array of targets (reviewed in Karre, 1993; Frederick et al., 1997). NK cells are large granular lymphocytes which express the surface markers CD16 or CD56 or both and lack the expression of surface CD3. LAK cells, which are related in function to NK cells, are derived from NK cells or subsets of MHC-unrestricted T cells, by treatment with various cytokines such as IL-2, IL-12 or IL-15 (Frederick et al., 1997). Recognition by NK and LAK cells is thought to be mediated through contributions of both inhibitory and stimulatory receptors. In particular, killer inhibitory receptors (KIRs) provide a dominant inhibitory signal when bound to MHC class I molecules on the surface of target cells (reviewed in Colonna, 1997; Moretta et al., 1996). During HSV infection, MHC class I expression is compromised (discussed below) and thus, infected cells become targets for lysis by NK or LAK cells. In addition, NK cells also express the FcγRIII receptor which binds to the Fc domain of IgG and transmits a positive signal for target cell lysis through ADCC (reviewed in Frederick et al., 1997). Late in HSV infection, however, target cells become resistant to lysis mediated by NK and LAK cells. Several studies have demonstrated that contact with HSV-infected fibroblasts renders NK or LAK cells incapable of destroying normally sensitive targets (Confer et al., 1990; York and Johnson, 1993). Fibroblasts infected with

HSV mutants lacking essential genes were unable to mediate this effect (York and Johnson, 1993). This result suggested that inhibition of lysis was mediated through the infection of NK and LAK cells after contact with HSV-infected targets. Detection of HSV antigens in LAK cells shortly after contact with infected cells confirmed this speculation (York and Johnson, 1993). Thus, it seems that the engagement of NK and LAK cell receptors with infected targets results in the formation of pseudo-cellular junctions which enables HSV to spread from one cell to the next. A similar phenomenon has also been observed with cytotoxic T lymphocytes (CTLs). CTL recognize infected targets through the binding of viral peptides presented in the context of MHC class I molecules. This interaction also seems to provide the intimate contact between effector and target cell required for HSV spread. Studies have demonstrated that following contact with HSV-infected cells, $CD8^+$ CTL are rendered inactive and unable to lyse susceptible targets (Posavad and Rosenthal, 1992; Posavad et al., 1993).

The mechanism of effector cell inactivation by HSV infection, however, remains poorly understood. Infection of cells with HSV leads to a myriad of cytopathic effects that may disrupt normal lytic cell function. In addition to dramatic changes in host cell transcriptional and translational pathways, HSV infection may also disrupt essential microtubular networks required for the efficient release of perforin and granzyme from lytic cells. Perforin and granzyme are cytotoxic compounds that are stored in lytic cell granules until recognition of an infected target triggers their directional release toward the point of cellular contact (reviewed in Janeway and Travers, 1997; Roitt, 1994). The organization and movement of granules is thought to be mediated by normal microtubular

traffic within the lytic cell. Upon entry of HSV into susceptible cells, microtubules become fragmented and this depolymerization is most pronounced at the cell periphery (Avitabile et al., 1995; Sodiek et al., 1997). This microtubule depolymerization may account for the inability of infected cytolytic cells to destroy susceptible targets. Alternatively, HSV may encode proteins that specifically function to inhibit various aspects of lytic cell function. For this to be a viable defense mechanism, the proteins in question would have to be induced immediately upon lytic cell infection and must either inactivate various cytotoxins or block their synthesis or release from the lytic cell.

1.5.4 Evasion of $CD8^+$ T Cell Recognition

Lysis of target cells by $CD8^+$ CTL is mediated through the specific recognition of viral antigens presented on the surface of infected cells. The specificity of this interaction makes CTL very potent killers of infected cells and thus, a likely target for immune evasion by HSV. Although HSV is capable of disrupting CTL function late in infection, the relevance of this in vivo remains unknown, as infected cells become susceptible to lysis immediately following the entry of virus into cells. In order for HSV to effectively inhibit CTL activation, recognition of viral antigens must be blocked early in infection.

In humans, the CTL response to HSV infection appears to be disproportionately represented by cytotoxic $CD4^+$ T cells (Schmid, 1988; reviewed in Schmid and Mawle, 1991; Yasukawa and Zarling, 1984a; Yasukawa and Zarling, 1984b). This is rather unexpected because the predominant source of antiviral CTL during infection typically arises from the $CD8^+$ T cell population. The few $CD8^+$ CTL that can be isolated from infected individuals seem to be

specific for structural components of the virion and not necessarily products of de novo protein synthesis (Tigges et al., 1992). Possibly related to this observation is the finding that HSV infection of human fibroblasts renders these cells resistant to lysis by HSV-specific $CD8^+$ CTL (Koelle et al., 1993; Posavad and Rosenthal, 1992; Posavad et al., 1993). This protection from lysis is observed within a few hours after infection, long before infectious virus is produced, so HSV infection of CTL cannot account for the defect in lysis. HSV infection of cells results in the rapid degradation of cellular mRNA by the Vhs protein, and thus, it was postulated that the resistance of HSV-infected cells to CTL lysis may be due to the absence of cell surface MHC class I proteins. On the contrary, however, when HSV-infected cells are pulsed with exogenous peptide, these cells become potent targets for CTL lysis, indicating that adequate levels of surface class I molecules do exist (Koelle et al., 1993). In addition, cells infected with mutant viruses lacking Vhs remain resistant to lysis by HSV-specific $CD8^+$ CTL (Posavad et al., 1993). Instead, these observations seemed to suggest that HSV may actively block the presentation of viral antigens via the MHC class I pathway.

Biochemical characterization of MHC class I molecules in HSV-infected cells revealed that shortly after infection class I complexes were retained in the ER (Hill et al., 1994; York et al., 1994). Class I complexes produced in TAP deficient cell lines also display a similar phenotype, and thus it was postulated that class I molecules in HSV-infected cells may also be peptide-empty and unstable. The stability of MHC class I complexes was examined by incubating detergent extracts from infected cells with various concentrations of exogenous peptide and then immunoprecipitating the class I proteins with a conformationally

dependent antibody that only recognizes stable HLA-A2 complexes (York et al., 1994). In the absence of peptide, very little class I was precipitated from extracts of HSV-infected cells. However, as increasing concentrations of peptide were added, a parallel increase in precipitated class I was observed. Thus, class I complexes from HSV-infected cells are stabilized by the addition of exogenous peptide. These results strongly suggest that the instability and ER retention of class I complexes during HSV infection is largely due to the failure of these complexes to be loaded with peptide.

1.5.4.1 HSV ICP47 is Required for ER Retention of MHC Class I

HSV expresses roughly 79 proteins, many of which have not been studied in any great detail. To limit the potential candidates responsible for this effect, the onset of class I instability in HSV infected cells was correlated with the induction of various stages in viral gene expression. Since ER retention of class I molecules was observed within 2 hours of infection (Hill et al., 1994; York et al., 1994), it appeared that either a structural virion component or an immediate early gene product was responsible for this effect. Exposure of HSV to UV light results in the crosslinking of genomic DNA such that all viral gene expression is blocked and only structural components are delivered into cells upon infection. Cells infected with UV inactivated HSV were unable to block CTL mediated lysis, indicating that immediate early gene expression was required to block class I presentation (York et al., 1994). Cells were then infected with a panel of mutant viruses, each lacking individual immediate early genes, and the only mutant unable to block class I transport was a virus deleted for the US12 gene, which encodes the immediate early protein, ICP47 (York et al., 1994). To verify this finding, ICP47

was expressed in cells, in the absence of any other HSV products, using a recombinant adenovirus and was found to mediate the retention of class I molecules in the ER (York et al., 1994). Furthermore, expression of ICP47 in cells rendered them resistant to lysis by $CD8^+$ CTL (York et al., 1994). Thus, ICP47 alone is sufficient to block presentation of viral antigens to $CD8^+$ T lymphocytes.

1.5.4.2 The Target of ICP47 Inhibition is TAP

Both HSV-1 and HSV-2 express ICP47 proteins, designated ICP47-1 and ICP47-2, respectively, which share approximately 42% amino acid identity. These proteins are relatively small, only 10 kDa in size, have no discernable signal or transmembrane sequences and share no homology with known cellular or viral proteins (Murchie and McGeoch, 1982). Expression of ICP47 during infection is largely restricted to the cytoplasm and nucleus (York et al., 1994). The observation that class I complexes in HSV infected cells are peptide-empty, coupled with the cytoplasmic distribution of ICP47, suggested that ICP47 may either interfere with the production of peptides, their delivery to the ER or their transport across the ER membrane. Peptides are generated in the cytosol through the degradation of polyubiquitinated proteins by the 26S proteasome. Thus, ICP47 may function to inhibit either the ubiquitination of cytoplasmic proteins or the proteolytic activity of the proteasome. However, when minigenes encoding class I peptides were expressed in cells, thereby effectively by-passing the requirement for antigen degradation by the proteasome, ICP47 was still capable of mediating the retention of class I molecules (Fruh et al., 1995). Thus, production of peptides in the cytosol does not seem to be the target of ICP47 action. In order for peptides to be loaded onto class I molecules, they must first

reach the lumen of the ER, where class I assembly takes place. Thus, peptides generated in the cytosol must first be delivered to TAP, followed by their transport across the ER membrane. To address the transport of peptides in ICP47-expressing cells, radiolabeled peptides containing a consensus glycosylation motif were introduced into permeabilized cells (Fruh et al., 1995; Hill et al., 1995). Translocation of radiolabeled peptides by TAP into the ER results in their rapid glycosylation and retention in the lumen. Thus, transport activity can be quantitated by incubating extracts with an immobilized lectin, concanavalin A-Sepharose (Con A), and measuring the recovery of glycosylated peptide. Expression of ICP47 in cells dramatically reduced the translocation of peptides into the ER, as indicated by the low recovery of radiolabeled peptide (Fruh et al., 1995; Hill et al., 1995). Thus, it seems that ICP47 functions either to inhibit the delivery of peptides to TAP or the function of TAP itself. To shed light on this issue, a series of co-immunoprecipitation experiments were conducted to determine whether ICP47 associated with any potential transport components, namely HSPs or TAP. Using the mild detergent digitonin to disrupt cells, ICP47 was found to co-immunoprecipitate with the TAP complex (Fruh et al., 1995; Hill et al., 1995). The interaction of ICP47 with TAP was also shown to require both TAP subunits. In cells expressing a single subunit, either TAP1 or TAP2, ICP47 could not be co-immunoprecipitated. However, when both subunits were co-expressed, leading to the assembly of functional heterodimers, ICP47 was found to form a tight interaction with TAP (Fruh et al., 1995; Hill et al., 1995). Together, these results suggest that the inhibition of peptide translocation by ICP47 is mediated through a specific interaction with the cytosolic domain of TAP.

1.6 Summary and Hypothesis

HSV establishes lifelong infections within its hosts and has the ability to reactivate in the face of a fully primed immune response. The capacity of HSV to replicate under these hostile conditions may be due in large part to various immune evasion tactics used by the virus, most striking of which, is the ability of HSV to block antigen recognition by $CD8^+$ T lymphocytes. Infection of cells with HSV renders them invisible to anti-HSV CTL, which typically recognize viral antigens presented on cell surface MHC class I proteins. The HSV immediate early protein, ICP47, was found to inhibit MHC class I antigen presentation by blocking the transfer of peptides across the ER membrane by TAP. Further studies demonstrated that ICP47 was capable of associating with TAP and that this interaction required both TAP subunits. The molecular mechanism by which ICP47 inhibits TAP activity, however, remained unknown until my studies began. Inactivation of TAP by ICP47 could theoretically occur through four principle effects. First, peptide translocation by TAP is ATP-dependent, and thus, ICP47 might interfere with either the binding of ATP to TAP or its hydrolysis. Second, ICP47 could potentially interact with the peptide binding domain of TAP, thereby precluding the binding of peptides. Third, ICP47 itself might function as a substrate for TAP, being partially translocated by the transporter before becoming lodged in the active transport channel. Finally, it remains possible that ICP47 may be interacting with TAP through an intermediary protein, such as a peptide chaperone, and that inhibition of peptide translocation might be mediated through this accessory component. One of the objectives of this thesis is to determine the molecular mechanism by which ICP47 blocked TAP.

Chapter 2

Stable binding of the herpes simplex virus ICP47 protein to the peptide binding site of TAP (EMBO Journal 15: 3256-3266, 1996)

2.0 Preface

In the following publication, I carried out the experiments in Figures 2.1A, 2.2, 2.3, 2.4, 2.6, 2.7 and 2.8. The peptide translocation assays using permeabilized cells in Figures 2.1B and 2.1C were conducted by Dr. Ann B. Hill at the Massachusetts Institute of Technology in Cambridge, MA. The crosslinking experiments with 8-azido-[^{32}P]ATP in Figure 2.5 were performed in our laboratory by Dr. David C. Johnson and Pieter Jugovic.

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Stable binding of the herpes simplex virus ICP47 protein to the peptide binding site of TAP

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The herpes simplex virus (HSV) ICP47 protein inhibits the MHC class I antigen presentation pathway by inhibiting the transporter associated with antigen presentation (TAP) which translocates peptides across the endoplasmic reticulum membrane. At present, ICP47 is the only inhibitor of TAP. Here, we show that ICP47 produced in bacteria can block human, but not mouse, TAP, and that heat denaturation of ICP47 has no effect on its ability to block TAP. ICP47 inhibited peptide binding to TAP without affecting ATP binding, consistent with previous observations that the peptide binding and ATP binding sites of TAP are distinct. ICP47 bound to TAP with a higher affinity (K_D ~5× 10^{-8} M) than did peptides, and ICP47 did not dissociate from TAP. ICP47 was not transported by TAP and remained sensitive to proteases added from the cytosolic surface of the membrane. Peptides acted as competitive inhibitors of ICP47 binding to TAP, and this inhibition required a 100- to 1000-fold molar excess of peptide. These results demonstrate that ICP47 binds to a site which includes the peptide binding domain of TAP and remains bound to this site in a stable fashion.

Keywords: herpes simplex virus/ICP47/peptide binding site/TAP

Introduction

CD8+ T lymphocytes recognize peptides, derived primarily from cytosolic proteins, that are presented on cell surface major histocompatibility complex (MHC) class I molecules (Rammensee *et al.*, 1993). Peptides are generated in the cytosol, largely by the action of proteasomes, and gain access to the lumen of the endoplasmic reticulum (ER), where they can associate with newly synthesized class I molecules (reviewed in Heemels and Ploegh, 1995; York and Rock, 1996). Transport of peptides across the ER membrane is mediated by the transporter associated with antigen presentation (TAP) which, like certain components of the proteasome, is encoded in the MHC (reviewed in Yewdell and Bennink, 1992). There is good evidence that TAP is the major source of peptides for class I antigen presentation (reviewed in Heemels and Ploegh, 1995; Howard, 1995; York and Rock, 1996), and mutant mice and human patients lacking TAP show significant defects in class I antigen presentation (van Kaer *et al.*, 1992; de la Salle *et al.*, 1994). In addition, TAP associates with MHC class I heavy chain (HC)–β_2-microglobulin (β_2-m) complexes and this co-localization in the ER membrane may enhance delivery of peptide to newly synthesized class I molecules (Androlewicz *et al.*, 1994; Ortmann *et al.*, 1994; Suh *et al.*, 1994).

TAP is a heterodimer composed of two proteins, TAP1 and TAP2, each with C-terminal hydrophilic domains that bind ATP and more N-terminal hydrophobic domains which may span the membrane six to eight times (reviewed in Townsend and Trowsdale, 1992; Monaco, 1992; Howard, 1995). TAP is a member of the ABC transporter family which includes the mammalian multi-drug resistance (mdr) glycoproteins, the cystic fibrosis transporter, a yeast mating factor transporter and several bacterial amino acid transporters (reviewed in Higgins, 1992). TAP has a relatively broad specificity for small peptides of ~7–12 amino acids, although there are preferences for peptides with hydrophobic or basic C-termini similar to those shown by MHC class I (reviewed in Heemels and Ploegh, 1995). Co-expression of both TAP1 and TAP2 is required for peptide binding and translocation (Androlewicz and Cresswell, 1994; van Endert *et al.*, 1994) and peptide substrates can be cross-linked to residues of both TAP1 and TAP2 (Androlewicz *et al.*, 1994). Hydrolysis of ATP is not required for peptide binding, but is necessary for peptide translocation, and the binding of ATP and peptide to TAP appear to be independent processes (Androlewicz *et al.*, 1993, 1994; Neefjes *et al.*, 1993; Shepherd *et al.*, 1993; Schumacher *et al.*, 1994; van Endert *et al.*, 1994).

TAP expressed in insect cells is capable of translocating peptide, demonstrating that mammalian factors other than TAP are not strictly required for peptide transport (Meyer *et al.*, 1994; van Endert *et al.*, 1994). Studies of TAP expressed in insect cells have suggested that TAP can exist in at least two states, one having a higher affinity for peptide and favoured in the absence of ATP and below 37°C, while the other, with lower affinity for peptide, is induced on binding of ATP (van Endert *et al.*, 1994). Similar observations have been made with other members of the ABC transporter family (Covitz *et al.*, 1994). However, at present, the molecular details of how TAP binds peptides and mediates their transport across the ER membrane are not well understood.

Herpes simplex virus (HSV) infection of human

fibroblasts causes rapid inhibition of MHC class I antigen presentation and class I HC–β_2-m complexes accumulate in the ER in a peptide empty form (Hill *et al.*, 1994; York *et al.*, 1994). We demonstrated that an HSV immediate early polypeptide, ICP47, is both necessary and sufficient to cause this inhibition (York *et al.*, 1994). ICP47 binds to TAP and inhibits peptide translocation in permeabilized mammalian cells (Fruh *et al.*, 1995; Hill *et al.*, 1995). ICP47 did not bind to TAP1 or TAP2 when the proteins were expressed individually (Hill *et al.*, 1995) and, thus, as with peptides, binding of ICP47 to TAP apparently requires contributions from both members of the heterodimer. ICP47 is the only known inhibitor of TAP, and it appears likely that analysis of the effects of ICP47 should provide valuable new information about TAP, as well as further elucidating an interesting strategy for evasion of the immune system.

Here we describe the mechanism by which ICP47 inhibits TAP-mediated peptide transport into the ER. ICP47 inhibited peptide binding to TAP, but there was no effect on ATP binding to TAP. Characterization of the binding of ICP47 to TAP indicated that ICP47 bound to a site which overlaps the peptide binding site, but with a higher affinity than peptides, and ICP47 remained accessible to proteases added from the cytoplasmic side of membranes. Therefore, ICP47 blocks TAP by binding in a relatively stable fashion to a domain of TAP which includes the peptide binding site.

Results

ICP47 produced in bacteria inhibits TAP

In order to study interactions between ICP47 and TAP, ICP47 was expressed in bacteria. The ICP47 gene was fused to glutathione-*S*-transferase (GST) sequences, so that the GST and ICP47 polypeptide sequences were separated by a thrombin-sensitive cleavage site. GST–ICP47 was purifed using glutathione–Sepharose and the ICP47 protein released from the GST by using thrombin which was then inactivated with phenylmethylsulfonyl fluoride (PMSF) (Figure 1A).

To determine whether ICP47 produced in bacteria (rICP47) was able to block TAP activity in cells, we introduced rICP47 into streptolysin O-permeabilized human fibroblasts as previously described (Hill *et al.*, 1995). The rICP47 inhibited TAP-mediated peptide transport and the half-maximal inhibitory concentration (IC_{50}) was ~0.3 μM (Figure 1B). When rICP47 was heated to 95°C for 30 min and cooled, the protein retained most or all of its ability to block TAP (Figure 1B). The inhibition of TAP function by rICP47 required the presence of intact rICP47 protein and was not mediated by residual thrombin because PMSF-inactivated thrombin alone, without rICP47, did not inhibit transport. In addition, ICP47 preparations treated with trypsin, followed by inactivation of the trypsin with trypsin inhibitor, abrogated the ability of rICP47 to interfere with peptide transport, demonstrating that the effect was due to the protein in the preparation.

The ability to introduce ICP47 directly into cells allowed us to address two questions raised by previous studies. First, is TAP-dependent transport in lymphoblastoid cell lines inhibited by ICP47? This question arose because of the previous observation that ICP47 expressed by HSV-1

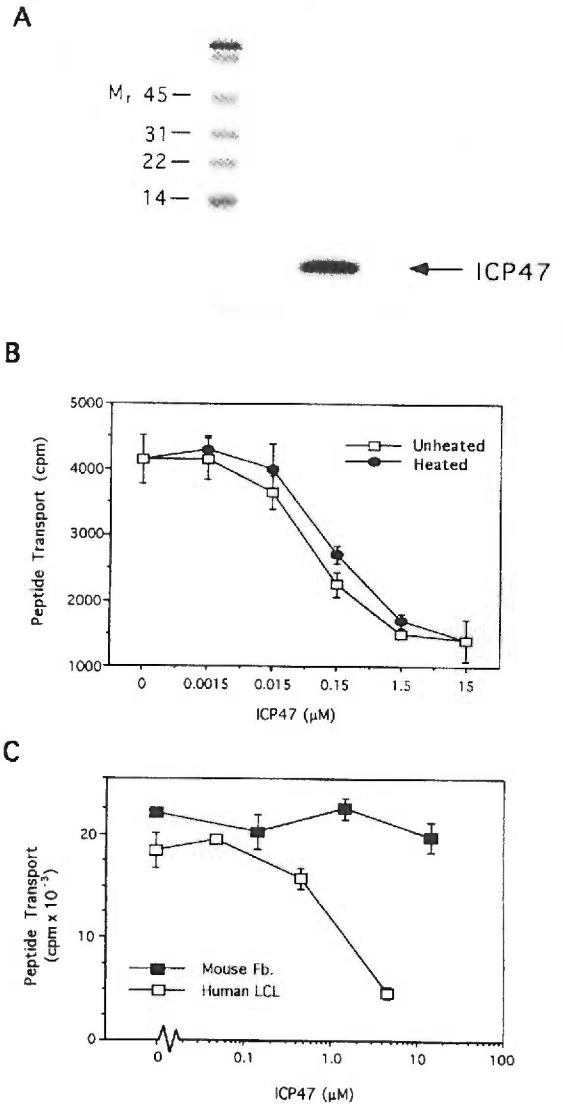

Fig. 1. Expression of HSV-1 ICP47 in bacteria and inhibition of TAP-mediated transport in permeabilized cells. (A) The GST–ICP47 fusion protein bound to glutathione–Sepharose was cleaved using thrombin. The thrombin was inactivated by PMSF and the rICP47 was subjected to SDS–gel electrophoresis and stained with Coomassie Blue. Marker proteins are shown on the left of the gel. (B) Human fibroblasts were permeabilized using streptolysin O and incubated with various quantitites of untreated rICP47 (open symbols), or rICP47 that had been heated to 95°C for 30 min (closed symbols) as well as the ^{125}I-labelled peptide library for 10 min at 37°C. (C) Mouse fibroblasts (closed symbols) or human B lymphoid cells (LCL) were permeabilized using streptolysin O and incubated with various quantities of rICP47 and ^{125}I-labelled peptide library for 10 min at 37°C. The cells were lysed using 0.5% NP-40 and cell lysates incubated with conA–Sepharose, the conA–Sepharose was washed and the glycosylated peptides were eluted with α-methylmannoside and counted.

or HSV-2 or using adenovirus (Ad) vectors inhibited antigen presentation in fibroblasts but not in human B lymphocyte lines (York *et al.*, 1994). When rICP47 was introduced into permeabilized B cells, peptide transport

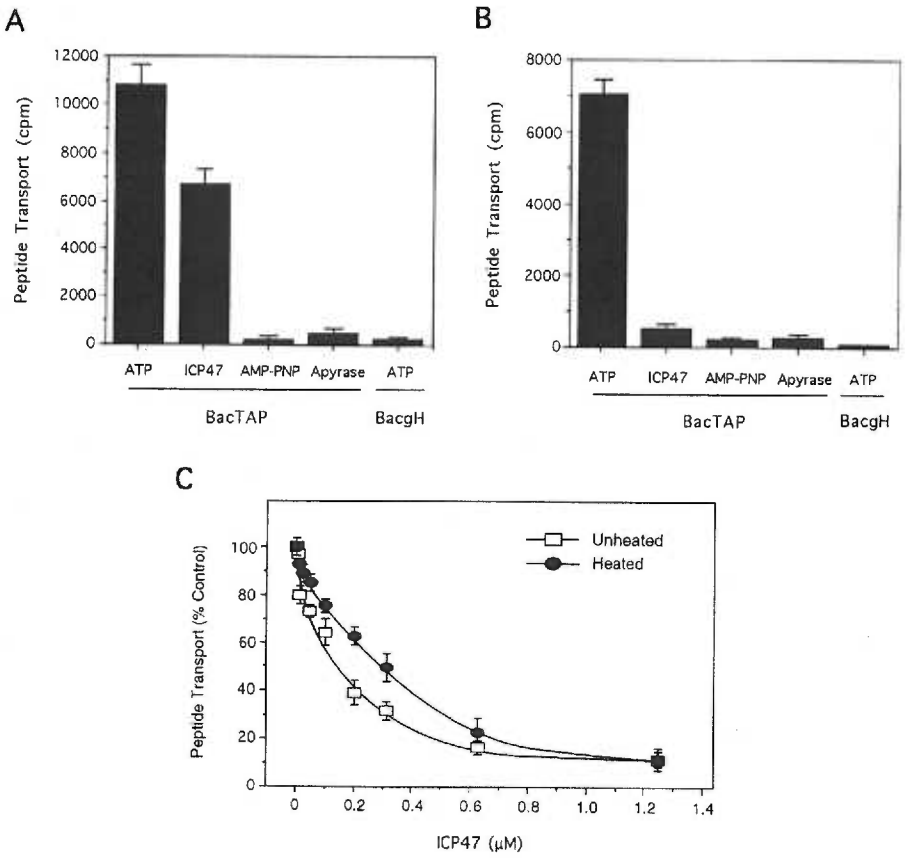

Fig. 2. Inhibition of TAP-mediated peptide translocation in insect microsomes by ICP47. Microsomes derived from insect cells infected with baculovirus vectors expresssing TAP1 and TAP2 (BacTAP) or HSV glycoprotein H (BacgH) were incubated with ATP (5 mM), rICP47 (1 μM in A and B) in the presence of ATP (5 mM), AMP-PNP (5 mM) for 5 min at 4°C or apyrase (20 U/ml) for 5 min at 37°C then the ^{125}I-labelled peptide library was added for 15 min at 37°C (**A**) or 23°C (**B** and **C**). In (C), the rICP47 was heated to 95°C for 10 min (closed symbols) or not heated (open symbols). The microsomes were washed twice, then lysed by using 1% NP-40, lysates clarified and incubated with conA–Sepharose. Radioactivity eluted with 200 mM α-methylmannoside was counted.

was inhibited and the IC_{50} was similar to, or perhaps marginally higher than, that observed with fibroblasts (Figure 1C). Therefore, the rICP47 effectively blocks TAP-mediated peptide transport in both human cell types. Second, is ICP47 able to block the function of mouse TAP? We had observed earlier that intracellular transport of class I molecules and presentation of viral antigens was normal in HSV-infected mouse cells (York *et al.*, 1994). When rICP47 was introduced into mouse fibroblasts (Figure 1C) or mouse EL4 lymphoid cells (not shown), there was little or no inhibition of TAP activity even when 15 μM rICP47 was used. Parenthetically, the ability of rICP47 to inhibit TAP in human cells and not mouse cells strengthens the conclusion that the inhibitory activity is due to specific protein–protein interactions, and is not attributable to non-specific toxicity of the preparation.

Inhibition of TAP activity in microsomes derived from insect or mammalian cells by rICP47

To characterize further the interaction between ICP47 and TAP, we expressed TAP in insect cells by using recombinant baculoviruses. Such vectors have the advantage of producing high levels of TAP in membranes that do not normally possess the TAP proteins. Microsomes were prepared from insect cells co-infected with baculoviruses expressing TAP1 (T1.5) and TAP2 (T2.12) as described previously (van Endert *et al.*, 1994). The membranes were incubated with a radiolabelled peptide library which can be glycosylated and retained in the ER (Heemels *et al.*, 1993). Peptide transport by the insect microsomes containing TAP was inhibited by apyrase treatment or the inclusion of AMP-PNP, a non-hydrolysable analogue of ATP (Figure 2A and B), and is, therefore, ATP dependent. In addition, transport was TAP dependent as indicated by use of microsomes from cells infected with a control baculovirus vector, BacgH. rICP47 effectively inhibited peptide translocation in TAP-expressing insect microsomes at 23°C and, again, when the protein was heated it retained its ability to block peptide transport (Figure 2B and C). Peptide transport in these insect microsomes was not affected by 10 μM GST, an irrelevant protein produced in bacteria (not shown). The IC_{50} of rICP47 inhibition of TAP-mediated peptide transport at 23°C was ~0.2 μM (Figure 2C), a value similar to that observed when ICP47 was introduced into permeabilized mammalian cells. However, rICP47 was less effective in inhibiting peptide

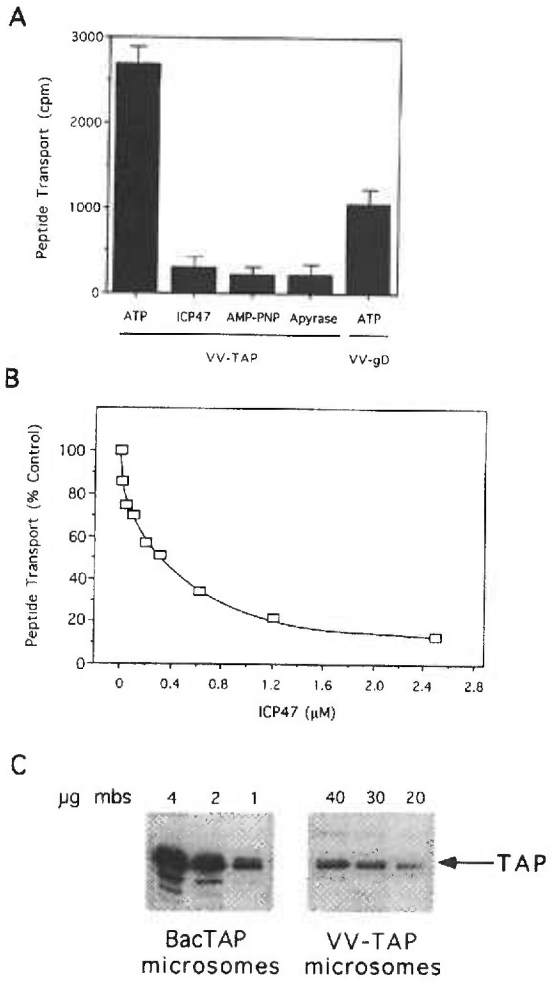

Fig. 3. Inhibition of peptide translocation in human microsomes by rICP47 and comparison of TAP levels in insect and human microsomes. (**A**) Microsomes derived from human KB cells infected with vaccina vectors expressing TAP1 and TAP2 (VV-TAP) or HSV glycoprotein D (VV-gD) were incubated with ATP (5 mM), rICP47 (1 uM) in the presence of ATP (5 mM), AMP-PNP (5 mM) for 5 min at 4°C or with apyrase (20 U/ml) for 5 min at 37°C then the ^{125}I-labelled peptide library was incubated with the membranes for 15 min at 37°C. (**B**) Microsomes from human cells infected with VV-TAP were incubated with various quantities of ICP47 in the presence of 5 mM ATP and radiolabelled peptide library for 15 min at 37°C. The membranes were washed, lysed in 1% NP-40 and radioactivity associated with conA–Sepharose counted. (**C**) Various quantities (μg of total membrane protein/lane) of insect microsomes containing TAP (BacTAP) or human microsomes containing TAP (VV-TAP) were subjected to electrophoresis using SDS–polyacrylamide gels, proteins transferred to nitrocellulose and the blots incubated with anti-TAP1 serum then washed and incubated with [^{125}I]protein A. The TAP1 band was quantified using a PhosphorImager and compared with a TAP1 standard that had been immunoprecipitated from insect cells and the quantity of TAP1 was determined by silver staining.

transport in these insect microsomes at 37°C, even though peptide transport was more efficient at the higher temperature (Figure 2A). This may be related to previous observations that peptide binds poorly to these membranes at 37°C (van Endert *et al.*, 1994) or that TAP can lose immunoreactivity when cell extracts are incubated at 37°C (Russ *et al.*, 1995), although there was no evidence of proteolysis of TAP in our microsomes at 37°C.

To examine rICP47's effects on TAP expressed in mammalian cell microsomes, we expressed TAP in human KB cells using a vaccinia virus vector, VV-TAP1&2 (Russ *et al.*, 1995) and prepared microsomes from the cells. Again, translocation of peptides was ATP dependent; however, with these human microsomes, there was significant endogenous TAP activity associated with microsomes from cells infected with a control vaccinia vector, VV-gD (Figure 3A). rICP47 inhibited the peptide transport by ~85% at 37°C (Figure 3B). Moreover, the IC_{50} of this inhibition was ~0.3 μM, similar to the value observed with insect microsomes at 23°C. Peptide translocation by the VV-TAP1&2 microsomes was also observed at 23°C, although at a reduced level, and ICP47 inhibited this translocation quite effectively (not shown). Therefore, ICP47 can inhibit TAP-mediated peptide translocation in both insect and human microsomes, albeit at different optimal temperatures.

We compared the quantity of TAP expressed in the human microsomes with that in the insect microsomes. The TAP proteins present in the insect microsomes could be visualized directly after samples were subjected to electrophoresis and then stained with silver reagent. In addition, TAP immunoprecipitated from insect cells was used to standardize Western blots. In Figure 3C, microsomes from insect cells infected with BacTAP1&2 or from human cells infected with VV-TAP1&2 were subjected to electrophoresis and blots probed with a polyclonal anti-TAP1 antiserum (Cromme *et al.*, 1994). The anti-TAP1 serum detects TAP1, the lower, more intense band, but also cross-reacts to some extent with TAP2, the upper, less intense band in these blots. PhosphorImager analysis indicated that there was ~60-fold more TAP1 in the insect microsomes than in the human microsomes (21 ng of TAP1/μg membrane protein versus 0.34 ng/μg). However, the total amount of peptide translocated by the human microsomes was only 4- to 6-fold lower than with similar quantities (micrograms of membrane protein) of insect microsomes, suggesting that the peptide translocation activity of TAP in human microsomes was 10- to 15-fold higher than in the insect cell membranes.

ICP47 inhibits peptide binding to TAP but not ATP binding

To study the effect of ICP47 on peptide binding to TAP, we used insect microsomes because the lower level of TAP in the human microsomes made analysis of peptide binding more difficult and control membranes devoid of TAP were available in the insect system. Insect microsomes were treated with apyrase and then incubated with the radioiodinated peptide library at 4°C, to ensure that peptide transport was kept to a minimum (van Endert *et al.*, 1994). Peptide binding was TAP dependent (Figure 4A) and was inhibited by rICP47 (IC_{50} ~0.2 μM) (Figure 4B), in agreement with the inhibition of peptide transport.

ATP binding to TAP was measured in the absence and presence of ICP47 by using 8-azido [^{32}P]ATP which can be cross-linked to TAP (Muller *et al.*, 1994; Russ *et al.*, 1995). Insect microsomes were incubated with 8-azido [^{32}P]ATP, then this reagent was cross-linked to TAP by using UV light and samples were immunoprecipitated

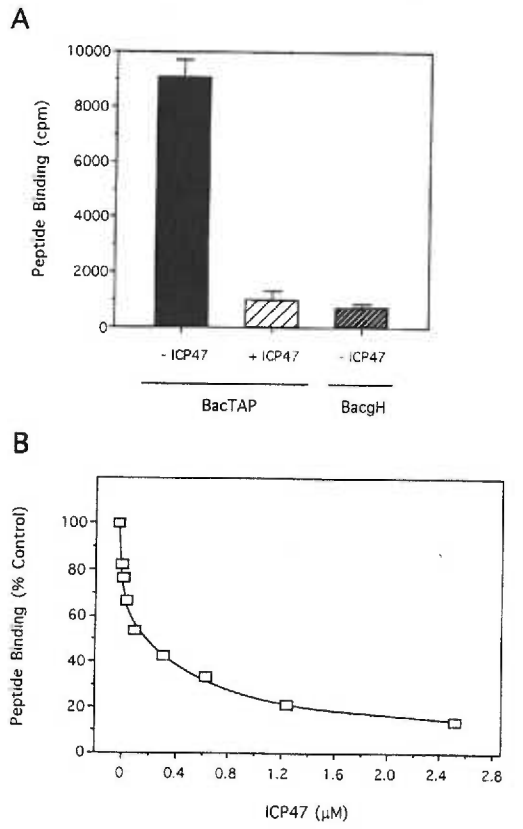

Fig. 4. Inhibition of peptide binding to TAP by rICP47. Microsomes derived from insect cells infected with BacTAP or with BacgH were treated with apyrase, washed in assay buffer, then incubated with or without rICP47 [1 μM in (**A**), or various concentrations in (**B**)] for 5 min at 4°C. The ^{125}I-labelled peptide library was added to the membranes for a further 20 min at 4°C. The membranes were washed twice in assay buffer, pelleted by centrifugation and radioactivity associated with the microsomes counted.

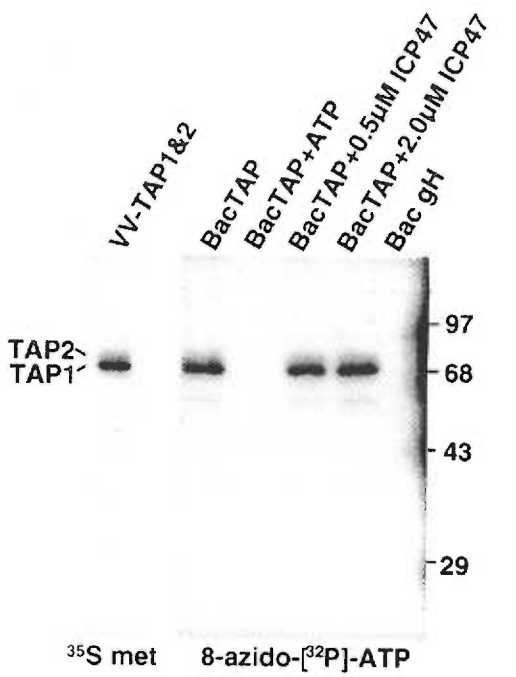

Fig. 5. Effects of rICP47 on ATP binding to TAP. Microsomes derived from insect cells infected with BacTAP or BacgH were washed in buffer and incubated with nothing, 5 mM ATP, 0.5 μM rICP47 or 2 μM rICP47 for 15 min at 4°C then 8-azido-[^{32}P]ATP was added for 15 min at 4°C in the dark. Samples were subjected to 254 nm light from a 1000 W source for 10 s then diluted with buffer containing 1% NP-40 and 0.5% DOC and TAP immunoprecipitated using anti-TAP1 antibodies. For comparison, the TAP complex was radiolabelled with [^{35}S]methionine in human cells infected with VV-TAP1&2 and immunoprecipitated using anti-TAP1 antibodies.

using anti-TAP1 antibodies. We observed radiolabelled TAP1 after immunoprecipitation with the anti-TAP1 antiserum, with only a small amount of TAP2 co-precipitated (Figure 5). 8-Azido [^{32}P]ATP labelling of TAP was inhibited by 5 mM ATP and was not observed with insect microsomes infected using a control baculovirus, BacgH (Figure 7), or in samples not photolysed (not shown). Incubation of insect microsomes with rICP47 had no effect on ATP binding to TAP (Figure 5), even at concentrations of rICP47 higher than required to inhibit translocation (0.5 and 2 μM). Therefore, while ICP47 inhibits binding of peptides to TAP, it does not affect ATP binding.

Properties of ICP47 binding to TAP and competitive inhibition by peptides

Since ICP47 could inhibit the binding of peptides to TAP, it was of interest to determine the properties of the ICP47 binding site on TAP. Microsomes derived from BacTAP-infected insect cells bound ^{125}I-labelled rICP47 and this binding was saturable (Figure 6A). Binding to control microsomes lacking TAP (derived from BacgH-infected cells) was <10% of that observed with TAP-containing microsomes (not shown), and this non-specific binding was subtracted from the binding observed with BacTAP membranes. Binding of rICP47 to BacTAP microsomes reached a plateau at ~1 μM ICP47 in assays involving ~0.3 μM TAP. Thus, it is possible that more than a single ICP47 molecule binds per TAP complex. Scatchard analyses of these data suggested that rICP47 bound to TAP complexes with a K_D of 5.2×10^{-8} M (Figure 6B). By comparison, peptides that are capable of binding to TAP with relatively high affinity can display K_D values in the range of 4×10^{-7} M, although most peptides bind with lower affinities (van Endert *et al.*, 1994). Gel electrophoresis of the ICP47 which bound to insect microsomes containing TAP did not reveal degradation of the protein (not shown) and previously we demonstrated that full-size ICP47 was associated with TAP in cells (Hill *et al.*, 1995) and, thus, there was no evidence that fragments of ICP47 were inhibiting TAP. Other experiments in which ICP47 binding was measured at 23°C revealed similar binding characteristics, although at 37°C the affinity of ICP47 for TAP-containing insect microsomes was reduced (results not shown).

Two types of competition experiments were performed to examine the relative affinities of ICP47 versus the peptide library for TAP. In the first, various quantities of labelled rICP47 were mixed with unlabelled peptide library and then immediately added to BacTAP microsomes and binding of rICP47 measured. The peptide library inhibited

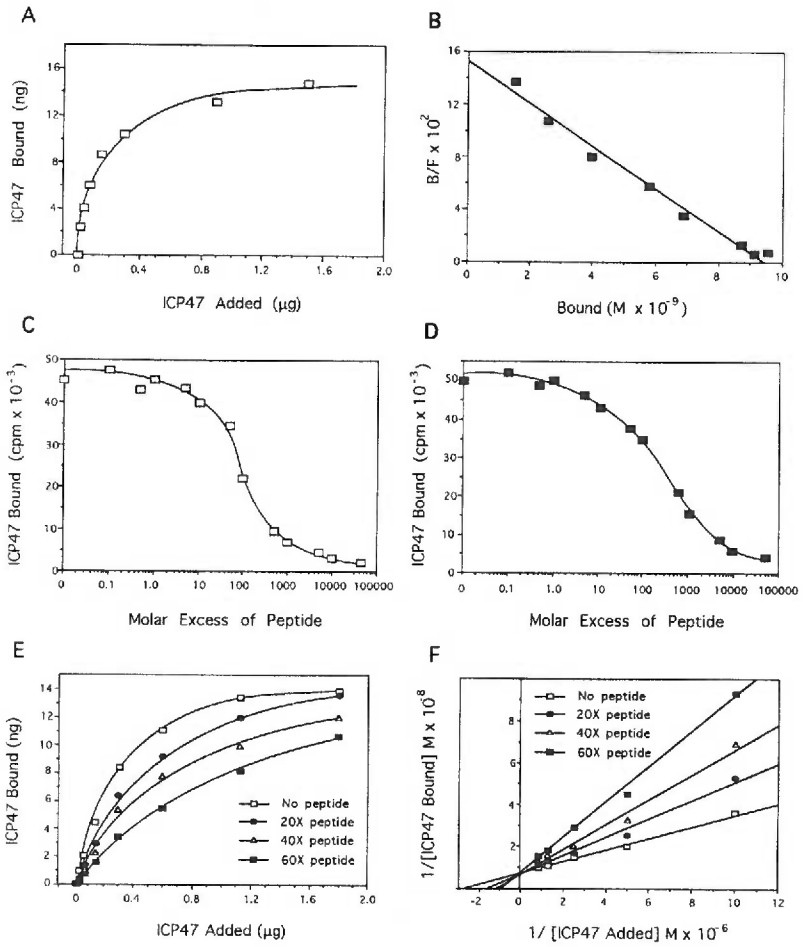

Fig. 6. Properties of ICP47 binding to TAP and competition for ICP47 binding by peptides. (**A**) ^{125}I-Labelled rICP47 was incubated with microsomes derived from insect cells infected with BacTAP or BacgH for 45 min at 4°C in assay buffer. The membranes were washed twice in assay buffer, pelleted by centrifugation and radioactivity associated with the microsomes was counted. For each concentration of ICP47, binding to the BacgH membranes was subtracted from the binding observed with BacTAP membranes. (**B**) Scatchard analysis of the binding of rICP47 was performed as described previously (Johnson *et al.*, 1990). (**C**) Insect microsomes containing TAP were mixed with ^{125}I-labelled ICP47 (0.2 μM) and, at the same time, the peptide library (unlabelled), at various molar ratios relative to the ICP47, for 45 min at 4°C. (**D**) ^{125}I-Labelled rICP47 was incubated with the microsomes for 15 min at 4°C, then various quantities of the peptide library were added for an additional 30 min at 4°C. The membranes were washed and radioactivity associated with the membranes counted. (**E**) Binding of radiolabelled rICP47 was measured as in (A) except that a constant amount of unlabelled peptide library: 6 μM (20×), 12 μM (40×) or 18 μM (60×) was present for each plot. (**F**) A plot of the reciprocal of substrate (ICP47) versus the reciprocal of substrate bound was performed on the data shown in (E).

binding of rICP47 to the microsomes in a dose-dependent manner, so that a 120-fold molar excess of peptide over ICP47 was required to inhibit ICP47 binding by 50% (Figure 6C). In a second experiment, rICP47 was incubated with microsomes for 15 min at 4°C then peptides were added for an additional 30 min and rICP47 binding was measured. In this case, a 450-fold molar excess of peptide over ICP47 was required to reduce binding of ICP47 by 50% (Figure 6D). Thus, ICP47 binds to TAP with a higher affinity than does peptide.

To test more directly whether ICP47 could bind to the peptide binding site of TAP, we determined whether peptides could act as competitive inhibitors of rICP47 binding. Binding experiments involving ^{125}I-labelled rICP47 similar to those described in Figure 6A were performed, except that ICP47 was titrated in the presence of a fixed concentration of peptide (Figure 6E). Plots of the reciprocal of substrate (ICP47) versus the reciprocal of the substrate bound (Segel, 1976) produced lines that intersected at a point close to the *y*-axis (Figure 6F). Therefore, at infinite concentrations of ICP47, its binding is unaffected by different quantities of competitor (peptide). This demonstrates classical competitive inhibition and is strong support for the hypothesis that ICP47 binds to a site on TAP which includes the peptide binding domain of TAP.

The off-rate of ICP47 and peptides from TAP

To examine the stability of the binding of ICP47 to TAP, we compared the dissociation of ICP47 from TAP with that of peptide from TAP. Insect microsomes were incubated with either radiolabelled rICP47 or radiolabelled

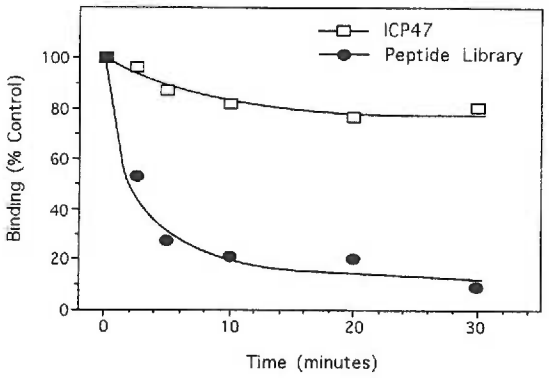

Fig. 7. Comparison of the off-rate of rICP47 versus peptides from TAP. Microsomes derived from insect cells infected with either BacTAP or BacgH were treated with apyrase, washed and then incubated with either radiolabelled rICP47 (open symbols) or radiolabelled peptide library (closed symbols), each at 0.2 μM, for 20 min at 4°C. Membranes were washed once and then resuspended in assay buffer containing 10 mM AMP-PNP. At various times, aliquots of the membranes were pelleted for 5 min and the radioactivity associated with the pellet counted. Binding of peptide or rICP47 to BacgH microsomes was subtracted from that observed with BacTAP microsomes.

peptide library, then the membranes were washed and suspended in buffer containing AMP-PNP. Previously, van Endert *et al.* (1994) showed that ATP or a non-hydrolysable ATP analogue increased the rate of peptide removal from TAP. At various times, the membranes were pelleted and the radioactivity associated with the membranes measured. More than 80% of the rICP47 was present after 30 min of incubation, whereas >90% of the peptides were displaced during this period (Figure 7). In other experiments, in which the microsomes were suspended in buffer containing ATP, similar results were obtained. Therefore, once bound, ICP47 remains associated with TAP in a stable fashion but peptides dissociate more readily.

Membrane topology of the ICP47 binding site

It is conceivable that ICP47 is itself translocated across the membrane, either entirely or in part, to yield the inactive ICP47–TAP complex. We performed proteolysis on TAP-containing microsomes incubated with rICP47 and assayed degradation of ICP47 and TAP. As a control, membranes containing a soluble form of HSV glycoprotein H (gH), which is transported across the ER and lacks a cytosolic domain, were similarly treated with protease. Western blots were performed to detect proteolysis of TAP and rICP47 in the BacTAP microsomes and of gH in the BacgH microsomes. ICP47 was completely destroyed following incubation with proteinase K for 5 min; a polyclonal serum directed to the entire protein did not recognize ICP47 fragments (Figure 8). Similarly, TAP was degraded after a 5 min incubation with proteinase K, although fragments of TAP1 were observed. The gH protein was largely resistant to proteolysis, although there was a faster migrating form which was sensitive to protease and was apparently not glycosylated and inserted into the membrane (Figure 8). gH was degraded when membranes were treated with proteinase K in the presence of detergents (not shown). The sensitivity of ICP47 to

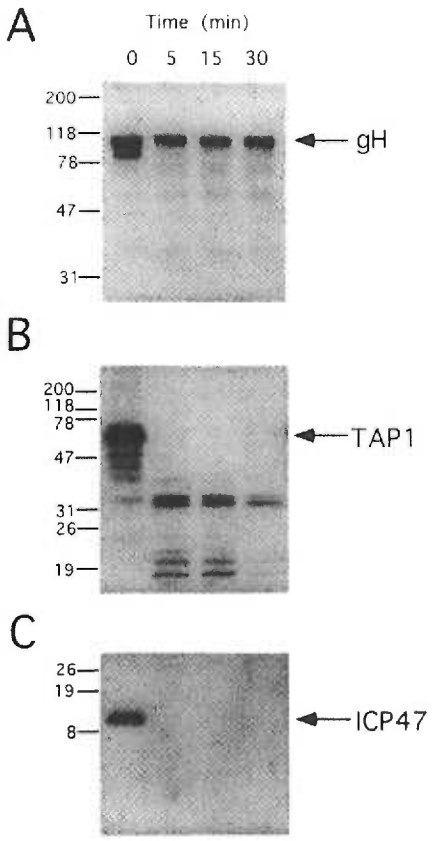

Fig. 8. Proteolysis of TAP and ICP47 bound to TAP in insect microsomes. (**A**) Microsomes from insect cells infected with BacgH were incubated for 30 min at 23°C in the presence of 5 mM ATP. (**B**) and (**C**) Microsomes from BacTAP-infected cells were incubated for 30 min at 23°C in the presence of 5 mM ATP and rICP47 (0.5 μM). Both sets of membranes were washed and resuspended in PBS containing 5 mM $MgCl_2$, 5 mM $CaCl_2$ and 1 mM DTT. Proteinase K (100 μg/ml) was added and incubated with the microsomes for 0, 5, 15 or 30 min then the proteinase K was inactivated by using 1 mM PMSF and the samples were mixed with 2% SDS and 2% β-mercaptoethanol and immediately heated to 100°C for 10 min. The samples were subjected to electrophoresis in SDS gels, proteins transferred to nitrocellulose and blots incubated with either anti-TAP1, anti-gH or anti-ICP47 serum, followed by incubation with [^{125}I]protein A. The blots were dried and exposed to X-ray film.

proteolysis suggests that ICP47 remains confined to the cytosolic side of the microsomal membrane.

Discussion

The role of TAP in mediating peptide transport into the ER for MHC class I antigen presentation is well established, and some of the biochemical and functional properties of TAP have been described (reviewed in Heemels and Ploegh, 1995; Hill and Ploegh, 1995; Howard, 1995). By analogy with other members of the ABC transporter family, TAP1 and TAP2 each span the membrane six or eight times and possess C-terminal, cytosolic domains which contain Walker box motifs that bind ATP (Russ *et al.*, 1995). Both TAP1 and TAP2 are required for peptide transport (Neefjes *et al.*, 1993; Meyer *et al.*, 1994;

van Endert *et al.*, 1994), and recent studies involving cross-linkable peptide analogues have indicated that the peptides bind to residues contributed by both TAP1 and TAP2 (Androlewicz *et al.*, 1994). However, at present, the detailed properties and composition of the peptide binding site of TAP and the mechanism by which peptide is translocated across the ER membrane are not well understood. Thus, studies of HSV ICP47 protein have the potential to provide valuable information on how TAP functions. In addition, by blocking TAP, ICP47 may be useful as a specific inhibitor of class I antigen presentation *in vitro* and *in vivo*.

Here, we have characterized the mechanism by which HSV ICP47 inhibits TAP-mediated peptide translocation across the ER membrane. ICP47 inhibited binding of peptide to TAP, yet there was no observed effect on ATP binding, consistent with earlier observations that peptide and ATP binding are independent (Shepherd *et al.*, 1993; Androlewicz and Cresswell, 1994; Schumacher *et al.*, 1994; van Endert *et al.*, 1994). In other experiments, we found no evidence that ICP47 could, itself, bind peptide or ATP (P.Jugovic and D.C.Johnson, unpublished). To characterize ICP47 binding to TAP, we measured the affinity of ICP47 for TAP and found that the K_D of this binding was $\sim 5\times10^{-8}$. This value is ~10-fold lower than for a peptide, R-9-L, ($K_D = 4\times10^{-7}$) (van Endert *et al.*, 1994). This specific peptide had a particularly high affinity for TAP, and the majority of other peptides tested in this previous report exhibited lower (10- to 1000-fold) affinities for TAP in competition assays. In our competition experiments involving a peptide library (composed of >2000 9mers), inhibition of ICP47 binding required peptide concentrations 100- to 1000-fold in excess of the ICP47 concentration. In addition, peptides were displaced from TAP much more rapidly than was ICP47, even at 4°C and under conditions where peptide was not transported. Therefore, it appears that ICP47 has a higher affinity than peptides for TAP and, once bound, ICP47 remains in that form for a relatively longer period.

The observation that ICP47 inhibited peptide binding to TAP combined with the finding that peptides inhibited ICP47 binding suggested that ICP47 binds to a site which includes the peptide binding site of TAP. However, to test this hypothesis more directly, we measured whether the binding of ICP47 was competitively inhibited by the peptide library. The results showed conclusively that ICP47 binds to a site on TAP which includes or overlaps the peptide binding site. However, one must consider that ICP47 is an 88 amino acid protein which presents a substantially larger surface for possible interactions with TAP than would be expected with 8–10 residue peptides. More extensive interactions with TAP may allow ICP47 to bind more tightly than peptides, and its size may preclude transport across the membrane. Indeed, ICP47 remained entirely sensitive to protease treatment after binding to TAP under conditions which promote peptide transport. This result is consistent with the notion that the peptide binding site of TAP is accessible to proteases added from the cytoplasmic surface. We conclude that ICP47 binds with relatively high affinity and in a stable fashion to a domain of TAP which includes the peptide binding site.

Remarkably, ICP47 could be heated to 100°C without loss of its ability to inhibit TAP. More recently, the entire 88 residue ICP47 was chemically synthesized and could also effectively block TAP in permeabilized cells (A.Hill and H.Ploegh, unpublished). These observations suggest that, if folding of ICP47 is necessary for its function, the protein must be able to refold after heating or chemical synthesis and attain a conformation that allows binding to TAP. It is also possible that ICP47 does not attain a terminally folded conformation until binding to TAP. Consistent with this hypothesis, circular dichroism measurements suggest that ICP47 is largely disordered in solution (R.Tomazin, A.Edwards and D.C.Johnson, unpublished). These results suggest that ICP47 or fragments of ICP47 might be the starting points for immunosuppressive drugs that would selectively and specifically inhibit TAP.

Previously, we reported that MHC class I presentation in human B lymphoid cells was not affected by ICP47 expression (York *et al.*, 1994). In these and other studies (Posavad and Rosenthal, 1992; Koelle *et al.*, 1993), ICP47 was delivered into the human B cells by infection with HSV or using recombinant Ad vectors. However, since HSV and Ad vectors infect human lymphoid cells poorly and expression of TAP is higher in lymphoid cells than in fibroblasts, it was conceivable that expression of ICP47 in the B cells did not attain levels sufficient to inhibit peptide transport. The studies described here demonstrate clearly that ICP47 can inhibit TAP in B lymphoid cells, although the IC_{50} was perhaps marginally higher than that observed in fibroblasts. In contrast to the results obtained with human B cells, high concentrations of ICP47 did not inhibit TAP in mouse fibroblasts or lymphoid cells, consistent with our previous observations that class I transport and antigen presentation was normal in mouse cells infected with HSV type 1 (York *et al.*, 1994).

The levels of TAP in the insect cell microsomes were apparently 60-fold higher than that observed in VV-TAP1&2-infected human cells. We found that this high level of expression was necessary to analyse accurately the interactions between ICP47 and TAP. Previously, we were unable to detect binding of ICP47, produced by *in vitro* translation, to microsomes derived from human cells (York *et al.*, 1994), although, when TAP expression was increased in these membranes by using VV-TAP1&2, binding of the *in vitro* translated ICP47 could be observed (R.Tomazin, unpublished). Peptide translocation in the human membranes appeared to display a 10- to 15-fold higher specific activity than that observed with the insect cell membranes, similar to previous observations (van Endert *et al.*, 1994). However, since peptide translocation was not measured directly, it is also conceivable that other components, e.g. glycosylation machinery, were limiting in these insect microsomes. These observations are intriguing and may relate to the absence of mammalian membrane proteins, e.g. MHC class I–β_2-m dimers or cytoplasmic factors, or to differences in lipid content and membrane fluidity between mammalian and insect microsomes.

Accompanying the reduced specific activity of TAP in the insect microsomes, we found that ICP47 inhibited TAP most effectively at 23°C, the optimal temperature for insect cell growth, whereas ICP47 was less effective in inhibiting TAP at 37°C. It has been suggested that TAP may exist in two conformations in the insect microsomes,

so that peptide binds better at lower temperatures and in the absence of ATP, whereas transport is maximal at higher temperatures (van Endert *et al.*, 1994). Together, these observations are consistent with the hypothesis that ICP47 functions more effectively under conditions that favour peptide binding, rather than transport. Nevertheless, ICP47 effectively inhibited peptide transport in both the insect (at 23°C) and mammalian microsomes (at 23 or 37°C) with an IC_{50} of ~0.2 μM, suggesting that, under these conditions, ICP47 could bind similarly to TAP in both mammalian and insect membranes. It is possible that binding of ICP47 to TAP is influenced by MHC class I–β_2-m in the complex or that there are defects in folding of TAP in insect cell membranes at 37°C, in the absence of mammalian proteins.

Given the results presented here, how should we view the effects of ICP47 during the course of an HSV infection in the human host? ICP47 is a member of the first class of proteins that is expressed in infected cells. By binding to TAP in a relatively stable manner and inhibiting peptide transport into the ER, it appears that ICP47 can inhibit MHC class I presentation of all other classes of HSV polypeptides, which make up the vast majority of proteins expressed in infected cells. This inhibition is observed in infected human fibroblasts and keratinocytes, which express relatively low levels of TAP, but not in B cells that express higher levels of TAP (Posavad and Rosenthal, 1992; Koelle *et al.*, 1993; York *et al.*, 1994), and which are not important hosts for the virus. The effects of ICP47, and other viral polypeptides, e.g. the vhs protein (Tigges *et al.*, 1996), appear to explain major defects in the CD8+ T lymphocyte response to HSV observed *in vivo* (reviewed in Schmid and Rouse, 1992; York and Johnson, 1995). Very few CD8+ T cells are observed during early stages of HSV lesions, and those that can be observed recognize primarily virion structural proteins which are delivered into cells on infection (Tigges *et al.*, 1992), and are most likely to bypass a TAP blockade. However, the effects of ICP47 on skin cells appear to be largely overcome by treating the cells with interferon-γ (IFN-γ) which up-regulates TAP and other components of the class I presentation pathway (Tigges *et al.*, 1996; R.Hendricks and D.C.Johnson, unpublished). *In vivo*, CD8+ T cells appear in HSV lesions coincident with or following the expression of IFN-γ (Cunningham and Noble, 1989). Thus, the effects of ICP47 may be important in conferring resistance to CD8+ T lymphocytes during early stages of virus replication, allowing virus replication and dissemination, but compensatory immune mechanisms may ultimately allow for recognition and control of virus spread.

Materials and methods

Production of recombinant ICP47 and radioiodination

The HSV-1 ICP47 coding sequences were fused downstream of the GST gene in the plasmid pGEX-2T (Pharmacia) producing plasmid pGEX-2T-47-1. Cultures of *Escherichia coli* (HB101) containing pGEX-2T-47-1 were grown overnight, induced with isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h then the cells were suspended in phosphate-buffered saline (PBS) containing an additional 350 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and a protease inhibitor cocktail (1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml chymostatin) and disrupted using a French press. Cell lysates were centrifuged at 12 000 *g* for 30 min then the supernatants were incubated with glutathione–Sepharose (Pharmacia) for 3 h with constant mixing at 4°C. The glutathione–Sepharose was loaded into a small column and washed three times with PBS containing an additional 350 mM NaCl and 0.1% Triton X-100, washed three times with PBS and then incubated with thrombin (Sigma, 0.35 μg/ml) for 45 min at 22°C in 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$. The ICP47 (100–400 μg/ml) was then removed from the column by washing with PBS and the thrombin inactivated with 1 mM PMSF. The ICP47 was aliquoted, snap-frozen in liquid N_2 and stored at –70°C. ICP47 (10 μg) was radioiodinated in 50 mM Tris–HCl, pH 7.5 (250 μl) containing 1 mCi Na[^{125}I] and a single Iodobead (Pierce) for 2–15 min, producing material with a specific activity of $1–6\times10^7$ c.p.m./μg.

Peptide translocation in permeabilized cells

Human lung fibroblasts (CCD-18LU) were obtained from ATCC. Mouse fibroblasts were prepared from mouse embryos and treated with recombinant IFN-γ (50 U/ml) for 24 h prior to the assay, as we previously found that peptide translocation was difficult to measure in these cells without IFN-γ treatment. Fibroblasts and human B lymphoid cells were permeabilized with streptolysin O (Bio Merieux, France) as previously described (Hill *et al.*, 1995); for each assay shown, at least 60% of the cells were unable to exclude Trypan Blue. The cells were incubated with a complex library of 2304 peptides (Heemels *et al.*, 1993) which had been radioiodinated using chloramine T (Hill *et al.*, 1995). ICP47 was either heated or not for 30 min at 95°C then cooled and introduced into the permeabilized cells in conjunction with the peptides in transport buffer (Hill *et al.*, 1995). The amount of peptide transported into the ER after 10 min at 37°C was assessed by lysing the cells with 0.5% NP-40 and applying lysates to concanavalin A (conA)–Sepharose. The conA–Sepharose was washed four times with lysis buffer and radioactivity associated with the conA–Sepharose was counted.

Preparation of microsomes containing TAP from insect and human cells

The construction and characterization of baculoviruses expressing human TAP1 (T1.5) and TAP2 (T2.12) has been described (van Endert *et al.*, 1994).

Sf9 cells were grown in Sf-900II medium (GIBCO) in suspension ($4–5\times10^6$ cells/ml) and 300–800 ml of these cultures were co-infected with either T1.5 and T2.12 baculoviruses or a baculovirus expressing a soluble form of HSV glycoprotein H (BacgH) for 68–72 h. BacgH expresses a truncated form of HSV type 1 gH in which the transmembrane and cytoplasmic domains of gH were removed (L.Hutchinson, C.Roop and D.C.Johnson, unpublished). The cell pellet was suspended in 10 mM Tris–Ac (pH 7.5), 1.5 mM MgAc, 1 mM DTT and protease inhibitor cocktail for 10 min on ice then the cells were disrupted using a Dounce homogenizer and 40 mM Tris–Ac (pH 7.5), 250 mM sucrose, 25 mM KAc, 3.5 MgAc, 0.5 mM CaAc were added before nuclei were removed by centrifugation at 800 *g* for 10 min. Mitochondria were removed by centrifugation at 10 000 *g* for 10 min then the crude microsomes were pelleted by centrifugation at 100 000 *g* for 60 min. The membranes were resuspended in 50 mM Tris–Ac (pH 7.5), 250 mM sucrose, 25 mM KAc, 5 mM MgAc, 0.5 mM CaAc, 1 mM DTT and the protease inhibitor cocktail (microsome buffer) and further purified by centrifugation through a 1.1 M sucrose cushion at 150 000 *g* for 4–5 h. The membrane pellets were resuspended in microsome buffer at 4–5 mg of membrane protein/ml, snap-frozen in liquid N_2 and stored at –70°C. Microsomes were also derived from human KB cells infected with VV-TAP1&2, a vaccinia virus containing the human TAP1 and TAP2 genes (Russ *et al.*, 1995) or infected with control vaccinia vector, VV-gD, which expresses HSV-1 gD (Cremer *et al.*, 1985). KB cells were grown in suspension in Joklik's-modified MEM containing 2% fetal bovine serum (FBS) and 10% horse serum (HS) at $3–6\times10^5$ cells/ml, concentrated 10-fold and infected with VV-TAP1&2 or VV-gD (using 5–10 p.f.u./cell) for 2 h. The infected cells were resuspended in Joklik's medium containing 1% FBS and 5% HS at 5×10^6 cells/ml and incubated for 10–12 h. Microsomes derived from the human cells were prepared as described for the insect cells except that the hypotonic buffer was 10 mM triethanolamine (TEA; pH 7.5), 1.5 mM MgAc, 1 mM DTT and the microsome buffer was 50 mM TEA (pH 7.5), 250 mM sucrose, 50 mM KAc, 2 mM MgAc, 0.5 mM EDTA, 1 mM DTT containing the protease inhibitor cocktail.

Analysis of TAP levels in microsome preparations

TAP expressed in insect microsomes could be visualized by silver staining (Bio-Rad). In addition, several micrograms of TAP were partially purified from insect cells by immunoprecipitation (York *et al.*, 1994) and used as a standard on Western blots. Western blot analysis of TAP1

expressed in insect and in human microsomes involved anti-TAP1 antibody (Cromme *et al.*, 1994) and [^{125}I]protein A as described (Brunetti *et al.*, 1994), then protein bands were quantified using a PhosphorImager.

Peptide transport into microsomes

A complex library of 2304 peptides described previously (Heemels *et al.*, 1993) was radioiodinated using chloramine T as described (Hill *et al.*, 1995). Peptide translocation was performed as described previously (Meyer *et al.*, 1994) using 20–25 µl of insect microsomes or 60–80 µl of human microsomes except that the conA–Sepharose incubation was performed for 4 h and elution with α-methylmannoside was for 8–12 h. Microsomes were treated with apyrase (Sigma, 20 U/ml) for 5 min at 37°C, or with AMP-PNP (Sigma, 5 mM).

Peptide and ICP47 binding to microsomes

Insect microsomes (20–25 µl) were treated with apyrase (20 U/ml) for 5 min at 37°C then washed and incubated with the ^{125}I-labelled peptide library for 20 min at 4°C in 150 µl of PBS containing 0.1% dialysed bovine serum albumin (BSA), 10 mM $MgCl_2$, 1 mM DTT and protease inhibitor cocktail mix without PMSF (assay buffer). The membranes were washed twice with assay buffer, pelleted by centrifugation at 20 000 *g*, then disrupted in 1% NP-40, 0.5% sodium deoxycholate (DOC), 50 mM Tris–HCl (pH 7.5), 100 mM NaCl (NP-40/DOC buffer) and radioactivity associated with the microsomes counted. For ICP47 binding, insect microsomes (20–25 µl) were incubated for 45–60 min at 4°C with varying amounts of radioiodinated ICP47 diluted in assay buffer. The membranes were washed and disrupted as above and radioactivity associated with the microsomes counted. The binding of ICP47 to microsomes derived from BacgH-infected cells was subtracted from the binding to microsomes derived from BacTAP-infected cells. Scatchard analysis was performed as described (Johnson *et al.*, 1990). Peptide competition for ICP47 binding was performed by incubating various quantitites of peptide, radiolabelled ICP47 (0.2 µM) and insect microsomes (25 µl) for 45–60 min at 4°C in assay buffer or, alternatively, by incubating microsomes with radiolabelled ICP47 for 15 min at 4°C then adding peptides for an additional 30 min at 4°C. To measure the off-rate of peptides and ICP47 from TAP, microsomes derived from insect cells infected with BacTAP or BacgH were treated with apyrase then incubated with radiolabelled ICP47 (0.2 µM) or radiolabelled peptide library (0.2 µM) at 4°C for 20 min. The microsomes were washed once and resuspended in assay buffer containing 10 mM AMP-PNP. Aliquots were taken at various times then the membranes were pelleted for 5 min using a microfuge and the radioactivity associated with the microsomes was counted.

Cross-linking of 8-azido-ATP to TAP

Insect cell microsomes (25–35 µl) were washed and suspended in 300 µl of 25 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT and the protease inhibitor cocktail (lacking PMSF). 8-Azido-[^{32}P]ATP (ICN; 3 µCi) was added and incubated with the membranes for 15 min at 4°C in the dark. The microsomes were transferred to cuvettes and photolysed with 254 nm light from a 1000 W mercury source for 10 s. The reactions were then diluted with 700 µl of NP-40/DOC buffer containing BSA (1 mg/ml) and 0.5 mM PMSF and the detergent extracts clarified by centrifugation at 20 000 *g* for 20 min. TAP was immunoprecipitated by using anti-TAP1 antiserum and protein A–Sepharose.

Proteolysis of microsomes

Insect microsomes were washed then incubated with ICP47 (0.5 µM) for 30 min at 23°C in assay buffer containing 5 mM ATP. The membranes were pelleted (20 000 *g* for 5 min) and suspended in PBS containing 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM DTT. The membranes were cooled on ice for 5 min then 100 µg/ml of proteinase K (Sigma, 20 mAnson U/mg) was added for 0, 5, 15 or 30 min at 4°C. Reactions were stopped by addition of 1 mM PMSF for 2.0 min then 2% SDS, 2% β-mercaptoethanol, 10% glycerol was added and the samples boiled for 10 min before loading on SDS–polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and TAP1 was detected using anti-TAP1 antiserum (Cromme *et al.*, 1994), gH was detected using R83 anti-gH serum (Roberts *et al.*, 1991) and ICP47 was detected using anti-ICP47-5 serum (Hill *et al.*, 1995).

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Materials and Methods (Data Not Shown):

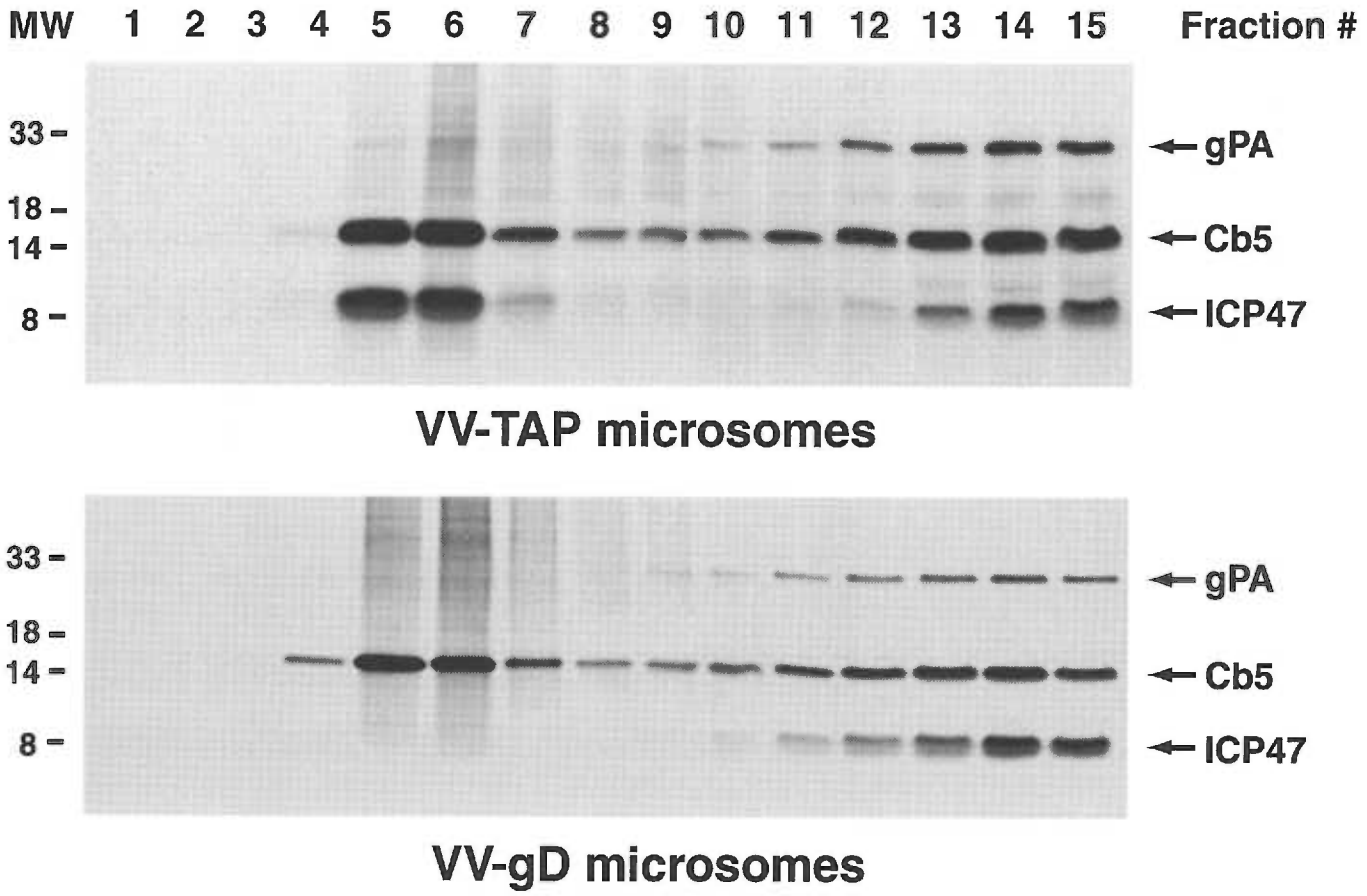
In Vitro Transcription and Translation: The ICP47 open reading frame was amplified by PCR from plasmid pRHP6 and inserted into pSPUTK to generate the plasmid pSPUTK-47. Plasmids pSPUTK-47, pSPUTK-gPA (Janiak et al., 1994a) and pSPUTK-Cb5 (Janiak et al., 1994b) were linearized by digestion with a suitable restriction enzyme prior to transcription in vitro. Briefly, linearized plasmids were resuspended in a HEPES buffer (80 mM HEPES pH 7.5, 15 mM $MgCl_2$, and 2 mM spermidine) supplemented with 10 mM DTT, RNasin, NTPs and SP6 polymerase and incubated at 37°C for 1 hr. Capped transcripts generated from the SP6 reactions were then normalized for RNA content using flourometric analysis.

For in vitro translations, approximately 1 µl of the transcription reaction was added to rabbit reticulocyte lysate supplemented with amino acids, ATP, a cocktail of protease inhibitors (PIN) and 10 µCi of ^{35}S-methionine. Reactions were incubated at 24°C for 1 hr followed by analysis of the translation products using SDS-polyacrylamide gel electrophoresis.

Microsome Binding and Gel Filtration Analysis: In vitro translated proteins, ICP47, globin-Protein A (gPA) or cytochrome b5 (Cb5) were added to microsomes purified from human KB cells infected with a vaccinia virus vector expressing TAP (VV-TAP) or a control protein, HSV glycoprotein D (VV-gD). Alternatively, iodinated recombinant ICP47 was added to microsomes purified

from Sf9 insect cells infected with baculovirus vectors expressing TAP1 and TAP2 (BacTAP) or a control protein, HSV glycoprotein H (BacgH). Samples were incubated at 24°C for 20 minutes and then subjected to gel filtration using a 1 ml Sepharose CL2B syringe column. Single drop fractions (approximately 100 µl each) were collected and subsequently analyzed using SDS-polyacrylamide gel electrophoresis.

Figure 9: Binding of in vitro translated ICP47 to TAP in human microsomes. ICP47, Cb5, which is capable of inserting into membranes, and gPA, a cytosolic protein, were translated in vitro using a rabbit reticulocyte lysate system. The proteins were then incubated with microsomes purified from human KB cells infected with a vaccinia virus vector expressing TAP (VV-TAP) or a control protein, HSV glycoprotein D (VV-gD), for 20 minutes at 24°C. The samples were then subjected to gel filtration using a Sepharose CL2B syringe column and single drop fractions were collected (indicated at the top of the figure). Excluded fractions containing microsomes were 4-7, while included fractions containing cytosolic proteins were 9-15.


MW
1
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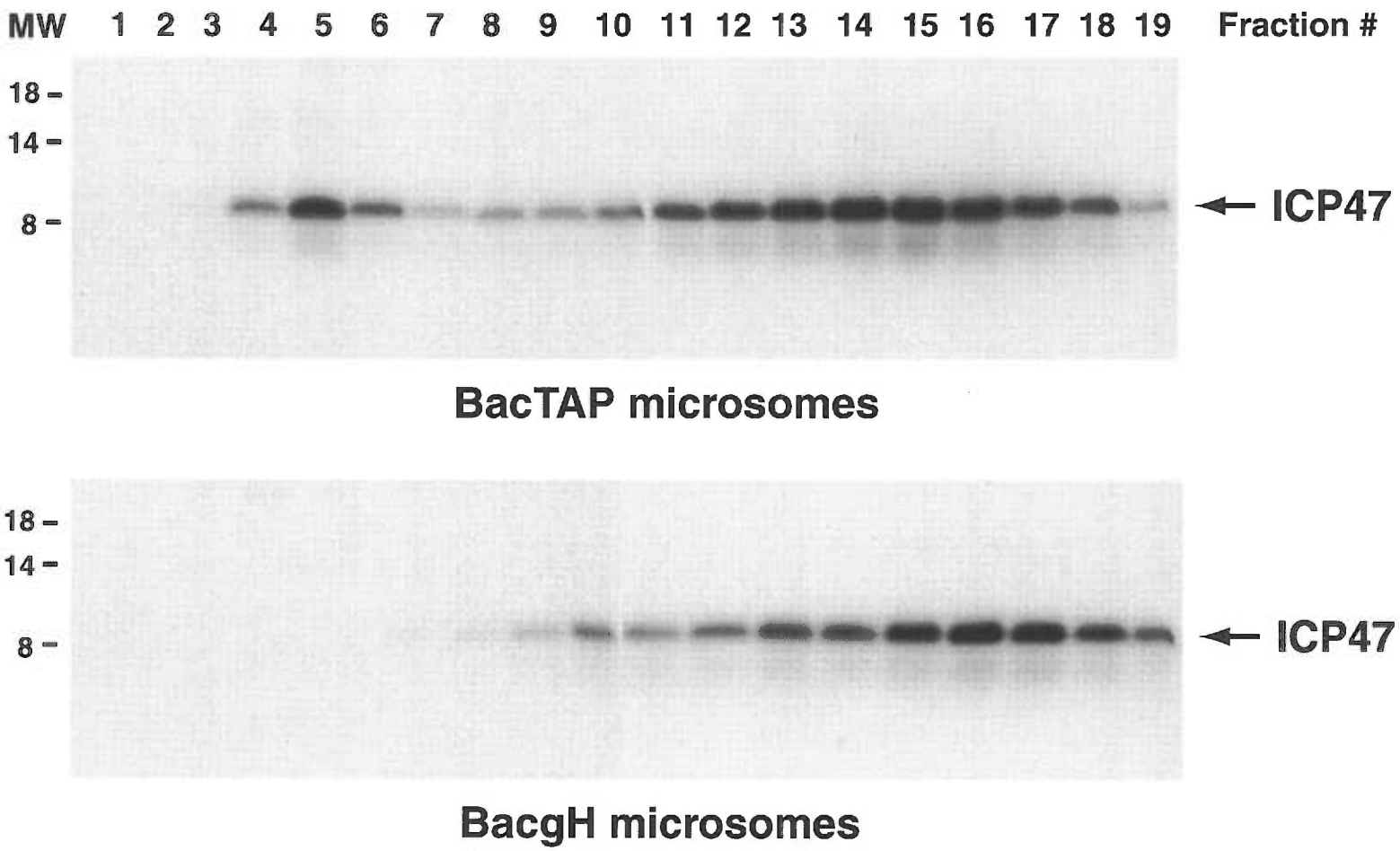
15
Fraction #
33 –
18 –
14 –
8 –
gPA
Cb5
ICP47
VV-TAP microsomes
33 –
18 –
14 –
8 –
gPA
Cb5
ICP47
VV-gD microsomes

Figure 10: Binding of recombinant ICP47 to TAP in insect microsomes. Recombinant ICP47 produced in bacteria was incubated with microsomes purified from insect cells infected with a baculovirus vector expressing TAP (BacTAP) or a control protein, HSV glycoprotein H (BacgH), for 20 minutes at 24°C. The samples were then subjected to gel filtration using a Sepharose CL2B syringe column and single drop fractions were collected (indicated at the top of the figure). Excluded fractions containing microsomes were 4-6, while included fractions containing cytosolic proteins were 9-18.


MW 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 Fraction #
18 –
14 –
8 –
← ICP47
BacTAP microsomes
18 –
14 –
8 –
← ICP47
BacgH microsomes

Chapter 3

Herpes simplex virus type 2 ICP47 inhibits human TAP but not mouse TAP

(Journal of Virology, 72: 2560-2563, 1998)

3.0 Preface

In the following publication, I carried out the experiments in Figures 3.3A, 3.4 and 3.5. The experiments in Figures 3.1, 3.2 and 3.3B were performed in our laboratory by Nico E. G. van Schoot under my direct supervision. Insect microsomes containing human TAP were purified by Cathy Wale at McMaster University in Hamilton, Ontario, Canada. Dr. Klaus Fruh at the Scripps Research Institute in La Jolla, CA provided the insect microsomes containing murine TAP.

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Herpes Simplex Virus Type 2 ICP47 Inhibits Human TAP but Not Mouse TAP

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Herpes simplex virus serotype 1 (HSV-1) expresses an immediate-early protein, ICP47, that effectively blocks the major histocompatibility complex class I antigen presentation pathway. HSV-1 ICP47 (ICP47-1) binds with high affinity to the human transporter associated with antigen presentation (TAP) and blocks the binding of antigenic peptides. HSV type 2 (HSV-2) ICP47 (ICP47-2) has only 42% amino acid sequence identity with ICP47-1. Here, we compared the levels of inhibition of human and murine TAP, expressed in insect cell microsomes, by ICP47-1 and ICP47-2. Both proteins inhibited human TAP at similar concentrations, and the K_D for ICP47-2 binding to human TAP was 4.8×10^{-8} M, virtually identical to that measured for ICP47-1 (5.2×10^{-8} M). There was some inhibition of murine TAP by both ICP47-2 and ICP47-1, but this inhibition was incomplete and only at ICP47 concentrations 50 to 100 times that required to inhibit human TAP. Lack of inhibition of murine TAP by ICP47-1 and ICP47-2 could be explained by an inability of both proteins to bind to murine TAP.

Previously, we showed that herpes simplex virus serotype 1 (HSV-1) ICP47 (ICP47-1) caused major histocompatibility complex (MHC) class I proteins to be retained in the endoplasmic reticulum (ER) of cells and that antigen presentation to $CD8^+$ T cells was inhibited after ICP47-1 was expressed in human fibroblasts (9). ICP47-1 blocked peptide transport across the ER membrane by TAP (2, 6), so that, without peptides, class I proteins were retained in the ER. By contrast, ICP47 did not detectably inhibit MHC class I antigen presentation in mouse cells (9) and inhibited murine TAP poorly (2, 6). ICP47-1 inhibited peptide binding to TAP without affecting the binding of ATP (1, 7) and bound with high affinity, and in a stable fashion, to human TAP (7). Peptides could competitively inhibit ICP47 binding to TAP, consistent with the hypothesis that ICP47-1 binds to a site which includes the peptide binding domain of TAP (7). Others have suggested that the present data do not exclude a distortion in TAP caused by the binding of ICP47 at a site distant from the peptide binding site (3). This seems improbable given our observations that ICP47 inhibits peptide binding and that peptides competitively inhibit ICP47 binding. In order for peptides to inhibit ICP47 binding and vice versa, one would have to invoke allosteric inhibition by both ICP47 and peptides, a highly unlikely prospect.

The predicted amino acid sequence of HSV type 2 ICP47 (ICP47-2) was recently described (3), and it was of some interest that ICP47-1 and ICP47-2 share only 42% amino acid identity (see Fig. 1A). Most of the homology is near the N termini and in the central regions of the molecules. A peptide including residues 2 to 35 of ICP47-1 blocked human TAP in permeabilized cells (3). This observation was somewhat surprising given that this peptide did not include residues 33 to 51, a sequence that is most homologous between ICP47-1 and ICP47-2. Presumably, this conserved domain, and even the C-terminal third of the protein, is important in virus-infected cells for stability or for functions that are not apparent in this in vitro assay involving detergent-permeabilized cells.

Given the differences between the primary structures of ICP47-1 and ICP47-2, we were interested in whether ICP47-2 might inhibit the murine TAP. If this were the case, it would make possible animal studies of the effects of ICP47. Here, we have produced a recombinant form of ICP47-2 and compared the effects of ICP47-2 and ICP47-1 on human and murine TAP proteins expressed in insect cell microsomes. Like ICP47-1, ICP47-2 efficiently blocked human TAP but even at high concentrations did not effectively block murine TAP. Moreover, there was little or no significant binding of either protein to insect microsomes containing mouse TAP.

The HSV-2 ICP47 gene was subcloned from plasmid pBB17, which contains a *Kpn*I-*Hin*dIII 8,477-bp fragment derived from the genome of HSV-2 strain HG52 inserted into pUC19, by using PCR to amplify ICP47-2 coding sequences. One PCR primer hybridized with the 5′ end of the ICP47-2 coding sequences and extended 5′ to generate a new *Bgl*II site just upstream of the initiation codon. The second PCR primer hybridized with 3′ sequences of the ICP47-2 gene, then diverged to produce an *Eco*RI site just downstream of the translation termination codon. After PCR, the DNA fragment was digested with *Eco*RI and inserted into the *Hin*cII (blunt) and *Eco*RI sites of pUC19, producing plasmid pUC47-2, which was subjected to DNA sequencing. The ICP47-2 coding sequences were excised from pUC47-2 with *Bgl*II and *Eco*RI and inserted into the *Bam*HI and *Eco*RI sites of pGEX-2T to generate a fusion protein with glutathione *S*-transferase (GST). The ICP47-GST fusion protein was expressed in bacteria and purified by using glutathione-Sepharose, and then the GST sequences were removed with thrombin as described previously for ICP47-1 (7). A comparison between the predicted amino acid sequences of ICP47-2 and ICP47-1 is shown in Fig. 1, with

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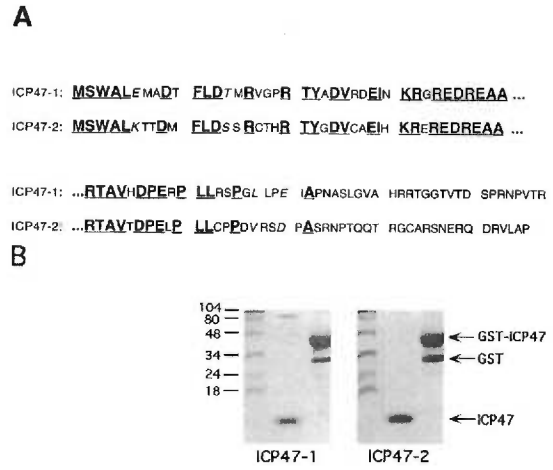

FIG. 1. Comparison of ICP47-1 and ICP47-2 protein sequences and preparation of purified proteins. (A) The predicted amino acid sequences of ICP47-1 derived from HSV-1 strain 17 (6a) and of ICP47-2 derived from HSV-2 strain HG52 (3) are shown. The boldface, underlined letters denote identical amino acids, and the italicized letters denote conserved residues. (B) ICP47-1 and ICP47-2 were produced in *Escherichia coli* by expressing the proteins as GST fusion proteins by fusing the ICP47 coding sequences to GST sequences in plasmid pGEX-2T as described previously (7). Lysates from bacteria were incubated with glutathione-Sepharose and washed several times, and then ICP47-1 or ICP47-2 was eluted by incubation with thrombin, which cleaves between the GST and ICP47 sequences (7). The thrombin was inactivated with phenylmethylsulfonyl fluoride, and the ICP47 preparations were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by Bradford protein analysis. The positions of GST-ICP47, GST, and ICP47 protein, as well as those of molecular weight markers 104, 80, 48, 34, 24, and 18 KDa in size, are indicated.

a comparative gel (Fig. 1B) showing the purified preparations of ICP47-1 and ICP47-2 from bacteria.

Microsomes purified from Sf9 insect cells infected with baculoviruses expressing human TAP1 and TAP2 have been described previously (7, 8), as were microsomes from *Drosophila* cells expressing murine TAP1 and TAP2 (1). We previously estimated that approximately 2% of the protein associated with the insect microsomes was human TAP (7), and the microsomes containing mouse TAP possessed similar TAP activity (see below). Peptide translocation by these microsomes was measured by using a library of ^{125}I-labelled peptides (5) that are glycosylated after transport into the ER. Radioactive peptides able to bind to concanavalin A were quantified as an indirect measure of peptide transport (6). Over a range of membranes from 2.5 to 20 μl, with protein concentrations of 10 to 12 mg/ml for human TAP microsomes and 5.0 to 7.0 mg/ml for mouse TAP microsomes, there was a linear increase in peptide transport (Fig. 2). Thus, peptides and ATP were not limiting. Peptide transport was specific because the transport observed with control membranes not containing TAP amounted to less than 1% of that observed when microsomes contained TAP. The levels of peptide transport associated with microsomes containing human or mouse TAP were also compared and standardized. Thus, in subsequent assays, 7.5 to 10 μl of microsomes exhibiting similar amounts of TAP activity were used.

ICP47-2 inhibited peptide transport by human TAP, and the inhibition was similar to that of ICP47-1; the 50% inhibitory concentration (IC_{50}) for ICP47-2 was 0.24 μM and for ICP47-1 was 0.27 μM (Fig. 3A). In other experiments the IC_{50} values for ICP47-1 and ICP47-2 varied from 0.15 to 0.35 μM, and there were no experiments in which there was a significant difference in the abilities of the two proteins to inhibit human TAP. Moreover, the binding properties of ICP47-2 to human TAP were similar to those of ICP47-1. Binding experiments were performed as described previously for ICP47-1 (7) by using membranes containing human TAP and ^{125}I-labelled ICP47-2. Specific binding of ICP47-2 was calculated by subtracting the binding to control microsomes derived from insect cells infected with a baculovirus expressing HSV gH (7). The binding of ICP47-2 was saturable, so that at a protein concentration of 1 μM approximately 16 ng of protein bound to human TAP (Fig. 4A). In previous experiments with a similar preparation of insect microsomes containing human TAP, the binding of ICP47-1 also saturated at 15 to 16 ng (7). The ICP47-2 binding data were analyzed in a standard Scatchard plot, and the K_D was calculated to be 4.8×10^{-8} M (Fig. 4B), compared with 5.2×10^{-8} M for ICP47-1 (7). These values are greater than those of high-affinity peptides that bind to human TAP with affinities reaching 4×10^{-7} M, though the vast majority of peptides bind to TAP with much lower affinities (8).

To determine whether ICP47-2 could inhibit the murine TAP, microsomes from insect cells expressing mouse TAP were incubated with various concentrations of ICP47-1 and ICP47-2 and TAP assays were performed. Inhibition of the mouse TAP was observed with both ICP47-1 and ICP47-2, but relatively high concentrations of both proteins were required (Fig. 3B). The IC_{50} values for ICP47-1 and ICP47-2 in this experiment were 10.8 and 16.2 μM, respectively. However, we were unable to reduce TAP activity beyond approximately 40% with ICP47-1 or ICP47-2 concentrations reaching 30 μM. This was 100 times the concentration required to inhibit human

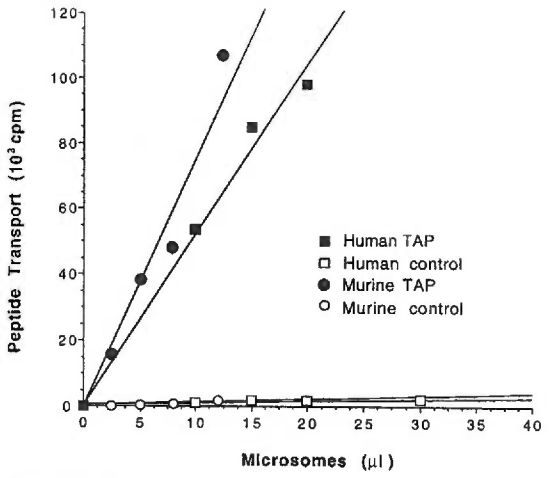

FIG. 2. Peptide transport by insect microsomes containing human or murine TAP. Microsomes were derived from insect Sf9 cells coinfected with BacTAP1 and BacTAP2 (Human TAP) (7) or from Sf9 cells infected with a control baculovirus, BacgH (Human control). Alternatively, microsomes were derived from *Drosophila* cells induced to express mouse TAP (Murine TAP) (1) or from *Drosophila* cells which were not induced to express mouse TAP (Murine control). Various concentrations of each microsome preparation were incubated with ^{125}I-labelled peptides and 5 mM ATP in a volume of 150 μl for 10 min at 23°C. The microsomes were washed, pelleted, and disrupted in detergent as described previously (7). Peptides able to bind to concanavalin A-Sepharose were eluted with alpha-methylmannoside and quantified (7).

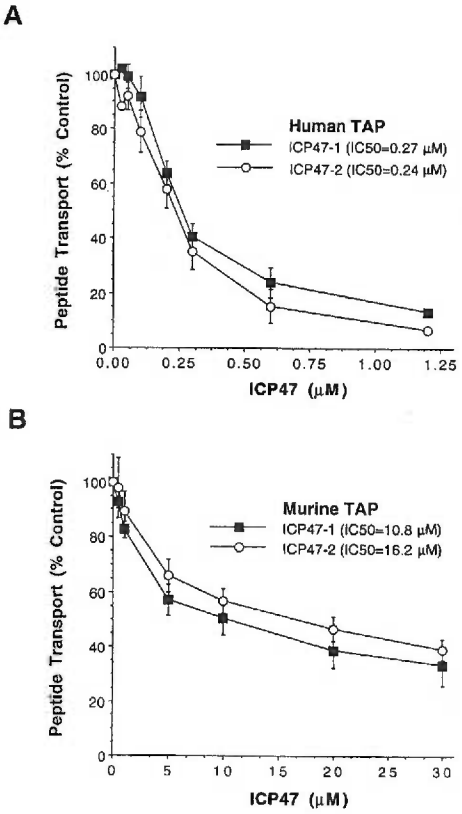

FIG. 3. Inhibition of human and murine TAP-mediated peptide transport by ICP47-1 and ICP47-2. TAP assays were performed as described in the legend for Fig. 2 by using insect microsomes containing human TAP (10 μl of membranes containing 12 mg of membrane protein per ml) (A) or murine TAP (7.5 μl of membranes containing 4.8 mg of membrane protein per ml but with equivalent levels of TAP activity compared with microsomes containing human TAP) (B) and various concentrations of ICP47-1 and ICP47-2. The results shown are combined from two separate experiments, each involving human and murine TAP.

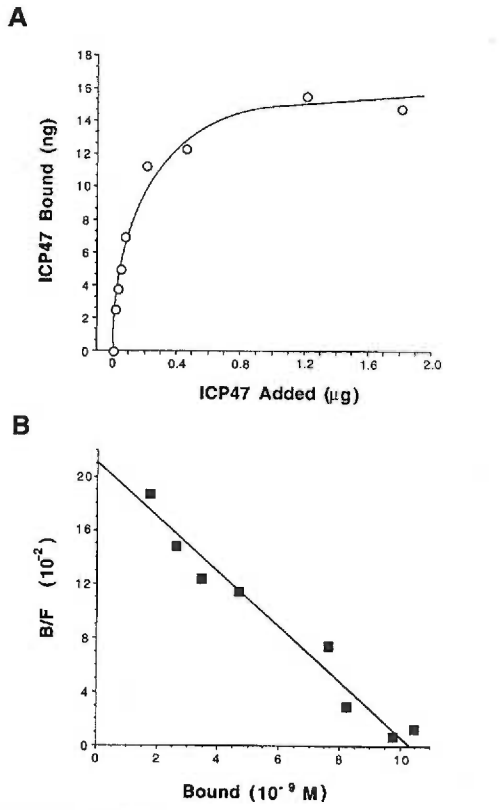

FIG. 4. Binding of ICP47-2 to human TAP. (A) Microsomes (15 μl of membranes with a 7.5-mg/ml concentration of membrane protein) derived from Sf9 cells expressing TAP1 and TAP2 or expressing HSV-1 gH (control membranes not containing TAP) were incubated with various amounts of ^{125}I-labelled ICP47-2 for 60 min at 4°C as described previously (7). Binding to control membranes was subtracted from binding to microsomes containing TAP at each point. (B) Scatchard analysis of the data in panel A. The K_D for ICP47-2 binding to TAP was calculated to be 4.8×10^{-8} M.

TAP by 50%. We attempted to measure the specific binding of radiolabelled ICP47-1 and ICP47-2 to microsomes containing mouse TAP in experiments similar to those shown in Fig. 4. However, there was little specific binding of ICP47-1 and ICP47-2, and it was difficult to measure binding at lower protein concentrations. We therefore measured binding at a single, higher protein concentration (2.75 μM), one sufficient to inhibit 10 to 20% of the mouse TAP activity and all of the human TAP activity. In this experiment, specific binding to microsomes containing murine TAP was determined by subtracting the binding to microsomes from insect cells that were not induced to express murine TAP (1). The binding of ICP47-1 and ICP47-2 to human TAP was easily measured (Fig. 5), although under these conditions it is important to note that ICP47-1 and ICP47-2 were present at concentrations beyond those required to saturate the TAP (Fig. 4A). By contrast, it was found that there was little or no significant binding of ICP47-1 or ICP47-2 to microsomes containing murine TAP when background binding to control membranes was subtracted. In the experiment shown, specific ICP47-2 binding was greater than zero, but in other experiments this binding was less than zero, and thus we concluded that there was no detectable binding overall. In every experiment, it was clear that the level of binding of ICP47-1 and ICP47-2 to murine TAP was at least 25-fold lower than to human TAP. However, the human TAP present in these microsomes was limiting in these experiments, and thus it is very likely that the 25-fold difference between the levels of binding to human and mouse TAP is an underestimate. More likely this difference is 50- to 100-fold. On the basis of the inhibitory concentrations required to block murine TAP and the binding studies described above, estimates of the binding affinities of ICP47-1 and ICP47-2 for murine TAP may fall in the range of 5×10^{-6} M. Therefore, ICP47-1 and ICP47-2 bind poorly to the murine TAP, and this largely accounts for their inability to block mouse TAP peptide transport.

In summary, ICP47-2 and ICP47-1 could block human TAP and bound to TAP with similar high affinities. It was interesting that these two proteins, whose primary structures are only about 40% identical, inhibit human TAP with indistinguishable profiles and bind to human TAP with virtually identical affin-

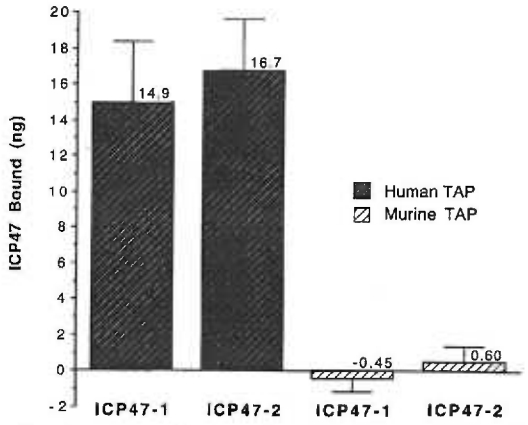

FIG. 5. Binding of ICP47-1 and ICP47-2 to microsomes containing murine TAP. Microsomes containing human TAP or control membranes without human TAP (100 μg of membrane protein per 150-μl assay) or microsomes containing mouse TAP or control membranes without mouse TAP (50 μg of membrane protein with the same TAP activity as with the human microsomes) were incubated with ^{125}I-labelled ICP47-1 or ICP47-2 at 2.75 μM for 60 min at 4°C. The microsomes were washed twice, pelleted, and disrupted with detergents as described previously (7). Radioactivity associated with the microsomes was quantified by gamma counting. "ICP47 bound" refers to specific binding, calculated by subtracting the binding to control membranes (without TAP) from that observed with microsomes containing human or murine TAP.

ities. Moreover, both proteins blocked murine TAP poorly and only at high protein concentrations and could not bind to murine TAP. These results, at face value, would suggest that mice will not be an appropriate model in which to test the effects of ICP47 on HSV replication or as a selective inhibitor of CD8$^+$ T-cell responses in other systems. However, we recently found that an HSV-1 ICP47 mutant showed dramatically reduced neurovirulence in mice, without altering the course of disease in the cornea (4). Therefore, ICP47 may attain sufficient concentrations in certain cells in the nervous systems of mice to inhibit TAP. This may be related to the fact that TAP and class I proteins are expressed at low levels in the nervous system. Alternatively, ICP47 may have other functions in the nervous system.

We are indebted to Aidan Dolan and Duncan McGeoch for communicating the DNA sequence of the ICP47-2 gene before publication and for providing us with a plasmid containing the gene. We thank Ian York for getting this work started and advice in later stages. We are grateful to Cathy Wale who prepared the microsomes containing human TAP.

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Table 1: Comparison of the biochemical properties of ICP47-1 and ICP47-2, as predicted from their primary amino acid sequence.

Biochemical Property	ICP47-1	ICP47-2
Polypeptide Length (# of amino acids)	88	86
Molecular Weight (Da)	9793	9785
Isoelectric Point (pH)	8.86	7.12
Ionic Charge at pH 7.0	+1.26	+0.14
Molar Extinction Coefficient	6970 +/- 5%	7450 +/- 5%
Absorbance of 1 mg/ml Solution at 280 nm	1.41	1.31

CHAPTER 4

HUMAN CYTOMEGALOVIRUS
INTRODUCTION

4.1 Human Cytomegalovirus

Cytomegalic inclusion disease (CID) was first reported in the early 1900's as a rare disease that affected premature infants often resulting in stillborn death or spontaneous abortion. Tissues of infants with CID, usually the kidneys and lungs, contained enlarged cells with extensive intranuclear inclusion bodies (reviewed in Britt and Alford, 1996; Ho, 1991). Several years later, it was noticed that the inclusion bodies associated with CID were very similar to those seen in cells infected with herpesviruses and thus, it was postulated that the disease may be caused by a virus closely related to the herpesvirus family (Lipshutz, 1921). It wasn't until 1956 that three independent laboratories isolated the causative agent of CID, a herpesvirus now known as human cytomegalovirus (HCMV) (Rowe et al., 1956; Smith, 1956; Weller et al., 1957). HCMV infection of humans has gone largely unnoticed throughout most of natural history due to the fact that the virus rarely causes overt disease in healthy individuals. However, in neonates or immunocompromised patients, the virus can become a life-threatening pathogen. Today, infection with HCMV is known to be endemic throughout much of the world and the virus is recognized as one of the leading causes of morbidity and mortality in AIDS patients and organ transplant recipients.

4.1.1 Structure

HCMV is a large, lipid-enveloped virus that is classified as a member of the *betaherpesvirinae* based on tissue tropism and replicative lifecycle (reviewed in Roizman, 1996). Also included in this subfamily are several closely related cytomegaloviruses (CMVs) that infect a wide range of species including primates,

horses, cats, guinea pigs, rats and mice. The genome of HCMV is the largest among the human herpesviruses and is comprised of a linear, double-stranded DNA molecule approximately 230 kbp in length (reviewed in Mocarski, 1996). Based on analysis of open reading frames greater than 100 amino acids, the coding capacity of the HCMV genome is thought to exceed 200 genes (Chee et al., 1990). Like HSV, the genome of HCMV is also divided into two unique segments of DNA: the unique long (U_L) and unique short (U_S) , which are flanked by inverted repeat sequences (reviewed in Mocarski, 1996). Inversion of these two segments of DNA occurs during productive infection, giving rise to four genome isomers.

4.1.2 Replication Cycle

In vivo, HCMV has been found associated with a large number of distinct cell types ranging from epithelial and endothelial cells to cells of myeloid lineage such as macrophages (reviewed in Sinzger and Jahn, 1996). Investigation of HCMV replication in these biologically relevant cells, however, has been difficult due to the inability to productively infect these cell types in vitro. Possibly, the isolation or manipulation of these cell populations in tissue culture may lead to subtle changes in gene expression or differentiation state which may render the cells nonpermissive for HCMV infection. To date, productive growth of HCMV in the laboratory is generally restricted to human diploid fibroblasts, and as a result, much of what is known about HCMV replication has been derived from studies using these cells as a model. In the past, the lack of in vivo evidence supporting a role for fibroblasts in HCMV infection has led to doubts as to the validity of this model. Recently, however, immunohistochemical analyses using

double-staining techniques have revealed that fibroblasts actually represent a major infected cell type in a variety of organs, including placenta, lung, and intestine.

The HCMV replication cycle is initiated through the attachment of virus to susceptible cells. The primary event in attachment is the binding of virus to cell surface heparin sulphate (Compton et al., 1993; Neyts et al., 1992), followed closely by more specific interactions with unknown cellular receptors (Adlish et al., 1990; Keay et al., 1989; Taylor and Cooper, 1990). Upon attachment of HCMV to cells, the virus fuses with the cellular membrane and deposits the nucleocapsid into the cytosol. The nucleocapsid is then transported to the nuclear envelope, followed by disassembly of the particle and delivery of the viral genome into the nuclear compartment. As with HSV, the transcription of HCMV genes is temporally separated into three main phases of expression: immediate-early (IE), early (E) and late (L) (reviewed in Mocarski, 1996; Stinski, 1990). During IE expression, two of the most abundant products are immediate early protein 1 (IE1) and immediate early protein 2 (IE2), which are essential transactivation factors and function to induce the expression of several of the E genes. The E genes typically encode enzymes or other proteins required for viral DNA synthesis. Following replication of the viral genome, transcription of the final class of genes, the L genes, is induced. L genes code for structural virion components and proteins involved in viral egress. Expression of structural proteins initiates the assembly of capsids in the nucleus, followed by the encapsidation of newly synthesized viral DNA. The maturation and egress of nucleocapsids from the nucleus remains one of the most controversial issues in

the field of HCMV replication. Similar to the models of egress proposed for HSV, HCMV nucleocapsids are thought to acquire their final lipid envelope either from the inner nuclear membrane (Campadelli-Fiume et al., 1991; Johnson and Spear, 1982) or early endosomal compartments (Radsak et al., 1990; Tooze et al., 1993). Regardless of the pathway of viral egress, progeny virus is efficiently released from the infected cell. A unique feature of HCMV infection is the formation of perinuclear inclusions within the cytosol of infected cells. These cytoplasmic inclusions are thought to be derived from lysosomal compartments into which electron dense particles have budded (Craighead et al., 1972; Smith and De Harven, 1978). These inactive particles, or so-called dense bodies, do not contain viral DNA or nucleocapsid but consist entirely of matrix and tegument structural proteins (Sarov and Abody, 1975). During HCMV infection, as much as 50% of the viral particles produced are in the form of dense bodies (Craighead et al., 1972; Sarov and Abady, 1975).

In addition to the lytic lifecycle, HCMV is also thought to establish latency in the infected host, but the cellular site and mechanism of latent infection remain extremely controversial. To gain insight into HCMV latency, several investigators have undertaken studies with nonpermissive cells as a model for restricted HCMV replication. The block in HCMV replication in nonpermissive cells usually occurs after penetration of the virus into the cell, but before the onset of late viral gene expression (DeMarchi, 1983; Misra and Hudson, 1977; Stinski, 1978). Most often, gene expression in nonpermissive cells is restricted to immediate-early and/or early gene products. The use of nonpermissive cells to study HCMV latency is rather limited, since conversion of these cells into the fully permissive

phenotype is extremely rare. In those few cases, however, stimulation of productive infection seemed to rely on the induction of cellular differentiation by treatment of nonpermissive cells with agents such as retinoic acid, hydrocortisone or phorbol esters. Recently, Nelson and colleagues have been able to reactivate latent HCMV from peripheral blood mononuclear cells (PBMCs) isolated from healthy seropositive donors (Soderberg-Naucler et al., 1997). The cell type thought to harbor latent HCMV was identified as a monocyte/macrophage and reactivation of virus was dependent on allogeneic stimulation of these cells. In nonpermissive monocytes, HCMV infection was found to be limited to immediate-early and early events, without the production of infectious virus. However, incubation of these monocytes with mixed lymphocytes from histoincompatible donors resulted in the potent activation of the cells and differentiation into fully permissive macrophages. Reactivation of HCMV from these macrophages typically occurred within 17 days poststimulation (Soderberg-Naucler et al., 1997). This model system is currently being studied to determine what factors are involved in the establishment and maintenance of HCMV latency.

4.1.3 Pathogenesis

As with HSV, HCMV infection is widespread, with current estimates for North America ranging from approximately 60-90% of adults infected, depending on geographic location (Mocarski, 1996). Humans represent the only known reservoir for HCMV and spread of virus is thought to occur through direct or indirect person-to-person contact. Although the most common vehicles of transmission are thought to be saliva and breast milk, HCMV has been detected in virtually all bodily fluids including cervical and vaginal secretions, semen, tears,

urine, feces and blood (Lang and Kummer, 1975; Reynolds et al., 1973; Stagno et al., 1980). Primary infection with HCMV usually occurs during early childhood, most likely as a result of casual contact in daycare centers or residential schools (Hutto et al., 1985; Pass et al., 1982). In particular, infants with acute HCMV infection are known to be a major factor in viral spread, as these children typically shed virus for several years after the primary infection (Alford et al., 1980; Kumar et al., 1973). In adults, the major route of primary infection remains sexual contact. In addition, hospitalized patients can also be infected with HCMV through exposure to contaminated blood products or organ allografts (reviewed in Britt and Alford, 1996).

In most cases, the primary exposure to HCMV occurs through contact of oropharyngeal membranes with saliva or breast milk from an infected individual actively shedding virus (Britt and Alford, 1996). Once within the salivary gland, the virus characteristically infects ductal epithelial cells, while sparing contiguous serosal and mucosal surfaces (Becroft, 1981; Ho, 1991). From this initial site of replication, HCMV is capable of spreading to distant organs throughout the body including the kidneys, liver, lungs, pancreas, intestines, and in extreme cases, the CNS (reviewed in Britt and Alford, 1996; Ho, 1991). The factors contributing to the dissemination of HCMV within the host remain unresolved. The recent observation that circulating monocytes harbor latent HCMV has led to the speculation that these cells may play an important role in viral dissemination (Soderberg-Naucler et al., 1997). Monocytes are often recruited to the site of HCMV infection in the salivary gland and may become latently infected upon exposure to virus. Circulation of these infected monocytes through the

bloodstream would then serve to disseminate virus throughout the body.

HCMV infection of immunocompetent individuals usually results in asymptomatic disease, although a mononucleosis syndrome may occur in rare cases (Klemola and Kaariainen, 1965; Klemola et al., 1967). Although primary infection with HCMV is controlled quite effectively by the host immune response, the virus is never truly cleared from the host. As with HSV, HCMV maintains a lifelong infection within the host by establishing latency in specific cell types. Evidence clearly supports a role for monocytes in latent infection, but other cell types may also contribute to the persistence of the virus (reviewed in Sinzger and Jahn, 1996). Periodically, throughout the life of the healthy host, HCMV reactivates from the latent state and goes on to produce infectious virus. Reactivation of virus usually goes unnoticed by the host and, depending on the site of reactivation, infectious virus may be shed into any one of several bodily secretions. As the host grows older, the frequency of reactivation and shedding seems to decrease (Britt and Alford, 1996).

Immunocompetent individuals infected with HCMV typically achieve a balance between viral replication and host immunity. However, when this balance is disrupted, as in the case of neonates or immunocompromised patients, HCMV can become a life-threatening pathogen. HCMV is currently the most common congenital viral infection in the United States, infecting approximately 40, 000 infants each year (Demmler, 1991; Stagno et al., 1982). Infection of the developing fetus with HCMV can have several serious clinical outcomes such as major organ dysfunction, microcephaly, chorioretinitis, neurological damage and death (reviewed in Britt and Alford, 1996; Ho, 1991). A major factor in the

frequency of transmission is thought to be maternal immunity. Transmission of HCMV to the fetus is approximately 100 times more likely during primary maternal infection than during episodes of secondary or recurrent infection (Medearis, 1982; Yow et al., 1988). Maternal immunity is also thought to influence fetal outcome, as women with prior exposure to HCMV rarely give birth to infants with clinically apparent disease (Stagno et al., 1982).

Transplant recipients are often placed on a regimen of potent immunosuppressive drugs in order to prevent allograft rejection. This suppression of the immune system, however, makes these patients extremely vulnerable to HCMV infection. HCMV is currently the leading cause of posttransplant infections in the United States (Britt and Alford, 1996). Transplant patients infected with HCMV may develop several life-threatening complications such as severe gastrointestinal disease, hepatitis, retinitis and pneumonitis (reviewed in Britt and Alford, 1996; Ho, 1991). HCMV-seronegative transplant recipients are at risk for primary infection from seropositive donors and thus, often develop more severe disease than seropositive recipients who experience reactivation of latent HCMV (Britt and Alford, 1996). In addition to opportunistic infections, HCMV has also been implicated as a contributing factor in graft rejection (Grattan et al., 1989; Richardson et al., 1981; Vierling and Fennel, 1985). Currently, the mechanism of this effect remains unknown, although an attractive model involves the viral mimicry of host antigens resulting in autoimmune attack of the transplanted organ.

The current AIDS epidemic has greatly increased the public's awareness of HCMV infection and disease. AIDS patients, due to a gradual reduction in $CD4^+$

T cells, become severely immunocompromised and as a result, show a keen susceptibility to opportunistic infection with HCMV. Post-mortem analyses of AIDS patients have shown that approximately 90% of these patients will develop an active HCMV infection during their lifetime (Gallant et al., 1992). The inability of AIDS patients to mount an appreciable immune response to HCMV permits the virus to replicate and spread uncontrollably throughout the host. Virtually every organ system within the body may become a target for infection, yet the most common sites include the GI system, the lungs and the eyes. Infection of these organs often leads to severe disease such as esophagitis, gastritis and enterocolitis in the GI tract, pneumonitis in the lungs and retinitis in the eyes (Heurlin et al., 1991; Jacobsen and Mills, 1988; Klatt and Shibata, 1988; Vinters et al., 1989). In particular, the development of HCMV retinitis seems to be unique to this adult population, with the incidence of disease reaching 20-25% of AIDS patients (Gallant et al., 1992; Jabs et al., 1989).

4.2 Immune Response to CMV Infections

Infection with HCMV results in an intimate association between the virus and the host immune system. Although HCMV infection is effectively controlled by the host immune response, the virus establishes latency in specific cell types and is never truly cleared from the infected host. Under certain conditions, especially in cases of immune suppression, HCMV reactivates from the latent state and goes on to produce progeny virus. As with HSV, reactivation of HCMV takes place in the face of a fully primed immune response. To better understand HCMV reactivation and how the virus might evade immune surveillance, various aspects of the host immune response to HCMV must be investigated. Unfortunately, due

to the extremely narrow host range of HCMV, animal models do not exist for the study of HCMV infection. Most information regarding host immunity to HCMV has been derived from human studies involving organ transplant recipients or AIDS patients. Post-mortem analyses of these patients, including infants with CID, have also provided additional information regarding the spread and extent of organ involvement associated with HCMV disease. As an alternative, many investigators have attempted to correlate HCMV disease with that of a related betaherpesvirus, murine cytomegalovirus (MCMV). MCMV and HCMV are genetically related viruses that share several properties in terms of their interaction with the host. Both viruses, for example, infect a similar spectrum of organs and tissues, and each virus is capable of causing life-threatening disease in the immunodeficient host. Unfortunately, in addition to these similarities, there are also important differences between the two viruses. In particular, HCMV has the ability to cross the placental barrier and cause congenital infection, whereas MCMV cannot. Also, the replication cycle of HCMV in permissive cells, such as macrophages, is much longer than that of MCMV. Therefore, based on these differences, any conclusions drawn from MCMV studies may not necessarily apply to HCMV. The following sections will provide a brief outline of what is currently known about HCMV immunity, followed by a more detailed description of the host immune response to MCMV.

4.2.1 Immune Response to HCMV

4.2.1.1 Innate Immunity

Human studies of the immune response to HCMV infection have largely focused on aspects of adaptive immunity rather than mechanisms of innate

defense. For this reason, information regarding the innate immune response to HCMV infection is lacking. As with HSV, however, NK cells are thought to play an important role in the early containment of HCMV infection. Two cases have been reported of patients with selective defects in NK cell activity and in both instances, the patients were extremely susceptible to herpesvirus infections, including HCMV, and often displayed severe recurrent infections (Biron et al., 1989; Jawahar et al., 1996). Macrophages comprise another important cellular component of the innate immune response and play an important role in the clearance of virus particles and infected cells. Unlike HSV, however, HCMV is capable of infecting monocytes/macrophages in vivo and may exploit these cells in order to disseminate virus throughout the host (Ibanez et al., 1991; Lathey and Spector, 1991; Pulliam, 1991). Monocytes infected with HCMV could serve to transport virus via the bloodstream to virtually any organ within the body. The recent observation that circulating PBMCs harbor latent HCMV provides additional support for this proposal (Soderberg-Naucler et al., 1997).

4.2.1.2 Adaptive Immunity

Studies with transplant recipients and AIDS patients have demonstrated that the adaptive immune response to HCMV is largely responsible for the clearance of active infections. In contrast to HSV, the humoral response to HCMV infection may be more effective in limiting viral spread. Unlike HSV, HCMV does not spread efficiently from cell-to-cell and the replication cycle of the virus is rather protracted (Britt and Alford, 1996). These factors are thought to contribute to the increased susceptibility of HCMV to the neutralizing effects of antibody and complement. Numerous studies with allograft recipients have demonstrated a

protective effect of passively administered immunoglobulin. Transplant recipients given intravenous immunoglobulins display a significant reduction in the extent and severity of HCMV disease when compared to those patients left untreated (Gale and Winston, 1991; Meyers, 1989; Snydman et al., 1987). Moreover, it has been repeatedly demonstrated that maternal immunity to HCMV plays a vital role in the acquisition and severity of HCMV disease in the newborn. Infants born to mothers with preconceptual antibody to HCMV rarely, if ever, display signs of clinically apparent disease (Fowler et al., 1992; Yeager et al., 1981). Although the manifestations of HCMV disease may be lessened in the presence of antibodies, the humoral response itself is unable to control viral replication. For the clearance of ongoing infections, the induction of specific cell-mediated immunity is thought to be essential.

The susceptibility of AIDS patients to HCMV disease has underscored the importance of $CD4^+$ T lymphocytes in the control of HCMV infection. HCMV represents one of the most important opportunistic infections in HIV-infected individuals and through the course of AIDS disease, approximately 90% of these patients will become infected (Gallant et al., 1992). The development of HCMV disease in AIDS patients also correlates directly with the reduction in viable $CD4^+$ T cells. When counts of $CD4^+$ T lymphocytes in the blood fall below $50/mm^3$, the frequency of invasive HCMV disease increases significantly (Gallant et al., 1992; Holland et al., 1990; Pertel et al., 1992). During this late stage in AIDS disease, when $CD4^+$ T cell function is severely impaired, infection with HCMV often results in early death.

As a counterpart to the studies conducted in AIDS patients, transplant

recipients have provided a wealth of information regarding the importance of $CD8^+$ T cells in clearing ongoing HCMV infections. Since allograft recipients are treated with potent immunosuppressive drugs to prevent graft rejection, these patients often suffer from severe HCMV disease during the posttransplant period. In an attempt to lessen the extent of HCMV disease in allograft recipients, Greenberg and colleagues have begun to explore the therapeutic potential of passive administration of specific T cell populations (Riddell et al., 1993). In particular, the transfer of anti-HCMV $CD8^+$ T cells has generated the most promising results . In clinical trials, the passive transfer of HCMV-specific $CD8^+$ T cells during the posttransplant period has been shown to significantly reduce the incidence of severe HCMV disease (Riddell et al., 1993). Moreover, similar results have been observed in transplant patients that manage to mount an intrinsic CTL response. Several studies have demonstrated that the generation of a detectable anti-HCMV CTL response in kidney or bone marrow transplant recipients is often associated with a more benign course of HCMV infection (Quinnan et al., 1982; Rook et al., 1984). Collectively, the data obtained from studies with AIDS patients and transplant recipients have clearly demonstrated an important role for T lymphocytes in the control of HCMV disease.

4.2.2 Immune Response to MCMV

4.2.2.1 Innate Immunity

All branches of host immunity are thought to be involved in the containment of MCMV infection. The first line of defense against infection involves various aspects of innate immunity. Mucosal membranes may provide some limited protection against primary infection, but in most cases, do not pose a

substantial barrier to entry of virus. Replication of MCMV in susceptible cells induces the secretion of various antiviral cytokines such as IFN-α and IFN-β. Several studies have demonstrated that prior treatment of immunocompetent mice with antibodies specific for IFN-α/β results in higher levels of MCMV replication and reduced survival (Chong et al., 1983; Grundy et al., 1982; Orange and Biron, 1996) . Additional cytokines may be secreted at the site of infection, depending on the cell type infected, and this typically results in the infiltration of various immune effector cells. Macrophages and NK cells, which comprise the innate cellular response, rapidly respond to the chemoattractant signals released by infected cells (reviewed in Janeway and Travers, 1996; Roitt, 1994). Macrophages are large mononuclear phagocytes capable of engulfing virus particles or infected cells. As with HCMV, MCMV is also capable of infecting monocytes/macrophages and thus, the infiltration of these cells to the site of infection may provide a means for dissemination of virus throughout the body. Using an MCMV recombinant virus tagged with β-galactosidase, Mocarski and colleagues were able to identify mononuclear phagocytes (possibly macrophages) as the major infected cell type in the blood, thereby implicating these cells in the dissemination of MCMV during infection (Stoddart et al., 1994). NK cells, however, may provide a more effective means of destroying MCMV infected cells. NK cells are cytolytic in nature and destroy infected targets which express low levels of MHC class I proteins on their surface (reviewed in Colonna, 1997; Moretta et al., 1996). As with HCMV, MCMV is known to express several proteins which function to downregulate MHC class I expression (reviewed in Hengel et al., 1999; Johnson and Hill, 1998), and thus, MCMV infected cells may

become potent targets for NK mediated lysis. In addition to lysis of infected cells, NK cells may also inhibit MCMV replication through the secretion of large amounts of IFN-γ, an extremely potent antiviral cytokine. Several studies have demonstrated a crucial role for NK cells in controlling MCMV infection. In mice depleted of NK cells and then infected with MCMV, viral titres often exceeded those in control mice by two orders of magnitude and MCMV-induced pathology was significantly enhanced (Bukowski et al., 1984; Orange and Biron, 1996; Welsh et al., 1991). Furthermore, a resistance gene to MCMV infection, known as Cmv-1, has been identified in the mouse that maps to the NK-gene complex on chromosome 6 (Scalzo et al., 1995). The enhanced resistance of Cmv-1^+ mice to MCMV infection appears to involve the early restriction of MCMV replication in the spleen through the cytotoxic action of NK cells (Scalzo et al., 1992). Although NK cells, macrophages and antiviral cytokines grossly alter the levels to which mice are susceptible to MCMV, these innate responses are not sufficient to eliminate virus in the absence of adaptive immunity.

4.2.2.2 Adaptive Immunity

Upon primary infection with MCMV, the production of neutralizing antibodies is typically delayed 4-6 days when compared to that of other acute viral infections (Manischewitz and Quinnan, 1980; Osborn et al., 1968). The relatively late development of these antibodies is thought to be due to the immunosuppressive effects of MCMV infection. As a consequence, neutralizing antibodies are not thought to play a significant role in the recovery from primary infection, as virus is generally cleared within 8-10 days before any significant increase in antibody levels (Mocarski, 1996). The main benefit of neutralizing

antibodies, then, may be in limiting viral dissemination after reactivation of latent MCMV. Passive administration of anti-MCMV immunoglobulins has been shown to protect mice from lethal challenge with MCMV (Araullo-Cruz et al., 1978; Farrell and Shellam, 1991; Shanely et al., 1981). During persistent MCMV infections, preexisting antibodies circulate in the blood and may provide a similar protective effect. Recent studies with B cell-deficient mice seem to support this hypothesis. Mice lacking B cells and antibodies were found to clear primary infection as quickly as their normal littermates, but reactivation of MCMV in these B cell-defienct mice resulted in 100-1000 fold higher viral titres in various organs (Jonjic et al., 1994).

As with HCMV, the clearance of active MCMV infections is dependent on the induction of specific cell-medicated immunity. Both $CD4^+$ and $CD8^+$ T lymphocytes are thought to be involved in this process. Most striking is the importance of $CD4^+$ T lymphocytes in the control of MCMV replication in the salivary gland (Jonjic et al., 1989; Jonjic et al., 1990). In immunocompetent mice, MCMV often establishes a transient infection in the salivary gland which is typically cleared within 4-8 weeks. In mice depleted of $CD4^+$ T cells, however, MCMV maintains a persistent salivary gland infection at relatively high viral concentrations (Koszinowski, 1991). Thus, in the absence of $CD4^+$ T cells, clearance of MCMV from the salivary gland is extremely inefficient. Moreover, several studies have demonstrated that adoptive transfer of $CD4^+$ T cells from the spleens of immune mice can protect animals from MCMV challenge (Jonjic et al., 1989; Shanely et al., 1987). $CD8^+$ T lymphocytes also appear to carry out critical functions during MCMV infection. Infection of immunocompetent mice with

MCMV rarely results in significant dissemination of virus to the lungs. However, in mice thymectomized and depleted of $CD8^+$ T cells, MCMV rapidly colonizes the lungs and replicates to high viral titres (Koszinowski, 1991). As with $CD4^+$ T cells, adoptive transfer of MCMV-specific $CD8^+$ T lymphocytes has also been shown to protect mice from lethal viral challenge. MCMV infection of mice immunosuppressed by total body γ-irradiation typically results in severe pneumonitis, splenitis and adrenalitis. However, transfer of $CD8^+$ T lymphocytes obtained from draining lymph nodes or memory cells of latently infected mice to naive animals provided protection when given prophylactically before infection or therapeutically after the onset of MCMV disease (Reddehase et al., 1987; Reddehase et al., 1988). Taken together, these observations support a primary role for T lymphocytes in conferring resistance to MCMV infection.

4.3 Immune Evasion Strategies of the CMVs

Throughout the lifetime of the infected host, HCMV and MCMV maintain an intimate relationship with the host immune system. As part of this lifelong interaction, a balance is achieved between viral replication and host immunity that enables the virus to persist within the host without causing any overt signs of disease. At no time is this balance more evident than during periods of reactivation when replication and shedding of virus largely go unnoticed by the host. The ability of HCMV and MCMV to replicate in the face of a fully primed immune response may be due in large part to the numerous immune evasion strategies employed by each virus. With coding capacities of greater than 200 proteins for both HCMV and MCMV, it is no surprise that each virus expresses several proteins dedicated to the task of suppressing host immunity. In particular,

the presentation of antigens by the MHC class I pathway is a major target for interference by both viruses. Indeed, the avoidance of $CD8^+$ CTL recognition may be espicaally important, since both HCMV and MCMV replicate very slowly within cells.

4.3.1 Chemokine Receptors

Both HCMV and MCMV encode homologues of the chemokine G-protein coupled receptor (GPCR) family. In HCMV these receptors are encoded by the genes US27, US28, UL33 and UL78 (Chee et al., 1990), while in MCMV they are encoded by the m33 and m78 genes (Rawlinson et al., 1996). Of these receptors, the HCMV US28 homologue is best characterized and is structurally similar to the human chemokine receptor CCR-1. US28 binds C-C chemokines including RANTES, MCP-1, MCP-3, MIP-1α and MIP-1β and has been shown to be a functional receptor in that stimulation with C-C chemokines results in a flux of intracellular Ca^{2+} and activation of mitogen-activated protein (MAP) kinase pathways (Kuhn et al., 1995; Neote et al., 1993). Intriguingly, many of the C-C chemokines, especially MCP-1 and MCP-3, function as chemoattractant signals for the migration of immune effector cells to the site of infection or injury. Therefore, a possible role for these viral GPCR homologues during infection may be to sequester chemokines, thereby inhibiting the chemotaxis of various immune effector cells. Consistent with this hypothesis, expression of US28 during HCMV infection results in the depletion of chemokines from the extracellular milieu (Bodaghi et al., 1998). Alternatively, since US28 is a functional GPCR, with the ability to transmit signals to the cell interior, this homologue may have functions apart from immune evasion. Streblow and colleagues (1999) have recently

demonstrated that migration of HCMV-infected smooth muscle cells in vitro is mediated by the US28 receptor homologue. Thus, the ability of US28 to induce chemotaxis in HCMV-infected cells may play a role in viral dissemination.

4.3.2 Fc Receptors in HCMV

Infection of human fibroblasts with HCMV results in the expression of receptors in the cytoplasm and on the cell surface of infected cells which are capable of binding the Fc region of antibodies (Furukawa et al., 1975; Rahman et al., 1976). To date, the proteins responsible for this activity have not been identified and this has made the study of the biological significance of these Fc receptors difficult. In vitro studies have demonstrated a potential role for the cell surface Fc receptor in the inhibition of antibody dependent cell-mediated cytotoxicity (ADCC). Mouse mammary (MM2) tumor cells become potent targets for adherent peritoneal exudate cells (PECs) when treated with MM2-specific antiserum. However, when these cells were incubated with HCMV-infected cells, killing by PECs was inhibited by approximately 80% and this was attributed to the binding of IgG to the Fc receptors of infected cells (Murayama et al., 1987). Recently, the second potential Fc receptor of HCMV has been localized to the tegument of viral particles (Stannard and Hardie, 1991). The presence of an Fc receptor in this location was unexpected, as it seems difficult to envision a role for this tegument protein in the protection of enveloped virus from antibody attack. Possibly, this receptor may aid in the entry of viral particles that have lost their envelopes through the binding of IgG on susceptible cells.

4.3.3 Fc Receptors in MCMV

Infection of cells with MCMV also induces the expression of a receptor

with Fc binding activity. The gene encoding this receptor was recently identified by Koszinowski and colleagues and has been designated fcr-1 (Thale et al., 1994). Cells microinjected with the fcr-1 gene bind antibody with high affinity at their cell surface and co-immunoprecipitation experiments demonstrate a direct association of the Fc receptor with antibody. The MCMV Fc receptor is a transmembrane protein approximately 88 kDa in size and is structurally related to the Fc receptors of HSV and VZV. Studies in mice, however, have not yet demonstrated a definitive role for this receptor in viral immune evasion. As expected, infection of immunocompetent mice with a deletion virus lacking the fcr-1 gene resulted in a significant reduction in viral replication when compared to wildtype virus (Crnkovic-Mertens et al., 1998). However, in mice deficient in B cells, the fcr-1 deletion virus displayed the identical phenotype. Thus, the attenuation of the virus deleted for the Fc receptor is not due to the binding of antibodies. As with HSV, the MCMV Fc receptor may have additional functions critical to the replication or spread of the virus.

4.3.4 Inhibition of NK Cell Recognition by HCMV

Sequence analysis of the HCMV genome revealed that the virus coded for a glycoprotein with homology to the MHC class I heavy chain (Beck and Barrel, 1988). This homologue was encoded by the UL18 gene and displayed an overall amino acid identity of 30-40% to classical class I proteins. Expression of UL18 in HCMV-infected cells, however, has been difficult to detect for unknown reasons. Preliminary studies in which UL18 was expressed using a vaccinia virus vector demonstrated that this homologue was capable of associating with cellular β_2-microglobulin (β_2m) (Browne et al., 1990) and that the complex formed between

UL18 and β_2m was capable of binding peptides and was expressed at the cell surface (Fahnestock et al., 1995). A functional role for UL18 during HCMV infection, nevertheless, has been difficult to establish. Based on the ability of UL18 to bind and 'present' endogenous peptides, it was postulated that this homologue may function to inhibit CTL activity. However, several experiments demonstrated that UL18 was not involved in the downmodulation of MHC class I expression or the protection of HCMV-infected cells from CTL recognition (Browne et al., 1992). The functional significance of UL18 remained quite elusive until it was discovered that NK cells recognize infected targets through the binding of class I proteins. Unlike T cells, however, NK cells selectively kill targets that lack expression of MHC class I proteins. Thus, it was postulated that UL18 may serve as a class I decoy to protect HCMV-infected cells from NK cell lysis. Initial studies seemed encouraging as expression of UL18 in class I deficient B cell lines led to protection against killing by a variety of NK-cell lines (Reyburn et al., 1997). These results, however, have recently been challenged. Using a virus deleted of the UL18 gene, Leong and colleagues (1998) have convincingly demonstrated that expression of UL18 during HCMV infection does not protect infected cells from NK lysis, but rather makes them more sensitive. These results have recently been verified using different strains of HCMV, with individual isolates increasing susceptibility to NK cells or having no effect at all (Fletcher et al., 1998). To complicate matters even further, despite the apparent inability of UL18 to protect HCMV-infected cells from NK lysis, this homologue has recently been shown to bind to a newly discovered NK receptor. Using a UL18 fusion construct in co-immunoprecipitation assays, Cosman and colleagues (1997) were

able to precipitate a 110-120 kDa receptor from various cells lines including B cells, monocytes and NK cells. This receptor is a member of the immunoglobulin superfamily and has been named the leukocyte immunoglobulin-like receptor-1 (LIR-1). Although LIR-1 shows significant homology to killer inhibitory receptors (KIRs) of NK cells, the function of this novel receptor remains unknown. The widespread distribution of LIR-1 on cells of monocytic lineage (Cella et al., 1997) has led to speculation that this receptor may play a role in monocyte activation or differentiation. Since HCMV infects these cells, expression of UL18 could influence various aspects of monocyte activation, possibly creating a more favorable environment for viral replication. Clearly, further invstigation is required to establish the true function of UL18 during HCMV infection.

4.3.5 Inhibition of NK Cell Recognition by MCMV

In contrast to the situation in HCMV, the function of the MCMV class I homologue, m144, seems to be better characterized. Several studies have demonstrated that m144 is capable of associating with β_2-microglobulin, but unlike UL18, this complex is unable to bind peptides (Chapman and Bjorkman, 1998). As with UL18, m144 has been proposed to function as a class I decoy during MCMV infection to protect infected cells from NK lysis. A significant advantage in the study of MCMV is the ability to conduct in vivo experiments. In mice infected with a deletion virus lacking m144, viral replication was significantly reduced and this was attributed to an inability of the virus to control antiviral NK cell activity (Farrel et al., 1997). These results strongly support a role for m144 in inhibiting NK function. The mechanism of this inhibition remains unknown, as m144 has not been shown to bind to known NK receptors. Current

efforts are directed at isolating a novel inhibitory receptor for m144.

In addition to m144, MCMV also encodes a second glycoprotein, m04 or gp34, that may play a role in the inhibition of NK cell activity. In pulse-chase experiments, gp34 was found to associate tightly with MHC class I molecules in the ER and then escort these complexes to the cell surface (Kleijnen et al., 1997). MCMV is known to downregulate MHC class I expression through the action of multiple proteins (discussed below) and thus, it was proposed that gp34 may function to rescue a small fraction of class I molecules for expression at the cell surface. Similar to the role proposed for UL18 or m144, expression of this limited set of class I molecules at the cell surface may protect infected cells from NK lysis. Unfortunately, recent studies have been unable to show a role for gp34 in protection of MCMV-infected cells from either NK or CTL recognition. Nonetheless, the association of gp34 with class I molecules at the cell surface strongly supports a role for this protein in immune evasion.

4.3.6 Prevention of MHC Class I Presentation of HCMV IE1 by pp65

Analysis of HCMV-specific $CD8^+$ CTL from infected individuals revealed that very few, if any, of these lymphocytes were specific for the major immediate early transcription factor, IE1 (Gilbert et al., 1993). This was rather unexpected as IE1 is not only among the most abundantly expressed proteins during HCMV infection, but is also one of the first. The low frequency of IE1-specific CTL suggested that IE1, or peptides derived thereof, were somehow resistant to presentation by the MHC class I pathway. HCMV is known to downregulate MHC class I expression (discussed below), but the proteins involved in this process are expressed during the E phase of viral gene expression, approximately

6-8 hours after the induction of IE1 expression. Thus, the failure of infected cells to present peptides derived from IEI was postulated to be an effect of a structural protein, possibly a component of the tegument. Two of the most abundant structural proteins in the virion are pp65 and pp150 (Mach et al., 1989). To determine which protein was responsible for this effect, Gilbert and colleagues (1996) co-expressed pp65 or pp150 with IE1 and then conducted chromium release assays with IE1-specific CTL. Using this approach, it was found that pp65 was quite effective in blocking IE1 presentation. This inhibitory effect was also specific for IE1, as presentation of other viral proteins was not affected by pp65. pp65 is the major structural component in the tegument and has previously been shown to possess serine/threonine protein kinase activity in vitro (Somogyi et al., 1990). Since expression or stability of IE1 was not affected by co-expression with pp65, it has been postulated that the phosphorylation of IE1 by pp65 or a cellular kinase that interacts with pp65 may limit access of this phosphoprotein to the proteolytic machinery in the cytosol. The precise mechanism of pp65 inhibition, however, remains unknown and awaits further investigation.

4.3.7 Inhibition of MHC Class I Presentation by HCMV

Infection of human fibroblasts with HCMV results in the potent downregulation of MHC class I expression (Browne et al., 1990). Immunoprecipitation of radiolabelled cells revealed that shortly after infection with HCMV, expression of class I heavy chains was rapidly reduced, while β_2-microglobulin remained stable (Beersma et al., 1993; Warren et al., 1994). Through a series of pulse-chase experiments, Beersma and colleagues (1993) demonstrated

that this reduction in class I expression was not due to an inhibition of protein synthesis, but was rather the likely result of accelerated degradation. Using a panel of HCMV mutants with deletions in the U_S component, Jones and colleagues (1995) identified two genes, US2 and US11, which were able to independently mediate the degradation of newly synthesized class I heavy chains. Surprisingly, the mechanism of action of both US2 and US11 was found to be virtually identical. Initial studies with US11 demonstrated that the half-life of class I heavy chains in US11-transfected cells was less than one minute (Wiertz et al., 1996a). However, when US11$^+$ cells were treated with various inhibitors of the proteasome, degradation of the class I heavy chain was blocked. Thus, US11 mediates the degradation of class I heavy chains via the proteasome. Class I heavy chains stabilized in the presence of proteasomal inhibitors, however, migrated more rapidly in SDS-polyacrylamide gels than mature class I heavy chains. Subsequent studies revealed that this lower molecular weight form of the class I heavy chain was in fact a deglycosylated intermediate. Since deglycosylation of proteins is thought to be mediated by a cytosolic N-glycanase, the generation of this intermediate was postulated to occur in the cytoplasm. Subcellular fractionation of US11$^+$ cells treated with inhibitors of the proteasome demonstrated that intermediates of the class I heavy chain were indeed localized to the cytosol. Thus, degradation of class I heavy chains mediated by US11 is thought to occur through the dislocation of newly synthesized heavy chains from the ER to the cytosol, where they are then deglycosylated by N-glycanase and degraded by the proteasome. The situation for US2 seems to be identical, except in one respect. A direct association of US11 with class I proteins has been

difficult to establish experimentally. However, since the degradation event mediated by US2 seems to be less efficient, US2 has been found not only to co-immunopreciptate with class I heavy chains, but also with components of the translocon, in particular Sec61α (Wiertz et al., 1996b). The translocon is a proteinaceous pore that extends through the ER membrane and facilitates the translocation of nascent proteins into the ER (Alberts et al., 1989). Thus, US2 and US11 are thought to mediate the retrograde translocation of class I heavy chains from the ER to the cytosol through specific interactions with components of the translocon.

Both US2 and US11 belong to gene families within the U_S component that share sequence homology. US2 is the prototype of the US2 gene family, which is comprised of two members, US2 and US3, while US11 belongs to the US6 gene family, which is made up of six members, including US6-US11 (Chee et al., 1990). The similarity of these related genes to US2 and US11 led to the speculation that the products they encode may also play a role in the inhibition of MHC class I antigen presentation. Subsequently, each reading frame within the US2-US11 region was transfected into cells and assayed for the ability to block class I presentation. From these studies, it was demonstrated that two additional reading frames, US3 and US6, were capable of inhibiting class I presentation.

In contrast to cells transfected with US2 or US11, expression of class I heavy chains in $US3^+$ cells remained quite stable (Ahn et al., 1996; Jones et al., 1996). However, upon further investigation, it was demonstrated that the class I heavy chains immunoprecipitated from $US3^+$ cells were sensitive to treatment with endoglycosidase H (endo H). As glycoproteins exit the ER and transit

through the Golgi apparatus, N-linked carbohydrates on the protein are modified from an endo H-sensitive to -resistant form. The failure of class I heavy chains to acquire endo H resistance in US3$^+$ cells suggested that these proteins were being retained in the ER. Colocalization experiments confirmed this hypothesis, as both US3 and class I heavy chains were found to accumulate in the ER. In addition, co-immunoprecipitation experiments demonstrated an association between US3 and class I heavy chains. Intriguingly, as the only IE protein within the US2-US11 region, US3 is expressed before US2 and US11, which are both E gene products. This sequential expression has led to the speculation that US3 may assist in the function of US2 and US11 by retaining class I heavy chains for subsequent degradation (Ahn et al., 1996). Using a combination of transfected cell lines and deletion viruses, Jones and Sun (1996) demonstrated that in the presence of US3, degradation of class I heavy chains by US2 or US11 was enhanced.

In contrast to the proteins described above, the inhibition of the class I presentation pathway by US6 is mediated through an entirely different mechanism. Class I proteins immunoprecipitated from US6$^+$ cells were found to be extremely unstable and displayed several characteristics reminiscent of those produced in TAP-deficient cell lines (Ahn et al., 1997; Hengel et al., 1997). This observation led to the speculation that US6 may inhibit TAP activity. To address this hypothesis, peptide transport assays were conducted in cells expressing either US6 or a control protein. In US6$^+$ cells, transport of peptides by TAP was reduced by almost 90% when compared to controls. Thus, US6 appears to block the TAP-mediated transport of peptides from the cytosol into the ER. The mechanism of TAP inhibition by US6 also seems to be distinct from that of

another viral inhibitor, HSV ICP47. ICP47 is a small, soluble protein that interacts with the cytosolic face of TAP. By contrast, US6 is a type I membrane glycoprotein and is thought to interact primarily with the ER lumenal domain of TAP (Ahn et al., 1997; Hengel et al., 1997). In co-immunoprecipitation experiments, US6 was found to associate with assembling MHC class I complexes in the lumen of the ER. At the core of these multimeric complexes is TAP, surrounded by tapasin, MHC class I and calnexin.

4.3.8 Inhibition of MHC Class I Presentation by MCMV

Similar to the effects observed in HCMV-infected cells, mouse fibroblasts infected with MCMV also display a significant reduction in class I expression (Thale et al. 1995). To identify viral proteins that may play a role in this effect, Koszinowski and colleagues conducted a series of co-immunoprecipitation experiments (Reusch et al., 1999). Precipitation of class I proteins from MCMV infected cells revealed an association with a 48 kDa protein. Antibodies raised against this precipitated protein were then used to identify the encoding gene, m06, which lies at the extreme left end of the MCMV genome. Cells transfected with m06 were found to express a 48 kDa glycoprotein, designated gp48, that associated tightly with class I proteins (Reusch et al., 1999). Pulse-chase experiments demonstrated that within a few hours after this association, both gp48 and class I proteins were degraded. However, unlike US2 and US11, the degradation of class I proteins mediated by gp48 did not involve the proteasome. Instead, gp48 was found to redirect class I molecules to the endosomal system for degradation within lysosomes. Inhibitors of lysosomal protease activity, such as chloroquine or ammonium chloride, effectively blocked the degradation of both

gp48 and class I proteins.

A second MCMV protein capable of inhibiting MHC class I presentation was identified by microinjecting cloned MCMV DNA fragments into cells and then screening these cells for surface expression of class I proteins (Ziegler et al., 1997). Using this approach, a single open reading frame, m152, was identified as having the capacity to block class I surface expression. m152 encodes a type I membrane glycoprotein that is approximately 37-40 kDa in size, designated gp37/40. Expression of gp37/40 in cells results in the retention of class I molecules in the ER-Golgi intermediate compartment (ERGIC) (Ziegler et al., 1997). The localization of class I proteins in this case is quite distinct from the ER retention observed with US3. In cells expressing gp37/40, class I proteins exit the ER, but are prevented from entering the medial-Golgi.

4.4 Role of the MHC Class II Pathway in the Biology of the CMVs

Cells capable of expressing MHC class II proteins, such as macrophages, endothelial cells and microglial cells, are thought to play important roles in the lifecycle of both HCMV and MCMV. Expression of MHC class II proteins is generally restricted to professional antigen presenting cells (APCs) such as macrophages, dendritic cells and B cells, but can be induced in many other cell types by treatment with IFN-γ. The primary role of the MHC class II pathway is to present exogenous anitgens to $CD4^+$ T lymphocytes (discussed in detail below). During active infections, HCMV and MCMV have been shown to replicate in a wide variety of cells, including epithelial, endothelial and microglial cells (reviewed in Sinzger and Jahn, 1997), cells that can be induced to express high levels of MHC class II proteins. In particular, endothelial cells in the bone marrow

stroma, the salivary gland and the lining of large blood vessels are thought to serve as sites for persistent infection (reviewed in Soderberg-Naucler et al., 1998). Dissemination of virus throughout the body and the establishment of latency, however, is thought to rely on the infection of monocyte/macrophages (Fish et al., 1995; Soderberg-Naucler et al., 1997; Stoddart et al., 1994). Macrophages belong to the subset of APCs and constituitively express class II proteins. A strategy of viral persistence for HCMV and MCMV is to remain quiescent in circulating monocytes until these cells enter tissues and differentiate into macrophages. Macrophages are permissive for HCMV and MCMV infection and reactivation of virus from these cells gives rise to productive secondary infections. Since many of the cells infected by HCMV and MCMV are capable of expressing MHC class II proteins, it seems likely that these cells would elicit responses from antiviral $CD4^+$ T cells. Indeed, several studies have demonstrated that $CD4^+$ T lymphocytes specific for the HCMV major regulatory protein, IE1, comprise a major fraction of the responding T cells in latently infected healthy blood donors (Alp et al., 1991; Davignon et al., 1995; Davignon et al., 1996). Thus, the presentation of viral antigens by the MHC class II pathway may play an important role in signalling the presence of HCMV and MCMV infection, especially following reactivation from macrophages.

4.5 Antigen Recognition by $CD4^+$ T Lymphocytes

Since the primary focus of my research dealt with the inhibition of $CD4^+$ T cell recognition by the HCMV protein US2, the following sections will describe the activation of $CD4^+$ T cells and the presentation of antigens by the MHC class II pathway.

4.5.1 CD4+ T Cell Activation

CD4+ T lymphocytes recognize antigenic peptides presented in the context of MHC class II molecules (reviewed in Castellino et al., 1997; Weenink and Gautum, 1997). The specific binding of peptide/MHC complexes by T cell receptors (TCRs) expressed on the surface of CD4+ T cells provides the initial signal for activation (reviewed in Janeway and Travers, 1997; Roitt, 1994). The second signal is mediated through the binding of costimulatory molecules, such as B7.1 and B7.2, which are expressed on the surface of the antigen presenting cell, to CD28 on the T lymphocyte. Naive CD4+ T cells typically require less costimulation to become activated than do naive CD8+ T cells (reviewed in Greenfield et al., 1998; Sharpe, 1995). The increased threshold for CD8+ T cell activation may reflect the need to protect self tissues from the destructive effects of autoimmune CTL. Upon activation, CD4+ T lymphocytes can differentiate into one of three distinct effector populations: T_H1 cells, T_H2 cells or CTL (reviewed in Janeway and Travers, 1997; Roitt, 1994). In the presence of IL-12 and IFN-γ, activated CD4+ T cells differentiate into T_H1 cells which function primarily to activate macrophages. In the presence of IL-4, activated CD4+ T cells differentiate into T_H2 cells which function to stimulate B cells to produce high affinity antibodies. CD4+ T cells can also give rise to CTL, but the conditions required to drive cells along this path remain poorly understood.

4.5.2 MHC Class II Antigen Presentation Pathway

As a counterpart to the MHC class I pathway, which presents endogenous peptides, the class II pathway has evolved primarily to present exogenous antigens that have been taken up by cells through either endocytosis or

phagocytosis (reviewed in Castellino et al., 1997; Weenink and Gautum, 1997). Thus, the class I and class II presentation pathways enable the immune system to effectively monitor both the intracellular and extracellular environments. In contrast to the ubiquitous expression of MHC class I proteins, class II proteins are expressed in a more restricted fashion, generally limited to a distinct subset of cells known as APCs which include macrophages, dendritic cells and B cells (reviewed in Castellino et al., 1997; Weenink and Gautum, 1997). APCs are highly speacialized cells that function primarily to process and present antigens to T lymphocytes. Since APCs are also capable of expressing high levels of costimulatory molecules (reviewed in Greenfield et al., 1998; Sharpe, 1995), these cells are potent activators of both naive $CD8^+$ and $CD4^+$ T lymphocytes. Although the constitutive expression of class II is restricted to APCs, many cells within the body can be induced to express class II upon treatment with IFN-γ (reviewed in Kappes and Strominger, 1988). In particular, endothelial, epithelial, and microglial cells can be induced to express high levels of MHC class II proteins. The assembly and loading of class II molecules with peptide is an intricate process that until recently remained poorly understood. Several key discoveries by Denzin and Cresswell, however, have shed considerable light on this process. A current model of the MHC class II presentation pathway, illustrated in Figure 1, is summarized below.

4.5.2.1 Assembly of MHC Class II Dimers in the ER

The MHC class II complex is a heterodimer of two transmembrane proteins, the α chain (35 kDa) and the β chain (27 kDa), which associate with one another through noncovalent interactions. After synthesis and translocation into the ER,

individual α and β chains associate with the chaperone calnexin which may aid in stabilization and folding of the proteins (Anderson and Cresswell, 1994; Schreiber et al., 1994). Although the precise order of assembly is not clear, ultimately, α and β chains associate separately or as heterodimers with a third transmembrane glycoprotein called the invariant chain (Ii) (Claesson-Welsh and Peterson, 1985; Scaiff et al., 1991; Anderson and Miller, 1992). The Ii is a chaperone-like protein that associates specifically with class II molecules and is thought to perform several vital functions in the maturation of class II dimers. First, the Ii may promote the proper folding and assembly of αβ dimers in the ER (Anderson and Miller, 1992; Layet and Germain, 1991). Second, the binding of the Ii to the peptide-binding groove of class II dimers prevents the premature loading of peptides in the ER intended for MHC class I molecules (Roche and Cresswell, 1990; Teyton et al., 1990). Third, the cytoplasmic tail of the Ii contains two leucine-based sorting signals (LI at positions 7-8 and ML at positions 16-17) which target the class II complex to the endosomal system and eventually to the MHC class II compartment (MIIC) (Claesson-Welsh and Peterson, 1985; Lotteau et al., 1990). Soon after synthesis and translocation into the ER, invariant chains oligomerize to form homotrimeric complexes with interchain disulfide bridges in their cytoplasmic tails (Roche et al., 1991). Once assembled, these Ii trimers serve as scaffolds onto which three αβ dimers assemble in a process that is thought to be facilitated by calnexin (Schreiber et al., 1994). As is common for the maturation of many multisubunit complexes, the stable assembly of this nonameric structure results in the dissociation of calnexin, followed shortly by the egress of the nonamer from the ER (Anderson et al., 1994; Schreiber et al., 1994). In this form,

MHC class II complexes are transported from the ER to the trans-Golgi network (TGN), where they are then diverted to the endosomal system for delivery to the MIIC.

4.5.2.2 Capture of MHC Class II Antigens

Antigens presented by MHC class II molecules are primarily derived from exogenous proteins taken up by cells through either endocytosis or phagocytosis. Capture of antigen by B cells and non-APCs is generally restricted to pinocytosis and receptor-mediated endocytosis (reviewed in Geuze, 1998). Pinocytosis involves the ingestion of fluid and solutes via small clathrin coated vesicles (Alberts et al., 1989). Since extracellular fluid is trapped in the coated pits as they invaginate to form vesicles, debris and proteins dissolved in the fluid are also internalized. Receptor-mediated endocytosis, however, provides cells with an efficient pathway for taking up specific macromolecules from the extracellular fluid. This process is initiated through the binding of macromolecules to cell surface receptors which results in their accumulation in clathrin coated pits (Alberts et al., 1989). These receptor-macromolecule complexes then become internalized within the cell and are delivered to endocytic vesicles. Capture and internalization of specific antigens by B cells occurs through receptor-mediated endocytosis via cell surface immunoglobulins (reviewed in Lanzavecchia, 1993). In addition to the mechanisms described above, macrophages and dendritic cells are capable of engulfing entire cells or pathogens through a process called phagocytosis (reviewed in Geuze, 1998). Phagocytosis is also receptor-mediated and involves the formation of large psuedopods that extend from the phagocytic cell and surround the particle to be internalized (Alberts et al., 1989; Geuze,

1998). To initiate this process, specific cell surface receptors must be triggered which transmit signals into the cell resulting in the mobilization of lipids and the remodelling of the cytoskeleton. Macrophages and dendritic cells express several receptors capable of triggering phagocytosis. In marcophages, these receptors recognize components such as the Fc region of IgG, complement factors C1 and C3, mannose-containing glycoproteins and various bacterial oligosaccharides (reviewed in Janeway and Travers, 1997; Roitt, 1994). Regardless of the mode of antigen uptake, particles and molecules ingested by cells usually end up in lysosomes. These compartments contain a wide variety of hydrolytic enzymes and function to degrade material that has been internalized by the cell (Alberts et al., 1989). As part of the endocytic pathway, the MIIC is thought to perform a similar function, by degrading exogenous proteins for presentation by class II molecules.

4.5.2.3 MHC Class II Compartment

The MIIC is a lysosomal-like vesicle that serves as the site for two essential events in the maturation of MHC class II molecules. First, antigenic peptides presented by class II molecules are generated within the MIIC (McCoy et al., 1989; Unanue, 1984; Ziegler and Unanue, 1982). Exogenous proteins taken up by cells through endocytosis or phagocytosis are delivered to the MIIC for proteolytic degradation into peptide fragments. The pH of the MIIC is extremely acidic, ranging between pH 4.5-5.5, and this facilitates the degradation of proteins by hydrolytic enzymes, such as the cathepsins, which reside in this compartment (Buus and Werdelin, 1986; Takahoshi et al., 1989). Second, the MIIC also serves as the site for peptide-loading onto class II molecules. Loading

of class II-peptides is mediated by a heterodimeric complex, known as HLA-DM, that is structurally related to classical class II proteins (Denzin and Cresswell, 1995; Denzin et al., 1994). By virtue of a tyrosine based sorting signal, HLA-DM accumulates intracellularly within the MIIC and represents the single most reliable marker for this organelle (Sanderson et al., 1994).

Although the functional parameters of the MIIC have been well defined, the intracellular identity of this organelle remains one of the most controversial topics in the field of class II antigen presentation. During the past decade, several investigators have made attempts to isolate and purify the MIIC from various APCs, such as B lymphoblasts and dendritic cells. Unfortunately, of the vesicles identified thus far, all seemed to differ in one respect or another in terms of morphology or expression of intracellular markers. The MIIC, first purified by Peters and colleagues (1991), was characterized as a multilamellar vesicle which stained positive for lysosomal markers such as β-hexoaminidase and lysosomal-associated membrane protein (LAMP)-1, but not for markers of early endosomes. Subsequently, a class II-containing vesicle (CIIV) was isolated from mouse A20 B cells by Amigorena and colleagues (1994) which stained positive for early endocytic markers such as the transferrin receptor, but was devoid of lysosomal protein LAMP-1. More recently, several laboratories have isolated a large multivesicular organelle that has been proposed to be the major site of peptide loading for class II molecules in various cell types (Castellino and Germain, 1995; Tulp et al., 1994; West et al., 1994). The compartment for peptide loading (CPL), however, did not display characteristics of either early or late endosomes or lysosomes. Intriguingly, many of the vesicles identified in these studies have been

proposed to be specilized compartments for class II processing and not necessarily general components of the endocytic pathway. This issue is a major point of contention, as several lines of investigation have yielded contradictory results. For example, an operational MIIC was recently reconstituted in a non-APC through the transfection of class II, Ii and HLA-DM (Karlsson et al., 1994). In transfected HeLa cells, these class II components accumulated in late endosomes giving rise to morphological structures which closely resembled the MIIC. This result seemed to suggest that conventioal endocytic vesicles could be transformed into functional class II compartments. Upon further analysis, however, presentation of antigens using this reconstituted system was found to be inefficient, indicating that additional components, quite possibly a specialized compartment, were required for effective presentation (Harding, 1995). Similar results were obtained using non-APCs treated with IFN-γ or transfected with the class II transactivator (CIITA) to induce expression of class II components (Muczynski et al., 1998). Although the cells in each case were able to present antigen, there was mislocalization of both HLA-DM and class II proteins and transport of class II to the cell surface was considerably delayed. In particular, the failure of HLA-DM to colocalize with class II proteins in these cells supports the notion that non-APCs lack a specialized compartment for class II processing. To reconcile the observations from the above studies, however, a compromise of some sorts may be necessary. It is now generally believed that in non-APCs, conventional endocytic compartments may suffice for class II processing, while in APCs, there might exist a specialized compartment that enables these cells to process and present antigen much more efficiently. Clearly, further study is

required before these issues can be resolved.

4.5.2.4 Peptide Loading of MHC Class II Complexes

During transit of α/β/Ii complexes through the endosomal system, the lumenal domain of the Ii is progressively degraded by various proteases that reside in the endocytic compartments (reviewed in Castellino et al., 1997; Weenink and Gautum, 1997). The initial cleavage events generate a truncated form of the Ii that remains bound to the MHC class II dimer and retains it within the MIIC. In the MIIC, the Ii is gradually degraded until all that remains is a 24 amino acid fragment (residues 81-104) known as the class II-associated invariant chain peptide (CLIP) (Donermeyer and Allen, 1989; Sette et al., 1992). CLIP remains bound to the class II dimer in the MIIC and occupies the peptide-binding groove (Ghosh et al., 1995). The association of CLIP with class II molecules precludes the binding of antigenic peptides. In order for class II dimers to be loaded with peptide, CLIP must either dissociate or be displaced from the peptide-binding groove. The removal of CLIP from class II dimers is mediated by a specific complex, HLA-DM, that resides in the MIIC (Denzin and Cresswell, 1995; Denzin et al., 1994). HLA-DM is a heterodimer of two transmembrane proteins, the α chain and the β chain, which share limited homology with classical class II proteins. HLA-DM functions as an enzyme to promote the release of CLIP from class II dimers, followed by the loading of antigenic peptides produced in the MIIC. The molecular mechanism by which HLA-DM induces the exchange of CLIP for peptide remains poorly understood. Recent evidence suggests that, under acidic conditions, the association of HLA-DM with class II/CLIP complexes induces a conformational change in the class II dimer that favors the dissociation

of CLIP (Denzin and Cresswell, 1995; Sanderson et al., 1996). Once CLIP is released from the complex, the αβ dimer is thought to adopt a second conformation which favors the binding of antigenic peptides. The αβ dimer is maintained in this conformational state by virtue of continued association with HLA-DM until peptide binding occurs (Denzin et al., 1996; Kropshofer et al., 1997). Once peptide is bound, HLA-DM dissociates from the loaded class II dimer and awaits the next available substrate. As a catalyst for peptide exchange, HLA-DM may also influence the repertoire of peptides presented by class II proteins (Kropshofer et al., 1997). In addition to CLIP, HLA-DM has also been shown to mediate the release of low affinity peptides from class II complexes (Kropshofer et al., 1996). Thus, a single class II molecule may undergo several rounds of peptide editing by HLA-DM until a high-affinity peptide is bound.

4.5.2.5 Export to the Cell Surface

Once class II molecules have been loaded with antigenic peptides, the complexes are ready to migrate to the cell surface for presentation to $CD4^+$ T lymphocytes. As with MHC class I complexes, the binding of peptide by class II dimers results in a dramatic increase in stability, giving rise to the formation of SDS-stable dimers (Germain and Hendrix, 1991; Neefjes and Ploegh, 1992; Wettstein et al., 1991). These dimers remain resistant to dissociation in 2% SDS at room temperature, whereas dimers loaded with CLIP or low affinity peptides rapidly unfold under these conditions. The acquisition of SDS-stability is thought to provide a signal for the export of loaded class II dimers to the cell surface (Germain et al., 1996; Castellino et al., 1997). Unfortunately, both the mechanism and route by which MHC class II molecules reach the cell surface remain poorly

understood. Currently, two potential pathways have been supported by experimental data. The first involves the direct fusion of the MIIC or related compartments with the plasma membrane. In human B cells, multivesicular MIICs have been shown to fuse directly with the plasma membrane and deliver internal vesicles called exosomes into the medium (Raposo et al., 1996). The second pathway involves the budding of small transport vesicles from the MIIC which would then serve to shuttle class II dimers to the plasma membrane. EM micrographs of MIICs often show extended tubular structures at either end of the organelle which may facilitate the budding of transport vesicles (Geuze, 1998).

4.5.2.6 Endogenous Presentation by the MHC Class II Pathway

Although the MHC class II pathway has evolved primarily to present exogenous antigens, there is increasing evidence that endogenous proteins can also be presented. One of the first reported examples of presentation of endogenous antigens by class II proteins arose from studies exploring the role of $CD4^+$ T cells in the control of measles virus infection. From these studies, it was found that $CD4^+$ T cells with cytotoxic activity were critical in limiting viral replication (Jacobsen et al., 1984). However, in order for $CD4^+$ CTL to be effective, these lymphocytes must be capable of identifying infected cells. $CD4^+$ T lymphocytes are restricted by class II proteins, and thus, the recognition of infected targets was speculated to involve the presentation of endogenous viral antigens by the class II pathway. To investigate this hypothesis, Jacobsen and colleagues (1989) transfected class II-expressing cells with various genes encoding measles proteins and then incubated the cells with measles-specific $CD4^+$ T cell clones. Through a series of elegant experiments, it was demonstrated

that the endogenously synthesized measles proteins were effectively presented to $CD4^+$ T cells. More recently, studies with the hepatitis C virus (HCV) have yielded similar results (Chen et al., 1998). The HCV core protein, when endogenously expressed in human B cells, was found to enter the class II pathway, resulting in the presentation of core protein peptides and the potent activation of specific $CD4^+$ T cells. Presently, there are three mechanisms by which endogenous antigen is thought to gain access to the class II pathway (reviewed in Aichinger and Lechler, 1995; Lechler et al., 1996). First, class II molecules may 'hijack' class I peptides while in the ER. Peptide binding by the $\alpha\beta$ dimer, in this case, would have to occur before association with the Ii. Second, cytosolic proteins may enter the MIIC through a process known as autophagy. Organelles within a cell are typically turned over by engulfment and degradation within autophagolysosomes (Alberts et al., 1989). These specialized compartments may fuse with the MIIC, thereby supplying abundant cytosolic antigens for presentation. The third mechanism involves the transit of glycoproteins through the endosomal system and possibly, the MIIC. This may occur through two pathways: glycoproteins may be sorted directly to various endosomal compartments via trafficking signals or these proteins may be endocytosed from the cell surface. This final mechanism of endogenous class II presentation may play an important role in the detection of infected cells by $CD4^+$ T lymphocytes. During infection with several herpesviruses, structural proteins and viral particles accumulate in the trans-Golgi network (TGN) and in endosomal compartments (Gershon et al, 1994; Jones and Grose, 1988; Radsak et al., 1990; Tooze et al., 1993). Thus, unlike proteins endocytosed at the cell surface,

these endogenously synthesized viral proteins may be delivered directly from endosomes to the MIIC. Peptides derived from the degradation of these viral proteins would then be presented via class II molecules to virus-specific $CD4^+$ T cells.

4.5.2.7 Diversity of MHC Class II Proteins

MHC class II proteins are encoded by three sets of α and β chain genes, designated HLA-DR, -DP and -DQ, located on human chromosome 6. The α and β chains of a single locus (DR, DP or DQ) can pair with one another, but cannot combine with chains expressed from different loci. As with MHC class I genes, the genes encoding the class II proteins, with the exception of DRα, are extremely polymorphic (see Figure 2; reviewed in Bodmer et al., 1991). The DRβ gene, for example, is comprised of at least 266 alleles which can be discriminated by PCR analysis. This diversity enables MHC class II proteins to bind a wide array of peptides. The requirements for peptide binding by class II proteins are not as well understood as those for class I. Unlike class I peptides, the peptides bound by class II proteins are not constrained in terms of length. Class II peptides generally vary in size from 13 to 30 amino acids (reviewed in Reizis et al., 1998). The reason for this variability lies in the open-ended structure of the class II peptide-binding groove. The amino and carboxy termini of class II peptides are not bound within the groove, but rather extend outwards from either end (Stern et al., 1994). This heterogeneity in peptide length has made analysis of peptide binding motifs extremely difficult. Considerable light was shed on this subject, however, following the crystallization of the HLA-DR complex bound to peptide (Stern et al., 1994). As with MHC class I, class II proteins were found to bind to a

core of 9 amino acids through specific interactions with anchor residues of the peptide. These anchor residues insert into pockets in the peptide binding groove that are lined with polymorphic amino acids (Rammensee et al., 1995; Stern et al., 1994). Therefore, allelic variants of class II proteins show different specificities for peptides. The diversity of peptide binding may be enhanced even further by the pairing of different alleles of class II proteins.

4.6 Downregulation of MHC Class II Gene Expression by the CMVs

Many of the cells infected by HCMV and MCMV are capable of expressing MHC class II proteins, and thus, are likely to elicit responses from antiviral $CD4^+$ T lymphocytes. As a countermeasure, both HCMV and MCMV have evolved mechanisms to downregulate the expression of class II genes at the transcriptional level (discussed below). These transcriptional effects, however, occur relatively late in infection and thus, may not be effective in blocking the presentation of IE and E gene products. The failure to do so would render infected cells susceptible to recognition by antiviral $CD4^+$ T lymphocytes. Before I began my studies, it was unclear whether HCMV expressed an IE or E protein capable of blocking class II antigen presentation.

4.6.1 Inhibition of MHC Class II Expression by HCMV

MHC class II expression can be induced in many cell types by treatment with IFN-γ. IFN-γ is a potent antiviral cytokine and one of its functions is to upregulate the expression of several components of the class II pathway to enhance presentation of antigens to $CD4^+$ T cells. Binding of IFN-γ to its receptor results in the activation of the Jak/Stat pathway followed by the induction of the class II transactivator (CIITA) (reviewed in Boss et al., 1997; Mach et al., 1996).

Figure 1: The MHC class II presentation pathway. The primary role of the class II pathway is to display exogenous antigens to $CD4^+$ T lymphocytes. 1. The α and β chains are initially translocated into the ER membrane. 2. Following glycosylation, the individual α and β chains associate with the chaperone calnexin (CXN). 3. The order of assembly is uncertain at this point, but eventually, the α and β chains associate with a trimeric invariant chain (Ii) complex. 4. Two additional $\alpha\beta$ heterodimers then associate with the trimeric Ii complex giving rise to a large $\alpha\beta$/Ii nonamer. Stable assembly of this nonameric structure results in the dissociation of calnexin and egress from the ER. 5. The nonamer then travels along the exocytic pathway until it reaches the trans-Golgi network (TGN), where it is diverted to the endosomal system. 6. In early endosomal compartments, the C-terminus of the Ii is partially degraded resulting in the dissociation of individual $\alpha\beta$/Ii complexes. These complexes are then delivered to the MHC class II compartment (MIIC). 7. Once in the MIIC, the Ii is further degraded until only a small remnant remains, called the class II-associated invariant chain peptide (CLIP), which binds to the peptide-binding groove. 8. The $\alpha\beta$-CLIP complex then associates with a class II-like heterodimer known as DM (encoded by HLA-DM). 9. The interaction with DM results in the removal of CLIP from the peptide-binding groove. 10. Empty class II complexes are then loaded with peptides derived from the degradation of extracellular proteins in the MIIC by cathepsins. 11. Although the precise route remains unclear, peptide-loaded class II complexes exit the MIIC and are transported to the cell surface for presentation to $CD4^+$ T cells.

MHC Class II Presentation Pathway

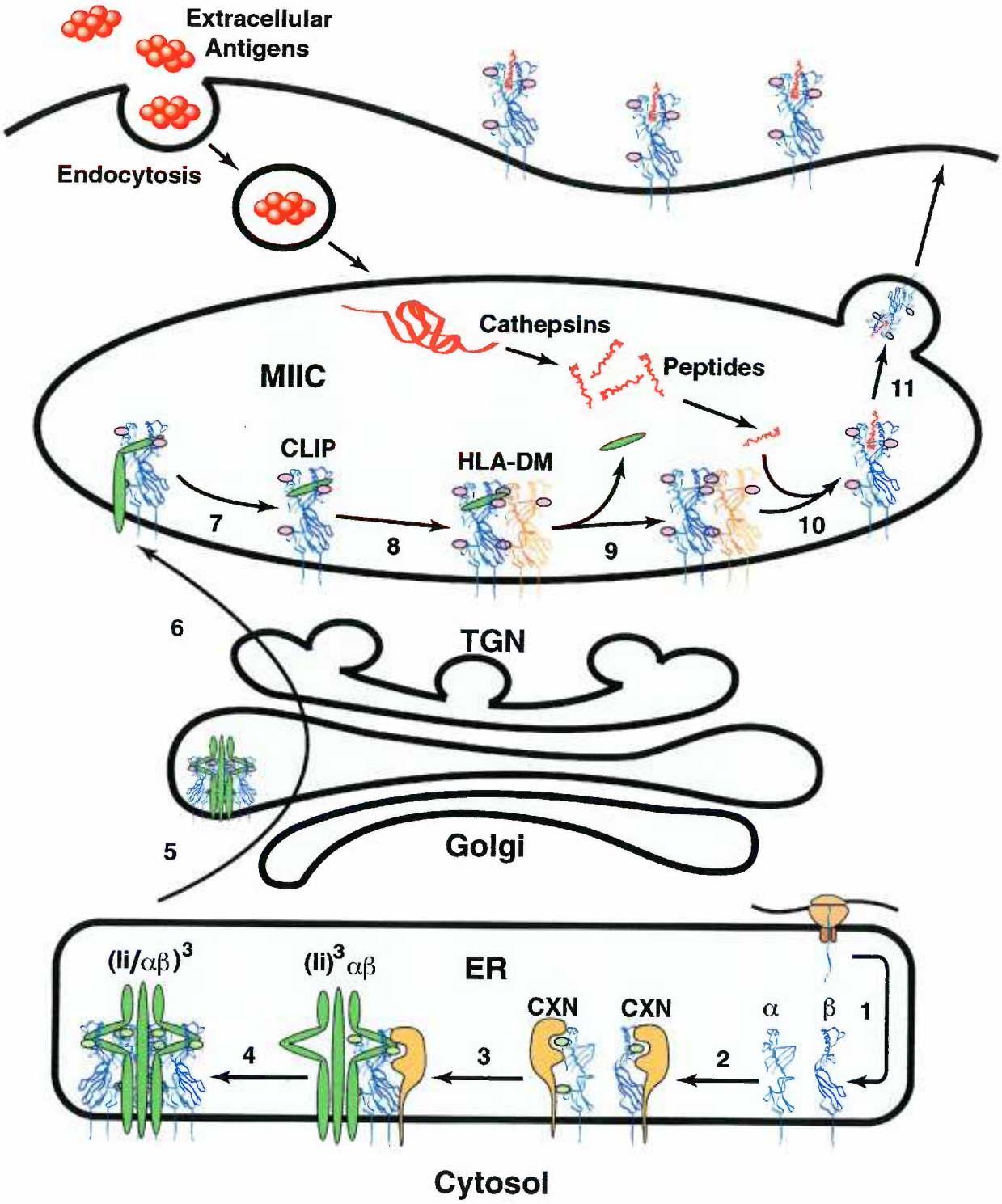
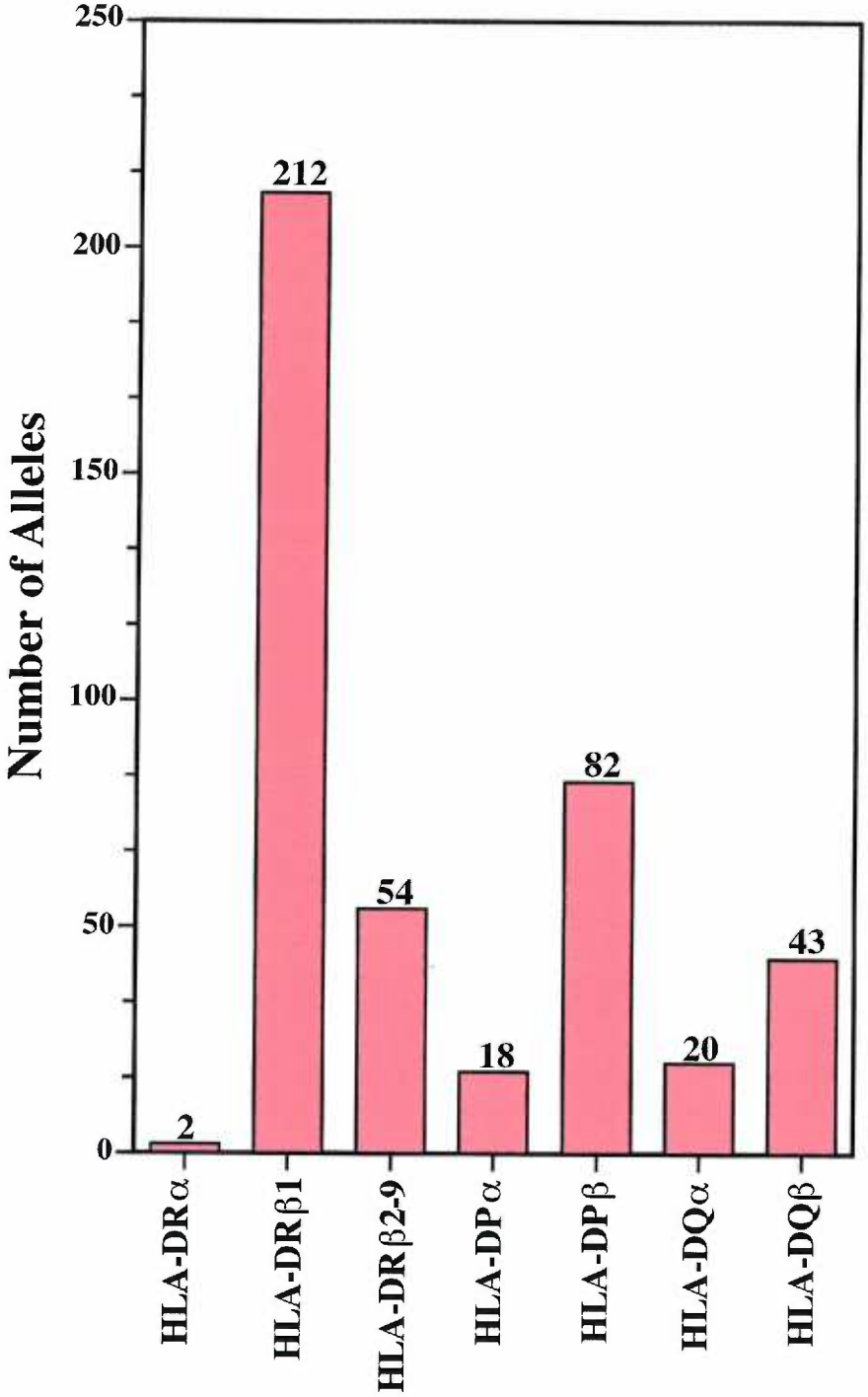


Figure 2: Polymorphism of MHC class II genes. Current number of alleles assigned to the HLA-DR, -DP and -DQ loci based on DNA sequence analysis. Data obtained from the HLA Informatics Group (www.anthonynolan.com).

Polymorphism of MHC Class II Genes



CIITA is a non-DNA binding transactivator that specifically induces the transcription of several genes involved in class II presentation (Steimle et al., 1994). During late times post infection, endothelial cells infected with HCMV become resistant to the transcriptional upregulation of class II genes induced by IFN-γ. Sedmak and colleagues demonstrated that this inability to induce class II expression was due to the accelerated turnover of the Janus kinase (Jak)(Miller et al., 1998). Treatment of cells with proteasome inhibitors increased the half-life of Jak and restored the ability of HCMV-infected cells to induce class II expression. The mechanism by which HCMV accelerates Jak turnover remains unknown.

4.6.2 Inhibition of MHC Class II Expression by MCMV

As with HCMV, MCMV also downregulates the expression of MHC class II genes induced by IFN-γ. However, in this case, the block in IFN-γ induction seemed to occur later in the Jak/Stat activation pathway (Heise et al., 1998). Analysis of early upstream events in the signalling cascade revealed that MCMV did not affect the phosphorylation or nuclear translocation of transcription factor Stat1α. Thus, MCMV may interfere with either the induction of CIITA or its incorporation into the class II promoter complex. Further studies are underway to define more closely the block in this pathway. In addition to the disruption of class II induction by IFN-γ, MCMV is also capable of blocking class II expression using another approach. Ghazal and colleagues have demostrated that infection of macrophages with MCMV results in the premature induction of interleukin-10 (IL-10) (Redpath et al., 1999). IL-10 is usually produced late during infections and serves to dampen the immune response by suppressing the production of various inflammatory cytokines, soluble mediators and cell surface receptors

(reviewed in Janeway and Travers, 1997; Roitt, 1994). In particular, IL-10 is quite effective in downregulating MHC class II expression. Thus, during infection with MCMV, the early induction of IL-10 is thought to function in an autocrine loop to reduce the expression of class II proteins on the surface of macrophages.

4.7 Summary and Hypothesis

HCMV is a ubiquitous herpesvirus that causes asymptomatic disease in healthy individuals, but can become a life-threatening pathogen in newborns and immunodeficient patients. HCMV establishes lifelong infections within its hosts, and during this time, develops an intimate relationship with the host immune system. A striking feature of HCMV infection is the ability of the virus to reactivate undetected within the host and produce infectious progeny. The ability of HCMV to replicate in the presence of a fully primed immune response may be due in large part to the numerous immune evasion tactics used by the virus. Several studies have demonstrated that HCMV is capable of evading $CD8^+$ T cell recognition by blocking the MHC class I antigen presentation pathway. However, the MHC class II presentation pathway can also signal the presence of viral infection, but is restricted to APCs such as B cells, dendritic cells and macrophages, cells that present antigen to $CD4^+$ T lymphocytes. Recent observations by Nelson and colleagues have suggested that monocytes/macrophages may play an important role in the lifecycle of HCMV. During primary infection with HCMV, the virus is thought to infect monocytes and establish latency in these cells. The ability of HCMV to remain quiescent in circulating monocytes may serve as an important means for dissemination of virus throughout the body. Once these monocytes enter tissues and differentiate into

macrophages, however, the virus reactivates and produces infectious progeny for transmission to new hosts. Remarkably, this reactivation not only takes place in the face of a fully primed immune response, but also within a macrophage that can present antigen through either the MHC class I or class II presentation pathways. Although the class II pathway normally presents exogenous antigens, there is evidence that during HCMV infection, endogenous viral proteins may transit through the endosomal system and reach the MIIC. Peptides derived from these viral proteins would be presented to antiviral $CD4^+$ T cells, signalling the presence of HCMV infection. Therefore, in order for HCMV to reactivate and replicate within a macrophage, it seemed likely that the virus might also encode inhibitors of the MHC class II pathway. Ideally, these inhibitors would be expressed during IE or E times post infection, thereby blocking the presentation of most viral antigens. Downregulation of class II gene expression late in infection, as has been described for HCMV, would not provide an effective means of immune evasion. Therefore, the second aim of this thesis is to identify and characterize potential HCMV inhibitors of the MHC class II presentation pathway.

Chapter 5

Cytomegalovirus US2 destroys two components of the MHC class II pathway, preventing recognition by CD4$^+$ T cells (Nature Medicine, 5: 1039-1043, 1999)

5.0 Preface

In the following publication, I carried out the experiments in Figures 5.1, 5.3, 5.4, 5.5, 5.6, 5.7 and 5.8. The Northern blot experiment in Figure 5.2 was conducted in our laboratory by Dr. Jessica Boname. The CD4$^+$ T cell assay in Figure 5.9 was performed by Dr. Nagendra R. Hegde in our laboratory and Dr. David M. Lewinsohn at the Portland VA Medical Center in Portland, OR.

Cytomegalovirus US2 destroys two components of the MHC class II pathway preventing recognition by CD4+ T cells

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Abstract

Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus that normally causes relatively asymptomatic infections. However, in patients that are immunosuppressed for bone marrow or tissue transplantation or with AIDS, HCMV can cause life-threatening disease[1]. As with other herpesviruses, HCMV establishes lifelong latent infections and, following periodic reactivation from latency, uses a panel of immune evasion proteins to survive and replicate in the face of robust, fully-primed host immunity[2,3]. Monocyte/macrophages are especially important cells in the lifecycle of HCMV; the virus establishes latency in these cells and, once activated, macrophages can produce virus and spread viral infection throughout the body[4]. Macrophages and other HCMV-permissive cells such as endothelial cells, can express MHC class II proteins and act as antigen presenting cells to $CD4^+$ T lymphocytes. Here, we show that HCMV expresses a protein, US2, that causes degradation of two key proteins in the MHC class II antigen presentation pathway, HLA-DR-α and DM-α. These observations were surprising given that US2 had previously been shown to cause degradation of MHC class I [5,6], which shows only limited primary sequence homology with class II proteins. Expression of US2 in cells reduced or abolished their ability to present antigen to $CD4^+$ T lymphocytes. Thus, US2 may allow HCMV-infected macrophages to remain relatively invisible to CD4+ T cells, a property that would be important following virus reactivation. Moreover, US2

or analogous herpesvirus proteins may have important consequences for immune-mediated containment of other viruses that can also infect macrophages e.g. HIV.

Introduction

$CD8^+$ and $CD4^+$ T lymphocytes are the principal line of defence against viral infections, recognizing small peptides derived from viral proteins and responding by rapidly killing virus-infected cells or orchestrating specific immune responses against the virus. Major histocompatability (MHC) class I proteins present peptides to $CD8^+$ T cells, peptides that are produced in the cytoplasm by proteasomes then translocated into the endoplasmic reticulum (ER) and loaded onto class I molecules for export to the cell surface[7]. Since MHC class I proteins are ubiquitous, $CD8^+$ T cells can detect the presence of viral proteins in most cells and eliminate infected cells by cytolysis. By contrast, MHC class II proteins are primarily expressed by professional antigen presenting cells, B cells, macrophages and dendritic cells, which can take up extracellular proteins by endocytosis or phagocytosis and present peptides derived from the proteins[8,9]. Class II proteins associate in the ER as a complex of α and β chains, bound to a third component, the invariant chain (Ii), which protects the peptide-binding site[10]. The α/β/Ii complexes are transported from the ER to a lysosomal-like compartment known as the MHC class II compartment (MIIC), where peptide loading occurs. Another class II component, DM, binds to the class II $\alpha\beta$ complex and catalyzes exchange

of a remnant of Ii for antigenic peptides, produced by protease degradation of proteins in the MIIC[11]. From the MIIC, peptide-loaded class II-αβ complexes move to the cell surface for recognition by $CD4^+$ T cells.

Herpesviruses are large DNA viruses that can establish lifelong latent infections in a variety of cell types and, as part of interactions with their hosts, interfere with immune recognition[2,3]. Immune evasion may be particularly important for successful virus replication following reactivation from latency, when the immune system has been fully primed to the virus. Several studies have identified mechanisms by which herpesviruses inhibit recognition by $CD8^+$ T cells. The herpes simplex virus ICP47 protein blocks the peptide transporter (TAP) preventing access of antigenic peptides into the class I pathway[12]. HCMV expresses at least 4 proteins that block different steps in the class I pathway[2,3], including US2 and US11 that bind the MHC class I heavy chain (HC) and cause dislocation of the protein into the cytoplasm and degradation by the proteasome[5,6,13].

HCMV infects a variety of cells including monocyte/macrophages and endothelial cells[14], that express MHC class II proteins. Monocyte/macrophages appear to play a crucial role in the lifecycle of HCMV, serving as a reservoir for latent virus and as a means of dissemination[4]. During productive HCMV replication in macrophages, HCMV structural proteins and particles accumulate in the trans-Golgi network and in endosomal compartments[15]. Thus, unlike proteins

endocytosed at the cell surface, these endogenously-synthesized HCMV proteins may be delivered directly from endosomes to the MIIC. Proteolysis of the viral proteins will yield peptides which can be presented to $CD4^+$ T cells, as has been described for measles virus[16]. By this scenario, virus-specific $CD4^+$ T cells could limit HCMV infection of monocytes/macrophages in vivo. Thus, we hypothesized that HCMV might also inhibit the MHC class II antigen presentation pathway to facilitate viral persistence in class II expressing cells.

Results and Discussion

To study potential effects of HCMV infection on MHC class II, we used a transformed glioblastoma cell line, U373-MG, which is permissive for HCMV infection and can be induced to express class II by interferon-γ (IFN-γ) treatment[17]. A dramatic reduction in expression or stability of class II was observed after as little as 12 h post infection by HCMV in IFN-γ-treated cells (Fig. 1A). An HCMV mutant unable to express a block of 12 genes, IRS1 and US1-US11[18], did not inhibit class II expression. The US1-US11 region of the HCMV genome encodes at least 4 proteins: US2, US3, US6, and US11, that can independently interfere with the stability, assembly or export of MHC class I molecules[2,3]. To further characterize the effect of HCMV infection on class II, a cell line that constitutively expresses MHC class II was constructed by transfecting U373 cells with the class II transactivator gene, CIITA[19]. Infection of

U373-CIITAHis cells with a panel of mutant viruses, each lacking one or more of the IRS1-US11 genes[18] indicated that US2 or US3 or both were necessary for this effect (Fig. 1B). The reduced expression of class II was not due to inhibition of IFN-γ mediated transcriptional enhancement of MHC class II genes, as described[20], because IFN-γ was not required to induce class II expression in the U373-CIITAHis cells and mRNAs specific for MHC class II-α, β and Ii were not reduced at these relatively early times after HCMV infection (Fig. 2). We also observed inhibition of the MHC class II pathway in human peripheral blood monocyte/macrophages after HCMV infection (not shown), but the results were variable, apparently due to incomplete virus infection of these cultured cells.

Stably-transfected cells expressing either US2 or US3 were constructed and we found that US2, but not US3, caused diminished expression of class II (Fig. 3). Previously, US2 had been shown to cause degradation of MHC class I heavy chains (HC), by causing translocation of HC into the cytoplasm and proteolysis by the proteasome[5,6]. To study US2's effects on class II proteins in more detail, a replication-defective adenovirus (Ad) vector expressing US2, AdtetUS2, was constructed by coupling the US2 gene to the tetracycline inducible promoter and inserting the gene into the E1 region of Ad. US2 expression could be induced in cells by coinfecting U373-CIITAHis cells with AdtetUS2 and a second virus, Adtet-trans, that produces a transcription factor able to activate the tet promoter[21]. In experiments where cells were radiolabelled

for 1 min then the label chased or 15 or 30 min, there was rapid loss of the MHC class II complex and this was dependent on the quantity of US2 delivered (Fig. 4). When the class II complex was denatured and individual proteins immunoprecipitated, class II-α was quickly degraded in US2-expressing cells. Some limited loss of β and Ii was observed in cells which was probably related to the loss of available α chains, as Ii and ß chains are less stable in the absence of α chains[22]. US2 also caused rapid degradation of the MHC class I HC as expected[5,6], but did not affect three cellular proteins: the transferrin receptor (TfR) (not shown), and two ER-resident proteins, GRP94 and calnexin (Fig. 4). In other experiments, class II-α was rapidly degraded in human brain endothelial cells infected with AdtetUS2/Adtet-trans (not shown).

In cells expressing US2 and treated with specific inhibitors of the proteasome, lactacystin[23] or carbonylbenzyl-leucyl-leucyl-leucyl-vinylsulphone (ZL_3VS)[24], class II-α chains were effectively stabilized (Fig. 5). Thus, proteasome activity is required for US2-induced degradation of class II-α. In US2-expressing cells treated with a proteasome inhibitor, class I HC is translocated into the cytoplasm and is deglycosylated (ref. 5; Fig. 5). By contrast, the class II-α that accumulated in US2-expressing cells treated with proteasome inhibitors remained primarily glycosylated (Fig. 5). Translocation of the class II-α chain into the cytosol was studied by fractionating cells into a nuclear/ER fraction (1,000 X g pellet), a dense vesicular fraction (10,000 X g pellet), a microsomal fraction

(100,000 X g pellet), and a cytosolic fraction (100,000 X g supernatant). In cells expressing US2 and treated with ZL_3VS, class II-α was primarily found in dense membrane fractions, as was the case with calnexin, an ER-resident protein, whereas a large fraction of class I HC was found in the cytosolic fraction (Fig. 6A). Therefore, in contrast to MHC class I HC, class II-α fails to accumulate in the cytoplasm in US2-expressing cells in which the proteasome is blocked. Recent reports have suggested that the type, number and location of oligosaccharides added to a polypeptide in the lumen of the ER can affect the half-life, and hence, the rate of degradation of certain proteins[25]. In order to determine whether hydrophilic oligosaccharides could affect the removal of class II−α chains from the ER membrane, US2-expressing cells were treated with tunicamycin, an inhibitor of N-linked glycosylation, and ZL_3VS. Once again, following subcellular fractionation, the majority of the class II-α chains remained in the ER membrane fractions (Fig. 6B). The failure of cytoplasmic intermediates of class II-α to accumulate in US2-expressing cells treated with proteasomal inhibitors suggests that the transfer of class II-α chains from the ER to the cytosol mediated by US2 requires the activity of the proteasome. Similar observations have been reported for the TCR α chain and class II-β chain when these proteins have been expressed without their respective binding partners[22,25]. In each case, the unstable protein is degraded, albeit more slowly than the class II-α chain with US2 present, but without the significant accumulation of cytosolic intermediates.

Proteosomes are known to be associated with the ER membrane[22] and thus, after destabilization by US2, the class II-α chain fed directly into the lumen of the proteosome.

The binding of US2 to class II-α, and other members of the class II complex, was measured by immunoprecipitating US2, denaturing the precipitated proteins then reprecipitating with anti-α, anti-β or anti-Ii antibodies. In order to stabilize transient interactions between US2 and class II proteins, cells were first treated with ZL_3VS then radiolabelled for 5 min and the label chased for 20 min. Under these conditions, there was obvious association of US2 with the class II-α chain, as well as with class I HC (Fig. 7A). However, there was also coprecipitation of β and Ii with US2, less than that observed with α. Similarly, anti-α, anti-β, and anti-Ii antibodies precipitated US2, although again there was less US2, especially the deglycosylated form, with anti-β and anti-Ii antibodies (Fig. 7B). These findings suggest that US2 binds to free α chains but also binds to the rapidly assembled class II α/β/Ii complexes, probably through direct interactions with a chains.

HLA-DM is an essential component of the MHC class II pathway that binds to the class II-α/β complex in the MIIC, stabilizing class II and catalyzing the loading of antigenic peptides[11]. The DM-α and DM-β chains share limited homology to the classical class II DR proteins (Fig. 8A), and thus, it was of interest to determine whether US2 affected DM. In US2-expressing U373-

CIITAHis cells, DM-α was rapidly degraded, whereas the stability of DM-β did not differ from that in control cells (Fig. 8B). Therefore, US2 causes degradation of DM-α, as well as class II DR-α and MHC class I HC. This destruction of two key components of the class II pathway as well as the class I HC was unexpected based on the limited primary sequence homology between the proteins (Fig. 8A). Other proteins that show similar sequence homology, i.e. class II DR-β and DM-β, are not affected by US2 (Fig. 8A). Similarly, US2 does not cause degradation of class I HLA-C and HLA-G[26], proteins that share extensive sequence homology with HLA-A and HLA-B, known targets of US2 (Fig. 8A). Class I, class II-DR, and class II-DM complexes all share an overall fold and domain organization with two membrane proximal immunoglobulin (Ig) constant domains and two membrane distal domains folded into peptide binding clefts[27]. Given that the Ig domains are shared by DR-β and β_2-microglobulin which are not degraded, it appears that US2 binds to the membrane distal α1 domains of DR-α, DM-α and class I HC. Therefore, this HCMV glycoprotein has apparently acquired the ability to block both the MHC class I and class II antigen presentation pathways by recognizing shared protein structures in these molecules that are rather dissimilar at the level of amino acid identity.

The US2-mediated degradation of both class II DR-α and DM-α suggested that US2 could inhibit class II antigen presentation to $CD4^+$ T lymphocytes. To test this, parental U373 or U373-CIITAHis cells were incubated for 18 h with

Mtb-39 antigen, a Mycobacterium tuberculosis protein, and an Mtb-39-specific $CD4^+$ T cell clone that produces IFN-γ upon antigen stimulation (Dillon, D.C. *et al.,* submitted). Untransfected U373 cells express low levels of MHC class II, and thus, stimulated minimal amounts of IFN-γ production (Fig. 9). Production of IFN-γ by the T cell clone in response to antigen-pulsed U373-CIITAHis cells was much higher and amounted to ≈ 75% that observed with antigen-pulsed dendritic cells. Thus, U373-CIITHis cells are highly efficient APCs. When U373-CIITAHis cells were infected with AdtetUS2/Adtet-trans to induce US2 expression, antigen-specific IFN-γ production by the T cells was reduced to 20% of that observed with controls infected with Adtet-trans alone, and to 30% of that seen with uninfected cells (Fig. 9). Therefore, US2 can effectively inhibit MHC class II antigen presentation to $CD4^+$ T cells. To date, we have been unable deliver US2 uniformly into cultured macrophages using either adenovirus vectors or HCMV. It is important to note that U373 cells are of myeloid origin, related in lineage to macrophages, and U373-CIITAHis cells express levels of class II that frequently exceed that expressed by macrophages in culture. Moreover, since U373-CIITAHis cells present antigen as efficiently as do dendritic cells, our results presumably extend to other APCs including macrophages.

This report provides the first evidence of a viral inhibitor of the MHC class II pathway. However, based on previous experience with viral inhibitors of the class I pathway, viruses able to infect macrophages, lymphocytes, or endothelial

cells are likely to express inhibitors of the class II pathway. This property may be especially important as viral membrane proteins and particles can traffic through endosomal compartments, and presumably enter the MIIC. Therefore, we propose that US2 can function to inhibit class II-mediated presentation of endogenous viral antigens, providing protection from $CD4^+$ T cell-mediatecl cytolysis, anti-viral cytokines and upregulation of costimulatory signals for $CD8^+$ T cells[28]. The effects of US2 may be important for HCMV replication in any one of several cells including endothelial cells, glial cells or macrophages. For monocyte/macrophages, the capacity of HCMV to block the MHC class II pathway would provide an important mechanism for survival, allowing the virus to remain invisible to both $CD8^+$ and $CD4^+$ T cells, especially after reactivation. Expression of US2 outside the context of the HCMV genome, eg. using viral vectors in cells or tissues, may make it possible to reduce detrimental $CD4^+$ and $CD8^+$ T lymphocyte responses in autoimmunity, transplantation or gene therapy.

Materials and Methods

Cells and viruses. The human glioblastoma cell line, U373-MG (ATCC), was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v fetal calf serum, penicillin/streptomycin and glutamine (PS/G). The U373-CIITAHis cell line was generated through the stable cotransfection of the U373 cell line with plasmids containing the sequences of the CIITA gene[29]

(CIITA.pcDNA1.Amp) and the histidinol resistance gene (pSV2His). U373-CIITAHis cells were cultured in DMEM lacking histidine, 10 % FCS, PS/G and 0.5 mM histidinol. The CIITAHisUS2Neo cell lines were generated through the stable cotransfection of the U373-CIITAHis cell line with plasmids containing the sequences of the HCMV US2 gene (pCA4US2) and the neomycin resistance gene (pSV2Neo). Similarly, the CIITAHisUS3Neo cell lines were generated as above except a plasmid containing the sequences of the HCMV US3 gene (pCA4US3) was used. Both sets of cell lines were cultured in DMEM lacking histidine, 10% FCS, PS/G, 0.5 mM histidinol and 200 μg/ml G418. All DNA transfections were carried out using 1-10 ug of linearized plasmids coprecipitated with calcium-phosphate as described[30] and added to 30-40 % confluent monolayers of cells in 60 mm dishes for 5 hours. 48 hours after transfection, the cells were replated in selective medium and individual colonies were isolated 3-4 weeks thereafter.

The propagation and construction of wildtype HCMV strain AD169 and the various AD169 deletion viruses, RV7186 (ΔIRS1-US11), RV7177 (ΔUS1-US6), RV5122 (ΔUS1), RV47 (ΔUS2,US3), RV69 (ΔUS6), RV725 (ΔUS7), RV61 (ΔUS9), RV67 (ΔUS10,US11), and RV35 (ΔUS6-US11) have been described[18].

Construction of adenovirus vectors. The shuttle vector pADtet7 was constructed by replacing the HCMV promoter of pADlox[31] with the minimal HCMV promoter fused to the tetracycline operator[21]. HCMV US2 coding

sequences were then PCR amplified and inserted downstream of the tetracycline inducible promoter generating the plasmid pADtet7-US2. pADtet7-US2 was transfected into 293-cre cells and subsequently infected with an (E1-) Ad helper virus (ψ5) which has loxP sites flanking the DNA packaging signal[31]. A recombinant virus, AdtetUS2, was derived from cre-mediated recombination between the shuttle plasmid and the helper virus and was propagated as described[31]. A second virus, Adtet-trans, expresses a transactivator protein that activates the tet-inducible promoter[21].

Immunoprecipitation experiments. U373 or U373-CIITAHis cells (1-3x10^6) were radiolabelled with ^{35}S-methionine/cysteine (150 μCi/ml) for 30 min then the label chased for 90-180 min in media containing excess methionine and cysteine. In other experiments, cells (5 X 10^6) were first detached from plates with trypsin/EDTA, transferred to Eppendorf tubes and incubated with ^{35}S-methionine/cysteine (500 μCi/ml) in the presence or absence of lactacystin (25 μM, Kamiya Biomedical Company, Seattle, WA), ZL_3VS (35 μM, obtained from Dr. Matthew Bogyo, Harvard Medical School, Boston, MA) or tunicamycin (5 mg/ml, Boeringher Mannheim, Indianapolis, IN). Immunoprecipitations were performed as described[12]. To immunoprecipitate individual class II proteins, proteins were first denatured in 25 mM Tris, pH 7.5, 150 NaCl (Tris saline) containing 1% SDS (SDS lysis buffer) and a cocktail of protease inhibitors and incubated at 95°C for 10 min. The SDS was diluted 10-fold in Tris saline

containing 1% Triton X-100 before immunoprecipitation. Sequential immunoprecipitation experiments were performed using cell extracts obtained using digitonin lysis buffer (1% digitonin, 10 mM HEPES, pH 7.5, 10 mM $CaCl_2$) containing a cocktail of protease inhibitors. Proteins were immunoprecipitated using primary antibodies, then denatured in SDS lysis buffer and the SDS diluted with Tris saline containing 1% Triton X-100 before secondary immunoprecipitation. A complete list of antibodies used in the experiments is available on request.

Northern blots. Total RNA was harvested from cells using Trizol Reagent (Gibco-BRL, Gaithersberg, MD). RNA (10 μg per well) was separated on 1% agarose-formaldehyde gels and transferred overnight to Hybond N+ membranes (Amersham, Arlington Heights, IL). Plasmid probes: pAR.2529.DRα, pAR.2529.DRβ1, pIi33, CIITA.pcDNA1.AMP, pcDNA3.gBNPS and pBSHuβactin (a gift from Dr. Richard Press, Oregon Health Sciences University, Portland, OR), were linearized, labelled with ^{32}P-ATP using a random primer labelling kit (Gibco-BRL, Gaithersberg, MD) and used to probe blots.

Subcellular fractionation. Radiolabelled cells ($1x10^7$) were homogenized and fractionated as described[5] with the following exceptions. Subcellular pellets were resuspended and denatured in SDS lysis buffer. The 100,000 X g supernatant was denatured by adding 1% SDS. Samples were heated to 95°C for 10 min, the SDS was removed using SDS-Out (Pierce Chemical Company, Rockford, IL) and

1% Triton X-100 was added before immunoprecipitation.

CD4$^+$ T Cell Assays. U373-CIITAHis cells were plated in 96-well microtiter plates and left uninfected or infected with Adtet-trans alone (200 PFU/cell) or coinfected with AdtetUS2 (150 PFU/cell) and Adtet-trans (50 PFU/cell). After 18 hr, the cells were incubated with 1 μg/ml of Mtb-39 protein (Corixa Corporation, Seattle, WA) and an Mtb-39-specific CD4$^+$ T cell clone (Dillon, D.C. *et al.*, submitted). Expression of IFN-γ in the culture medium was measured after 18 hr by using a quantitative sandwich ELISA as described (Dillon, D.C. *et al.*, submitted).

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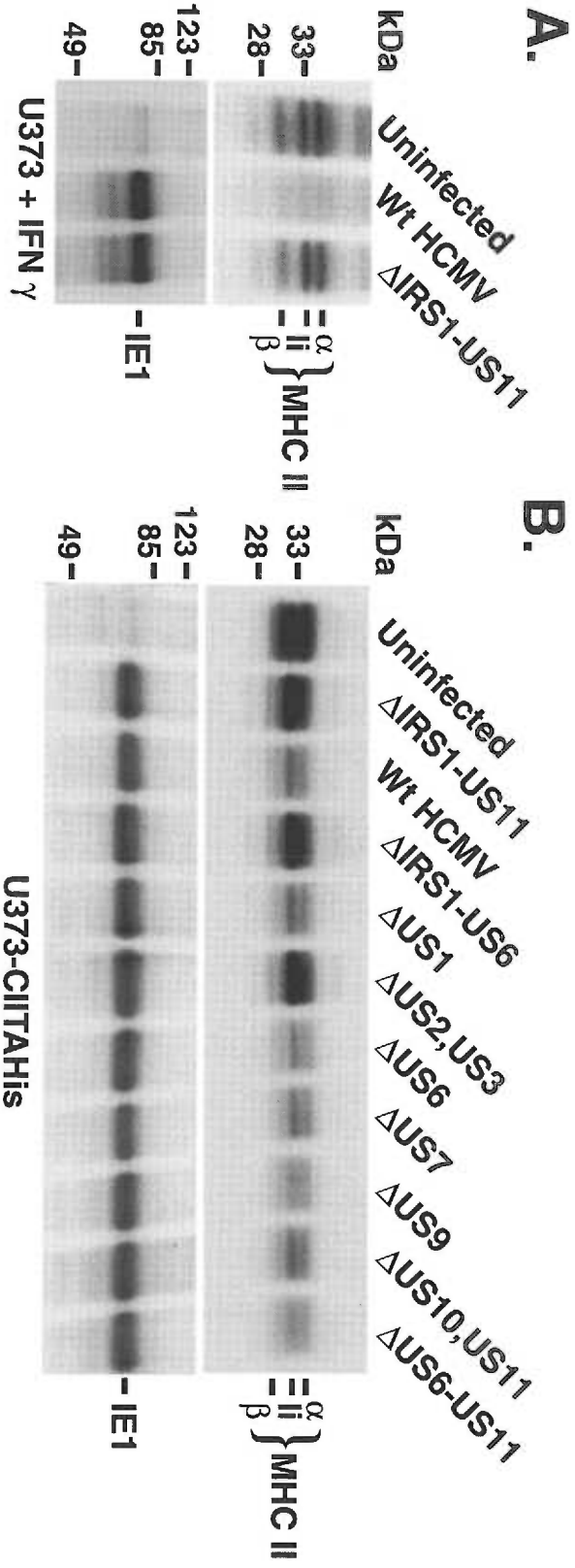
Figure 1: MHC class II expression is reduced in HCMV-infected cells. A. U373 cells were treated with IFN-γ for 72 hrs then left uninfected or infected with wild type HCMV (strain AD169) or a mutant HCMV lacking the IRS1-US11 genes. After 12 hrs of infection the cells were radiolabelled with ^{35}S-methionine/cysteine for 30 min and the label chased for 2 hrs. MHC class II complexes were immunoprecipitated from cell extracts using MAb DA6.147, specific for class II DR-α, or HCMV major immediate early protein (IE1) was precipitated using a rabbit polyclonal antiserum. Samples were separated using 14% SDS-polyacrylamide gels. **B.** U373 cells transfected with CIITA (U373-CIITAHis) were left uninfected or infected with wild type HCMV or HCMV mutants lacking the indicated genes for 36 hrs. Cells were then radiolabelled and class II or IE1 immunoprecipitated as described in A.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Wt HCMV
ΔIRS1-US6
ΔUS1
ΔUS2,US3
ΔUS6
ΔUS7
ΔUS9
ΔUS10,US11
ΔUS6-US11
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49-
α
Ii
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MHC II
IE1
U373-CIITAHis

Figure 2: Reduction in MHC class II expression in HCMV-infected cells is not due to transcriptional downregulation. U373-CIITAHis cells were left uninfected or infected with wild type HCMV or the HCMV mutant unable to express IRS1-US11 for 36 hrs. Total RNA was harvested from the cells and separated using electrophoresis with a 1% agarose gel containing 2.2 M formaldehyde. The RNA was then transferred to Hybond-N+ membranes and the membranes probed with linearized, ^{32}P-labelled plasmids containing the DR-α, DR-β, Ii, CIITA, HCMV glycoprotein B (gB), or β-actin genes.

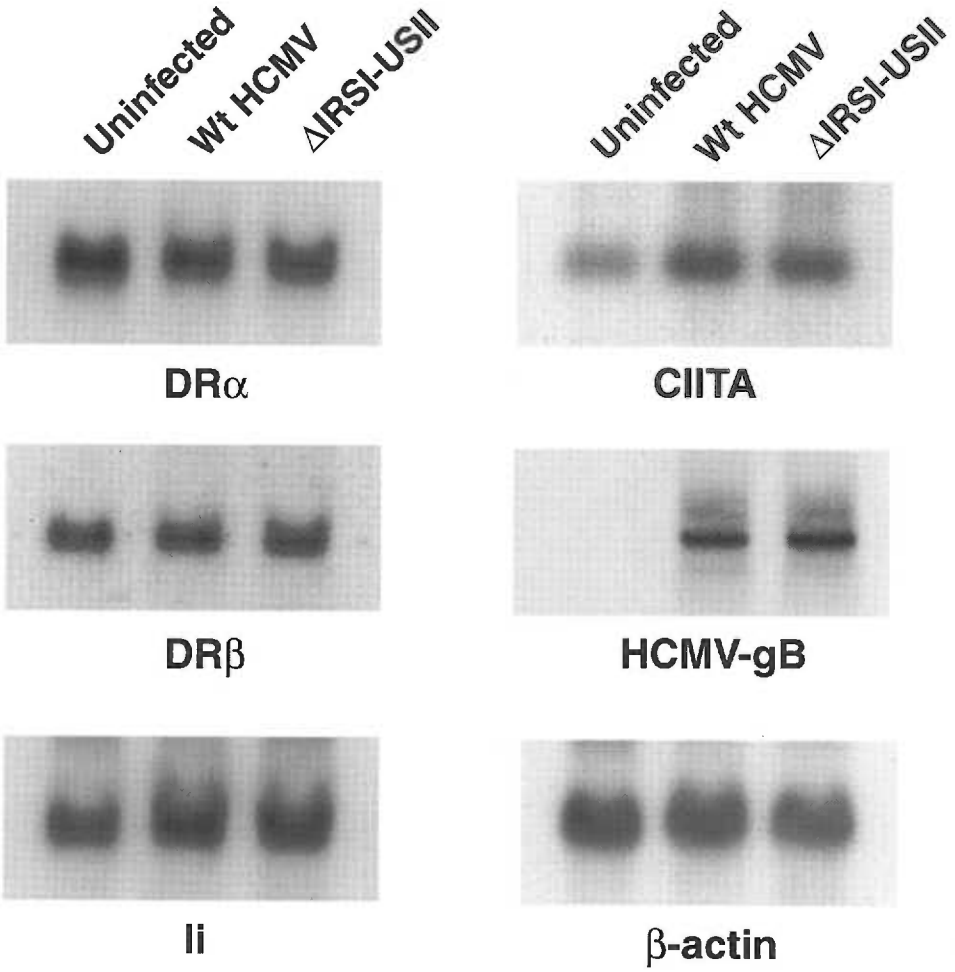

Uninfected
Wt HCMV
ΔIRSI-USII
Uninfected
Wt HCMV
ΔIRSI-USII
DRα
CIITA
DRβ
HCMV-gB
Ii
β-actin

Figure 3: Cells expressing US2 , and not US3, show reduced MHC class II expression. **A.** Screening of CIITAHisUS2Neo transfectants for expression of US2. Cells were radiolabelled with ^{35}S-methionine/cysteine for 1 hr, then lysed in NP40/DOC buffer and immunoprecipitated for US2 using a rabbit anti-peptide polyclonal serum. The asterisk denotes the cell line used to assess MHC class II expression below. **B.** Screening of CIITAHisUS3Neo transfectants for expression of US3. Cells were radiolabelled and immunoprecipitated as above, except a rabbit anti-peptide polyclonal serum directed against US3 was used. The asterisk denotes the cell line used to assess MHC class II expression below. **C.** MHC class II expression was measured in the CIITAHisUS2Neo and CIITAHisUS3Neo cell lines by immunoprecipitation. The cells were radiolabelled with ^{35}S-methionine/cysteine for 30 min, the label was then chased for 30 or 90 min in media containing excess methionine/cysteine followed by immunoprecipitation for MHC class II proteins with a monoclonal antibody directed to the invariant chain, PIN.1.

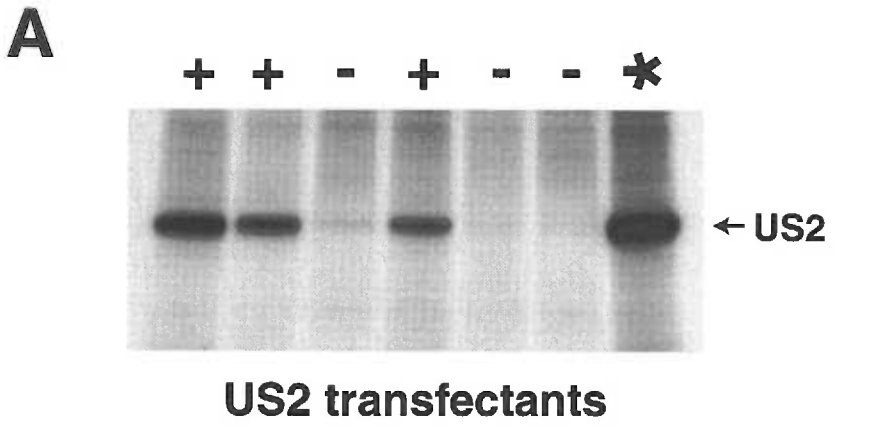
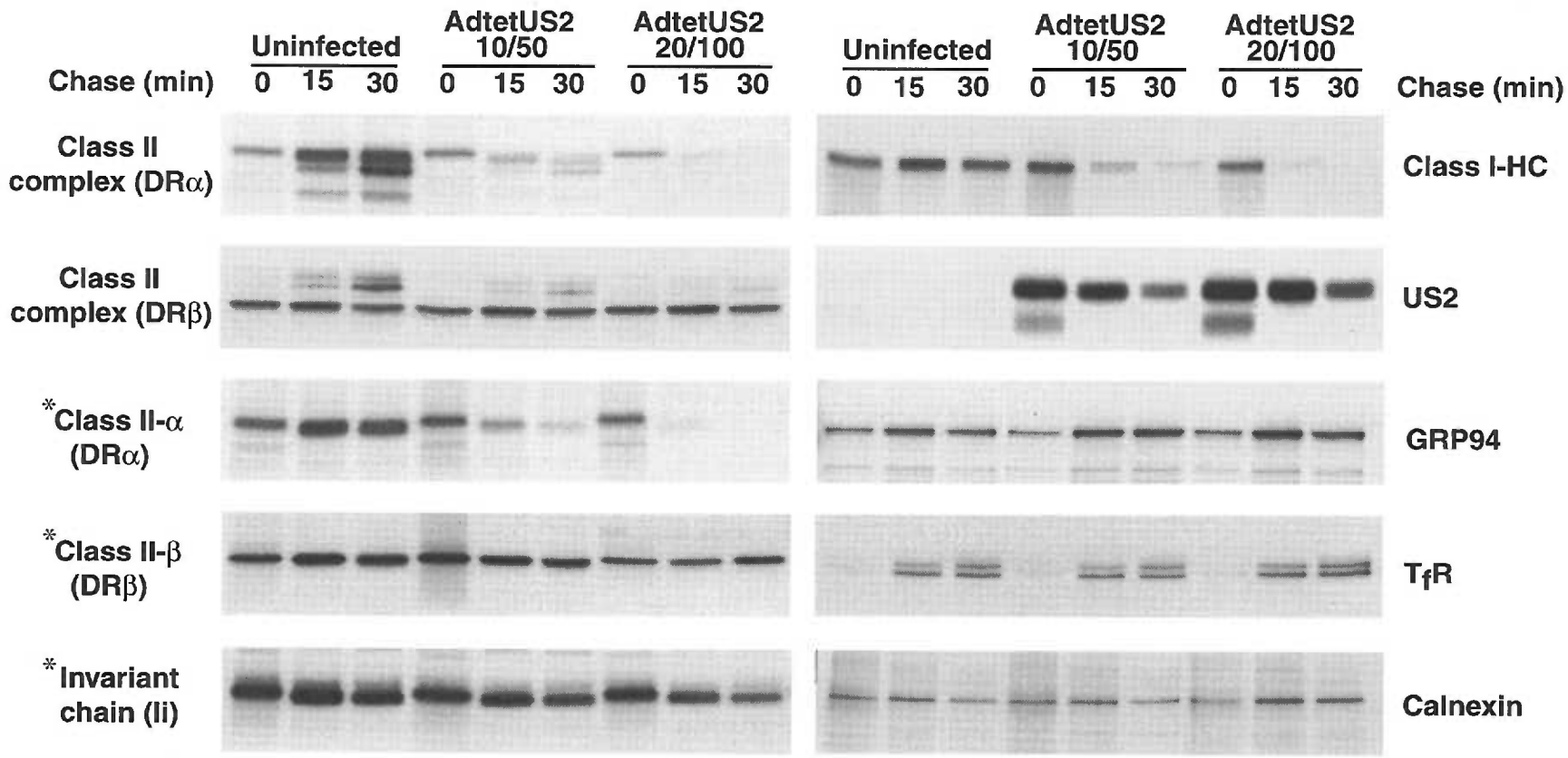

A
+ + - + - - *
← US2
US2 transfectants
B
+ - - - * - + -
← US3
US3 transfectants
C
Control
US2+
US3+
P 30 90 P 30 90 P 30 90 min chase
← MHC II

Figure 4: MHC class II-α is unstable in US2-expressing cells. U373-CIITAHis cells were left uninfected or infected with AdtetUS2 and Adtet-trans using two quantities of virus: 10 PFU/cell of Adtet-trans and 50 PFU/cell of AdtetUS2, or 20 PFU/cell of Adtet-trans and 100 PFU/cell of AdtetUS2 for 12 hrs. The cells were radiolabelled with ^{35}S-methionine/cysteine for 1 min then the label chased with excess methionine/cysteine for 0, 15 or 30 min before cell extracts were prepared. Immunoprecipitations were then performed under non-denaturing conditions using antibodies specific for class II-α (monoclonal antibody DA6.147), class II-β (monoclonal antibody HB10A), US2 (rabbit antibody against US2N), class I heavy chain (rabbit antibody against HC), transferrin receptor (TfR) (monoclonal antibody B3/25), calnexin (rabbit antibody against Cxn/c), and GRP-94 (monoclonal antibody 9G10). Alternatively, samples were denatured using SDS, then the SDS diluted and extracts immunoprecipitated with antibodies specific for class II-α (monoclonal antibody DA6.147), class II-β (monoclonal antibody HB10A) and invariant chain (Ii) (monoclonal antibody PIN.1).



*Samples were denatured in 1 % SDS and boiled for 10 minutes.

Figure 5: US2 induces degradation of MHC class II-α chains by the proteasome. U373-CIITAHis cells were left uninfected or infected with Adtet-trans and AdtetUS2 using 20 and 100 PFU/cell, respectively. After 11 hrs of expression, proteasome inhibitors (25 μM lactacystin or 35 μM ZL_3VS) were added for 1 hr, then the cells were radiolabelled with ^{35}S-methionine/cysteine for 1 min and the radiolabel chased for 0, 20 or 40 min. Class II-α and class I HC were immunoprecipitated after the samples were denatured as described in Figure 4. As a reference for the removal of immature N-linked carbohydrates, additional immunoprecipitates of class II-α and class I HC from uninfected cells were treated with endoglycosidase H (Endo H). + CHO and - CHO indicate glycosylated and nonglycosylated proteins, respectively.

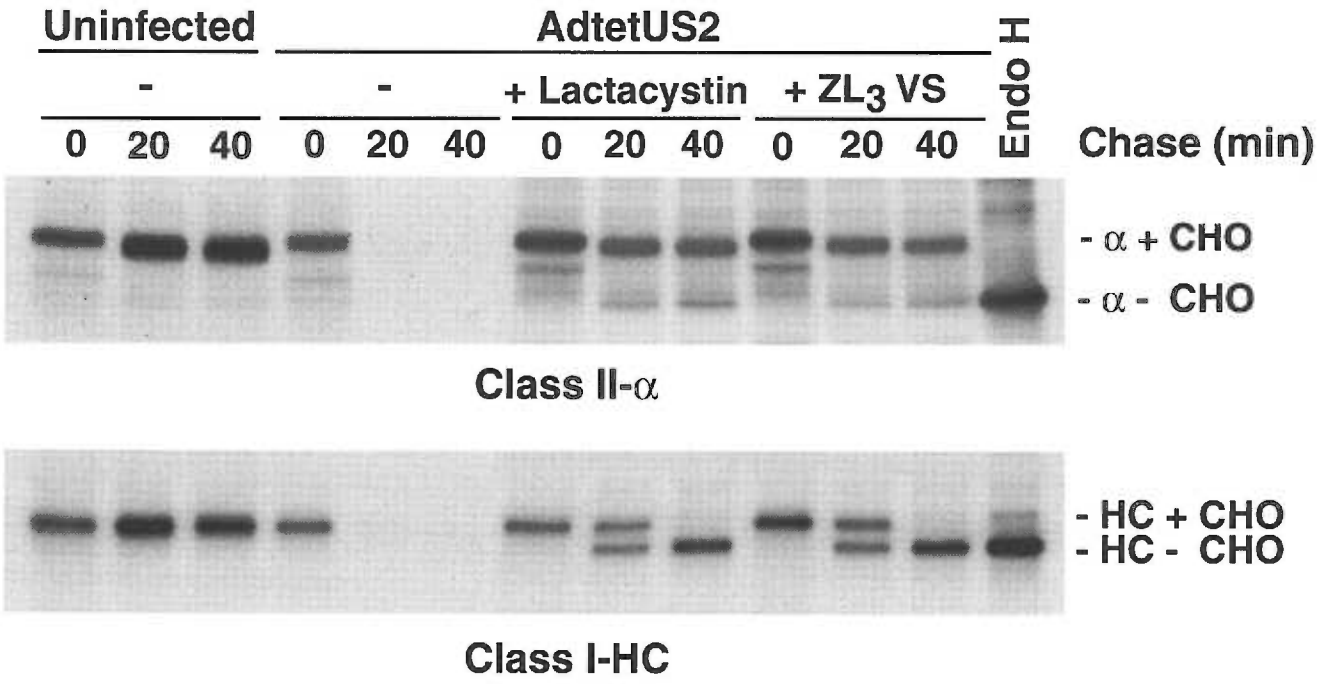
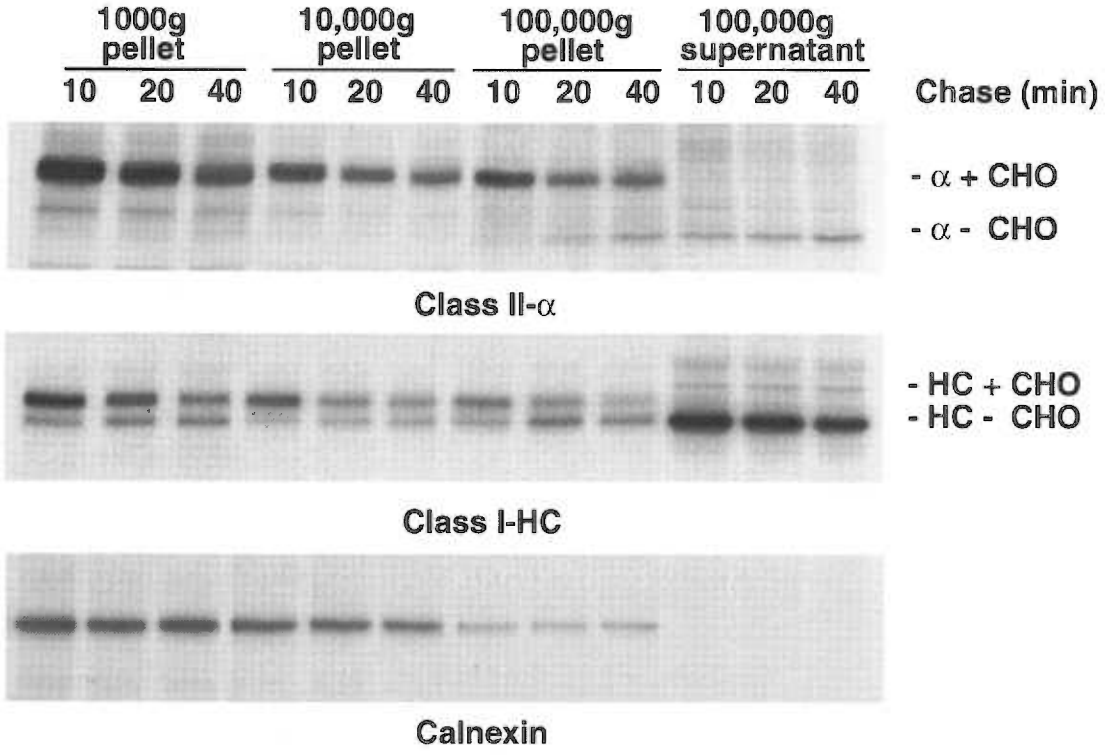

Uninfected
AdtetUS2
-
-
+ Lactacystin
+ ZL3 VS
Endo H
0 20 40 0 20 40 0 20 40 0 20 40
Chase (min)
- α + CHO
- α - CHO
Class II-α
- HC + CHO
- HC - CHO
Class I-HC

Figure 6: The class II-α chain is not dislocated to the cytosol in US2-expressing cells in which the proteasome is blocked. A. U373-CIITAHis cells were left uninfected or infected with Adtet-trans and AdtetUS2 using 20 and 100 PFU/cell, respectively. Cells were treated with ZL_3VS (35 μM) and radiolabelled as described in Figure 5. The cells were then subjected to crude subcellular fractionation such that 1,000 X g, 10,000 X g and 100,000 X g pellets and 100,000 X g supernatants were prepared. Membrane pellets or supernatant fractions were denatured using SDS before MHC class II-α, MHC class I HC or calnexin were immunoprecipitated. **B.** U373-CIITAHis cells were infected as above and treated 1 hr prior to radiolabelling with tunicamycin (5 μg/ml) , an inhibitor of N-linked glycosylation, and ZL_3VS (35 μM). Cells were then radiolabelled, fractionated and immunoprecipitated as described in A.

A.


1000g pellet
10,000g pellet
100,000g pellet
100,000g supernatant
10 20 40 10 20 40 10 20 40 10 20 40
Chase (min)
- α + CHO
- α - CHO
Class II-α
- HC + CHO
- HC - CHO
Class I-HC
Calnexin

B.

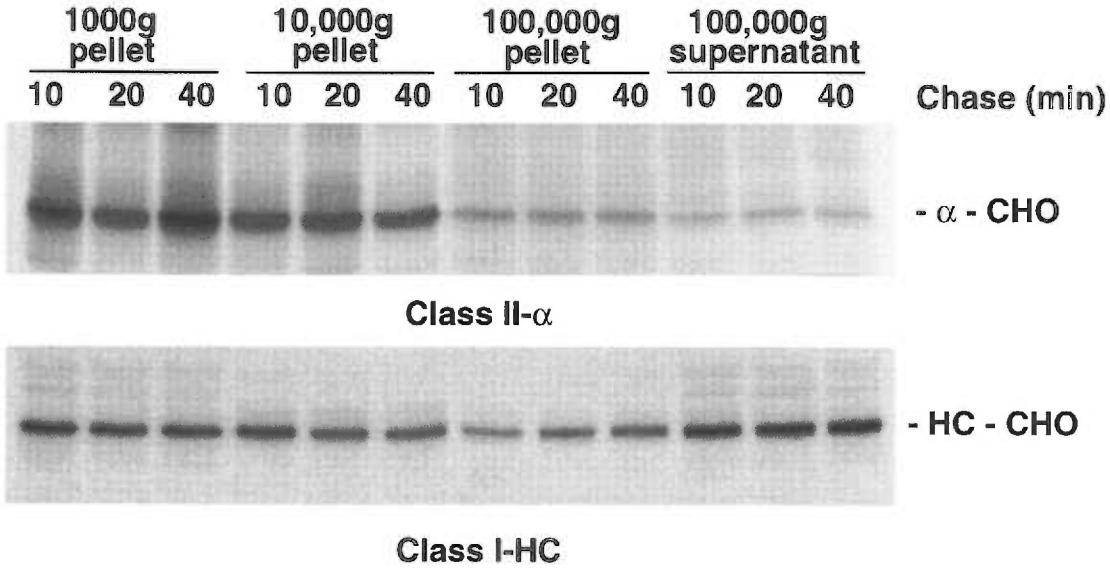
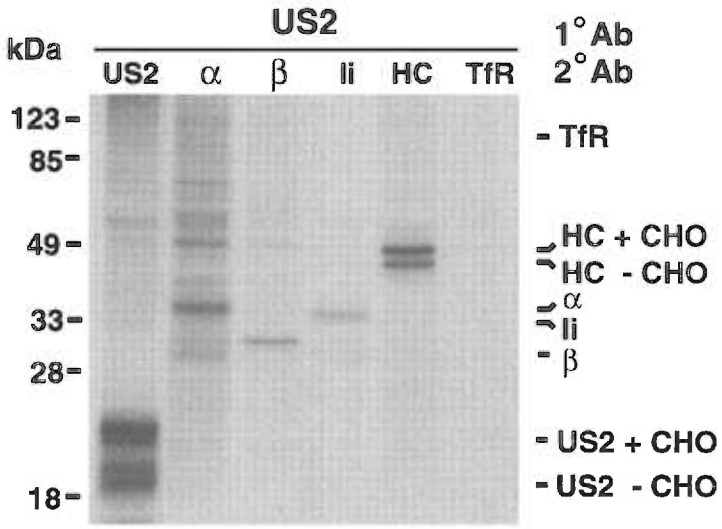

1000g pellet
10,000g pellet
100,000g pellet
100,000g supernatant
10 20 40 10 20 40 10 20 40 10 20 40
Chase (min)
- α - CHO
Class II-α
- HC - CHO
Class I-HC

Figure 7: US2 binds to MHC class II proteins. AdtetUS2 infected U373-CIITAHis cells were treated with ZL_3VS (35 μM), labelled with ^{35}S-methionine/cysteine for 5 min, then the label chased for 20 min. Sequential immunoprecipitations were then performed as follows: **A.** US2 was immunoprecipitated from cell extracts prepared with the mild detergent digitonin using a polyclonal serum. The precipitated proteins were then denatured with SDS, the SDS diluted and samples re-immunoprecipitated using anti-US2, anti-class II-α, anti-class II-β, anti-Ii, anti-class I HC or anti-TfR antibodies. **B.** Digitonin cell extracts were immunoprecipitated using anti-class II-α, anti-class II-β, anti-Ii, anti-class I HC or anti-TfR antibodies, the samples denatured and then re-immunoprecipitated using anti-US2 antibodies or antibodies directed to α, β, Ii, HC or TfR.

A.


kDa
US2
1° Ab
2° Ab
US2
α
β
Ii
HC
TfR
123
85
49
33
28
18
- TfR
HC + CHO
HC - CHO
α
Ii
- β
- US2 + CHO
- US2 - CHO

B.

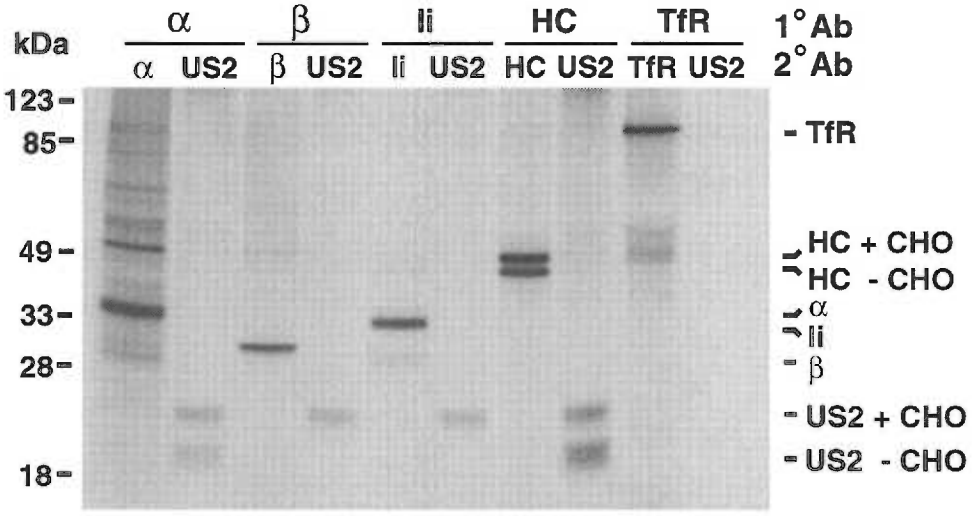
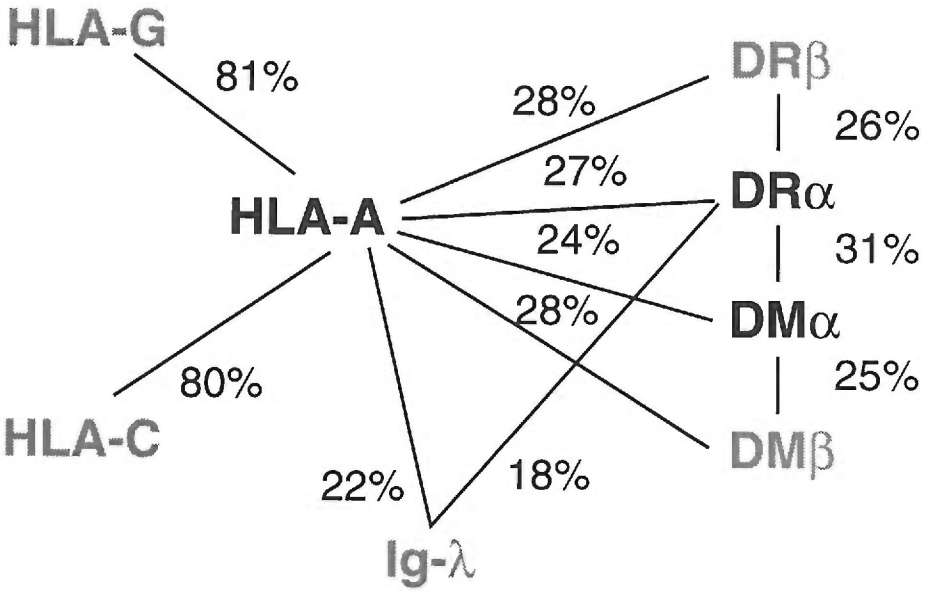

kDa
α
β
Ii
HC
TfR
1° Ab
2° Ab
α US2
β US2
Ii US2
HC US2
TfR US2
123
85
49
33
28
18
- TfR
HC + CHO
HC - CHO
α
Ii
- β
- US2 + CHO
- US2 - CHO

Figure 8: US2 induces degradation of HLA-DM. **A.** Homology tree showing the amino acid identity (%)[32] between classical MHC class I proteins HLA-A2 and HLA-C, non-classical class I HLA-G, MHC class II proteins DR-α, DR-β, DM-α, and DM-β and immunoglobulin light chain lambda (Igλ). **B.** U373-CIITAHis cells were left uninfected or infected with Adtet-trans and AdtetUS2 using two quantities of virus: 10 and 50 PFU/cell, or 20 and 100 PFU/cell, respectively, for 12 hrs. The cells were radiolabelled with ^{35}S-methionine/cysteine for 1 min then the label chased with excess methionine/cysteine for 0, 15 or 30 min. Cell extracts were denatured in SDS and class II DM-α and DM-β were immunoprecipitated using the monoclonal antibodies 5C1 and DMB-C, respectively.

A.


HLA-G
81%
HLA-A
HLA-C
80%
Ig-λ
22%
18%
28%
27%
24%
28%
DRβ
26%
DRα
31%
DMα
25%
DMβ

B.

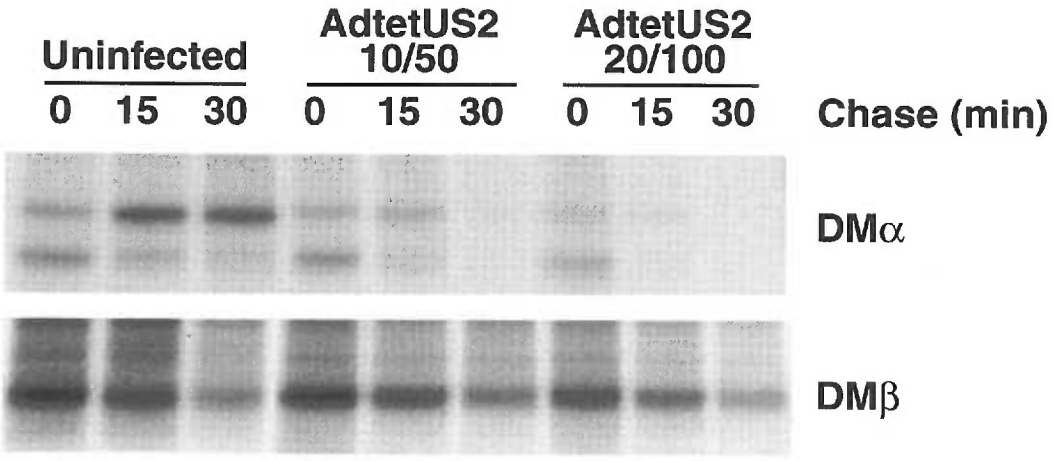
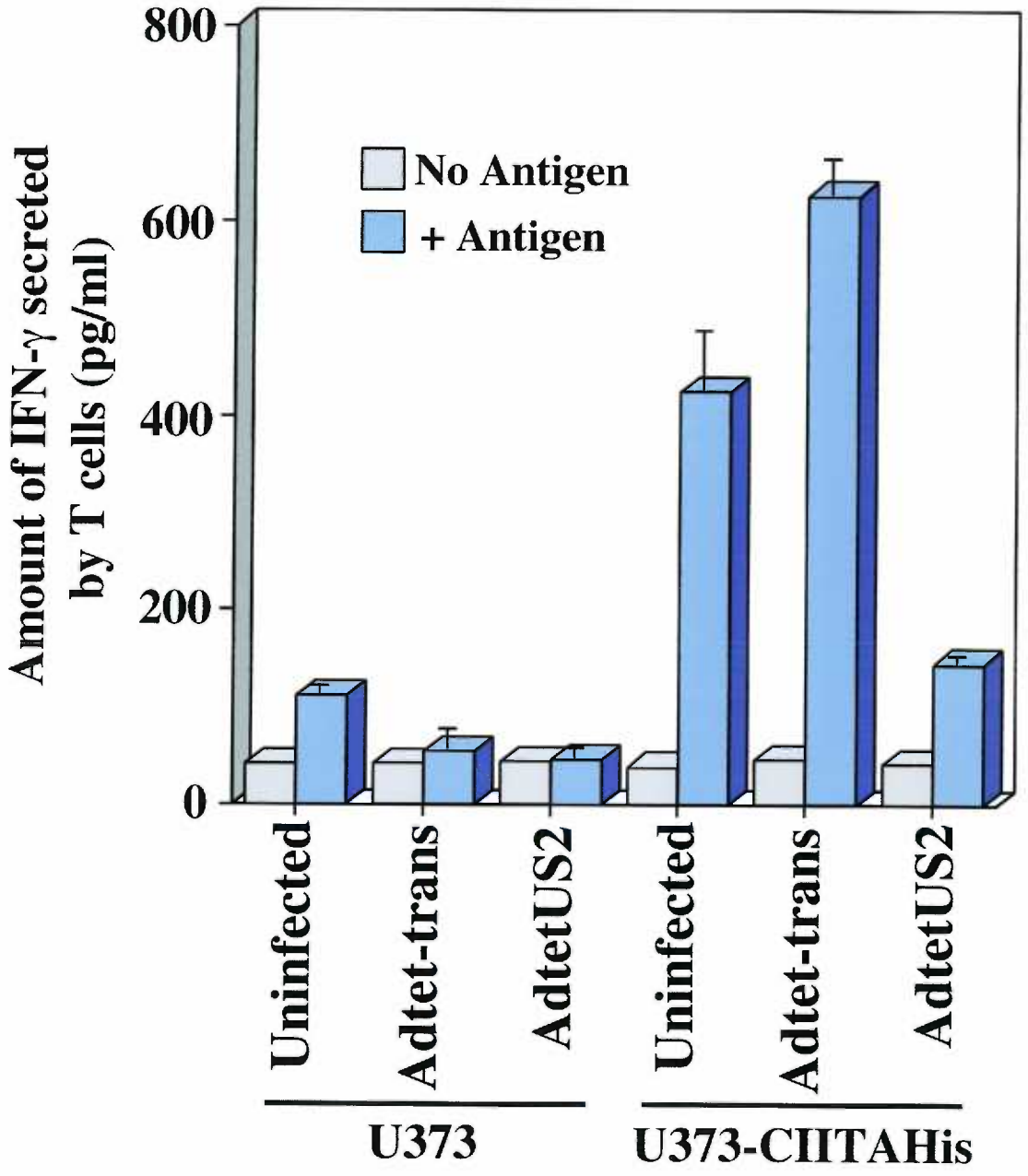

Uninfected
AdtetUS2 10/50
AdtetUS2 20/100
0 15 30 0 15 30 0 15 30
Chase (min)
DMα
DMβ

Figure 9: US2 inhibits presentation of exogenous antigen to $CD4^+$ T cells. U373 cells or U373-CIITAHis cells were left uninfected or infected with Adtet-trans alone or with AdtetUS2 and Adtet-trans. After 18 hrs, the cells were incubated with 1μg/ml of Mtb-39 protein and an Mtb-39-specific $CD4^+$ T cell clone. Secretion of IFN-γ into the culture supernatant was measured 18 hrs later by ELISA.


800
600
400
200
0
Amount of IFN-γ secreted by T cells (pg/ml)
No Antigen
+ Antigen
Uninfected
Adtet-trans
AdtetUS2
Uninfected
Adtet-trans
AdtetUS2
U373
U373-CIITAHis

CHAPTER 6

SUMMARY AND DISCUSSION

6.1 Inhibition of MHC Class I Antigen Presentation by HSV ICP47

HSV is a ubiquitous herpesvirus that establishes lifelong latent infections within its hosts. Periodically, in response to stress, UV light or other unknown factors, HSV reactivates from the latent state and produces infectious progeny for transmission to new hosts. Reactivation represents a rather intriguing feature of HSV infection, as replication of virus takes place in the face of a fully primed immune response. The ability of HSV to reactivate and replicate under these hostile conditions may be due in large part to the numerous immune evasion strategies used by the virus. In particular, HSV encodes an immediate early protein, ICP47, that enables the virus to avoid detection by $CD8^+$ T lymphocytes.

Previous investigations had demonstrated that individuals infected with HSV display a disproportionately high ratio of $CD4^+$ to $CD8^+$ CTL (reviewed in Schmid and Mawle, 1991; Yasukawa and Zarling, 1984a; Yasukawa and Zarling, 1984b). This was quite surprising since $CD4^+$ T lymphocytes typically give rise to helper cells and not necessarily CTL. In addition, several in vitro studies demonstrated that human fibroblasts infected with HSV were resistant to lysis by antiviral $CD8^+$ CTL (Koelle et al., 1993; Posavad and Rosenthal, 1992; Posavad et al., 1993). Collectively, these observations suggested that HSV might express a protein capable of blocking $CD8^+$ CTL recognition. Ian York, a former graduate student in our laboratory, set out to identify this protein using a combined biochemical and genetic approach. The inability of $CD8^+$ CTL to lyse HSV infected targets was shown to involve the retention of MHC class I molecules in the ER in an unstable, peptide-empty form (York et al., 1994). Using various HSV deletion mutants, the protein responsible for this effect was identified as the

immediate early gene product, ICP47. Further studies performed by Ann Hill and Pieter Jugovic, also a former graduate student, demonstrated that ICP47 bound to the transporter associated with antigen presentation (TAP) and was capable of blocking the translocation of peptides into the ER (Hill et al., 1995). At this time, I began to investigate the mechanism by which ICP47 inhibited TAP function.

Previously, Ian had demonstrated that ICP47 itself, expressed in the absence of other viral proteins, was capable of blocking the transport of peptides into the ER by TAP (York et al., 1994). This result, however, did not preclude the possibility that other cellular factors, in addition to TAP, were required to mediate this effect. Indeed, several observations from our laboratory indicated that ICP47 could interact with proteins other than TAP. In co-immunoprecipitation experiments, ICP47 was found not only to associate with TAP, but also with several other unidentified proteins, quite possibly heat shock proteins (HSPs), that may play a role in ICP47 function. In addition, a GST-ICP47 fusion protein produced in bacteria was found to precipitate a small cytosolic protein called calcyclin from human cell extracts. Calcyclin is a member of the S-100 family of calcium binding proteins, yet its function within the cell remains unknown. To characterize the mechanism of ICP47 inhibition of TAP, and to determine whether additional factors were required to mediate this effect, I developed an in vitro system to examine the interaction of ICP47 with TAP. Microsomes containing human TAP were purified from insect cells co-infected with baculovirus vectors expressing the TAP1 and TAP2 subunits. Since insects do not possess a mammalian-compatible immune system, components involved in class I antigen processing, and thus, potential cofactors for ICP47, should not be present in the

insect microsomes. HSV-1 ICP47 was expressed as a GST fusion protein in bacteria and purified using a glutathione-Sepharose column. Full length ICP47 was then released from the column by cleavage of a thrombin sensitive site engineered between the ICP47 and GST sequences. In binding studies, this recombinant ICP47 bound specifically to microsomes containing human TAP and not those purified from insect cells expressing a control protein, HSV glycoprotein H (gH) (see Figure 10, Chapter 2). More importantly, similar to the effects observed in translocation assays with permeabilized mammalian cells, ICP47 was found to effectively block the transport of peptides by TAP in the insect microsomes. Thus, in the absence of other mammalian proteins, ICP47 retained its ability to block TAP activity. This result indicated that the inhibition of TAP by ICP47 did not require additional mammalian cofactors, but was most likely mediated through direct interactions between TAP and ICP47. Of course, it remains possible that the insect membranes contained cellular components in addition to TAP which are involved in ICP47 function.

Using this in vitro system, I went on to characterize the molecular mechanism by which ICP47 inhibited TAP. Initially, I conducted binding assays with peptides and ATP to determine whether ICP47 was capable of blocking their association with TAP. In the presence of ICP47, binding of peptides to TAP was dramatically reduced. The IC_{50} of ICP47's effects on peptide binding was virtually identical to that observed in the translocation assays, suggesting that the mechanism of ICP47 inhibition of TAP was most likely mediated through the blockage of peptide binding. By contrast, ICP47 had no effect on the binding of ATP to TAP, even at concentrations 10-fold greater than those required for

effective inhibition of peptide transport. Next, I measured the affinity of ICP47 for TAP and found that the K_d of this interaction was approximately 5.2 x 10^{-8} M. By comparison, the vast majority of peptides that interact with TAP in vivo have affinities approximately 100-1000 fold lower than this value. Thus, ICP47 binds to TAP with a much higher affinity than do peptides. Competition experiments using a complex library of over 2000 peptides revealed that in order to abolish the binding of ICP47 to TAP, a 1000-fold molar excess of peptide was required. Peptides, at high relative concentrations, were also found to act as competitive inhibitors of ICP47 binding to TAP. Competitive inhibition implies that the binding of ICP47 and peptides to TAP are mutually exclusive, and therefore, their binding sites must overlap to some extent. Based on this observation, we concluded that ICP47 binds to a site which includes or overlaps the peptide-binding domain of TAP. In experiments measuring the off-rate of ICP47 or peptides from TAP, ICP47 dissociated much more slowly than did peptides, indicating that ICP47 binds to TAP in a stable fashion. Finally, in proteolysis experiments with TAP microsomes, ICP47 remained accessible to proteases added from the cytosolic surface and was not transported into the ER. Together, these results demonstrate that ICP47 binds to a site which includes the peptide binding domain of TAP, remaining bound to this site in a stable fashion, such that the transport of peptides into the ER is blocked.

In addition to the biochemical data, a rather surprising observation was made concerning the functional stability of ICP47. Remarkably, even after heat denaturation at 95°C for 10 minutes, ICP47 was found to retain most, if not all, of its inhibitory activity. In terms of ICP47 secondary structure, two possible

explanations may account for this result. First, if folding is necessary for ICP47 function, then the protein itself must have the capacity to refold into its native conformation following heat denaturation. Alternatively, it remains possible that ICP47 may not achieve its final active conformation until binding to TAP. This latter hypothesis seems to be supported by circular dichroism (CD) measurements made by Aled Edwards in collaboration with our laboratory. In these experiments, purified recombinant ICP47 was found to be largely unstructured in solution.

To date, my research describing the mechanism of ICP47 action remains the only detailed biochemical characterization of a viral inhibitor of the MHC class I presentation pathway. Ahn and colleagues (1996) attempted similar studies with ICP47 but were unable to measure the direct binding of ICP47 to TAP. In their studies, a recombinant form of ICP47 was used in which the protein had been tagged with 6 histidine residues to aid in protein purification. The incorporation of this highly charged tag prevented this group from conducting binding assays with TAP as the tagged ICP47 bound non-specifically to the negatively charged phospholipids in the microsomes. Thus, much of the biochemical data describing the interaction of ICP47 with TAP in this study was derived entirely from competition assays with various peptides. This indirect means of assessing ICP47 affinity for TAP, however, is inherently flawed, as competition with different peptides may yield conflicting results. As the only detailed characterization of ICP47 function, my research has also contributed to the understanding of HSV immune evasion at the molecular level. Since ICP47 binds to TAP with a much higher affinity than do peptides, and its association with TAP remains quite stable, it seems rather unlikely that peptides at

intracellular concentrations would be capable of disrupting this interaction. This suggests that even under circumstances of low ICP47 expression, this inhibitor may have a profound effect on the presentation of viral antigens. In particular, this may be especially important during reactivation, at the onset of viral gene expression, when levels of ICP47 may be exceedingly low.

As a continuation of my studies, I went on to investigate the binding of ICP47 from both HSV serotypes 1 (ICP47-1) and 2 (ICP47-2) to human and murine TAP. Previous reports examining the effects of ICP47-1 indicated that this protein was unable to inhibit TAP in murine cells (Fruh et al., 1995; York et al., 1994). This was unfortunate as it meant that mice could not be used as an animal model to study the effects of ICP47 in vivo. ICP47-2, however, shares only 42% amino acid identity with ICP47-1, and thus, it was of interest to determine whether ICP47-2 could inhibit murine TAP. Initial studies indicated that both ICP47-1 and ICP47-2 inhibited human TAP at similar concentrations. Using Scatchard analysis, I calculated the affinity of ICP47-2 for human TAP to be 4.8 x 10^{-8} M, almost identical to that measured for ICP47-1 (5.2 x 10^{-8} M). In studies with murine TAP, however, both proteins were found to be significantly less effective in blocking peptide translocation. Some limited inhibition of murine TAP by both ICP47-1 and ICP47-2 was observed, but only at concentrations 50-100 times greater than those required to block human TAP. Moreover, both ICP47-1 and ICP47-2 were found to bind to murine TAP very poorly. Therefore, the failure of ICP47-1 and ICP47-2 to inhibit TAP in murine fibroblasts could be explained by the inability of these proteins to bind to the transporter. The results of this study, nonetheless, were rather surprising, considering that human and murine

TAP share extensive sequence homology, approximately 75% identity at the amino acid level (see Figures 1 and 2). However, a major difference between the two transporters is their preference for peptide binding and translocation. Human TAP favors peptides with hydrophobic or positively charged residues at the C-terminus, while murine TAP favors only those with hydrophobic C-termini (see Table 1). Examination of the amino acid sequences of ICP47-1 and ICP47-2 reveals that both proteins are highly charged, especially in the conserved regions at the amino terminus of each protein. Therefore, these charged residues may play an essential role in binding of ICP47 to human TAP, but may actually interfere with the association with murine TAP. HSV is a human virus and as such, has faced no evolutionary pressure to block murine TAP.

As an extension of these studies, Pieter Jugovic examined the ability of ICP47-1 and ICP47-2 to block TAP in cells from a variety of animal species (Jugovic et al., 1998). Pieter conducted translocation assays in cells permeabilized with streptolysin-O and incubated with various concentrations of recombinant ICP47. From these studies it was found that ICP47-1 and ICP47-2 displayed virtually identical species specificity for TAP; both proteins were able to block TAP in human, monkey, cow, pig or dog cells, but not in cells from rabbits, guinea pigs, mice or rats. A limitation of this study, however, was the inability to introduce high concentrations of ICP47-1 or ICP47-2 into the permeabilized cells. Thus, at these relatively low ICP47 concentrations, no inhibition of TAP whatsoever was observed in rabbit, guinea pig or mouse cells. However, at the highest concentrations of ICP47-1 and ICP47-2 used in the study, there was a reproducible 20% inhibition of peptide transport in the rat cells.

Figure 1: Amino acid sequence comparison of human and murine TAP1 subunits. The protein sequences of human TAP1 (top line) and murine TAP1 (bottom line) were aligned using the program GAP (global alignment of proteins) of the GCG Wisconsin sequence analysis package. Only mismatched residues are indicated for the murine TAP1 sequence. Potential transmembrane domains are boxed and the Walker A and B consensus sites for ATP binding are underlined.

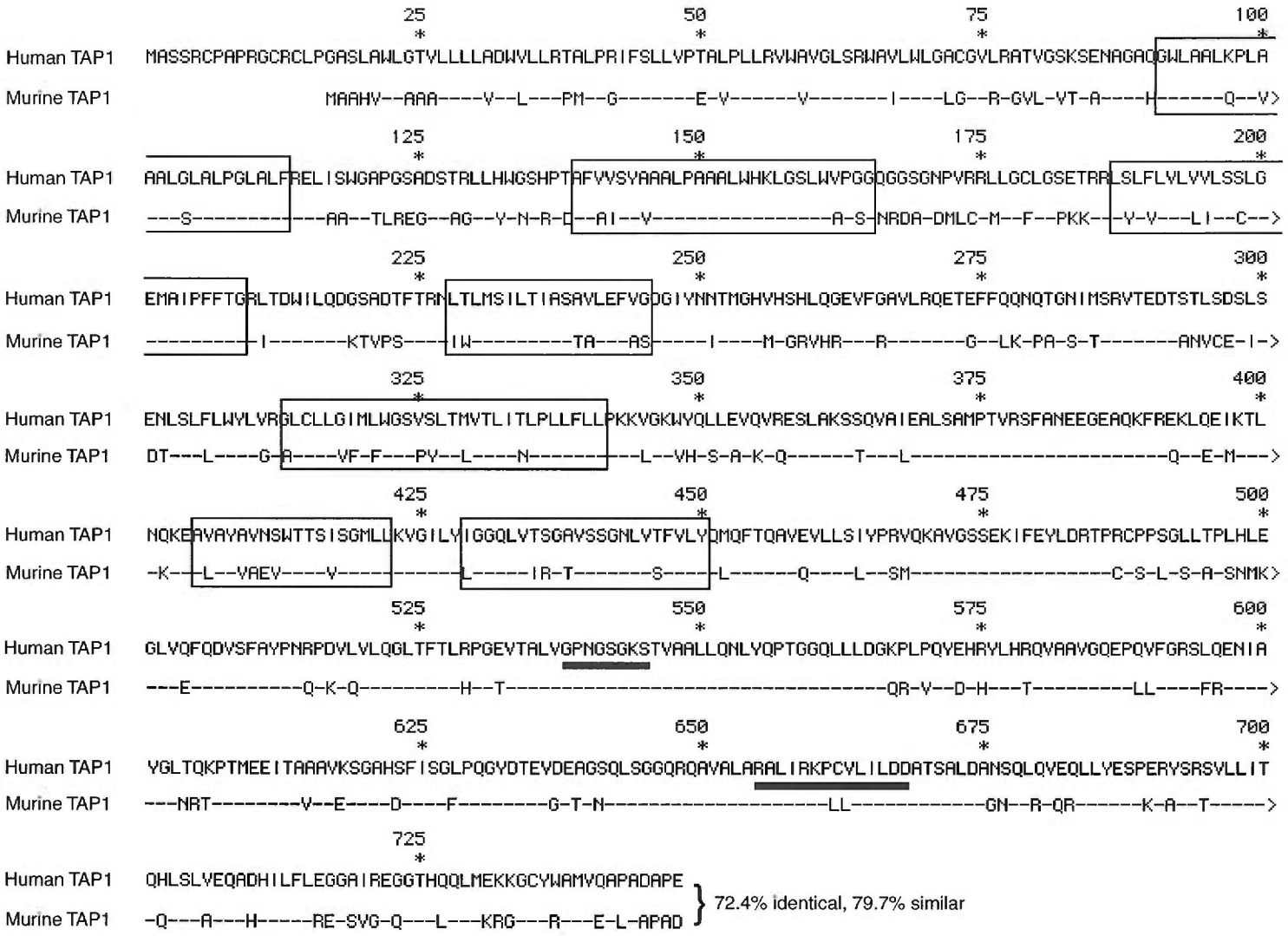

Human TAP1
Murine TAP1
72.4% identical, 79.7% similar

Figure 2: Amino acid sequence comparison of human and murine TAP2 subunits. The protein sequences of human TAP2 (top line) and murine TAP2 (bottom line) were aligned using the program GAP (global alignment of proteins) of the GCG Wisconsin sequence analysis package. Only mismatched residues are indicated for the murine TAP2 sequence. Potential transmembrane domains are boxed and the Walker A and B consensus sites for ATP binding are underlined.

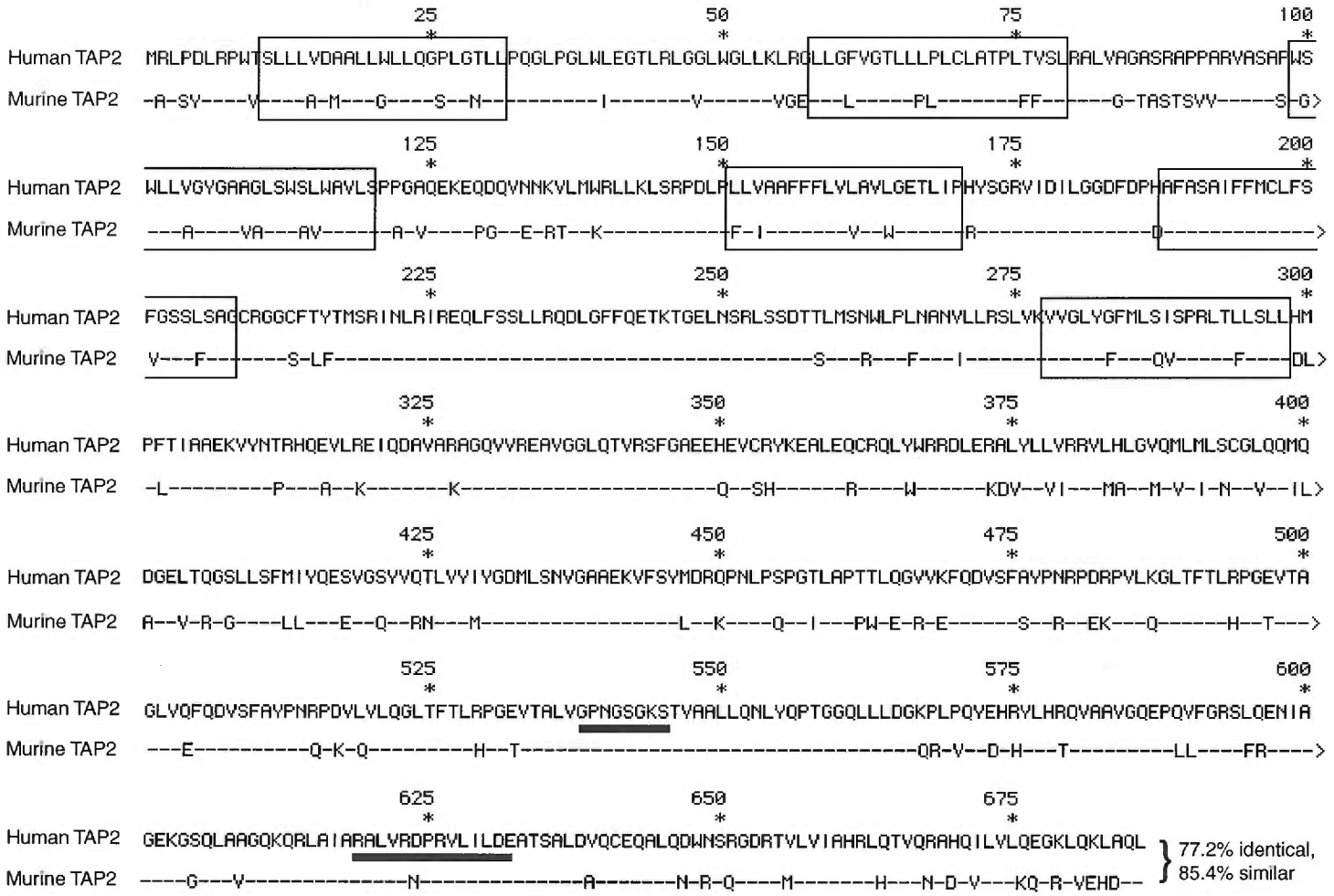

Human TAP2
Murine TAP2
77.2% identical,
85.4% similar

Table 1: Comparison of the molecular characteristics of the human and murine TAP subunits, as predicted from their primary amino acid sequence.

Molecular Characteristic	Human TAP		Murine TAP	
	TAP1	TAP2	TAP1	TAP2
Polypeptide length (# amino acids)	748	686	724	702
Molecular weight (Da)	80964	75663	79005	77444
Isoelectric point (pH)	7.04	8.73	9.21	6.94
Ionic charge at pH 7.0	+0.18	+4.09	+9.36	-0.09
# of cysteine residues	9	10	6	8
Potential trans-membrane domains	7	6	7	6
Potential glycosylation sites (NXT/S)	4	1	3	2
Peptide preference (COOH-terminus)	Hydrophobic or basic residues		Hydrophobic residues	

Since polymorphism of rat TAP has been shown to influence peptide specificity (Heemels et al., 1993: Momburg et al., 1994), one might speculate that this inhibition may be due to the incorporation of an allele that is sensitive to ICP47. Rats are known express four alleles of the TAP2 subunit, and based on sequence homology, these alleles can be classified into one of two groups, cim^a or cim^b. With cim^a alleles, rat TAP shows a preference for peptides with hydrophilic residues at the C-terminus, similar to that of human TAP. In the presence of cim^b alleles, however, the peptide preference of rat TAP shifts to peptides with hydrophobic C-termini, more closely resembling that of murine TAP. Thus, with the incorporation of cim^a alleles, as opposed to cim^b, ICP47-1 and ICP47-2 may bind to rat TAP with a higher affinity, thereby blocking peptide translocation. Of course, in a heterozygous rat, only partial inhibition of transport activity would be observed, as TAP complexes with cim^b alleles would remain resistant to ICP47 function.

Although a great deal of progress has been made in characterizing the mechanism of ICP47 inhibition of TAP, a number of unresolved issues remain. Shortly after the publication of my work, two independent groups mapped the active domain of ICP47-1 to the first 34 amino acids of the protein (Galocha et al., 1997; Neumann et al., 1997). To identify functional regions within ICP47, both groups synthesized various truncated forms of ICP47 in vitro and conducted translocation assays using TAP-containing microsomes or permeabilized cells. A large peptide spanning the residues 3 to 34 of ICP47 was found to be as active as the full length protein in these in vitro assays. This region is highly conserved between ICP47-1 and ICP47-2 and contains several charged residues that were

shown to be critical for ICP47-1 function. However, it seems rather interesting that the most homologous region between ICP47-1 and ICP47-2 lies outside the proposed active domain between residues 33 to 52 (see Figure 1A, Chapter 3). Within this stretch of 19 amino acids, the two proteins differ at only 2 positions. Based on this high degree of homology, its seems likely that this region plays an important role in ICP47 function. However, since the mapping of functional domains of ICP47 was conducted largely in vitro, it seems quite possible that an in vivo function for this second homologous domain might have been overlooked. In particular, one might speculate that this region may be involved in limiting access of ICP47 to the proteasome. The cytosolic distribution of ICP47, coupled with its rather extended structure, make this protein a prime target for proteasomal degradation. Thus, in order for ICP47 to mediate the inhibition of TAP, it might be essential for the protein to escape degradation by the proteasome. Alternatively, ICP47 may have a secondary function unrelated to immune evasion, and this conserved region may play a role in that process. Regardless, it seems highly unlikely that the C-terminal half of ICP47 would be maintained without an essential function for this domain.

Recently, a great deal of effort has been directed at determining the secondary structure of ICP47. Our preliminary CD experiments indicated that ICP47 was largely disordered in solution and may only achieve a functional conformation upon binding to TAP. However, Tampe and colleagues have demonstrated that the induction of this active conformation may also involve an association with membranes (Beinart et al., 1997). Consistent with our previous observations, this group found that ICP47 in aqueous solution was largely

unstructured. However, in the presence of membrane mimetics or lipid membranes, an α-helical structure was induced in the amino terminus of the protein. Thus, the authors propose that upon docking of ICP47 to membranes, a conformational change is induced in the protein that may facilitate binding to TAP. Unfortunately, due to the artificial conditions used in this study, the induction of this α-helical structure in ICP47 may not be relevant in vivo. In my binding studies with mammalian or insect microsomes, ICP47 was not found to significantly associate with membranes unless TAP was present (see Figures 9 and 10, Chapter 2).

From the time ICP47 was first identified as a inhibitor of $CD8^+$ T cell recognition, it has been proposed that this protein would make an ideal immunosuppressive agent for use in organ transplantation or gene therapy. However, to date, much of the evidence supporting a role for ICP47 in immune evasion has come from in vitro studies. Whether ICP47 can mediate these same effects in vivo is not known. A single animal study conducted by our laboratory in collaboration with Robert Hendricks, however, has provided some insight regarding the role of ICP47 in vivo (Goldsmith et al., 1998). In this study, an HSV-1 mutant virus lacking ICP47 was found to be significantly less neurovirulent than wildtype virus in mice. The reduced neurovirulence of the $ICP47^-$ mutant was due to a protective $CD8^+$ T cell response. Thus, from these observations it appears that ICP47 is capable of blocking $CD8^+$ T cell recognition in the nervous system of the mouse. This result was rather unexpected since my previous studies demonstrated that ICP47 bound to murine TAP poorly and was approximately 50-100 times less effective in blocking murine TAP than human TAP. However, it

remains possible that ICP47 might attain sufficient concentrations in certain cells in the nervous system of the mouse to inhibit TAP. Neurons are known to express low levels of TAP and MHC class I proteins, and thus, this impaired capacity to present viral antigens may contribute to the enhanced activity of ICP47 in these cells. Alternatively, ICP47 may have other unknown functions in the nervous system which play a role in HSV neurovirulence. To further characterize the role of ICP47 in immune evasion, additional in vivo studies must be performed. Ideally, these studies should be conducted in animals in which the native TAP has been shown to be sensitive to the effects of ICP47 such as monkeys, cows, dogs, or pigs. However, as discussed above, it may be worthwhile to re-examine the rat as a potential animal model, as rats homozygous for cim^a alleles of the TAP2 subunit may be susceptible to the effects of ICP47.

6.2 Inhibition of MHC Class II Antigen Presentation by HCMV US2

Unlike HSV, which has a short replication cycle, HCMV replicates very slowly in cells, often requiring several days to produce infectious progeny. Due to this rather extended replication cycle, cells infected with HCMV may be more vulnerable to attack by various components of the immune system, such as $CD8^+$ CTL. This added pressure by the immune system may be reflected by the fact that HCMV encodes at least five proteins dedicated to the task of blocking MHC class I presentation. Unlike HSV, however, HCMV also infects numerous cell types capable of expressing MHC class II proteins, such as monocytes/macrophages, endothelial cells and microglial cells. In particular, monocytes/macrophages appear to play a crucial role in the lifecycle of HCMV. Following primary infection, HCMV is thought to establish latency in circulating

monocytes and use these cells to disseminate virus throughout the body. Once these monocytes enter tissues and differentiate into macrophages, however, the virus is thought to reactivate from the latent state and produce infectious progeny for transmission to new hosts.

An intriguing aspect of HCMV reactivation is the ability of the virus to replicate within a macrophage that can present antigens to both $CD8^+$ and $CD4^+$ T cells. Macrophages belong to a subset of cells known as professional antigen presenting cells (APCs) and these cells constitutively express high levels of both MHC class I and class II proteins. Although the MHC class II pathway normally presents exogenous antigens, there is evidence that during HCMV infection, endogenous viral proteins, such as glycoprotein B (gB), transit through the endosomal system (Fish et al., 1998) and may reach the class II compartment. These endogenous viral proteins would then be degraded and loaded onto class II proteins for presentation to $CD4^+$ T lymphocytes. Therefore, in order for HCMV to reactivate and replicate within a macrophage, it may be essential for the virus to block endogenous antigen presentation by both the class I and class II pathways. Since HCMV is known to express several proteins capable of downregulating class I presentation, it seemed likely that the virus might also encode inhibitors of the class II pathway. As the second aim of this thesis, I investigated whether HCMV expressed a viral inhibitor of the MHC class II presentation pathway.

The initial impetus for my research in HCMV immune evasion came from observations made by our collaborators, Beth Walters and Stan Riddell, suggesting that HCMV was capable of blocking the presentation of class II

antigens. They found that primary macrophage cultures infected with HCMV were unable to present tetanus toxin to an antigen-specific $CD4^+$ T cell clone. This observation suggested that HCMV might inhibit or downregulate the MHC class II pathway such that exogenous antigen was not presented to the $CD4^+$ T cell clone. Based on this speculation, Jennefir DeKoning, a post doc in our laboratory, and I attempted to characterize this effect and identify the gene(s) responsible. Jennefir produced $CD4^+$ T cell clones specific for tetanus toxin and tested their ability to recognize antigen presented by primary macrophage cultures infected with HCMV. This approach met with limited success, however, as the primary macrophage cultures could not be adequately infected with HCMV in order to assess class II inhibition.

As a counterpart to these functional studies, I adopted a biochemical approach. Instead of using primary macrophage cultures, I made use of a human transformed microglial cell line, U373-MG, that can be efficiently infected by HCMV. To induce class II expression in these cells, I initially treated the cells with IFN-γ. In later experiments, I transfected the cells with the class II transactivator (CIITA) so that there was constitutive expression of class II proteins. In U373 cells infected with HCMV, expression or stability of MHC class II proteins was dramatically reduced after as little as 12 hours of infection. Using deletion viruses constructed by Tom Jones, this effect was mapped to the US2 gene in the U_S region of the HCMV genome. Previously, US2 had been shown to play a role in the inhibition of the MHC class I pathway by mediating the degradation of class I heavy chains by the proteasome (Jones and Sun, 1997; Wiertz et al., 1996). To further characterize the effects of US2 on the class II

pathway, I made use of a replication-defective adenovirus vector capable of expressing high levels of US2 in an inducible fashion. In cells expressing US2, the class II complex rapidly disappeared and this was dependent on the amount of US2 delivered. In experiments designed to determine whether the entire class II complex was affected or a single component, US2 was found to specifically target the α chain, leaving the β and Ii chains intact. The destruction of the class II-α chain by US2 was examined in greater detail and was found to involve the activity of catalytic proteasomes in the cytosol. Inhibitors of the proteasome, such as lactacystin and ZL_3VS, effectively blocked degradation of the α chain in US2-expressing cells. However, unlike the class I heavy chain, the α chain was not translocated to the cytosol in US2-expressing cells in which the proteasome was blocked. Under these conditions, the majority of the α chain remained anchored in the ER membrane in the fully glycosylated form. Proteasomes are known to be associated with the ER membrane (Dusseljee et al., 1998; Yang et al., 1998), and thus, after destabilization by US2, the α chain may be fed directly into the lumen of the proteasome. US2 was also found to mediate the degradation of the α chain of the HLA-DM complex. HLA-DM functions as an enzyme to load class II dimers with antigenic peptide in the MIIC and shares some limited homology with classical class II proteins (Denzin et al., 1995). Moreover, in functional T cell assays, the expression of US2 in cells was found to reduce or abolish their ability to present exogenous antigen to a helper $CD4^+$ T cell clone. Therefore, together, these results demonstrate that US2 is capable of mediating the degradation of class II DR-α and DM-α chains via the proteasome, and by doing so, has the capacity to block the presentation of antigens to $CD4^+$ T cells.

The observations that US2 causes degradation of class II DR-α and DM-α chains, as well as class I heavy chains, including several mouse alleles (Machold et al., 1997), were somewhat surprising considering these molecules show only very limited primary sequence homology, approximately 25% identity at the amino acid level (see Figure 8, Chapter 5). However, from crystallographic data, all three complexes share a similar secondary structure and overall domain organization. Each complex can be divided into four separate domains consisting of two membrane proximal conserved immunoglobulin domains and two membrane distal domains folded into a peptide binding cleft. In light of US2's ability to interact with a specific component of each complex, it seems likely that a determinant shared between the targeted proteins may act as a binding site for US2. Since the highly conserved membrane proximal immunoglobulin domains are shared between all members of the MHC complexes, including β_2m and the class II-β chain which are not targeted by US2, this domain seems an unlikely site for US2 binding. Therefore, of the remaining domains, the membrane distal α1 region of class I heavy chain is most similar to the α1 domains of class II DR-α and DM-α and, thus, this region is most likely the potential binding site for US2. However, complicating this simplistic conclusion is the recent finding that US2 does not cause the degradation of HLA-C and HLA-G (Schust et al., 1998). Both HLA-C and HLA-G have α1 domains that are very similar to classical class I HLA-A and HLA-B molecules (approximately 80% identical), yet these proteins remain resistant to the effects of US2.

For successful evasion of host immunity by HCMV, the class II-α chain, as compared to the β chain, makes an ideal target for the disruption of the class II

presentation pathway. In humans, there exists three isotypes of genes that code for classical class II proteins involved in peptide presentation: HLA-DR, -DP and -DQ. In each case, the gene for the α chain is considerably less polymorphic than that of the β gene (see Figure 2, Chapter 4). For example, the DR-α gene is essentially monomorphic with only two alleles differing by a few amino acids, while the DR-β gene consists of approximately 266 different alleles. By specifically targeting the α chains of the class II complex, HCMV ensures that a broad spectrum of class II products will be destroyed in any given population. The α chain of the HLA-DM complex also shows very little polymorphism, but this target represents an additional advantage for the virus. As mentioned previously, HLA-DM is a class II-like heterodimer that functions in an enzymatic manner to load class II molecules with antigenic peptide in the MIIC. In the absence of the HLA-DM complex, class II molecules do not acquire antigenic peptide, and thus, cannot elicit viral specific responses from $CD4^+$ T cells. With the degradation of the DM-α chain, HCMV disables a vital step in the presentation of class II peptides, and safeguards against the presentation of viral antigens by class II products that may have escaped destruction in the ER. Furthermore, due in part to the enzymatic nature of the HLA-DM complex, the DM-α and -β chains are typically expressed to much lower levels than classical class II proteins which may result in a more rapid depletion by US2.

With the disruption of the class II pathway in monocytes/macrophages, HCMV has the potential to evade considerable $CD4^+$ T cell recognition and thereby, limit many of the roles $CD4^+$ T cells play in orchestrating and mounting an immune response to viral infection. Although this hypothesis has not been

tested directly, my research to date is consistent with the notion that HCMV may be capable of disrupting normal $CD4^+$ T cell functions. Analogous to cytotoxic $CD8^+$ T cells, a fraction of the $CD4^+$ T cell population has the ability to destroy infected cells on contact. These cytolytic $CD4^+$ T cells destroy infected targets either through the release of perforin and granzymes or through the engagement of the fas death receptor. The cytolytic $CD4^+$ T cell response during HCMV infection may actually play a more significant role than first thought due in part to the potent downregulation of the class I pathway and thus, the inhibition of $CD8^+$ CTL recognition. An increase in the frequency of cytolytic $CD4^+$ T cells could compensate for the absence of a strong $CD8^+$ CTL response. Moreover, by blocking the MHC class II pathway and avoiding recognition by $CD4^+$ T lymphocytes, HCMV may limit the $CD8^+$ CTL response even further. Activated $CD4^+$ T cells are the major producers of IL-2 during an immune response and the secretion of this cytokine may play an essential role in priming a strong $CD8^+$ CTL response. Both $CD4^+$ and $CD8^+$ T cells require IL-2 to undergo the massive proliferation and expansion required to mount an immune response to viral infection. However, $CD8^+$ T cells do not produce considerable quantities of IL-2 and therefore, rely on the activation of neighboring $CD4^+$ T cells to provide much of the cytokine needed for their proliferation (Janeway and Travers, 1997; Roitt, 1994). In the absence of this $CD4^+$ T cell activation, the secreted levels of IL-2 may become limiting, resulting in the reduced proliferation of $CD8^+$ T cells and a weak CTL response.

$CD4^+$ T cells also augment the CTL response in a different manner. Due in part to the destructive nature of cytotoxic T cells, these cells typically require

higher levels of co-stimulatory molecules on target cells in order to become activated and acquire effector functions (Janeway and Travers, 1997; Roitt, 1994). Naive $CD4^+$ T cells, however, require less co-stimulatory activity to become activated and once bound to an infected target, have the ability to induce the expression of higher levels of co-stimulatory molecules on that cell. In this situation, $CD8^+$ T cells recognizing antigen on weakly co-stimulating cells may become activated only in the presence of $CD4^+$ T cells bound to the same target cell (Schoenberger et al., 1998). Therefore, in the absence of MHC class II presentation, HCMV may subvert this mode of T cell activation and nullify considerable antiviral CTL activity. Thus, the inhibition of class II presentation by US2 in HCMV-infected cells may have a profound effect on both the antiviral $CD4^+$ and $CD8^+$ T cell response.

The very existence of US2, with the capacity to destroy class II proteins within infected cells, indicates that presentation of endogenous antigens by the class II pathway may play a significant role in signalling the presence of viral infection. Typically, the MHC class II pathway presents exogenous antigens that have been taken up by cells through either endocytosis or phagocytosis. However, during infection of class II-expressing cells, viral proteins and/or particles transit through the endosomal system (Fish et al., 1998) and may arrive in the MIIC. Peptides derived from these viral proteins would then be presented by MHC class II proteins, signalling the presence of viral infection to $CD4^+$ T lymphocytes. Therefore, viral proteins synthesized during infection may provide an abundant source of endogenous antigen for presentation by the MHC class II pathway. Thus, in order for a virus to infect and replicate within a class II-

expressing cell, it may be essential for the virus to block endogenous presentation by both the class I and class II pathways. With this in mind, it seems very likely that other viruses capable of infecting macrophages, dendritic cells, B cells or endothelial cells may express proteins with similar functions to US2.

In the future, several key issues concerning the function of US2 should be investigated in greater detail. For instance, the domains in US2 required for binding and/or dislocation of class I and class II proteins have not yet been defined. Mutational studies conducted by Ploegh and colleagues have demonstrated that a soluble form of US2 is capable of binding class I heavy chains, but is unable to mediate their dislocation into the cytosol (Tortorella et al., 1998). Thus, the ER lumenal portion of US2 appears to be sufficient for class I binding, whereas the transmembrane and cytosolic domains may play a more active role in dislocation. Related to this is the finding that two cysteine residues in the cytosolic tail of US2 seem to be critical for the dislocation of class I heavy chains into the cytosol (Hidde Ploegh, personal communication). Intriguingly, the membrane proximal cysteine residues of many proteins are covalently modified with fatty acids and this may serve to stabilize the proteins in the lipid bilayer. Both the class I heavy chain and the class II-α chain are modified in this way. In my own preliminary experiments with US2, however, I could not detect any fatty acid modification of the protein. Therefore, it remains possible that the cysteine residues in the cytosolic tail of US2 may actually interfere with the fatty acid modification of the class I heavy chain and the class II-α chain, thereby destabilizing the proteins. This destabilization in the lipid bilayer may then facilitate the dislocation of the proteins into the cytosol. Clearly, this hypothesis is

purely speculative and requires further investigation.

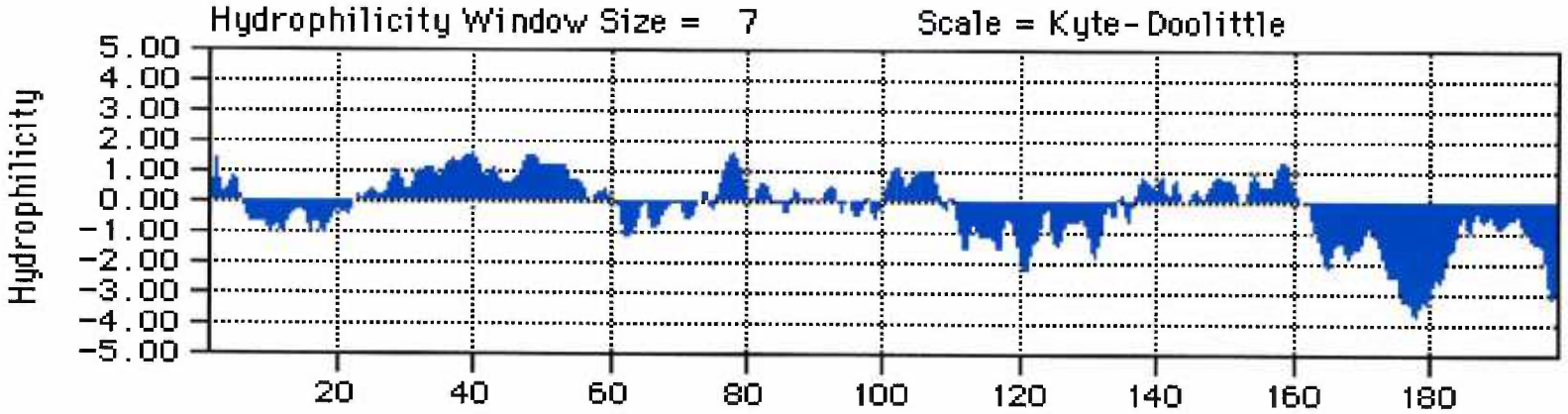
As proposed by my research, the role of US2 during HCMV infection is to block the presentation of endogenous viral antigens via the MHC class II pathway. However, to date, this hypothesis has not been directly tested. All functional $CD4^+$ T cell assays conducted thus far have used exogenous antigen to stimulate a $CD4^+$ T cell response. Therefore, to investigate whether US2 is capable of blocking the presentation of endogenous antigens by the class II pathway two approaches become readily apparent. First, functional assays could be conducted with $CD4^+$ T cells specific for either E or L HCMV proteins in the context of viral infection. Unfortunately, these antiviral $CD4^+$ T cells may be difficult to isolate and/or propagate once in tissue culture. Alternatively, a recombinant HCMV virus could be constructed to express a protein such as ovalbumin or hen egg lysozyme, for which well characterized $CD4^+$ T cells exist. In either case, the failure to elicit a $CD4^+$ T cell response to these endogenous antigens would indicate that US2 is capable of blocking their presentation via the MHC class II pathway.

Within the U_S region of the HCMV genome, US2 shares homology with two other genes, namely US3 and US11 (see Figure 3; Table 2). US2 and US3 are the only members of the US2 gene family and share approximately 27% identity at the amino acid level. US11, by contrast, belongs to the US6 family of genes, yet this protein shares approximately 19% amino acid identity with US2 (see Figure 4). Previously, both US3 and US11 have been shown to play roles in the downregulation of the MHC class I pathway. US3 binds to and retains class I heavy chains in the ER (Ahn et al., 1996; Jones et al., 1996), while US11, in a

manner virtually identical to that of US2, mediates the degradation of class I heavy chains by the proteasome (Jones and Sun, 1998; Wiertz et al., 1996). Therefore, based on the homology with US2, it is possible that US3 and US11 may also bind class II proteins. Effects of US3 or US11 on class II presentation may have been missed in my earlier experiments due to the presence of US2 or the failure to express adequate amounts of these viral proteins during infection. Unfortunately, a role for US11 in class II downregulation seems less likely, as previous degradation assays with mutant HCMV viruses did not uncover an effect for this protein. Nonetheless, experiments are underway to express US3 and US11 individually using recombinant adenovirus vectors in order to assess whether these proteins are capable of blocking class II presentation.

Figure 3: Structural characteristics of US2. A. Kyte-Doolittle hydrophilicity plot of US2. **B.** Secondary structure of US2 as predicted by the program PLOTSTRUCTURE (Garnier-Oeguthorpe-Robson algorithm) of the GCG Wisconsin sequence analysis package. The putative signal sequence and transmembrane domains are boxed in blue. Regions of homology shared with US3 and US11 are boxed in yellow. Homology region #1 spans residues 47 to 70, homology region #2 spans residues 94 to 113 and homology region #3 spans residues 133 to 143.

A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Scale = Kyte-Doolittle
Hydrophilicity
5.00
4.00
3.00
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180

B

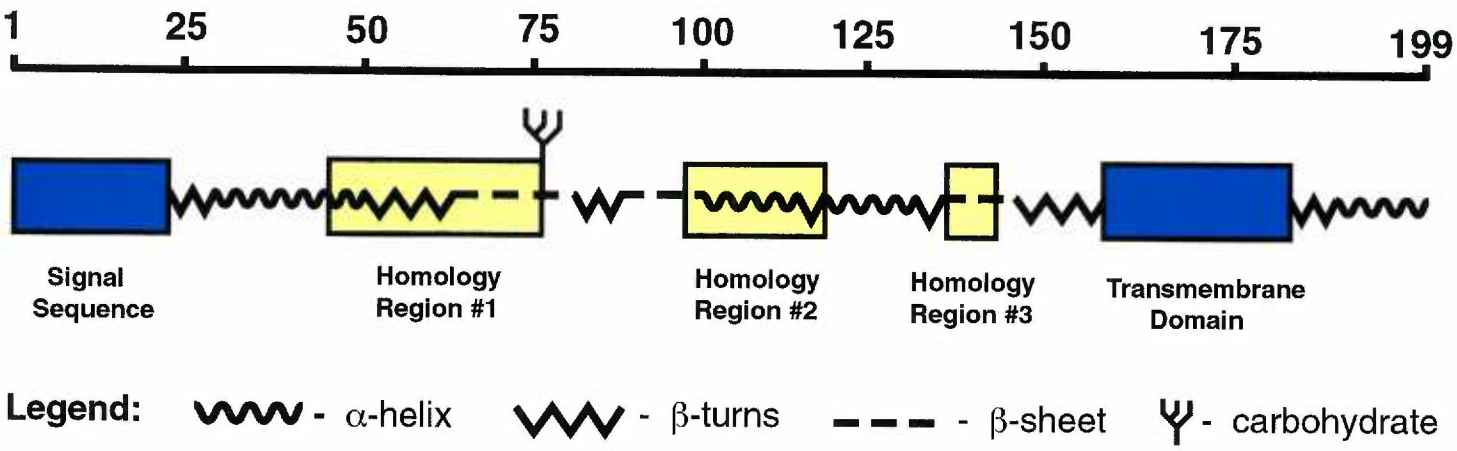
1
25
50
75
100
125
150
175
199
Signal Sequence
Homology Region #1
Homology Region #2
Homology Region #3
Transmembrane Domain
Legend:
- α-helix
- β-turns
- β-sheet
- carbohydrate

Figure 4: Amino acid sequence comparison of US2, US3 and US11. The protein sequences of US2, US3 and US11 were aligned using the program PILEUP of the GCG Wisconsin sequence analysis package. Vertical lines and dots represent identical and similar amino acids, respectively. Putative signal sequences and transmembrane domains are underlined. Regions of relatively high homology are boxed and an N-linked glycosylation site conserved among all three proteins is shown in black.

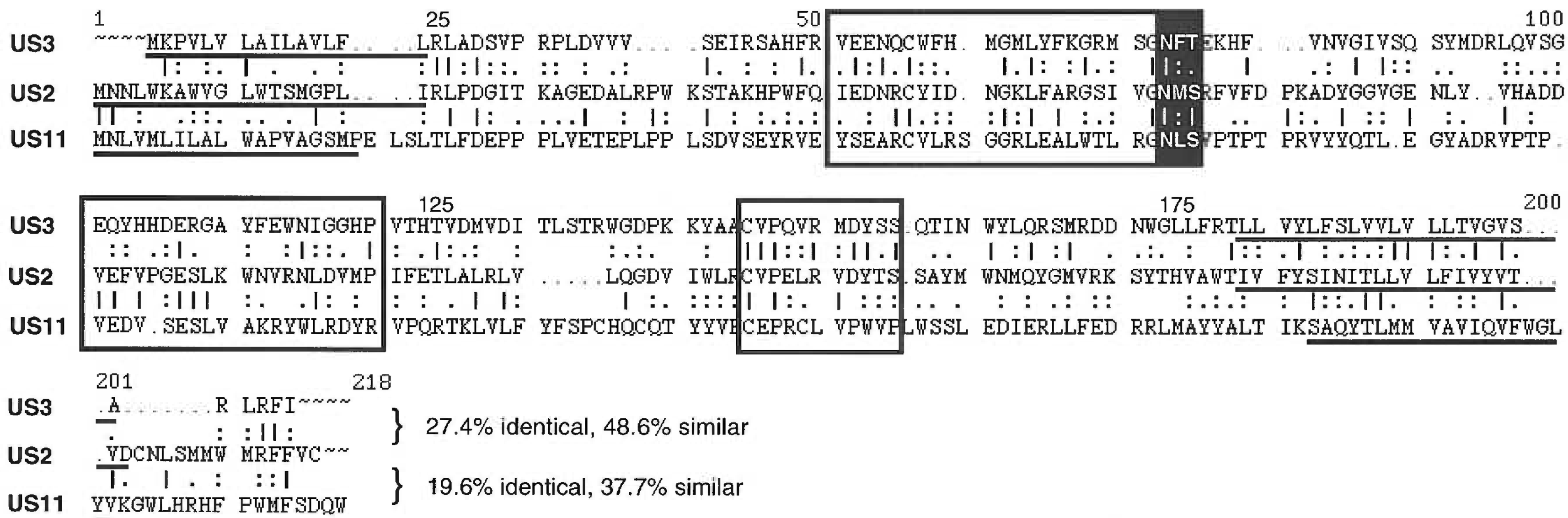
1 25 50 100
US3 ~~~~MKPVLV LAILAVLF.. ..LRLADSVP RPLDVVV.... .SEIRSAHFR VEENQCWFH. MGMLYFKGRM SGNFTEKHF. ..VNVGIVSQ SYMDRLQVSG
US2 MNNLWKAWVG LWTSMGPL.. ..IRLPDGIT KAGEDALRPW KSTAKHPWFQ IEDNRCYID. NGKLFARGSI VGNMSRFVFD PKADYGGVGE NLY..VHADD
US11 MNLVMLILAL WAPVAGSMPE LSLTLFDEPP PLVETEPLPP LSDVSEYRVE YSEARCYLRS GGRLEALWTL RGNLSVPTPT PRVYYQTL.E GYADRVPTP.
125 175 200
US3 EQYHHDERGA YFEWNIGGHP VTHTVDMVDI TLSTRWGDPK KYAACVPQVR MDYSS.QTIN WYLQRSMRDD NWGLLFRTLL VYLFSLVVLV LLTVGVS...
US2 VEFVPGESLK WNVRNLDVMP IFETLALRLV .....LQGDV IWLRCVPELR VDYTS.SAYM WNMQYGMVRK SYTHVAWTIV FYSINITLLV LFIVYVT...
US11 VEDV.SESLV AKRYWLRDYR VPQRTKLVLF YFSPCHQCQT YYVECEPRCL VPWVPLWSSL EDIERLLFED RRLMAYYALT IKSAQYTLMM VAVIQVFWGL
201 218
US3 .A.......R LRFI~~~~
US2 .VDCNLSMMW MRFFVC~~
US11 YVKGWLHRHF PWMFSDQW
27.4% identical, 48.6% similar
19.6% identical, 37.7% similar

Table 2: Comparison of the molecular characteristics of the HCMV proteins, US2, US3 and US11, as predicted from their primary amino acid sequence.

Molecular Characteristic	US2	US3	US11
Polypeptide length (# amino acids)	199	186	215
Molecular weight (Da)	23109	21753	25263
Isoelectric point (pH)	7.23	8.93	5.32
Ionic charge at pH 7.0	+0.27	+2.64	-3.72
# of cysteine residues	4	2	5
Potential trans-membrane domains	1	1	1
Potential glycosylation sites (NXT/S)	1	1	1

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Why go I thus heavily, while the enemy oppresseth me?
My bones are smitten asunder as with a sword:
While mine enemies that trouble me cast me in the teeth;
Namely, while they say daily unto me:
Where is now thy God?
-Psalm 42, v. 11

What though the field be lost?
All is not lost; the unconquerable will,
And study of revenge, immortal hate,
And courage never to submit or yield.
-J.M. (1667)

Without shedding of blood is no remission.
-Hebrews, ch. 9, v. 22

Life's but a walking shadow, a poor player
That struts and frets his hour upon the stage
And then is heard no more: it is a tale
Told by an idiot, full of sound and fury,
Signifying nothing.
-W.S. (1606)

Beast of burden, savage and cruel,
Withered my dreams and cast me a fool.
-C.R. (1994)

Mercy and truth are met together:
Righteousness and peace have kissed each other.
Truth shall flourish out of the earth:
And righteousness hath looked down from heaven.
-Psalm 85, v. 10

It is only that which cannot be expressed otherwise
that is worth expressing in music.
-F.D. (1920)

Talk and song from tongues of lilting grace,
Whose sounds caress my ear.
Not a word I heard could I relate,
But the story was quite clear.
-R.P. (1974)