CHARACTERIZATION OF A HERPES SIMPLEX VIRUS T CELL IMMUNE EVASION STRATEGY

By

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ABSTRACT

Herpes simplex virus (HSV) infections are common in all human populations and for most people they represent relatively mild lifelong infections. To facilitate the persistent infection of hosts, HSV has evolved immune evasion strategies which suppress various aspects of the immune response including the actions of complement and antibodies. Previously in our laboratory, an HSV immediate early protein called ICP47 was shown to inhibit the MHC class I antigen presentation pathway and thereby block recognition of virus infected cells by CD8+ cytotoxic T lymphocytes (CTL). This thesis explores the potential cellular targets of ICP47. Using immunoprecipitation I found ICP47 associates with the transporter associated with antigen presentation (TAP). By blocking the transport of peptide antigens into the endoplasmic reticulum, MHC class I molecules become unstable and are subsequently degraded before displaying HSV antigens on the cell surface. Thus, CTL destruction of cells infected with HSV is blocked. In addition, an interaction between an ICP47 bacterial fusion protein, called GSTICP47-1 and a cellular protein, calcyclin, was examined. The functions of calcyclin are largely unknown. However, based on its association with ICP47, it was possible that calcyclin might play a role in the class I pathway - perhaps as the peptide shuttle. Nevertheless, the results of several experiments were consistent with the notion that calcyclin and ICP47 may not interact *in vivo* and that calcyclin may not play a role in the MHC class I antigen presentation pathway.

DEDICATION

For their unending support throughout my research career, I would like to dedicate this body of research to both my grandparents and parents.

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LIST OF ABBREVIATIONS

AddlE1	A replication deficient adenovirus lacking its E1 region and used in these experiments as a control virus.
AdICP47-1	A replication deficient adenovirus with the US12 gene from a KOS strain of Herpes Simplex Virus inserted into the E1 region of the Adenovirus which only expresses ICP47.
CTL	Cytotoxic T-lymphocytes
FCS	Fetal Calf Serum
GST	Glutathione S-Transferase
GSTICP47-1	A fusion protein composed of Glutathione S-Transferase and ICP47 from HSV-1 separated by a thrombin cut site.
GSTICP47-2	A fusion protein composed of Glutathione S-Transferase and ICP47 from HSV-2 separated by a thrombin cut site.
HSV-1	Herpes Simplex Virus type 1
HSV-2	Herpes Simplex Virus type 2
IC_{50}	A concentration of inhibitor allowing only 50% of maximal activity in a functional assay.
ICP47-1/2	Infected Cell Protein 47 from HSV type 1/2; Product of the immediate early gene US12.
IFN γ	An immunocytokine called interferon gamma which upregulates the expression of MHC genes.
^{125}I	An isotope of iodine which can be covalently linked to tyrosine residues to radiolabel proteins or peptides.
MHC	Major Histocompatibility Complex; a set of genes containing class I (A,B and C), class II (SB, DC and DR) and Class 3 on chromosome 6.

MOI	Multiplicity of Infection; the number of plaque forming units per cell.
PBS	Phosphate-buffered saline
rICP47-1	Recombinant ICP47 type 1 made by cleaving GSTICP47-1 fusion protein with thrombin.
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TAP	Transporter Associated with Antigen Presentation; a member of the ATP binding cassette family of membrane transporters.
Tris-HCl	2-Amino-2-hydroxymethyl-1,3-propanediol; used to buffer solutions.
TYNRTRALI	A peptide consisting of nine amino acids in the following sequence: Threonine, Tryosine, Asparagine, Arginine, Threonine, Arginine, Leucine and Isoleucine.
VV-TAP1	A vaccinia vector expressing the TAP 1 subunit.
VV-TAP2	A vaccinia vector expressing the TAP 2 subunit.
VV-TAP1&2	A vaccinia vector expressing both the TAP 1 and TAP 2 subunits.
v/v	volume per unit volume
w/v	weight per unit volume

INTRODUCTION

Herpesviridae

Herpesviruses form a large group of enveloped doubled-stranded DNA viruses (reviewed in Roizman and Sears, 1996). Over the last fifty years, at least 80 distinct herpesviruses have been identified, in a variety of species from fish to mammals. The Herpesviridae family share three important characteristics including: a common mode of replication, the ability to establish latent infections and a similar DNA genome and particle structure. For example, all herpesviruses have a dense fibrillar DNA core surrounded by an icosahedral capsid composed of 12 pentameric and 150 hexameric capsomeres. The capsid is then encompassed in an amorphous protein matrix called the tegument which in turn is enveloped by a membrane containing several glycoproteins.

Based on differences in biological properties, the Herpesviridae family can be divided into three subfamilies including alphaherpesvirinae, betaherpesvirinae and gammaherpesvirinae. Subfamilies share similarities in genome structure, tissue tropism, cytopathology and sites of latency (reviewed in Roizman and Sears, 1996). Alphaherpesvirinae cause lytic infections and often establish latency in sensory ganglia. Generally, they have short replication cycles and variable host ranges (reviewed in Roizman and Sears, 1996). Herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2), varicella zoster virus (VZV) and pseudorabies virus (PRV) are some members of this family. HSV-1 and HSV-2 can cause cold sores and genital lesions while VZV causes chicken pox and sometimes shingles. Betaherpesvirinae are characterized by slow replication cycles and have

genus or species specific host ranges. Cells infected with betaherpesvirinae often undergo cytomegalia - an enlargement of the nuclei and cytoplasm characterized by deposits of DNA inclusion bodies (reviewed in Roizman and Sears, 1996). Secretory glands, lymphoreticular cells and kidney cells are sites of latent infections for betaherpesvirinae (reviewed in Roizman and Sears, 1996). Examples of this subfamily include human cytomegalovirus (HCMV), which can cause a mononucleosis-like disease and human herpesvirus 6 and human herpesvirus 7. Finally, the gammaherpesvirinae have a restricted host range but have a tropism for lymphoblastoid cells and are often latent in B or T cells (reviewed in Roizman and Sears, 1996). Their replication cycles and cytopathology are variable. Epstein-Barr virus is an example of a gamma herpes virus which causes mononucleosis.

Herpes Simplex Virus And Its Lifecycle

Herpes simplex type 1 and 2 are the most extensively studied herpesviruses. They are enveloped viruses approximately 150 nm in diameter containing a linear double-stranded DNA genome which is 152 kilobase pairs long (reviewed in Roizman and Sears, 1996). The HSV genome is arranged into a unique long (U_L) and a unique short (U_S) region each of which is flanked by terminal repeat sequences. At least 78 proteins are encoded by the genome and used throughout its infection cycle.

HSV initiates infection by first attaching to cells then fusion with the host membranes, usually the plasma membrane, releases the tegumen and the nucleocapsid into the cytoplasm (reviewed in Roizman and Sears, 1996). The capsid is transported to the pores of the nuclear

membrane where viral DNA is released into the nucleus (reviewed in Roizman and Sears, 1996). Two sequential waves of transcription and translation follow which produce additional viral transcription factors and replication machinery consecutively. Viral DNA is replicated by the rolling circle mechanism and after the production of structural components in a third round of transcription and translation, the DNA is packaged into preassembled capsids (reviewed in Roizman and Sears, 1996). Finally, the capsid binds to viral glycoproteins aggregated in cellular membranes and the capsids acquire an envelop of these glycoproteins and cellular membranes. The newly enveloped virion is then transported into the extracellular space where it is released to infect new hosts and begin the cycle anew.

HSV Infections And Host Immune Responses

HSV infections typically occur on the superficial mucosal membranes of the oropharynx or genitals where they cause vesicular lesions of the epidermis (reviewed in Samuelson and Lichtenberg, 1994; York and Johnson, 1994; Roizman and Sears, 1996). Eventually sensory ganglia become infected via sensory neurons and virus establishes a latent infection in neurons of ganglia. Occasionally, latent HSV reactivates and reinfects cells at the mucosal site of entry. Herpes simplex virus infections are common in all human populations and for most people they represent relatively mild infections which usually resolve within a few weeks (reviewed in Samuelson and Lichtenberg, 1994). More severe pathologies including gingivostomatitis, encephalitis, esophagitis, hepatitis and corneal blindness are sometimes associated with herpes infections especially in infants or immunocompromised patients

(reviewed in Samuelson and Lichtenberg, 1994).

The host's immune response to HSV infections is thought to involve all non-specific, humoral and cell-mediated aspects of the immune system (reviewed in York and Johnson, 1994). The importance of each of these branches of the immune system in controlling HSV spread is not clear. However, while a positive correlation between viral clearance and a strong humoral response has not been established, it may be possible to prevent HSV infections by rapidly neutralizing extracellular virion particles with a strong humoral response mounted against the whole virus or its protein components (reviewed in Corey and Spear, 1986; reviewed in York and Johnson, 1994). After entering a host cell, HSV can spread directly from cell-to-cell and can thus escape the humoral arm of the immune system (reviewed in York and Johnson, 1994). Macrophages and natural killer cells control HSV spread during the period in which more specific cell-mediated immune responses develop (reviewed in Domke-Optiz and Sawatzkym, 1990; York and Johnson, 1994). An effective T cell response to HSV is critical in clearing HSV infections as evident in immunocompromised patients who experience very severe HSV infections (Corey and Spear, 1986; Schmid and Rouse, 1992). Surprisingly, HSV can reactivate and replicate in healthy hosts despite the hostile environment created by strong humoral and cell mediated immune responses. This fact is linked to the unique ability of HSV to remain quiescent in neurons and to its immune evasion strategies which will be further discussed below.

Passive Immune Evasion Strategy Of HSV

HSV Latency and Its Role in Immune Evasion

All herpesviruses have evolved means to conceal themselves from the assaults of the host immune system so that a life-long latent or persistent infection can be established. This unique but poorly understood ability is called latency. After release of HSV particles from the initial mucosal site of infection, the virions infect the sensory neurons. Subsequently, the virions are translocated by retrograde axonal transport to the dorsal root ganglia where, after a short period of replication, the virus establishes a latent infection (Kritensson *et al.*, 1986; Lycke *et al.*, 1984; reviewed in Roizman, 1982; Roizman and Sears, 1996). In latently infected cells, the viral genome is present in multiple copies but transcription is very limited and the production of infectious viral particles is absent until reactivation occurs. Most latent herpes simplex virus infections are restricted to neurons (Cook *et al.*, 1974; Roizman and Sears, 1996).

The mechanism by which viral latency is established is not well understood but the circular episomal HSV genome is known to express three different overlapping latency-associated transcripts (LATs) that map to the long repeat of the viral genome (reviewed in Roizman and Sears, 1996). They are antisense to the transcript of ICP0 and are 1.45 kb, 1.5 kb and 2.0 kb in size. It appears that none of these transcripts are translated into corresponding proteins *in vitro* or *in vivo* in mice, rabbit or human neurons (Mador *et al.*, 1995). Transcriptional control of the major 2.0 kb LAT species is regulated by the LAT2 promoter during latent infections (Chen *et al.*, 1995). Transcription of this LAT in latently

infected trigeminal ganglion cells is thought to be constant over the life time of the host and does not change significantly after virus reactivation (Hill *et al.*, 1996). There is little replication of the HSV genome during latency and infectious virions can be produced from a limited number of viral genomes upon reactivation. HSV mutants lacking LAT transcription were defective in reactivation but not in replication or in the establishment and maintenance of latency (Perng *et al.*, 1996). Various physical and emotional stresses, menstruation and hormonal imbalances have been implicated in triggering reactivation (reviewed in Roizman and Sears, 1996).

LATs may function as an antisense inhibitor of ICP0 which ultimately prevents the expression of immediate early genes necessary to initiate reactivation. Only *in vitro* evidence supports this hypothesis (reviewed in Roizman and Sears, 1996). Alternatively, inhibition of viral immediate early gene transcription might occur in latent neurons because of an absence of essential host or viral trans activating factors. Neuronal transcription factors like Oct-1 as well as viral ICP0 and ICP4 are thought to be essential activating elements (reviewed in Roizman and Sears, 1996). Their absence may restrict viral growth and promote latency in neurons. Perhaps physical, emotional or hormonal stresses result in imbalances or excesses of these factors and potentiate the reactivation of latent HSV (reviewed in Roizman and Sears, 1996). However, these cellular and viral transcriptional activators can be identified in latently infected neurons and so the reactivation process may be more complicated (reviewed in Roizman and Sears, 1996). Future investigations are necessary to identify other factors responsible for this poorly understood dormant state.

Latency serves in several capacities as an immune evasion strategy. First, the intracellular environment of neurons cloaks HSV antigens from immune cell detection and protect the virion particles from the deleterious effects of cytokines induced during infection (Spriggs, 1992). Viral protein expression does not occur during latency and this restricts presentation of immunogenic peptides or proteins. Finally, normal healthy neurons lack Major Histocompatibility Complex (MHC) class I molecules (Neumann *et al.*, 1995). Thus, neurons may serve as sanctuary from the immune system. However, damaged neurons treated with interferon gamma (IFN γ) recently were found to express MHC class I molecules (Neumann *et al.*, 1995). Perhaps HSV latency is a means of controlling the viral infection and preventing extensive cellular damage that could induce MHC class I expression. Therefore HSV may have evolved to use latency in neurons as a strategy to avoid the immune system.

The Immune Evasion Strategies Of HSV During Its Lytic Cycle

Overcoming the Complement Cascade

The immune system has several nonspecific as well as specific mechanisms which challenge the success of all infectious pathogens including herpes simplex viruses. One of these mechanisms is the complement system, which is divided into two branches; the classical pathway and the alternative pathway (reviewed in Abbas *et al.*, 1994). There are over twenty different proteins in this system which remain dormant in plasma until triggered by a sequence of activation events. This typically begins in one of two ways depending on the pathway. For example in the classical pathway, a protein called C1 is activated when it

binds to antigen-antibody complexes of IgG or IgM. Activated C1 catalyzes the production of classical C3 convertase (C4b2a) from C2 and C4 subunits. This convertase cleaves C3 into C3b molecules which then interact with other cellular factors to form C5 convertase (C42a3b). In the alternate pathway, microbial surfaces, endotoxins and complex polysaccharides initiate the formation of alternate C3 convertase (C3bBb) which is stabilized by properdin. The alternate C3 convertase cleaves C3 into C3b which then begins the formation of the alternate C5 convertase enzyme (C3bBb3b). Though the C5 convertases of each pathway are structurally distinct, they catalyse identical reactions. Each C5 convertase can activate other complement proteins including C6, C7, C8 and C9 which form a pore-like structure called the membrane attack complex (MAC). Cells affected by the formation of these MAC pores eventually lyse due to osmotic pressure. Also, both pathways can affect the destruction of pathogens by: increasing vasodilation and vascular permeability at the site of infection, augmenting leukocyte chemotaxis and enhancing opsonization and phagocytosis.

Complement functions in a localized manner especially in oral and genital secretions. HSV may have evolved mechanisms to overcome the complement defense cascade in order to infect mucosal cells. Several studies have shown that HSV infected endothelial, epithelial and fibroblastic cells bind C3 complement components (Cines *et al.*, 1982; Freidman *et al.*, 1984; Smiley *et al.*, 1985 and Kubota *et al.*, 1987). This C3 receptor protein was shown to be an envelope glycoprotein called gC-1 and gC-2, in HSV-1 and HSV-2, respectively. An *in vitro* experiment using cells transfected with the gC-1 gene demonstrated that gC-1 is sufficient to bind C3b (Einsenberg *et al.*, 1987). Further investigations have characterized

gC-1's capacity to bind both C3b and iC3b (McNearney et al, 1987; Harris *et al.*, 1990; reviewed in Johnson and York, 1994). Structurally, gC-1 is slightly larger than gC-2 as it has four domains important for C3 binding versus the three homologous domains found in gC-2 (reviewed in York and Johnson, 1994). HSV-1 and HSV-2 infections express similar levels of related gC molecules (Jennings *et al.*, 1987) but it is unclear why gC-2 binds C3b less efficiently than gC-1 (reviewed in York and Johnson, 1994).

Several investigations have shown how gC provides protection against complemen-mediated neutralization and cytolysis through the alternative pathway (McNearney *et al.*, 1987; Harris *et al.*, 1990; Hidaka *et al.*, 1991; reviewed in York and Johnson, 1994). A recent study by Freidmann and associates (1996) showed gC mutant strains were unable to bind C3b and were 5000 fold more susceptible to complement neutralization than wild-type virus. This neutralization was not enhanced with antibodies recognizing HSV proteins which supports the hypothesis that the protection provided by gC works on the alternative C3 convertase enzyme. HSV is thought to avoid the classical pathway using a virally encoded Fc receptor which can bind to anti-HSV antibodies required for activation of the classical C3 convertase. However, to inhibit the alternative pathway, gC-1 destabilizes the alternative convertase by inhibiting the binding of properdin. In contrast, gC-2 does not affect the hemolytic ability of the alternative complement cascade as it does not inhibit properdin binding (Fries *et al.*, 1986; Eisenberg *et al.*, 1987; Hung *et al.* 1994; reviewed in York and Johnson, 1994). Complement inhibition may be augmented even further when gC-1 binds to heparin sulphate - another molecule known to downregulate complement activity (Freidmann

et al., 1996).

Furthermore, *in vivo* experiments have demonstrated that the alternative complement pathway is an important first line defense against HSV infections prior to the development of specific humoral or cell-mediated immunities (Van Strip *et al.*, 1989). Results of preliminary experiments using a guinea pig model have shown that gC-1 mutants grew to much lower titers and the resulting vaginitis was much less severe than that produced by wild-type HSV-1 (Freidmann *et al.*, 1996). The same study showed that guinea pigs deficient in C3 production were equally infected by both gC-1 mutants and wild-type HSV-1. This confirmed the role of gC-1 *in vivo*. Moreover, the ability of gC-1 to bind to another complement component called iC3b is thought to prevent opsonization and phagocytosis (Kubota *et al.*, 1987; Tal-Singer *et al.*, 1991). Future experiments are necessary to elucidate gC's role in abrogating the complement cascade *in vivo*, to determine whether or not gC plays an important role in protecting HSV from the humoral immune system.

Resistance to Antibodies

HSV has evolved proteins that bind to IgG perhaps as a means to avoid antibody responses. Several studies have shown that cells infected with HSV-1 and HSV-2 express a receptor protein that can bind to IgG (Westmoreland and Watkins, 1974; Nakamura *et al.*, 1978) at the CH2 and CH3 regions of the Fc domain (Johansson *et al.*, 1984). These unique Fc receptors can bind IgG expressed by humans, rabbits, goats, sheep and cows but not from chickens, mice, rats, cats or horses (Johansson *et al.*, 1985).

The HSV Fc receptor was shown to involve a complex of two HSV-1 glycoproteins gE and gI (Baucke and Spear, 1979; Johnson and Feenstra., 1987) encoded by the US8 and US7 genes respectively (McGeoch *et al.*, 1985; Johnson *et al.*, 1988). Expression of both gE and gI was sufficient for Fc receptor activity (Hanke *et al.*, 1990; reviewed in York and Johnson, 1994). In addition, gE alone can bind to both monomeric IgG and IgG bound to antigens, but with less efficiency than the gE-gI complex (reviewed in York and Johnson, 1994).

A double binding or bi-polar bridging hypothesis suggests that the Fc receptor may inhibit the cytotoxic effects of antibodies, lymphocytes and complement (Frank and Freidmann, 1989). In this model, the Fab regions of anti-HSV antibodies bind to an HSV antigen, while the Fc regions are bound by gE-gI complexes. By binding the Fc region of anti-HSV antibodies, an interaction with C1, the first component of the classical cascade, is prevented and consequently a classical complement response is avoided (Frank and Freidmann, 1989; reviewed in York and Johnson, 1994). In the same manner, bi-polar bridging could prevent the destruction of HSV-infected cells by some leukocytes whose activity is mediated by Fc receptors expressed on their cell surfaces (Frank and Freidmann, 1989; reviewed in York and Johnson, 1994). In support of this model, one study had shown that HSV Fc receptors can prevent the activation of the classical complement cascade by reducing the binding of C1q (Dubin *et al.*, 1991). Moreover, experiments with gE mutants have demonstrated a role of the Fc receptor in resistance to antibody dependent cellular cytotoxicity (ADCC) and complement mediated neutralization (reviewed in York and Johnson, 1994). The exact role the viral Fc receptor has in immune evasion *in vivo* is unclear

but a loss of both gE and gI significantly reduces neurovirulence (Neidhardt *et al.*, 1987; Rajcani *et al.*, 1990). Whether the reduction of neurovirulence is related at all to a loss of this immune evasion mechanism is uncertain, but it is clear that gE and gI function in cell-to-cell spread and this is very important in neurovirulence (Dingwell *et al.*, 1995).

HSV Infection of T Lymphocytes and NK cells by Direct Cell-to-Cell Spread

Late in the infection cycle, it was observed that HSV infected cells became resistant to lysis by cytotoxic T lymphocytes (CTL), lymphokine-activated killer (LAK) cells and natural killer (NK) cells (Posavad *et al.*, 1993; York *et al.*, 1993). After initial contact with HSV infected targets, the lymphocyte effectors were unable to lyse other target cells (Confer *et al.*, 1990; Posavad *et al.*, 1992; Posavad *et al.*, 1993; Koelle *et al.*, 1993). It was suggested that the lymphocytes themselves become infected with HSV after coming into intimate contact with their targets (York *et al.*, 1993). In support of this hypothesis, immunofluoresence confirmed the presence of HSV antigens in LAK cells that were first incubated with HSV infected fibroblasts (York *et al.*, 1993). The exact mechanism that abrogates effector cell function is unknown but the HSV infection may alter the cytoskeleton, adhesion molecules or cause anomalies in transcription and translation in their lymphocyte hosts (reviewed in York and Johnson, 1994). Alternatively, a specific HSV protein may inhibit effector cell functions. Moreover, one investigation showed that an HSV infection in monocytes interfered with its accessory cell functions such as the display of antigens to T cells (Hayward, 1993).

Inhibition of Recognition by Anti-HSV Cytotoxic Lymphocytes

Several studies have observed a bias in the T cell populations found in humans infected with HSV. Until recently, it was not known why the T cell responses generated by an HSV infection were primarily due to CD4+ T cells (reviewed in Schmid *et al.*, 1992). The few CD8+ T cells that were generated primarily recognize structural virion components and not newly synthesized viral proteins (Tigges *et al.*, 1992). However, mice infected with HSV can easily generate CD8+ T cells specific to immediate early, early and late HSV gene products (reviewed in York and Johnson, 1994). Furthermore, some investigations have demonstrated that CD8+ CTL cannot lyse syngeneic human fibroblasts infected with HSV (Posavad and Rosenthal, 1992; Posavad *et al.*, 1993; Koelle *et al.*, 1993).

Until recently, the mechanism responsible for this unique phenomenon remained a mystery. Protection from lysis occurs early in the infectious cycle long before HSV particles are produced (York et al., 1994; reviewed in York and Johnson, 1994). Therefore, CD8+ T cells were not themselves infected by HSV. It was possible that the virion host shutoff protein (vhs) which is known to cause degradation of mRNA could inhibit MHC class I expression. However, this hypothesis can not explain why HSV infected fibroblasts treated with appropriate peptide antigens can be lysed by CD8+ CTL. Exogenous peptides can complex with some species of empty MHC class I molecules on the surface of cells (reviewed in York *et al.*, 1996). Therefore, the HSV-infected cells must process some class I on their surfaces to bind peptide and sensitize the cells to CD8+ CTL lysis. This suggests that MHC class I molecules are properly expressed in HSV-infected cells. In addition, fibroblasts

infected with vhs mutants were not significantly more sensitive to CTL lysis than fibroblasts infected with wild-type HSV (Posavad *et al.*, 1993).

The viral protein responsible for this phenomenon was thought to be either a structural component or an immediate early gene product as CTL resistance was demonstrated 2h postinfection during immediate early gene expression. Treating human fibroblasts with UV inactivated HSV did not inhibit CTL lysis (York *et al.*, 1994). This established that a virion structural component was not responsible for MHC class instability. However, the inhibition of MHC class I processing blockade was relieved in human fibroblasts infected with an HSV-1 mutant lacking the immediate early gene US12 which encodes a protein, ICP47. An adenovirus vector expressing only the ICP47 protein was shown to destabilize MHC complexes so that class I accumulated in the ER (York *et al.*, 1994). Thus, ICP47 was sufficient to inhibit MHC class I processing. When tested in functional CTL assays, expression of ICP47 by an adenovirus vector blocked the lysis of human fibroblasts superinfected with HCMV (York *et al.*, 1994). Therefore the immediate early gene product, ICP47, blocked class I antigen presentation to CD8+ T cells (York *et al.*, 1994).

The Immediate-Early Gene US12 And ICP47 Its Protein Product

In HSV-1 and HSV-2, ICP47-1 and ICP47-2 are encoded by the immediate early gene US12. Both ICP47 protein products are approximately 10 kDa in size and share 42% amino acid identity (Table 1 and Figure 1). They have no obvious signal sequences, transmembrane domains or homologies to any other known cellular or viral proteins. ICP47-1 is not localized

to a specific cellular compartment as immunofluorescence has shown the distribution of ICP47 to be ubiquitous as early as 2h postinfection (York *et al.*, 1994). Little is known about the protein structure of either ICP47-1 or ICP47-2. However, ICP47-1 can still function to inhibit antigen presentation after being boiled (Tomazin *et al*, 1996). This suggests that ICP47 has little if any secondary structure or that the protein can refold after being denatured. However, it is also possible that denatured ICP47 can refold into a functional conformation after binding to its cellular target. Despite the robust thermal stability, both types of ICP47 are highly sensitive to protease degradation (Tomazin *et al.*, 1996). Finally, another surprising feature of ICP47-1 was the association identified between a GSTICP47-1 fusion protein and a cellular protein called calcyclin (York *et al.*, unpublished results). It is not known what the significance of this association is with respect to ICP47-1's ability to inhibit the class I pathway. Since most of this work focuses on the suppression of MHC class I by ICP47, the following sections will describe the MHC class I antigen presentation pathway.

Table 1: A Comparison of Characteristics of ICP47 from HSV Type 1 and Type 2 Predicted By Their Primary Sequences.

Analysis	ICP47 Type 1	ICP47 Type 2
Molecular Weight (kDa)	9.793	9.785
Length (amino acids)	88	86
Molar Extinction Coefficient	6970+-5%	7450+-5%
Absorbance at 280nm (mg/ml)	1.41	1.31
Isoelectric Point	8.86	7.12
Charge at pH 7.0	+1.26	+0.14

ICP47-1: **MSWAL***E* MA**D**T **FLD***T* M**R**VGP**R** **TY**A**DV**RD**EI**N **KR**G**REDREAA** ...

ICP47-2: **MSWAL***K* TT**D**M **FLD***S* S **R**CTH**R** **TY**G**DV**CA**EI**H **KR**E**REDREAA** ...

ICP47-1: ...**RTAV**H**DPE**R**P** **LL**RS**P**G*L* LP*E* I**A**PNASLGVA HRRTGGTVTD SPRNPVTR

ICP47-2: ...**RTAV**T**DPE**L**P** **LL**CP**P**D*V* RS*D* P**A**SRNPTQQT RGCARSNERQ DRVLAP

FIGURE 1: A comparison of shared primary sequence homology between ICP47 from HSV type 1 and HSV type 2. The enlarged bold underlined letters correspond to homologous amino acid residues. Italicized letters are conservative residue changes.

The Roles of the MHC Class I and Class II Antigen Presentation Pathways

The immune systems of vertebrate organisms must cope with cells affected by oncogenic transformation or infected by viral pathogens. The intracellular nature of the proteins expressed in these situations make it important for the immune system to have a mechanism to detect proteins expressed inside cells. To overcome these difficulties, higher organisms have evolved at least two different antigen presentation pathways that involve a cluster of polymorphic genes known as the major histocompatibility complex (MHC) (reviewed in Abbas *et al.*, 1994).

The MHC class I pathway is constitutively active in most somatic cells and can be upregulated by proinflammatory cytokines. For the most part, MHC class I proteins form stable complexes with fragments of endogenously expressed proteins which are then exported to the cell surface to display these protein antigens to T cells. Normally, CD8+ T cells ignore MHC class I molecules complexed with autologous antigens. However, cytotoxic killer T cells are activated and will affect the destruction of cells displaying tumour or viral antigens on their MHC class I molecules. Thus the MHC class I pathway provides an effective window into the intracellular environment (Figure 2a).

In contrast to MHC class I antigen presentation, MHC class II has evolved to display antigens which are captured by endocytosis from the extracellular mileau. Professional antigen display cells of the immune system express MHC class II molecules. These immune cells coordinate immune responses by interacting with CD4+ T cells .

The Ubiquitin-Proteosome Proteolytic Pathway - Providing A Source of Peptides For Class I Antigen Presentation

Before MHC class I molecules are exported to the cell surface to display antigens to the immune system, they must first bind to short antigenic peptides. This binding facilitates the correct folding of MHC class I molecules prior to their export. The exact origin of the peptides used by this pathway has been investigated and at least two alternative sources have been proposed. First, antigenic peptides may be directly generated as peptons by translation of aberrant short mRNA transcripts or by initiation and termination of translation at unconventional mRNA sites (Boon and Van Peel, 1989). This hypothesis explains why some pepton fragments are presented from genes where the antigenic sequence was not in the translational reading frame (Fetten *et al.*, 1991). The pepton hypothesis was further supported by finding that the MHC class I proteins display of antigenic sequences that follow stop codons or that come from genes lacking promoters or translational start sites (Chomez *et al.*, 1992).

However, protein degradation is probably the main source of antigenic peptides presented by MHC class I proteins. Mammalian cells have several methods of degrading intracellular and extracellular proteins into peptide fragments. Some of these proteolytic systems include lysosomal enzymes, calpains, the ATP/ubiquitin-independent nonlysosomal pathways and the ATP/ubiquitin-dependent proteosome pathways. These proteolytic systems play important roles in the regulation of many subcellular functions but it is often not clear what their relative contributions are. Recent studies have shown a potential role of

ATP/ubiquitin dependent proteosomes in the MHC class I antigen presentation pathway (Einsenlohr *et al.*, 1992.; Shastri *et al.*, 1995; Goldberg,1992; Goldberg and Rock, 1992).

Proteolysis by proteosomes is a five step process. First, short lived or abnormal proteins are conjugated to a small, 8.5 kD, highly conserved protein called ubiquitin (Goldberg, and Rock, 1992; Monaco, 1992; Michalek *et al.*, 1993; Hershiko *et al.*, 1992; reviewed in Rechsteiner, 1987). This covalent modification is controlled by three enzymes E1, E2 and E3. The E1 enzyme hydrolyses ATP and activates the carboxy terminal glycine residue of ubiquitin into a high energy thiol ester intermediate. Next, the ubiquitin conjugating enzyme, E2, transfers the activated intermediate from E1 to the target protein bound by the ubiquitin protein ligase or E3 enzyme. The resulting isopeptide bond covalently links the activated glycine residue of ubiquitin to an amino terminal lysine residue on the target protein. Polyubiquitation of the target protein continues and this ubiquitation enhances recognition and degradation by the 26S proteosome complex (Ciechanover *et al.*, 1984; Gropper *et al.*,1991). Degradation of target proteins is followed by the recycling of ubiquitin molecules carried out by isopeptidases (Chau *et al.*, 1989).

The factors that determine what antigens are degraded by the proteosome are still unclear. However, the ubiquitination of a protein can be influenced by its amino terminal residue. Proteins with acidic or basic amino terminal residues like aspartate or arginine are ubiquinated and degraded very quickly as compared to proteins that start with methionine or glycine residue (Varshovsky, 1992). Since E3 binds to target proteins, it follows that it must play an important role in selecting proteins for degradation perhaps by reading the amino

Figure 2a: The MHC Class I Antigen Presentation Pathway. The primary role of this pathway is to display internal proteins to CD8+ T cells. 1. The majority of proteins that enter the MHC class I antigen presentation pathway are those that are endogenously expressed by intracellular pathogens like viruses or antigens produced by tumours. 2. The pathway begins with the ubiquitination of antigens which can enhance the degradation of antigens by tagging them for hydrolysis by multisubunit cylindrical structures known as proteosomes. 3. Peptides resulting from this proteolysis are conveyed to the surface of the endoplasmic reticulum, by unknown mechanisms, perhaps involving a specific protein shuttle. 4. At the ER membrane, the peptides bind to Transporter associated with Antigen Processing (TAP) and are transported into the lumen of the ER. 5. The peptides are released and assemble with the MHC class I heavy chain and β_2-microgobulin complex, which is also bound to the lumenal face of the TAP molecule and interacts with tapasin and the chaperonin, calreticulin. 6. After binding peptides, the MHC class I molecule achieves its correct conformation and with the completion of further posttranslational modifications in the Golgi apparatus, the mature complex of class I heavy chain, β2M and peptide is transported to the cell surface. 7. At the cell surface the antigens are displayed CD8+ T lymphocytes which use their Tcell receptors to recognize class I-peptide complexes.

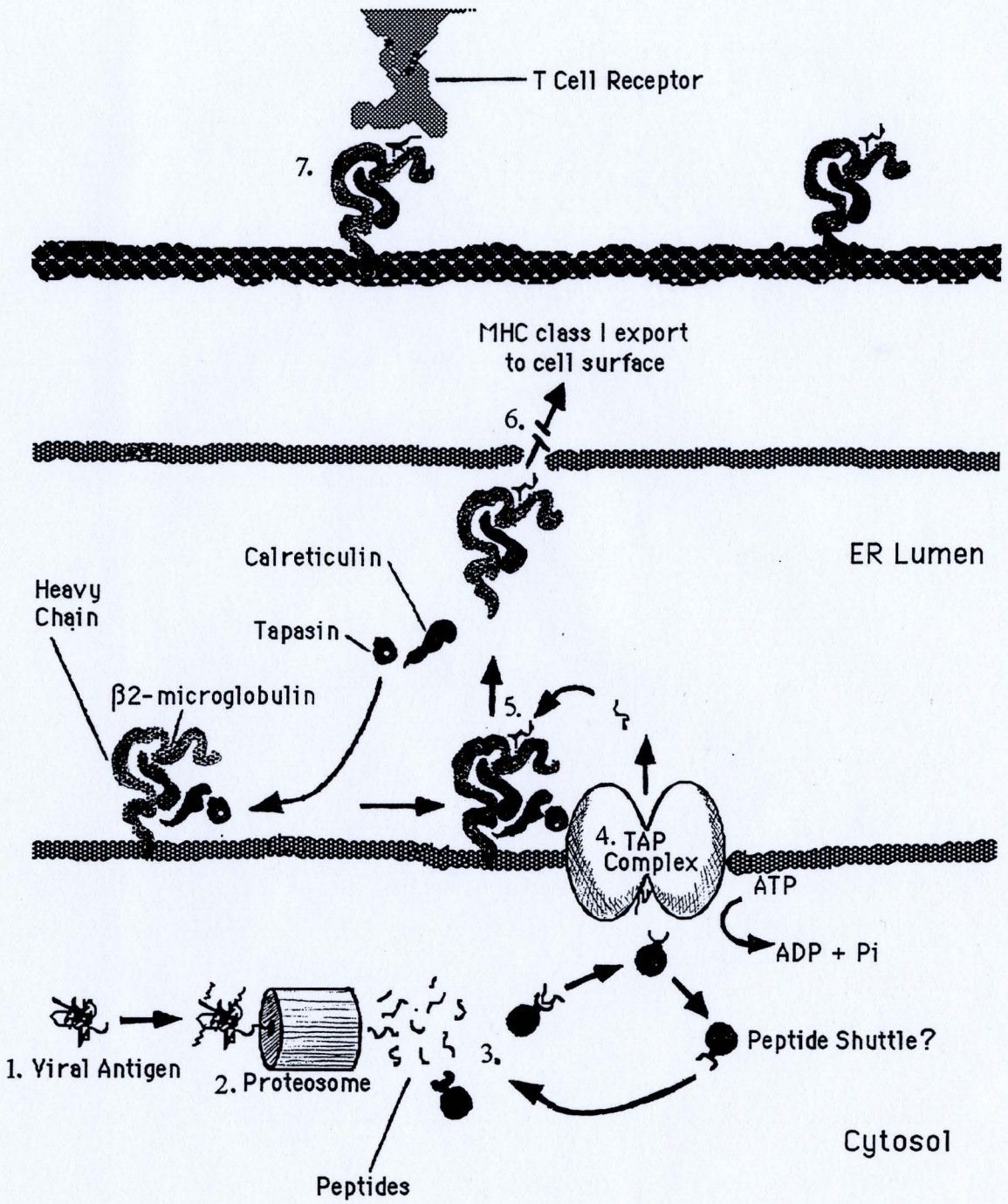
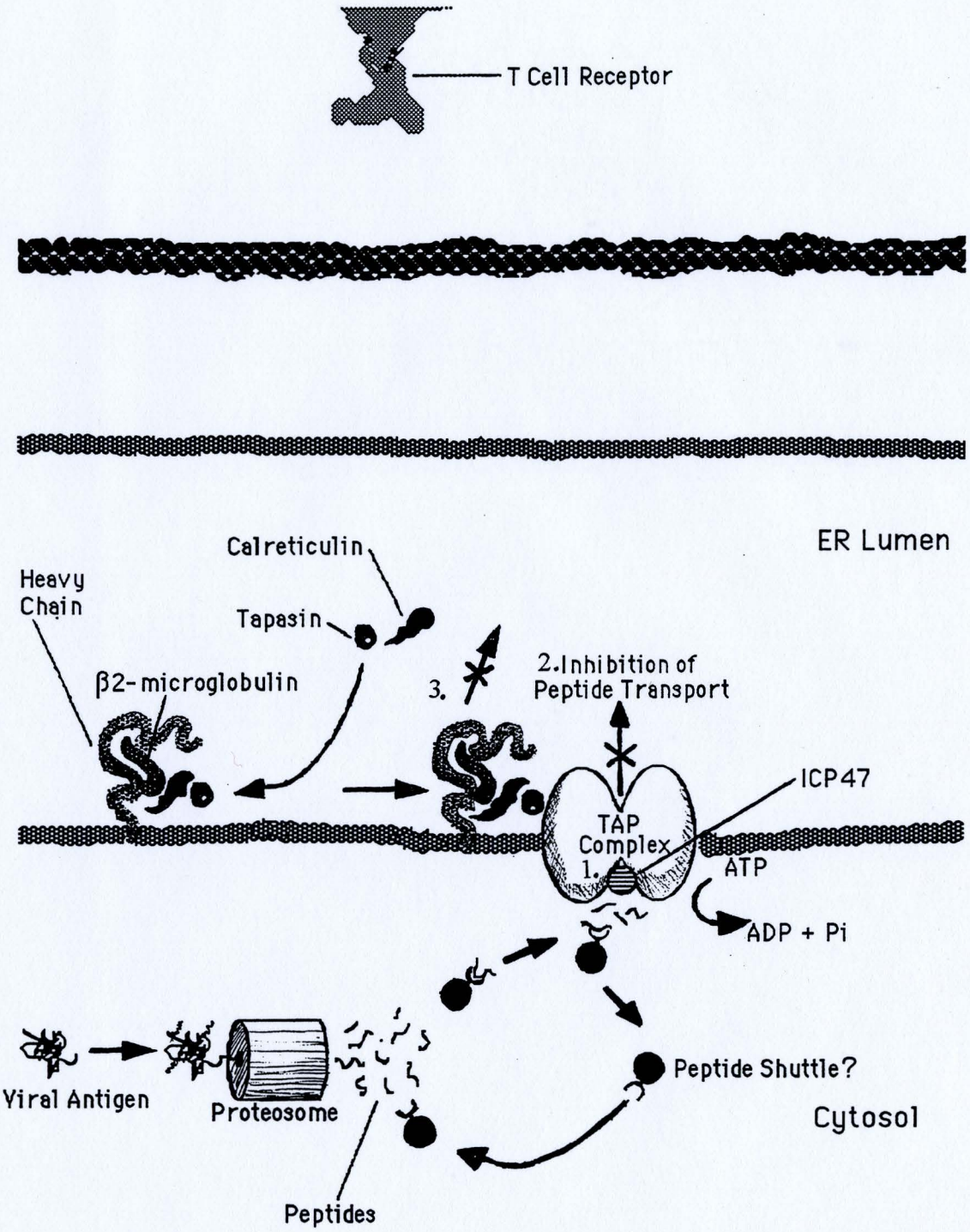

T Cell Receptor
7.
MHC class I export
to cell surface
6.
ER Lumen
Calreticulin
Heavy
Chain
Tapasin
β2-microglobulin
5.
4. TAP
Complex
ATP
ADP + Pi
Peptide Shuttle?
1. Viral Antigen
2. Proteosome
3.
Peptides
Cytosol

Figure 2b: 1. HSV Inhibition of MHC Class I Antigen Presentation. 1. When a cell is infected with HSV, the immediate early gene product ICP47 is made and binds to the cytosolic face of two subunits that comprise the TAP complex. 2. The binding of ICP47 to TAP effectively blocks the transport of the peptides. 3. Without peptides, the proper assembly of class I complexes cannot not be accomplished and the result is retention and subsequent degradation of class I molecules in the lumen of the ER. Thus, the number of MHC class I complexes which are exported to the surface is diminished and HSV-infected cell become invisible to the cell-mediated immune responses of CD8+ T lymphocytes.


T Cell Receptor
ER Lumen
Calreticulin
Heavy Chain
Tapasin
β2-microglobulin
3.
2.Inhibition of Peptide Transport
ICP47
TAP Complex
1.
ATP
ADP + Pi
Peptide Shuttle?
Cytosol
Viral Antigen
Proteosome
Peptides

terminal residues. Several different species of E2 enzymes have been identified and these different species may make selections of target proteins for degradation (Chau *et al.*, 1989).

Though recent evidence supports the function of proteosomes in the MHC class I pathway, the role of ubiquination is still unclear. One study has shown that the display of an ovalbumin peptide could be inhibited at a nonpermissive temperature in a mutant mouse cell line, ts20, which expresses a thermal labile E1 enzyme (Goldberg and Rock, 1992). The same study restored proper antigen display in ts20 cells at nonpermissive temperatures when the ovalbumin peptide was expressed from a vaccinia vector. They concluded that ubiquination is a necessary step in MHC class I antigen presentation. In contrast, a second group found that presentation of six endogenous and two exogenous antigens was not blocked at nonpermissive temperatures for a mouse L929 mutant cell line, tsA1S9 which expresses a temperature sensitive mutation in the E1 gene (Ciechanover, 1994). However, studies using these temperature sensitive mutants are misleading as ubiquination may not be completely inhibited at nonpermissive temperatures.

Other studies have enhanced the ubiquination of antigens to determine the effect on antigen presentation (Bachmir *et al.*, 1986). One group enhanced the display of an B-galactosidase epitope by changing its amino terminus to an acidic or basic residue . As mentioned above, these alterations would destabilize the protein and augment ATP/ubiquitin dependent proteolysis. The same study blocked the ubiquitin/proteosome pathway and subsequent antigen presentation by methylating potential ubiquination sites. They also showed that antigen degradation was dependent on ATP and ubiquitin. Enhancing or

diminishing the rate of degradation correlated to the rate of antigen presentation *in vivo* which suggests that ubiquination is a vital rate limiting step. Whether this conclusion is true for other less stable antigens has yet to be determined. If ATP/ubiquitin dependent proteolysis is indeed a rate limiting step then the peptide species presented by the MHC class I pathway may be biased toward abundant, fast degrading antigens (Ciechanover, 1994).

In recent years, two related multicatalytic proteases have been shown to act as the major source of MHC class I peptide antigens. These highly conserved structures, denoted the 20 S and 26 S proteosome, function as endopepidases and are found in the cytoplasm and nuclei of eukaryotic cells (Rechsteiner *et al.*, 1993). Both proteasomes have related structural features but different substrate specificities.

The 20S proteasome has also been called the multicatalytic protease or MCP. Electron microscope studies have shown this proteasome resembles a four layer rectangle with a length of 15 nm and a width of 11 nm (Baumeister *et al.*, 1988). Alternatively, it appears as a hollow cylinder composed of four rings . The rings are 11 nm in diameter and form a 2-4 nm pore (Rechsteiner *et al.*, 1993; Kopp *et al.*, 1993). Eukaryotic MCP rings are composed of six or seven α and β subunit dimers (Kopp *et al.*, 1993). The size of each subunit type ranges from 20 kDa to 32 kDa (Kopp *et al.*, 1993). A highly conserved region of twenty amino acids in the amino terminus of the α subunit is thought to play a role in MCP assembly (Zwickl *et al.*, 1992). Several α subunits have highly charged carboxy terminal extensions which are thought to bind associated regulatory elements of the proteosome (Rechsteiner *et al.*, 1993). β subunits are believed to degrade denatured proteins (Rechsteiner *et al.*, 1993).

The heterogeneity of these subunits imparts the broad substrate specificity of 20S proteasomes though a complete absence of disulfide bonds and complete unfolding of substrates are prerequisites for their degradation (Wenzel and Baumeister, 1995). Inhibitor studies and the analysis of conserved amino residues suggest that the MCP acts as a typical serine protease (Rechsteiner *et al.*, 1993). Kinetic studies of the proteosomes suggest that the active sites exist along the internal surface of the proteosome core (Dick *et al.*,1991).

Based on electron microscopy studies, the 26S proteosome was found to include both mushroom and barbell-like shaped particles thought to be composed of two interacting mushroom structures (Rechsteiner *et al.*, 1993). The structure of the active form of the 26S proteosome remains a mystery. Each mushroom shaped structure is composed of two rings which in turn are composed of six $\alpha\beta$ subunits identical to the subunits of the MCP. Up to fifteen subunits with molecular weights that range from 42 kDa to 110 kDa make up the cap of the mushroom structure. The roles each of these subunits is currently under investigation. The 26S proteosome was shown to bind ubiquitin conjugated substrates (Dubiel *et al.*, 1992). Subunit 4 (S4), subunit 5 (S5) and subunit 7 (S7) of the human proteosome are members of a newly characterized ATPase family and have a preference in binding to proteins conjugated with more than four ubiquitin molecules (Dubiel *et al.*, 1992; Dubiel *et al.*, 1993; Deveraux *et al.*,1994). It has been suggested that ubiquitin enhances the binding of protein substrates to the S4, S5 and S7 components of the 26S proteosome (Deveraux *et al.*,1994) which in turn enhances protein degradation (Deveraux *et al.*,1994; reviewed in York *et al.*, 1996).

Several pieces of evidence support a major role for proteasomes in antigen presentation.

One study indirectly assayed the effect of proteasome inhibitors on antigen presentation (Rock *et al.*, 1994). Two peptide aldehydes, MG115 and LLnL, acted as specific competitive inhibitors of both the 20S and 26S proteasome, and these peptide aldehydes arrested most intracellular proteolysis. Cells exposed to these peptides aldehydes where also deficient in presenting ovalbumin antigens. Antigen presentation was restored when these inhibitors were removed or when a source of peptides was supplied by expressing a peptide minigene. In contrast, the inhibition of lysosomal enzymes and calpains did not affect MHC class I presentation (Rock *et al.*, 1994).

Perhaps the strongest evidence implicating the proteasomes in antigen presentation was the identification of two MHC gene products LMP2 and LMP7 (van Endert *et al.*, 1992). These subunits have a close homology to the catalytic β subunits of proteasomes (Monaco and McDevitt, 1986). Interferon γ (a cytokine known to modulate immune responses) upregulates their expression and causes their preferential incorporation into newly synthesized proteasomes (Monaco and McDevitt, 1986). This incorporation biases the activity of proteasomes such that peptide substrates are cleaved after hydrophobic and basic residues while cleavage after acidic residues remains the same or in some cases is reduced (reviewed in York *et al.*, 1996). Coincidentally, the peptides displayed by MHC class I molecules terminate primarily in basic or hydrophobic residues (Engelhard, 1994). Thus, the induction and incorporation of LMP subunits serves to alter proteasome activity favouring the generation of immunogenic peptides. Further, this is evidence that the proteosome itself is involved in MHC class I antigen presentation.

Peptide Stability in the Cytoplasm

Cytosolic peptides are known to have very short half lives in the milieu of proteolytic activity found in the cytoplasm (Momburg *et al.*, 1994.; Hill *et al.*, 1995). These degradative processes make safe passage for antigenic peptides to the endoplasmic reticulum (ER) very difficult. However, antigenic peptides may overcome this problem by associating with chaperone molecules like heat shock protein (HSP) 70. Some antigenic peptides have been found to associate with HSP70 though many proteins and peptides are known to bind to heat shock proteins nonspecifically (Flynn *et al.*, 1989). A gene for an HSP70 protein has been identified in the cluster of genes that encode the MHC class I proteins but the role of this HSP70 in antigen presentation is not clear (Sargent *et al.*, 1989). However, it has been suggested that HSP70 and HSP90 act in a relay system to transfer antigenic peptides through the cytosol until they reach the ER (reviewed in Srivastava *et al.*, 1994). Recent evidence has shown an association between HSP70 and the transporter associated with antigen presentation (TAP) (Okada *et al.*, personal communication). Perhaps HSP90 binds to cytosolic peptides, then passes these peptides along to HSP70 and then to the TAP complex which facilitates their import into the ER. Alternatively, proteosomes may associate with the TAP complex or other integral ER membrane proteins either directly or indirectly. In this juxtaposition, the source of antigens is located in close proximity to the TAP complex which would enhance both safe intracellular passage and translocation efficiency. However, evidence supporting this type of interaction has not yet been published. Thus the mechanisms

by which peptides are stabilized in the cytosol and travel to the ER remains a mystery.

Peptide Translocation into the Endoplasmic Reticulum - The Transporter Associated with Antigen Presentation (TAP)

Before binding and stabilizing MHC class I complexes, cytosolic peptides generated by the proteosomes must first be transported into the lumen of the ER. The molecule responsible for this transport is called the Transporter associated with Antigen Presentation (TAP) and it is a member of the ATP Binding Cassette (ABC) family of transporters (Kelly *et al.*, 1992). Each functional complex is a heteroduplex composed of two subunits encoded in the MHC gene cluster called TAP1 (69 kDa) and TAP2 (74 kDa) (Russ *et al.*, 1995; Kleijmeer, 1992). Both subunits are integral membrane proteins and span the ER membrane with six transmembrane domains (Kelly *et al.*, 1992; Russ *et al.*, 1992). Both TAP subunits are essential for peptide transport (Neefjes *et al.*, 1993). Heteroduplexes rapidly form and are restricted to the ER membrane and possibly to the membranes of the cis-Golgi Apparatus (Russ *et al.*, 1995; Meyer *et al.*,1994).

The cytosolic carboxy terminal domains of each TAP subunit have consensus ATP binding sites (Russ *et al.*, 1995). However, both ATP sites also have significant affinity for GTP and to a lesser extent for the other trinucleotides (Kelly *et al.*,1992; Russ *et al.*, 1995). ATP binding is dependent on divalent cations including magnesium and calcium and there are magnesium binding sites in the ATP-binding regions (Russ *et al.*, 1995). ATP hydrolysis is essential for the translocation of peptides into the lumen of the ER but not for peptide

binding (Androlewicz and Cresswell, 1994; van Endert *et al.*, 1994). The interaction of both TAP subunits is essential to form a peptide binding site which appears to be independent of the ATP sites (Androlewicz and Cresswell, 1994; Uebil *et al.*, 1995).

The accumulation of peptides in the lumen of the ER closely parallels the affinity of peptides for the peptide binding site (van Endert *et al.*, 1994). There are several intrinsic factors that affect this affinity including peptide length, carboxy terminal residues and internal amino acid flanking sequences. Peptides which bind strongly are 8-12 amino acids long, very small peptides less than 8 amino acids long and those which are 13-25 residues long bind weakly and are translocated less efficiently (Androlewicz and Cresswell, 1994; van Endert *et al.*, 1994). Different species have different carboxy terminal residue requirements that enhance their affinity to TAP. For example, mouse TAP molecules prefer hydrophobic C-terminal residues while human TAP complexes bind peptides with both hydrophobic and basic C-terminal residues (Heemels *et al.*, 1993). In rats, TAP affinity for specific carboxy termini depends on two different TAP alleles called cim^a and cim^b. Cim^a preferentially transports peptides with hydrophobic or basic C-terminal residues while cim^b is biased toward hydrophobic residues (Heemels *et al.*, 1993). To date, differences in N-termini residues have not been linked to differences in peptide binding or transport (Heemels *et al.*, 1993).

The Major Histocompatibility Complex (MHC) Class I Molecules

Almost all somatic cells express MHC class I complexes which can interact with the surface receptors of CD8+ T cells (reviewed in Abbas *et al.*, 1994). The structure of a

mature MHC class I complex consists of a 45 kDa α heavy chain, a 12 kDa light chain called beta2-microglobulin (β2-M), and a short peptide antigen (8-10 aa). The heavy chain is composed of 340 amino acids. Its structure is folded into a peptide binding cleft (composed of two homologous domains α1 and α2), an immunoglobulin-like domain (called α3), a transmembrane region and a short cytoplasmic domain (reviewed in Abbas *et al.*, 1994). β2-M is a secreted protein of approximately 100 amino acids and can be membrane retained by binding to the heavy chain (reviewed in York *et al.*, 1996). Both proteins are co-translationally transported into the lumen of the rough ER where the heavy chain immediately associates with BiP, the first of three chaperones molecules (reviewed in Haas, 1991). The precise role of BiP in class I assembly is unclear. Next, the second chaperone protein, called calnexin, binds to the heavy chain to aid its folding and assembly (reviewed in Bergeron *et al.*, 1994). This stabilizes the heavy chain and facilitates the binding of β2-M. The heteroduplex of heavy chain and β2-M then binds to the lumenal face of the TAP complex as mediated by a chaperone called calreticulin and by tapasin (Sadasivan *et al.*, 1996). The binding of class I proteins to TAP is thought enhance the subsequent association of class I complexes with antigenic peptides (Ortmann *et al.*, 1994; Suh *et al.*, 1994). Upon peptide binding, the trimeric complex achieves its proper conformation and is then exported through the Golgi apparatus where final modification of glycosylated residues and export to the plasma membrane occurs (reviewed in York *et al.*, 1996).

An important feature of the MHC class I complex is the polymorphic nature of the

peptide binding cleft in the class I heavy chain. Amino acid variations in this cleft equip the MHC class I complexes with the means to bind and present numerous peptides from a wide spectrum of intracellular pathogens. Human MHC class I genes have been studied extensively and several allelic families have been identified at the three distinct loci (HLA-A, B and C) on chromosome 6. The HLA-A family for example includes the six alleles A2, A3, A9, A10, A19 and A80 (Lawlor *et al.*, 1995). Each family member binds a distinct but overlapping set of peptide sequences, eight to ten amino acids long (Falk *et al.*, 1991), which show preference for types of specific residues at position two and at the C-terminus (Sidney *et al.*, 1995). For example, members of the HLA-B family were found to bind peptides with a proline at position two and hydrophobic or aromatic C-terminal residues. These residues interact with the deep pocket structures of MHC class I molecules found in the peptide binding cleft (reviewed in Bjorkman and Parham, 1990). The affinity of different peptides for class I complexes is very dependent on the specific amino acid side chains present in the binding gooves of the MHC class I heavy chain. Indeed, mutating the residues of these regions alters the repertoire of peptides bound by the MHC class I molecules (Rohen *et al.*, 1993). Also, other regions of the class I molecule are thought to influence peptide binding specificity (reviewed in Bjorkman and Parham, 1990). Recently, it was shown that different CTL can differentiate between individual MHC subtypes by detecting specific class I-peptide complexes (Smith *et al.*, 1995). Thus, the peptide specificities of each class I subtype not only alters the efficiency of antigen presentation but also coordinates the destruction of infected cells by specific CTL effectors (Smith *et al.*, 1995).

EXPERIMENTAL RATIONALE

The destabilization of class I molecules by HSV ICP47 was ascribed to a lack of antigenic peptides (York *et al.*, 1994). Inhibiting any one of several steps in the class I antigen presentation pathway could potentially diminish the pool of peptides in the ER which were available to MHC class I proteins. For example, if ubiquination or proteosomes were inhibited then viral antigens would not be degraded to antigenic peptides. Alternatively, ICP47 could potentially have functioned to inhibit TAP so that viral peptides are not imported into the ER for proper assembly. It was also possible that ICP47 inhibited translocation of peptides through the cytoplasm from the proteosomes to the TAP so as to reduce the pool of antigenic peptides. Determining the cellular target of ICP47 and determining how antigen presentation was blocked by this viral protein were the objectives of this thesis.

Prologue: The results described in Chapter 1 of this thesis have been published in Hill *et al.*, 1995. My contribution to this publication was demonstrating a physical association between ICP47 and the TAP.

CHAPTER 1: Identifying A Cellular Target Of ICP47

Our lab had shown that the MHC class I complexes of fibroblasts infected with wild-type HSV-1 were retained in the endoplasmic reticulum in an unstable peptide-empty state; as early as 2 h after virus infection (York *et al.*, 1994). ICP47-1 was shown to be sufficient for this inhibition but the mechanism of action of ICP47-1 was not known. Thus, the first objective of this project was to determine the cellular target(s) of ICP47. Several experiments were performed to identify a physical association between ICP47 and potential cellular targets including the proteosome, the TAP complex and heat shock proteins. Results from some of these experiments suggested that ICP47 did not associate with proteosomes or heat shock proteins. However, an important observation made by Dr. Ann Hill lead us to investigate a possible physical association between ICP47 and TAP. Using a functional assay which could evaluate peptide transport by TAP, Dr. Hill discovered that transport of peptides in HSV-infected cells was almost completely abolished. The following section describes experiments I performed to characterize binding of ICP47 to the TAP complex.

I.1 MATERIALS AND METHODS

I.1.a. Cells.

All cells lines including HeLa (derived from a tumour from a patient, Helen Lane) cells and normal human fibroblasts derived from skin biopsies (used between passage 10 and 20) were passaged in a minimal media or Dulbecco's minimal media supplemented with 10% v/v FCS, 450 ug/mL of glutamine, 100 ug/mL of penicillin and 100 ug/mL of streptomyocin. Cell cultures were grown at 37°C in an atmosphere enriched with a 5% CO_2 final concentration.

I..1.b Viruses and Viral Expression Vectors.

Herpes simplex virus (type 1) infections were done using wildtype HSV-1 strain F or with R3631 (a mutant lacking the part of the US11 and US12 gene coding sequences, as described by Mavromara-Nazos *et al.*, 1986). All HSV viral stocks were grown up and titered on Vero cells. The adenovirus vector AdICP47-1 produces ICP4-1 by expressing the HSV type 1 US12 gene under the HCMV promoter, in the E1 region of the adenovirus genome as previously described by York *et al.*, 1994. The control adenovirus vector AddlE1 lacks the E1 region and also the HSV type 1 US12 gene insert. All adenovirus vectors were propagated and titered on 293 cells. The vaccinia vectors expressing either TAP1 or TAP2 subunits (VV-TAP1 and VV-TAP2 respectively) under a p7.7 promoter were constructed by homologous recombination of TAP subunit cDNA into the coding region of the vaccinia thymidine kinase gene (Russ *et al.*, 1995). The vaccinia vector, VV-TAP1&2 expresses both

TAP subunits and was constructed by inserting the TAP 2 cDNA into the HindIII site of VV-TAP1 (Russ *et al.*, 1995). All vaccinia vectors were grown and propagated in thymidine kinase-deficient human 143B osteosacroma cells.

I.1.c Expression of TAP and ICP47 in HeLa Cells

HeLa cells were treated with 100 units/mL of IFN-γ (Gibco-BRL) for 12 h to induce TAP and then infected with a AdICP47-1 using 100 pfu/cell for 36 h to induce expression of ICP47-1. The first 2 h of all adenovirus infections were done in Dulbecco's minimal media containing 2% FCS and 100 units/mL IFN γ. The media then was changed to Dulbecco's minimal media containing 10% FCS and 100 units/mL IFN γ for the duration of the infection.

Recombinant vaccinia virus vectors were used to express TAP in HeLa cells which were first infected with AdICP47-1 or AddlE1 as described above except that IFN γ was not present. After 36 h of the adenovirus infection, the cells were coinfected with a vaccinia vector expressing the TAP1 subunit (VV-TAP1), the TAP2 subunit (VV-TAP2) or both subunits (VV-TAP1&2). Infections with each vaccinia vector were done in Dulbecco's minimal media supplemented with 10% FCS using 10 pfu/cell. HeLa cells infected with both VV-TAP1 and VV-TAP2 were done at an MOI of 7 for each vector. The duration of all vaccinia infections was 6 h prior to radiolabelling.

I.1.d Metabolic Radiolabelling of Cells.

HeLa cells induced with IFN γ and infected with adenovirus vectors for 36 h were washed 3 times with 10 mL of Dulbecco's minimal media lacking methionine and cysteine. The radiolabelling media consisted of Dulbecco's minimal media lacking methionine and cysteine and was supplemented with 100 uCi/mL of ^{35}S-methionine and ^{35}S-cysteine, 100 units/mL of IFN γ, 1% dialyzed FCS and 5% Dulbecco's minimal media. The labelling media was then added to the cells and incubated for 6 h. HeLa cells coinfected with adenovirus and vaccinia vectors were radiolabelled using Dulbecco's minimal media lacking methionine and cysteine, and supplemented with 100 uCi/mL of ^{35}S-methionine and ^{35}S-cysteine and 1% dialyzed FCS for 4 h.

I.1.e Immunoprecipitation Reactions.

At the end of the metabolic radiolabelling period, the radioactive media was aspirated off and approximately $1.0x10^7$ cells were lysed in the 15 mL of digitonin (Sigma) lysis buffer. This detergent solution was prepared by dissolving digitonin at 1% w/v in 100 mM NaCl, 50 mM Tris (pH 7.5) at 90°C. The solution was then cooled and filtered in a 45 uM filter. In addition, the digitonin lysis solution was supplemented with 2 mg/mL of BSA and 0.5 mM PMSF to inhibit nonspecific proteolysis. The detergent cell lysates were centrifuged at 83000 X g for 45 min then divided into 1.5 mL aliquots. Each aliquot was incubated for 90 min on ice with 5 uL of one of the following antisera: anti-ICP47-5; a polyclonal rabbit antiserum produced against whole recombinant ICP47 produced as a Glutathione-S-transferase fusion protein in bacteria (York *et al.*, 1994), anti-CP47-1; a polyclonal rabbit antiserum produced

against a carboxy terminal peptide of ICP47 (York *et al.*, 1994), preimmune control antisera and anti-TAP-C (Ortmann *et al.*, 1994) and anti-TAP-P (Cromme *et al.*, 1994); two different polyclonal rabbit antisera recognizing the human TAP1 subunit. To precipitate the immune complexes, 50 uL of protein A Sepharose beads were added to each tube and incubated for 2 h at 4 ℃ with constant inversion. The protein A Sepharose beads were then washed three times with 0.1 % w/v digitonin detergent solution. SDS PAGE electrophoresis (using 10% DATD crosslinked gels) was used to examine all recovered protein A Sepharose immune complexes.

I.1.f Sequential Immunoprecipitation Reactions.

To sequentially immunoprecipitate ICP47 and then MHC class I or TAP, ICP47 was first immunoprecipitated with anti-ICP47-5 and anti-CP47-1. The immune complexes were then denatured by adding an equal volume of denaturing buffer (25mM Tris (pH6.2), 2% v/v SDS and 600mM B-mercaptoethanol) followed by boiling for 5 min. Denatured samples were diluted 10 fold using 1% digitonin buffer and then reimmunoprecipiated using 10 uL of either anti-TAP-P or HC10 antisera as described above. All samples were analyzed by SDS PAGE using 10% v/v polyacrylamide gels.

I.1.g Protein Electrophoresis.

All labelled protein samples were combined with gel loading buffer so that a final concentration of 10% v/v glycerol, 50mM Tris (pH 6.8), 100 mM dithiothreitol, 2% v/v

sodium dodecyl sulfate and 0.1% v/v bromophenol blue was attained and samples were boiled for 5 min. Protein samples were then loaded onto a SDS-polyacrrlamide gel consisting of 5% DATD-crosslinked polyacrylamide, 125mM Tris (pH6.8), 0.1% SDS, 5% w/v ammonium persulphate (APS) and 0.03% v/v N,N,N',N'-tetramethylethylenediamide (TEMED); and a separating gel of 10% DATD-crosslinked polyacrylamide, 375mM Tris (pH8.8), 0.1% SDS, 0.05% w/v ammonium persulphate (APS) and 0.03% v/v TEMED. The gels were subjected to electrophoresis at a constant voltage of 60 volts for about 14 h. After protein electrophoresis, the gels were incubated in a fixing buffer consisting of 8% v/v acetic acid and 46% v/v methanol for 1 h and then in Enlightening solution (Dupont-NEN) for 1 h. Finally, the gels are dried at 65°C under a vacuum for 2 h and then exposed to X-ray film (Dupont-NEN).

I.2 RESULTS

I.2.a Discovery of the Physical Association of ICP47 and the TAP complex.

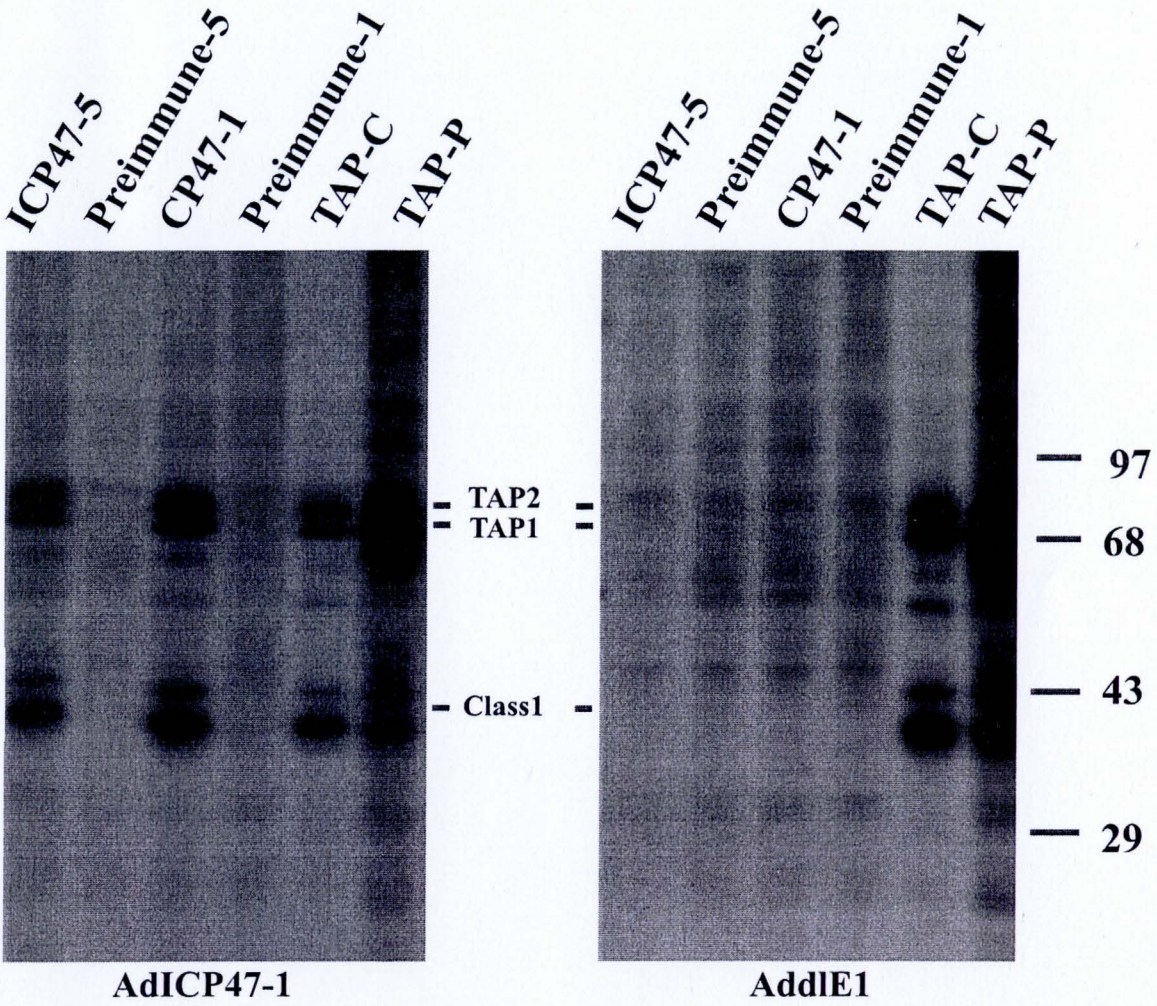
I attemped to determine whether ICP47 interacts directly with the TAP by using immunoprecipitation experiments. Other investigations had previously established an association between TAP molecules and the class I complex, by using immunoprecipitation reactions under very mild lysis conditions involving the detergent digitonin (Ortmann *et al.*, 1994 and Suh *et al.*, 1994). We hypothesized that under similar detergent conditions it could be possible to identify an association between ICP47 and the TAP complex if one existed.

To this end, HeLa cells were first induced with IFN-γ to enhance the expression of

proteins of the MHC class I pathway. I found that HeLa cells were a good choice for these experiments because they produced relatively high levels of MHC proteins after induction with IFN-γ. Induced cells were infected with one of two adenovirus vectors including AdICP47-1 or AddlE1. AdICP47-1 is a replication defective adenovirus vector which exclusively expresses ICP47-1 from a US12 gene inserted into its E1 region, while AddlE1 is the corresponding control vector that does not express ICP47 (York *et al.*, 1994). After radiolabelling the cells and immunoprecipitating the cell lysates a physical interaction of ICP47 and the TAP complex was discovered.

Two different polyclonal sera produced against ICP47 called anti-CP47-1 (directed to a terminal peptide of ICP47) and anti-ICP47-5 (directed to the entire protein produced in bacteria) co-precipitated two pairs of bands with relative molecular weights of 70 kDa and 43 kDa (Figure 3). This interaction was specific as doublet bands were not seen when preimmune serum was used. The 70 kDa size of the larger doublet corresponds to the size of the TAP units which were precipitated directly by using anti-TAP antibodies (Figure 3). The smaller doublet of 43 kDa was identical to the heavy chain of the class I complex immunoprecipitated using anti-MHC class I antibodies. In digitonin lysates of HeLa cells infected with AddlE1 there were noTAP or class I proteins which confirmed that the expression of ICP47 was necessary.

Figure 3: ICP47 associates with the TAP complex. HeLa cells were first treated with IFN-γ and then infected with AdICP47-1 or AddlE1 using 100 pfu/cell. After 36 h of infection, cells extracts were made using buffer containing 1% digitonin in 150 mM NaCl, 50 mM Tris (pH7.5), 2 mg/mL BSA and 0.5 mM PMSF. Cell extracts were incubated with anti-ICP47 sera, preimmune sera or anti-TAP antibodies. All immunocomplexes were precipitated using protein A-Sepharose. The immunoprecipitated proteins were subjected to SDS-PAGE using 10% DATD-crosslinked acrylamide gels.


ICP47-5
Preimmune-5
CP47-1
Preimmune-1
TAP-C
TAP-P
TAP2
TAP1
Class1
97
68
43
29
AdICP47-1
AddlE1

Additionally, it was possible to coprecipitate ICP47 using anti-TAP-P and anti-TAP-C antibodies. ICP47 is approximately 10 kDa in size. Faint 10 kDa bands were coprecipitated by both anti-TAP antibodies from cells infected with AdICP47-1, but not from AddlE1-infected cells (Figure 4). A 14 kDa band that appears in the anti-TAP lanes and anti-ICP47 lane represents β2M - the light chain of the MHC class I complex. This data when taken together with the coprecipitations of ICP47 from anti-TAP antibodies further support the association of ICP47 and the TAP complex.

1.2.b Sequential Immunoprecipitation of TAP and MHC Class I by Anti-ICP47 antibodies.

Sequential immunoprecipitation was used to confirm the identity of proteins that associated with ICP47. In this procedure, proteins immunoprecipitated with anti-ICP47 antibodies were eluted from the anti-ICP47 antibodies and reimmunoprecipitated with either anti-TAP-P or anti MHC class I (HC10) antibodies. HC10 antibodies recognize denatured MHC class I heavy chain molecules and the anti-TAP antibodies were prepared against a TAP-1 peptide. The smaller member of the 70 kDa doublet was identified as the TAP1 subunit (Figure 5a) and the coprecipitating 43 kDa doublet was precipitated by the anti-class I heavy chain and antibody HC10 (Figure 5b). The doublet in the range of 43 kDa was related to differences in glycosylation or other posttranslational modifications of the MHC class I heavy chain protein. Therefore, the association of ICP47 and the TAP-class I complex was confirmed.

Figure 4: The TAP complex coprecipitates ICP47. A fraction of the samples from the immunoprecipitation reactions described in Figure 3 were subjected to SDS-PAGE on a 16% DATD acrylamide gel to demonstrate the presence of ICP47, that coprecipitated with TAP transporter. The 14 kDa protein is β2-microglobulin, the light chain of the MHC class I complex.

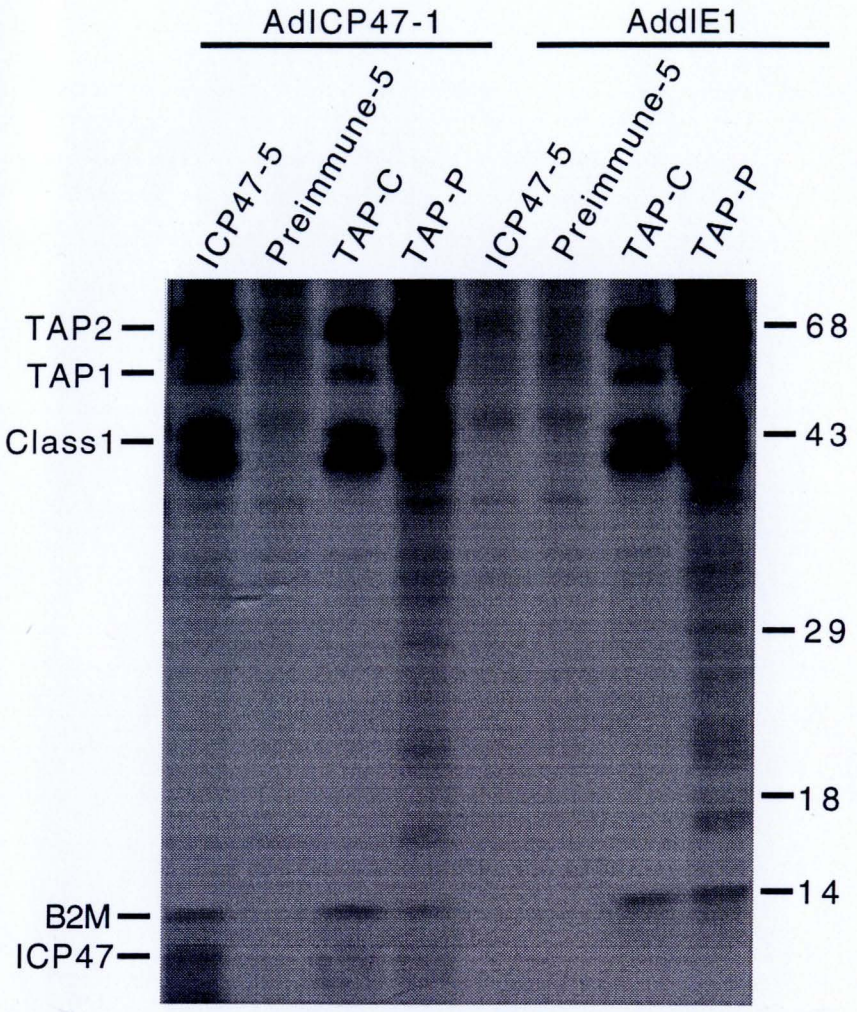
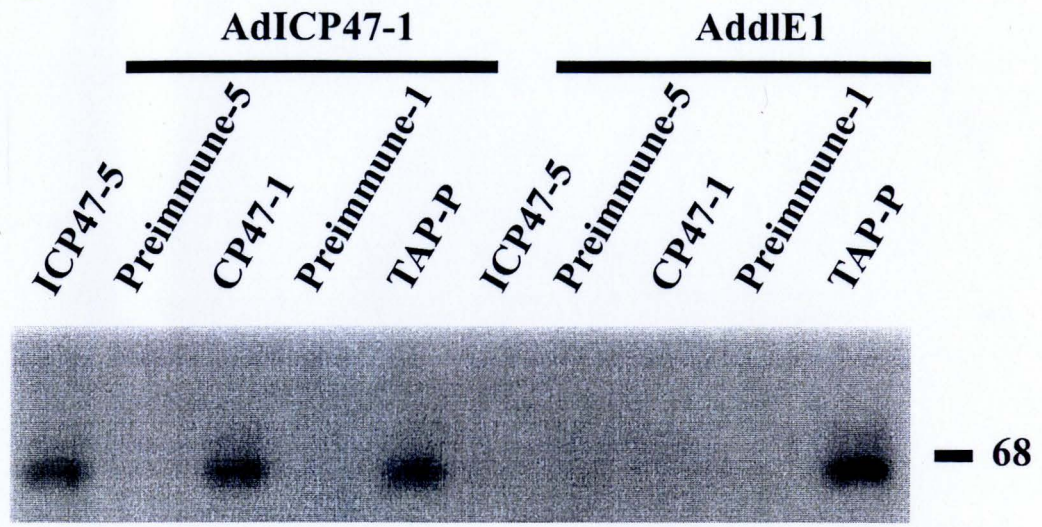
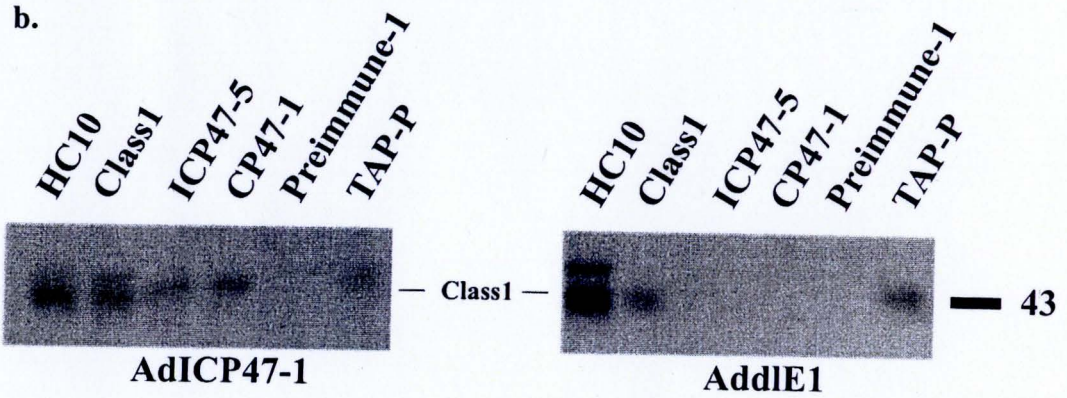

AdICP47-1
AddIE1
ICP47-5
Preimmune-5
TAP-C
TAP-P
ICP47-5
Preimmune-5
TAP-C
TAP-P
TAP2
TAP1
Class1
B2M
ICP47
68
43
29
18
14

Figure 5 a, b: The identity of the TAP subunits and class I heavy chain was confirmed by sequential immunoprecipitation reactions. First, the immune complexes were precipitated as described in Figure 3 and then the precipitated proteins were disassociated by boiling for 5 min in an equal volume of 50 mM Tris buffer (pH6.8) containing 2% SDS and 600 mM β–mercaptoethanol. Samples were diluted with 1% digitonin buffer, immunoprecipitated with either anti-TAP-P or HC10 antibody specific against denatured class I heavy chain and finally separated by SDS-PAGE, using 10% DATD-crosslinked acrylamide gels. The gels were exposed to film for 6 days.

a.


AdICP47-1
AddlE1
ICP47-5
Preimmune-5
CP47-1
Preimmune-1
TAP-P
ICP47-5
Preimmune-5
CP47-1
Preimmune-1
TAP-P
68

b.

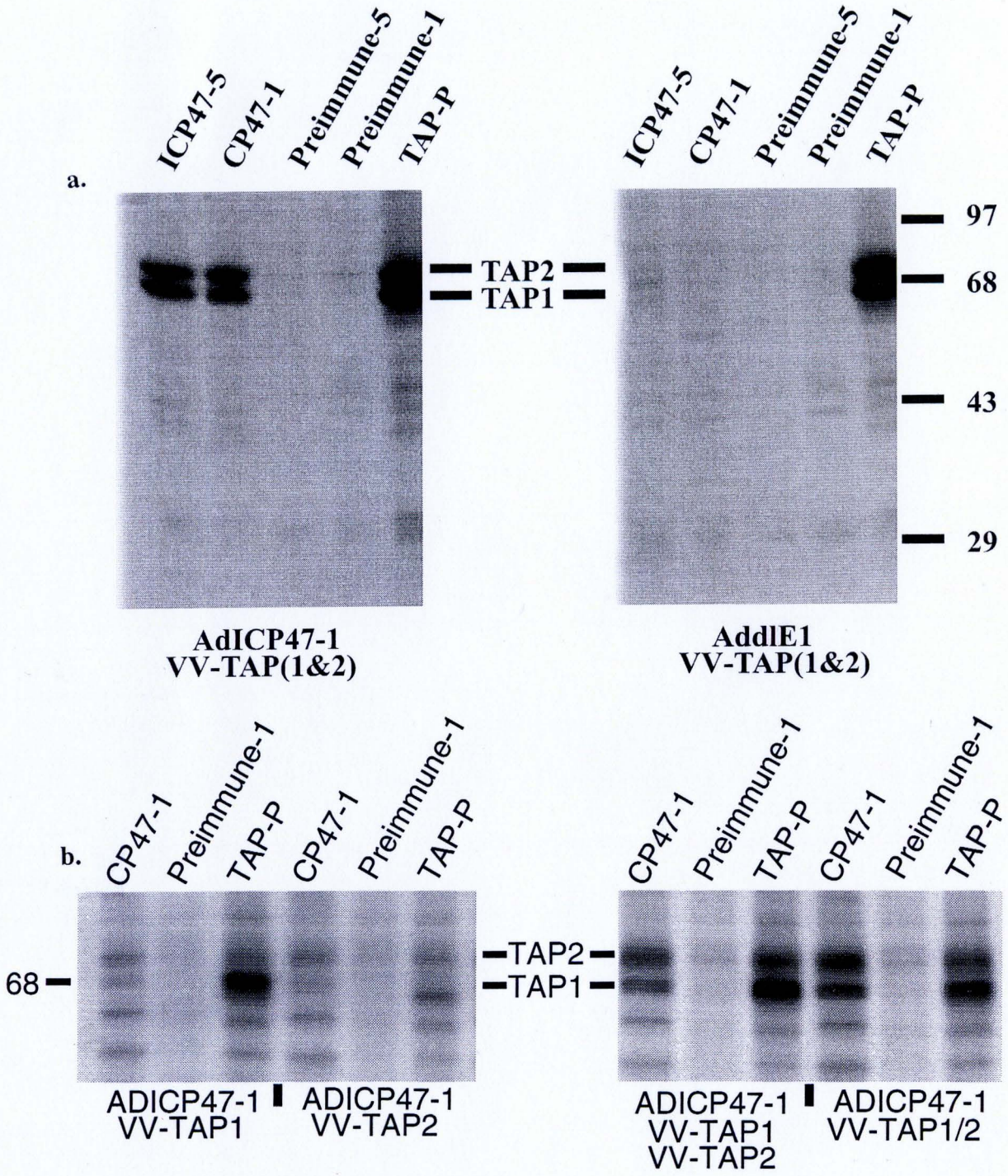

HC10
Class1
ICP47-5
CP47-1
Preimmune-1
TAP-P
HC10
Class1
ICP47-5
CP47-1
Preimmune-1
TAP-P
Class1
43
AdICP47-1
AddlE1

1.2.c Requirement for TAP1 and TAP2 for ICP47 Binding.

It was not clear to us whether ICP47 was associating with one or both of the TAP subunits or with the class I heavy chains as both the 70 kDa and 43 kDa proteins coprecipitated with anti-ICP47 antibodies. To explore the nature of this association and determine whether both TAP1 and TAP2 were necessary, we used vaccinia vectors to express TAP1, TAP2 or both. A vaccinia vector, VV-TAP1&2, was used to express both TAP subunits. HeLa cells were infected with AdICP47-1 or its control AddlE1 and then coinfected with VV-TAP1&2. Digitonin extracts of these coinfected HeLa cells were immunoprecipitated with anti-ICP47 antibodies and again the pair of 70 kDa proteins coprecipitated with ICP47 (Figure 6a). However, there were much lower or even undetectable amounts of the class I heavy chain coprecipitating with the TAP subunits. This is likely due to the fact that ICP47-1 and TAP have been overexpressed in the cells and suggests that ICP47 interacts directly with the TAP complex rather than through class I complexes.

To further elucidate the association of ICP47 and the TAP complex and to determine which subunit facilitated the interaction, HeLa cells were once again infected with AdICP47-1 or its control AddlE1 then coinfected with vaccinia vector that expressed either TAP 1 (VV-TAP1), TAP2 (VV-TAP2) or both subunits in concert (VV-TAP1&2). It was not possible to coprecipitate either TAP1 or TAP2 using anti-ICP47 antisera when the coinfection expressed ICP47 and only one of the TAP subunits (Figure 6b).

Figure 6: An association between ICP47 and the TAP complex is dependent on the co-expression of both TAP subunits. HeLa cells were infected with AdICP47 (or AddlE1) as described in Figure 3 and then coinfected with the vaccinia vectors (VV) expressing one or both TAP subunits for 10 h using 10 pfu/cell. After 6 h of co-infection, the cells were radiolabelled with ^{35}S-methionine and ^{35}S-cysteine for 4 h, then immunoprecipitated and separated by SDS-PAGE on 10% DATD acrylamide gels as described in Figure 3. *a.* The VV-TAP1&2 was used to overexpress both TAP subunits in cells infected with AdICP47-1 or AddlE1 to show that ICP47-1 was associating with the TAP complex and not with one of the class I subunits. *b.* To further characterize the TAP-ICP47-1 interaction and determine which of the TAP subunits was binding to ICP47-1, each of the TAP subunits was expressed individually in HeLa cells. Thus, AdICP47-1 infected HeLa cells were co-infected with VV-TAP1, VV-TAP2, VV-TAP1&2 or both VV-TAP1 and VV-TAP2 vectors, then cell extracts were immunprecipitated using C47-1, preimmune or TAP-P antibodies.


a.
ICP47-5
CP47-1
Preimmune-5
Preimmune-1
TAP-P
TAP2
TAP1
97
68
43
29
AdICP47-1
VV-TAP(1&2)
AddlE1
VV-TAP(1&2)
b.
CP47-1
Preimmune-1
TAP-P
68
TAP2
TAP1
ADICP47-1
VV-TAP1
ADICP47-1
VV-TAP2
ADICP47-1
VV-TAP1
VV-TAP2
ADICP47-1
VV-TAP1/2

However, when cells were coinfected with VV-TAP1 and VV-TAP2, anti-ICP47 serum precipitated the TAP complex. Thus, it is clear that ICP47 requires the expression of both subunits in concert to form an association with the TAP complex.

Furthermore, these data support the notion that the N-terminus of ICP47 is associating with the TAP. Anti-CP47-1 antibodies recognize and bind to a C-terminal peptide sequence of ICP47, and this binding might prevent the C-terminus from interacting with the TAP subunits.

I.3 DISCUSSION

Previous investigations had suggested that there was a high ratio of CD4+ to CD8+ T lymphocytes reactive with herpes simplex virus *in vivo* (reviewed in Schmid *et al.*, 1992; reviewed in York and Johnson, 1994). In addition, HSV-infected fibroblasts had been shown to acquire resistance to lysis by CD8+ CTL (reviewed in York and Johnson, 1994). The bias in CD4+ T cell populations and resistance to CD8+ CTL remained a mystery until a recent study described the effects of an HSV immediate early gene product, ICP47 (York *et al.*, 1994). This study determined that ICP47 was both necessary and sufficient to cause retention of class I molecules in the ER so that the display of viral peptide antigens on MHC class I proteins was inhibited. MHC class I molecules complexed with antigenic peptides are essential to activate CD8+ T lymphocytes (Rammensee *et al.*, 1993) which in turn affect the destruction of HSV compromised cells. Thus, the inhibition of the MHC class I antigen presentation pathway appears to be an effective means by which HSV evades CD8+ T cell

immune responses. It also accounts for the predisposition of activated HSV-specific CD4+ T cell populations as CD4+ T lymphoctyes recognize MHC class II complexes.

But where in the MHC class I pathway was ICP47 acting? There are at least four places in the MHC class I pathway that could theoretically be inhibited by ICP47. The first site is at the proteosome - the source of most antigenic peptides. Without peptides, MHC class I molecules cannot achieve their correct conformation for subsequent export to the cell surface (reviewed in Abbas *et al.*, 1994). Preliminary studies, however, did not identify a physical association of ICP47 with the subunits of the proteosome or show a functional effect of ICP47 blocking the degradation of antigens into peptides (R. Tomazin, D. C. Johnson and M. A. Recksteiner, personal communication). It appeared that ICP47 did not block the proteosome.

A second potential site which could be inhibited by ICP47 is a shuttle protein that might act to transport peptides from proteosomes to TAP complexes in the membrane of the ER. If ICP47 could block such a shuttle then it may competitively inhibit peptide binding which in turn could result in inefficient peptide translocation and eventual degradation in the milieu of the cytoplasm. Heat shock proteins (HSPs) have the potential to bind peptides (Sargent *et al.*, 1989; reviewed in Srivastava *et al.*, 1994) and there is evidence that such peptides can play a role in MHC class I antigen presentation (reviewed in Srivastava *et al.*, 1994) If HSPs are responsible for peptide translocation, and this has not been established, they probably are not affected by ICP47 as we could not find a physical association between HSP 60 or HSP 70 and ICP47 (P. Jugovic and D. C. Johnson, unpublished data).

Moreover, it is conceivable that ICP47 might have affected the stabilization of the class I heavy chain and β2-M complex by disrupting the interactions of chaperone molecules like calnexin, calreticulin or tapsin. After failing to achieve the correct conformation, MHC class I complexes would be degraded before their export. Interactions between ICP47 and these chaperones were not investigated in this study. Preventing the binding of peptides to the cleft of class I heavy chain could also result in the destabilization of MHC class I molecules. However, we could find no evidence that ICP47 entered the lumen of the ER and thus it is unlikely that ICP47 binds chaperones or precludes peptide binding.

The fourth site at which ICP47 could inhibit the presentation of antigens by the class I pathway is at the TAP complex. This complex is encoded in the MHC gene cluster and is composed of two subunits that translocate peptides from the cytosol to the lumen of the ER (reviewed in Abbas *et al.*, 1994). This heteroduplex represents the minimal functional unit which is necessary for peptide translocation as demonstrated by functional assays using insect microsomes containing human TAP complexes (van Endert *et al.*, 1994). To date, most investigations agree that the TAP is the fundamental means of translocating antigens into the ER for complexing to MHC class I molecules and eventual display to CD8+ T cells (reviewed in Hill and Ploegh, 1995 and York *et al.*, 1996). In fact, patients lacking functional TAP complexes have difficulty in class I antigen presentation and have major defects in immunity (de la Salle *et al.*, 1994). Thus, if the proper functioning of the TAP is disrupted there are serious consequences for the detection and elimination intracellular pathogens and tumours by CD8+ T cells. Hence the TAP complex is an ideal target for an immune evasion strategy

of HSV.

We found that ICP47 associates with TAP and that both TAP subunits are required for ICP47 binding (Figures 3, 4, 5a & b and 6a & b). There at least three ways that ICP47 could inhibit peptide transport by TAP. First, ICP47 may affect hydrolysis of ATP by this complex. TAP is a member of the ATP Binding Cassette (ABC) family of transporters (Kelley *et al.*, 1992). Each TAP subunit has a consensus ATP binding domain (Russ *et al.*, 1995) which facilitates ATP hydrolysis essential for peptide transport but not for peptide binding (reviewed in Hill and Ploegh, 1995; Russ *et al.*, 1995). Second, ICP47 could affect peptide transport by binding to the ATP domains of each TAP subunits. Third, ICP47 could bind and sequester ATP from TAP. However, ICP47 was shown not to bind to the ATP consensus regions of the TAP (Tomazin *et al.*, 1996; Ahn *et al.*, 1996) or to ATP (P. Jugovic and D. C. Johnson, unpublished).

ICP47 might affect the TAP by associating with the peptide binding region. Previous investigations have shown that both TAP subunits are required for peptide transport (Neefjes *et al.*, 1993; Androlewicz and Cresswell, 1994; van Endert et al., 1994) as both subunits interact to form the peptide groove (Androlewicz and Cresswell, 1994; Uebil *et al.*, 1995). The results discussed above have shown that both subunits are required for ICP47 to bind to TAP molecules (Figure 6a & b). Subsequent investigations have shown that ICP47 has a strong affinity for TAP (K_D =5.2X10^{-8}M) (Tomazin *et al.*, 1996; Ahn *et al.*, 1996). The affinity of ICP47 for TAP is approximately 10 to 1000 fold higher than the affinity of peptides for TAP (Tomazin et al., 1996). Moreover, kinetic binding studies have confirmed that

ICP47's association with TAP is stable (Tomazin *et al.*, 1996). These investigations in concert with data from competitive binding studies have demonstrated that full length ICP47 acts as a classical competitor of peptides by associating with the peptide binding groove on the cytosolic face of TAP molecules with a 10 to 1000 fold greater affinity than most peptides (Hill *et al.*, 1995; Tomazin *et al.*, 1996).

The flow of peptides into the ER could be obstructed if ICP47 was fully or partially transported by TAP. TAP has been shown to transport a broad spectrum of peptides that are 8 to 12 amino acids (reviewed in York et al., 1996). ICP47 is 10 times greater in size than most translocated peptides. However, larger peptides (approximately one third the size of ICP47) can be transported by TAP complexes but very inefficiently (Androlewicz and Cresswell, 1994; van Endert *et al.*, 1994). Moreover, much like peptides, ICP47 has no secondary structure based on circular dichroism measurements (unpublished R. Tomazin, A., Edwards and D. C. Johnson). In addition, ICP47 is distributed throughout the cystol in HSV infected cells (York et al., 1994) and therefore has access to the peptide binding site of TAP. Thus, it is conceivable that ICP47 is transported very inefficiently by TAP which consequently could diminish the translocation of other peptides. However, in assays where the membrane topology of the ICP47 binding site was studied, ICP47 was shown to remain confined to the cytosolic face of TAP molecules (Tomazin et al., 1996).

By inhibiting TAP function, ICP47 effectively blocks MHC class I assembly which in turn prevents antigen presentation. The association of ICP47 with the TAP complex has been shown to inhibit TAP function in permeabilized cell peptide transport assays (Hill *et al.*,

1995). In these transport assays, HSV or ADICP47-1vectors were used to express ICP47-1 in human cells that were subsequently permeabilized with streptolysin O and incubated with radiolabelled peptides. As peptides were transported into the ER they were glycosylated and could be recovered by binding to an immobilized lectin, concanavalin A Sepharose. By measuring the amount of radiolabelled peptide recovered, it was possible to determine how much peptide was translocated into the ER by TAP. However, when ICP47 was expressed in human cells, the recovery of radiolabelled peptide diminished, suggesting that TAP function was inhibited.

Furthermore, a recombinant ICP47-1 (rICP47-1) inhibited translocation of radiolabelled peptides in human and mouse cells (Tomazin *et al.*, 1996; Ahn *et al.*, 1996). Human fibroblasts and human B lymphoblastoid cells were inhibited by ICP47 with an IC_{50} of approximately 0.3 uM rICP47-1 (Tomazin et al., 1996; Ahn *et al.*, 1996). Approximately, the same IC_{50} was found in *in vitro* TAP assays using either insect or human microsomes containing human TAP molecules (Tomazin *et al.*, 1996). In contrast, TAP was not inhibited in permeabilized mouse fibroblasts and mouse EL4 lymphoid cells treated with 10 uM of rICP47-1.

To date, very little information is known about the actions of ICP47 in tissues or whole organisms. Most of our *in vivo* investigations have used normal or transformed human fibroblasts and epithelial cells. Attempts to inhibit peptide transport in mouse cell lines have failed using concentrations of rICP47 up to 75 fold greater than those needed to inhibit human TAP (Tomazin *et al.*, 1996). Moreover in recent experiments, rICP47-1 and rICP47-2 did

not inhibit TAP function in primary fibroblasts derived from rats, guinea pigs and rabbits (P. Jugovic and D. C. Johnson, unpublished). Thus, the effects of ICP47 appear to be species specific and possibly limited to primates or mammals more closely related to primates (Tomazin *et al.*, 1996; Ahn *et al.*, 1996; P. Jugovic and D. C. Johnson, unpublished). This specificity has limited our ability to study the effects of ICP47 in animals and specifically on pathogenesis of HSV. Currently our investigations are screening other mammalian primary fibroblasts including canine, porcine, bovine, equine and primate fibroblasts for inhibitory effects of ICP47-1 and ICP47-2. We hope to find a suitable animal model in which to study the effects of ICP47 in tissues and on the immune system of a whole organism.

CHAPTER II: Calcyclin, Another Potential Cellular Target of ICP47

Shortly after discovering that ICP47 was responsible for the inhibition of MHC class I antigen presentation, Ian York found an association between the ICP47 bacterial fusion protein called GSTICP47-1 and a small protein of approximately 10 kDa, that was present in the lysates of different human fibroblasts and epithelial cells but not in B or T cell lines or in 293 cells. After purifying and microsequencing the protein, it was identified as calcyclin.

Calcyclin is a member of the highly conserved, S-100 family of calcium-binding proteins including S-100α, S-100β, p11 and migration-inhibitory factor-related proteins (Dorin *et al.*, 1990). The calcyclin gene has been localized to chromsome 1q21 in humans and to chromosome 3 in mice (Dorin *et al.*, 1990). It is a unique copy gene composed of 3 exons encoding a protein approximately 11 kDa (Ferrari *et al.*, 1987). The promoter controlling calcyclin expression has a TATA box and an enhancer region with strong homology to the SV40 enhancer core (Ferrari *et al.*, 1987). Expression of calcyclin is upregulated by growth and mitogen factors including epidermal growth factor and platelet-derived growth factor but not by insulin (Calabretta, 1986). In addition, IFN–γ does not enhance the expression of calcyclin (unpublished, P. Jugovic and D. C. Johnson).

Calcyclin is very homologous to other S-100 proteins (Calabretta, 1986; Emoto *et al.*, 1992) and is usually found as a homodimer (Emoto *et al.*, 1992). Results from NMR spectroscopy reveal that calcyclin has a symetric homodimeric fold which is unique among

calcium-binding proteins (Potts *et al.*, 1995). The dimerization is mediated by hydrophobic contacts in highly conserved N-terminal residues which could be an important structural paradigm of proteins in the S-100 family (Potts *et al.*, 1995). Calcyclin binds to zinc ions which reduces conformational changes needed for calcium binding (Filipek *et al.*, 1990). The biological activities of calcyclin are thought to be modified by Ca^{2+} and Zn^{2+} (Filipek *et al.*, 1990) and by binding to the sialic acid residues of cellular glycoproteins (Zeng *et al.*, 1991).

The biological roles of S-100 proteins, including calcyclin, are not well characterized. In mice, expression of calcyclin has been identified in proliferating cells of the stomach, brain, testes, prostate, vesicular gland and in whole embryos (Guo *et al.*, 1990). Furthermore, calcyclin expression is elevated in melanomas (Calabretta, 1986) and in lymphoblast, myelogenous and monocytic leukemia cells (Zeng *et al.*, 1991) The expression of calcyclin in rapidly dividing cells suggests that calcyclin may play a role in cell proliferation - perhaps by affecting calcium signal transduction (Zeng *et al.*, 1991). However, specific roles for calcyclin and other S-100 proteins in cell growth have not been determined.

Furthermore, calcyclin may participate in membrane trafficking events mediated by a calcium-dependent association with calcyclin-associated protein (CAP50 or annexin XI), a protein belonging to family of proteins called annexins (Tokumitsu *et al.*, 1992; Minami *et al.*, 1992; Watanabe *et al.*, 1993). Annexins are a group of proteins which often localize to the plasma membrane by binding to anionic phospholipids in a calcium-dependent manner (reviewed in Raynal and Pollard, 1994). This family of proteins may be universal mediators of endocytosis and exocytosis but their exact roles in these membrane trafficking events have

not been well established (reviewed in Raynal and Pollard, 1994). Thus, the interactions between calcyclin and annexin XI may regulate the import and export of proteins.

These properties of calcyclin and the fact that ICP47 binds to it may suggest that calcyclin may serve as part of the MHC class I antigen presentation pathway. The second objective of this thesis was to determine if the binding of ICP47 to calcyclin had an effect on MHC class I antigen presentation and whether calcyclin, itself, is important for the MHC class I pathway. I attempted to determine if calcyclin could interact with ICP47 in cells expressing both proteins by attempting to coprecipitate the proteins from cell extracts. I also attempted to determine whether calcyclin could play a role in the MHC class I pathway by binding peptides.

II.1 MATERIALS AND METHODS

II.1.a Cells.

HeLa cells, 293 cells and normal mouse fibroblasts (derived from Balb/c mice) were passaged in a minimal media or Dulbecco's minimal media supplemented with 10% v/v FCS, 450 ug/mL of glutamine, 100 ug/mL of penicillin, 100 ug/mL of streptomyocin. T1, T2, MR and Jurkat cells were grown in RPMI media supplemented with 10% v/v FCS, 450 ug/mL of glutamine, 100 ug/mL of penicillin, 100 ug/mL of streptomyocin and 25uM beta-mercaptoethanol (βMe). All cell cultures were grown at 37°C in an atmosphère enriched with a 5% CO_2 final concentration.

Figure 7: Identifying cell lines that express the 11 kDa ICP47 associating protein, calcyclin. Approximately $2.0X10^7$ cells from HeLa, 293, T1, T2, MR and Jurkat cells were boiled for 5 min in an equal volume of loading buffer and were subjected to SDS-PAGE on a 16% DATD polyacrylamide gel. All separated proteins were transferred to an immobilon-P membrane. The membrane blots were probed with anti-calcyclin antibodies and visualized with iodinated protein A. Finally, the blot was dried and exposed to X-ray film for 12 h.

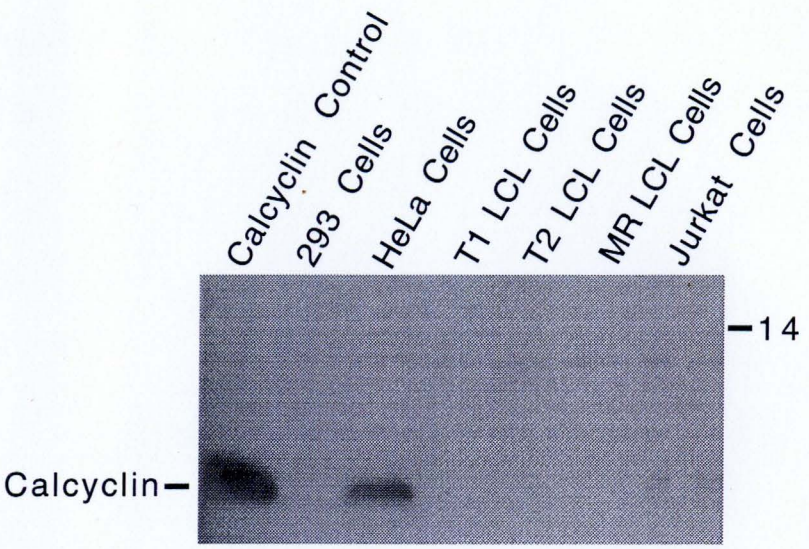
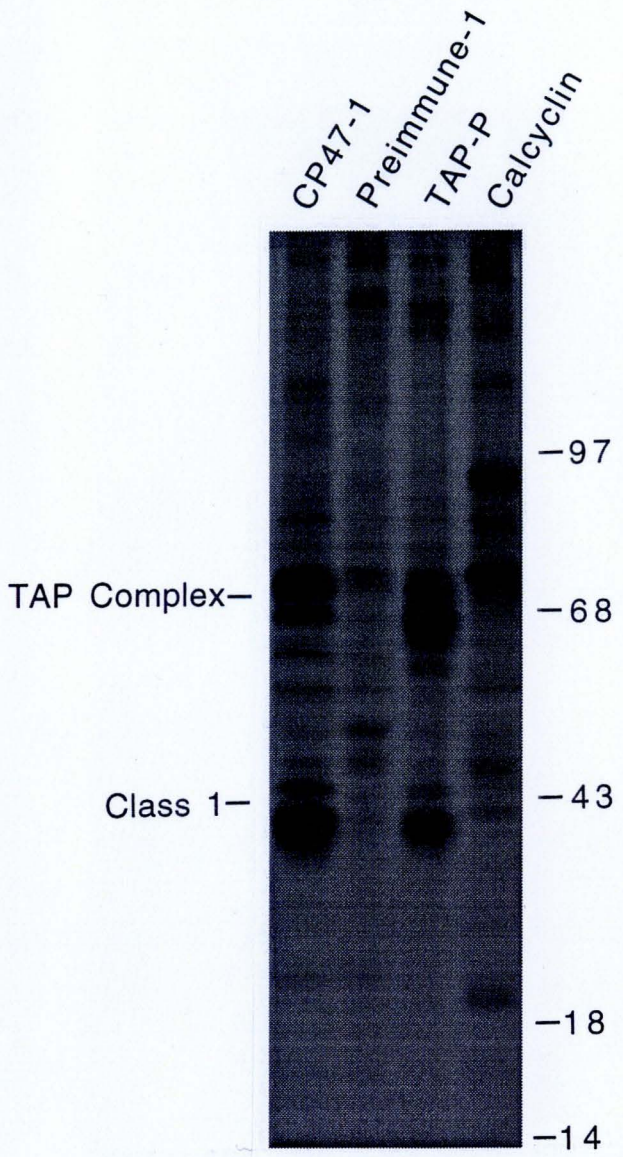

Calcyclin Control
293 Cells
HeLa Cells
T1 LCL Cells
T2 LCL Cells
MR LCL Cells
Jurkat Cells
14
Calcyclin

Figure 8: ICP47 and calcyclin do not coimmunopreciptate from cell extracts. To search for an *in vivo* association, HeLa cells were infected with AdICP47-1, metabollically radiolabelled with ^{35}S-methionine and ^{35}S-cysteine and then lysed with a buffer containing 1% digitonin. The lysates were incubated with anti-CP47-1, preimmune or anti-calcyclin antibodies for 90 min on ice. Next, all immune complexes were precipitated with protein A Sepharose and analysed by SDS-PAGE.

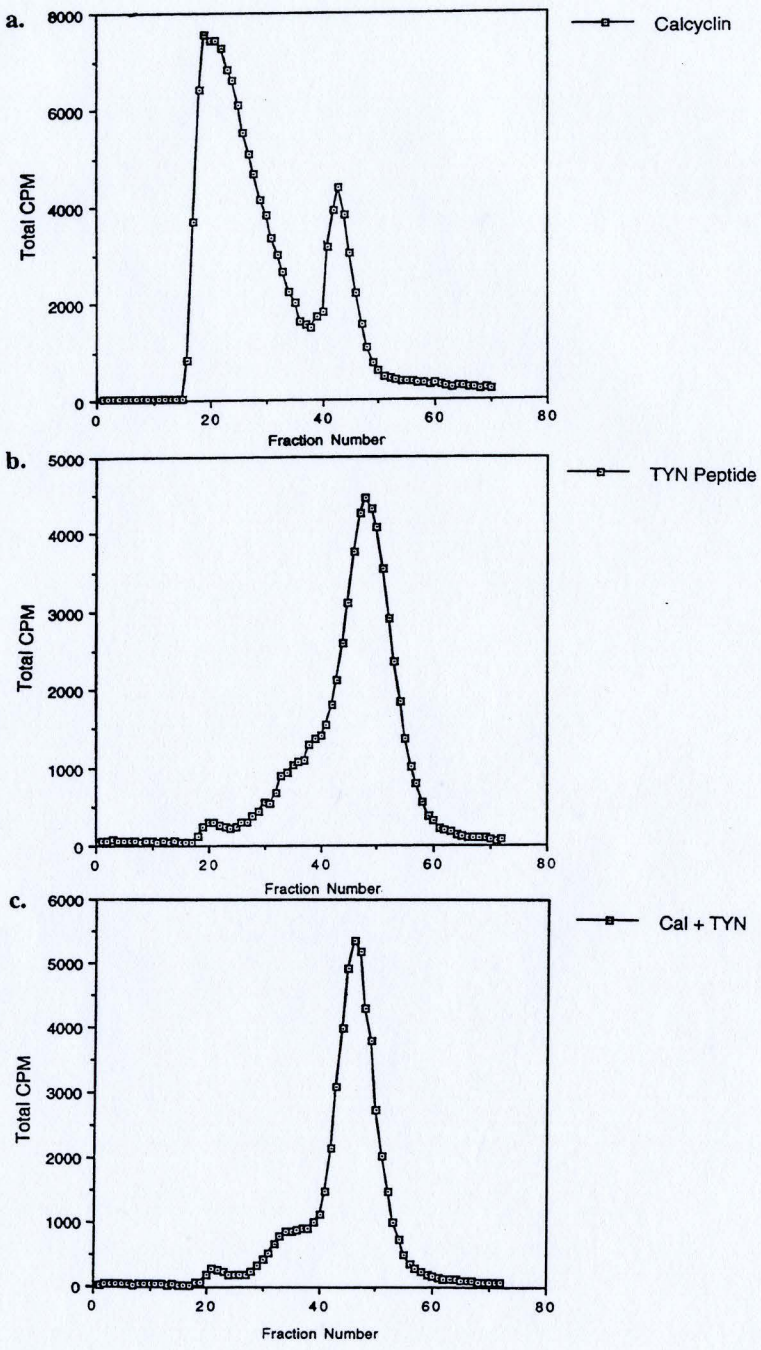

CP47-1
Preimmune-1
TAP-P
Calcyclin
TAP Complex
Class 1
97
68
43
18
14

to both anti-calcyclin and anti-ICP47-5 immunoprecipitates (Figure 8). This is consistent with the notion that viral ICP47-1 and calcyclin do not associate in detergent extracts of cells. This is in contrast to the results of Ian York who found calcyclin can bind to immobilized ICP47 - ICP47-GST bound to gluthathione-Sepharose.

II.2.c Binding of Peptides to Calcyclin.

Our inability to show an association between ICP47 and calcyclin in detergent extracts, did not exclude the possibility that ICP47 binds to calcyclin in cells and that calcyclin was a part of the MHC class I pathway. We hypothesized that calcyclin might function to bind peptides, stabilizing these peptides from nonspecific proteolysis or be involved in peptide translocation from proteosomes to TAP. Gel filtration was used to determine if calcyclin binds peptides. Calcyclin is approximately 10 times the size of a 9 amino acid peptide (TYNRTRALI) used in this experiment, and therefore calcyclin was expected to elute before the peptide in Sephadex G-25 gel filtration chromatography. As seen in Figure 9a and b, radiolabelled calcyclin eluted from this column primarily in fractions 18 to 40, though there was a second smaller peak that eluted in fractions 40 to 50. This smaller peak was probably a degradation product of calcyclin, another protein contaminant or small amounts of free $Na^{125}I$ remaining in the preparation. ^{125}I-peptide eluted in fractions 40 to 50. Radiolabelled peptide and unlabelled calcyclin were combined and incubated overnight at 4°C. If peptides were associating with calcyclin, it was expected that a fraction of the radiolabelled peptides would elute in the fractions 18 to 40 with calcyclin. However, the elution profile of the

Figure 9: Binding of peptides to calcyclin. Calcyclin and the peptide TYNRTALI were iodinated. G25 Sephadex resin was equilibrated in PBS containing 0.1% CHAPS detergent and 0.5 mg/mL of BSA and a column was prepared. a. ^{125}I-calcyclin was chromatographed onto the G25 Sephadex column and 50 200 uL fractions were collected and counted using a gamma counter. b. ^{125}I-peptide was similarily chromatographed onto this column. c. Finally, unlabelled calcyclin was mixed with ^{125}I-peptide, incubated overnight and then chromatographed using the same column.

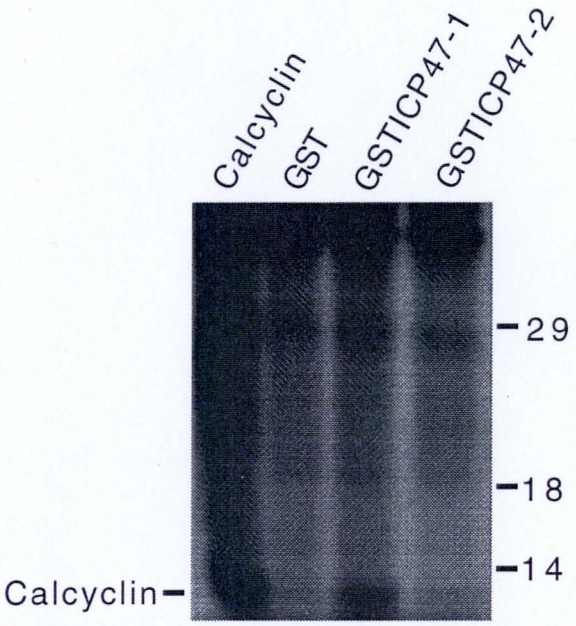

a.
Total CPM
8000
6000
4000
2000
0
0
20
40
60
80
Fraction Number
Calcyclin
b.
Total CPM
5000
4000
3000
2000
1000
0
0
20
40
60
80
Fraction Number
TYN Peptide
c.
Total CPM
6000
5000
4000
3000
2000
1000
0
0
20
40
60
80
Fraction Number
Cal + TYN

radiolabelled peptide did not alter after incubation with calcyclin (Figure 9c). Consequently, we could not establish that calcyclin binds to peptides in the size range of those used in the MHC class I pathway.

II.2.d Establishing a GSTICP47-2 association with calcyclin.

To determine if HSV-2 ICP47 binds to calcyclin, a GST fusion protein in which HSV-2 ICP47 was fused to GST sequences at the C-terminus was purified from bacterial extracts. GSTICP47-2 was tethered to glutathione-Sepharose and incubated with radiolabelled extracts from Hela cells. To our surprise, the GSTICP47-2 did not bind calcyclin under these conditions (Figure 10). In contrast, the GSTICP47-1 did bind to calcyclin. Therefore, although HSV-2 ICP47 inhibits MHC class I antigen presentation, it does not bind to calcyclin. This result sheds doubt on the hypothesis that the binding of ICP47 to calcyclin is important role in the inhibition of the MHC class I antigen presentation pathway.

Figure 10: Calcyclin associates with ICP47-1 but not with ICP47-2. HeLa cells were radiolabelled for 3 h with media containing ^{35}S-methionine and ^{35}S-cysteine. The cells were then lysed with a buffer containing 0.2% octylglucoside. GST, GSTICP47-1 and GSTICP47-2 proteins were prepared from bacterial extracts and bound to glutathione-Sepharose beads. The extracts from radiolabelled HeLa cells were incubated with GST and the GSTICP47 fusion proteins for 1 h and washed with a buffer containing 0.2% octyglucoside. Calcyclin was immunoprecipated from Hela cell extracts using a polyclonal antiserum recognizing calcyclin (Minami *et al.*, 1992). Finally, the fusion protein samples were boiled with SDS loading buffer for 5 min and the eluted proteins were separated on a 16% DATD-crosslinked SDS-polyacrylamide gel.


Calcyclin
GST
GSTICP47-1
GSTICP47-2
29
18
14
Calcyclin

II.3 DISCUSSION

Calcyclin is a member of the S100 family of calcium binding proteins and has a predicted weight of 11 kDa (Ferrari *et al.*, 1987). It has been found in both cytoplasmic and nuclear fractions in human and mouse primary and tumor cells lines (Calabretta, 1986; Guo *et al.*, 1990; Zeng *et al.*, 1991). However, our investigations found that some cell lines including 293s, T1 LCLs, T2 LCLs, MR LCLs and Jurkat cells did not express calcyclin. Calcyclin expression can be upregulated by mitogens like epithelial growth factor and platelet derived growth factor but not by IFN–γ. Very little is known about the functions of calcyclin but it has been shown to associate with some members of the annexin family, specifically annexin XI, which is thought to function in cell trafficking events such as exocytosis and perhaps endocytosis (Minami *et al.*, 1992; Watanabe *et al.*, 1993; Mamiya *et al.*, 1994). The exact role calcyclin plays in cell trafficking is uncertain but its interactions with annexin XI are mediated in a calcium dependent manner as facilitated by two EF hand structures (Minami *et al.*, 1992; Watanabe *et al.*, 1993). Since MHC class I must traffick to the cell surface to present peptide antigens, it is conceivable that calcyclin and annexin XI are involved in antigen presentation.

We hypothesized that calcyclin might play a role in the class I antigen display pathway because calcyclin associated with a GST fusion protein containing ICP47 sequences and ICP47 inhibits the class I pathway. Furthermore, because calcyclin may have functions related to trafficking of intracellular components we investigated whether calcyclin could bind peptides. However, results from two types of experiments argued against the ideas that

calcyclin binds peptides or associates with ICP47 *in vivo*, at least in detergent extracts of cells. However, another cellular target for ICP47, TAP, had already been identified and it was clear that inhibition of TAP blocked antigen presentation.

When exposed to IFN γ, the expression of most genes belonging to the MHC family is augmented. As was mentioned above, HeLa cells express calcyclin and upregulate MHC class I genes in response to IFN γ. However, IFN-γ did not increase the levels of calcyclin expressed by HeLa cells (results not shown). This suggests that calcyclin is not part of the family of "MHC-like" genes.

Moreover, if calcyclin did have a biologically significant role in antigen presentation then one would expect to find a stable association between ICP47-1 and calcyclin in infected cells. I was unable to coprecipitate calcyclin and ICP47-1 from digitonin extracts of cells. However, it is conceivable that the detergent conditions used in the immunoprecipitation protocol inhibited or disrupted interaction. It is also possible that the anti-calcyclin or anti-ICP47 antibodies used in these experiments recognized and sterically hindered the relevent epitopes needed for the ICP47-1 and calcyclin interaction.

We also investigated the possibility that calcyclin bound peptides acting as a peptide shuttle. This possibility was based on two important features of calcyclin. First, calcyclin is a soluble protein present in the cytosol where it has access to peptides generated by proteosomes. In addition, calcyclin associates with annexin XI (Tokumitsu *et al.*, 1992; Minami *et al.*, 1992; Watanabe *et al.*, 1993) which could in turn facilitate calcyclin's binding to the membranes of the ER. Based on these characteristics, one could envision calcyclin

facilitating the trafficking of peptides from the proteosome to the cytosolic face of the ER, where peptides would be released and bound by the TAP. It has been suggested that heat shock proteins perform this function (Srivastava *et al.*, 1992), but this has not been solidly established. The results of the gel filtration experiments suggested that calcyclin does not directly associate with antigenic peptides.

After screening several cell lines, I determined that calcyclin expression was extremely low in 293 cells (Figure 7) but present at higher levels in the parental human embryonic kidney (HEK) cells (results not shown). 293 cells were derived by transforming HEK cells with Ad5 DNA (Graham *et al.*, 1977). Steve Primorac, a techician in our laboratory, observed that the majority of the class I complexes were retained in ER in 293 cells. If one assumes that calcyclin is involved in the MHC class I pathway, then the lack of calcyclin could perhaps inhibit the full processing of MHC class I molecules. However, when 293 cells were transfected with the calcyclin gene and calcyclin expression was restored, MHC class I molecules were still retained in the ER. Other investigations have previously shown that annexin XI is not expressed in kidney cells (reviewed in Raynal and Pollard, 1994). Since we didn't reestablish expression of annexin XI and calcyclin together in 293 cells, it is not clear that we have completely tested the hypothesis that calcyclin has a role in antigen presentation which could be affected by ICP47. However, reestablishing calcyclin expression did not, on its own, fix the defects in MHC class I processing in 293 cells.

Finally, we attempted to determine if ICP47-2 could bind to calcyclin. Using a GST fusion protein containing ICP47-2 sequences (similar to the GSTICP47-1 construct), there

was no binding of calcyclin, though calcyclin bound to GSTICP47-1. There is 42% amino acid identity between the coding sequences of ICP47-1 and ICP47-2, mostly near the amino terminal and central regions of the proteins. If calcyclin was a part of the antigen presentation pathway, it would seem likely that ICP47-2 should also retain the same ability to affect calcyclin, since both proteins block the pathway effectively. However, it is possible that ICP47-1 and ICP47-2 block the MHC class I pathway entirely through their effects on TAP. When taken together, these data suggest that the interaction between GSTICP47-1 and calcyclin may be an artifact and have no biological significance with respect to inhibiting MHC class I antigen presentation. However, other experiments are required to determine definitively whether calcyclin plays a role in the MHC class I pathway and whether ICP47 binding blocks calcyclin's role in the pathway.

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