Immune evasion by varicelloviruses : the identification of a new family of TAP-inhibiting proteins

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CHAPTER 1

Introduction

The families of herpesviruses

The general outlook in the herpesvirus field has gradually changed the last 5 years, predominantly due to the discovery of a number of herpesviruses in fish, amphibians and mollusks (1-3). This has led to the recent proposal to create an order of *Herpesvirales* divided into three families: the *Herpesviridae*, the *Alloherpesviridae* and the *Malacoherpesviridae* (4). The *Alphaherpesvirinae*, the *Betaherpesvirinae* and the *Gammaherpesvirinae* still remain the only subfamilies of the *Herpesviridae*.

Besides their large double stranded DNA genome (ranging from 124 kbp to 295 kbp), all members of the *Herpesvirales* share another common property, namely their virion architecture. The DNA genome is found in an icosahedral (T=16) capsid of 115 to 130 nm with a specific portal complex. The capsid is surrounded by a highly ordered structure named tegument that supports the surrounding envelope (5). Beside structural proteins, the tegument contains such molecules as the virion host shutoff (vhs) protein, which functions as soon as the virus enters the cell (6).

At present, 56 complete herpesvirus genomes have been published. The genomes have 70 to 165 genes (but this number may go up to 200 when the larger viruses have been fully sequenced). At least one gene, encoding a DNA packaging enzyme, has a homolog in all families (2). Within the family of *Herpesviridae*, 43 genes are conserved in all members and were obviously inherited from a common ancestor (7). These conserved genes are named ´core´ genes and they encode proteins that function predominantly in DNA replication, capsid formation and capsid egress, or are part of the tegument or envelope. The UL49.5 or UL49A gene that encodes homologs of glycoprotein N (gN) belongs to the ´core´ genes and is thus found in all presently sequenced members of the *Herpesviridae*. Core genes are thought to play a fundamental role in the replication of the *Herpesviridae*, in contrast to the ´non-core´ genes that have a more accessory function (2). The UL41 gene encoding the vhs protein is an example of a ´non-core´ gene that is only found in *Alphaherpesvirinae*.

The last 10 years it has become clear that herpesviruses have developed many mechanisms to counteract the host's innate and acquired immune responses. For example, the gD protein of alphaherpesviruses can postpone apoptosis before the virus enters the cell (8). The vhs protein present in the virion not only down-regulates the host gene expression, it can also specifically counteract the interferon type I response of the host cell (9). For example, the product of the immediate-early gene of bovine herpesvirus 1 ICP0 (infected cell protein 0; BICP0) not only acts on viral promoters, but also inhibits interferon-dependent promoters (10). Herpesviruses have developed many additional mechanisms to evade the acquired immune response, as will be discussed later on. The fact that immune evasion genes belong to the ´non-core´ or ´accessory´ genes may reflect a more extensive form of adaptation to the host defense mechanisms.

Co-evolution of herpesviruses with their hosts

Viruses co-evolve with their hosts through adaptation to the host's cellular processes/pathways that they exploit. The best adapted virus will successfully maintain co-existence with its host (reservoir) and it will consequently ensure the survival of both. For example, the ability to escape their host's immune response represents an advantageous evolutionary adaptation from the virus's perspective.

The common ancestor of all the *Herpesvirales* is thought to have existed 570-505 million years ago (mya), around the time the first vertebrates appeared (4). The most recent common ancestor of the *Herpesviridae* is estimated to have emerged 400 mya (11). This is

long before the appearance of the most recent ancestor of the mammals (around 220 mya). The phylogenetic trees of the *Herpesviridae* and their hosts have roughly the same form, implying a co-evolution between viruses and hosts. This is particularly convincing for clades of the *Alphaherpesvirinae* and their hosts (12). This synchronous development implies a co-adaptation between the virus and its host.

Co-evolving with their hosts, herpesviruses have acquired elaborate strategies for evading the host's immune response. For this purpose, herpesviruses have exploited multiple cellular pathways, including the MHC class I antigen-processing pathway. This appears to be the principal means by which herpesviruses interfere with CD8^{+} T cell function, and it is certainly the best characterized until now. Numerous herpesviruses-encoded proteins that interfere with MHC class I antigen presentation have been described and it appears that these viral proteins are able to interfere at essentially every step of this pathway (reviewed in 13 and 14).

The MHC class I antigen processing and presentation pathway

T-lymphocytes or T-cells recognize antigenic peptides presented on the surface of host cells by MHC molecules (reviewed in 15). T-cells owe their specificity to the T-cell receptor (TCR). The TCR interacts with two types of MHC molecules: MHC class I and MHC class II. TCR-mediated recognition of class I and class II molecules works in combination with one of two co-receptors, CD8 or CD4, respectively.

MHC class I molecules are type I membrane glycoproteins associated with the small subunit β2microglobulin (β2m) found on all nucleated cells in vertebrates. MHC class I molecules mainly present peptides of endogenous proteins to CD8^{+} cytotoxic T-cells that scan for displayed peptide repertoire (figure 1). Detection of non-self peptides derived from endogenous pathogens or mutated proteins will trigger the elimination of antigen-presenting cells.

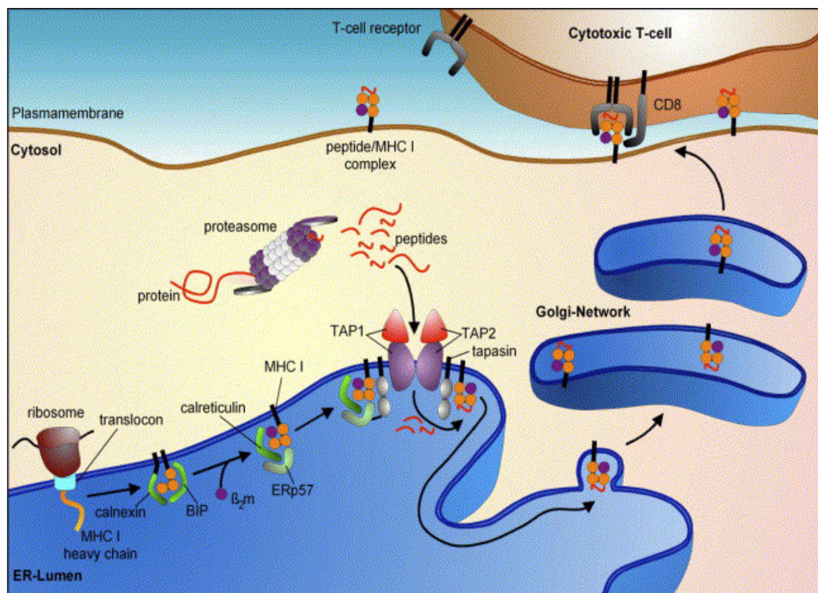

Fig. 1. Model of the MHC class I antigen presentation pathway. MHC class I molecules folding and complex formation and assembly of peptide loading complex (see text for further description). *Adopted from Abele & Tampe FEBS Lett. 2006 580:1156-1163*

MHC class II molecules exist as heterodimers of two transmembrane glycoprotein subunits, the α and β chain and are found only on the cell surface of specialized immune cells (dendritic cells, monocytes, macrophages, B-cells and thymic epithelial cells). They present peptide fragments of exogenous proteins. $CD4^{+}$ T helper cells recognize peptide-MHC class II complexes and stimulate B-cells to produce antibodies.

Generation and processing of antigenic peptides

Most MHC class I molecules acquire their peptide cargo in the ER, and most of these peptides are generated from cytosolic substrates. Proteins can reach the cytosol either by being synthesized by ribosomes or by penetrating the plasma membrane through internalization. Endogenous peptides appear to originate primarily from defective forms of newly synthesized proteins (termed DRiPs, for defective ribosomal products) (16) that are rapidly degraded by the ubiquitin-proteasome pathway (17-19). In general, ubiquitination targets misfolded proteins as well as DRiPs for degradation by the proteasome (20). Proteasomes are large multi-subunit complexes with protease activity (21, 22). They are responsible for the bulk turnover of proteins in the cell, therefore maintaining homeostasis between anabolic and catabolic pathways (15). A direct function for proteasomes in the generation of antigens for class I presentation has been demonstrated to be essential for this process (18, 23, 24). Additional cytosolic proteases can further cut and trim proteasome-generated peptides (e.g. tripeptydil peptidase II – TPPII; 25). Treatment of cells with interferon-γ or its production due to inflammation stimulates the formation of immuno-proteasomes, where the catalytically active subunits are replaced by LMP2, LMP7, and MECL1 (low molecular mass polypeptides 2 and 7; multi-catalytic endopeptidase complex-like 1) (26, 27). The resulting exchange gives a higher quantity of antigenic peptides with suitable carboxyl-termini and of the approximate length (6 to 11 amino acids) preferred by MHC class I molecules (28). However, only a fraction of these peptides makes its way to the lumen of the ER (29). Through the Transporter associated with Antigenic Processing (TAP)-mediated transport (30), peptides reach the endoplasmic reticulum (ER) (figure 1) where they subsequently face the aminopeptidase associated with antigen processing (ERAAP, also named ER aminopeptidase 1 ERAP1), which is capable of trimming the amino-termini of certain peptides. This increases the binding affinity of these peptides to MHC class I and the fraction of ER luminal peptides available for stable presentation (31, 32).

MHC class I/peptide-loading complex assembly

Proper folding and assembly of MHC class I/β2m molecules with peptides is supposed to be controlled by a number of accessory proteins and chaperones that are, to a varying degree, dedicated to this process (figure 1). The present model is that MHC class I heavy-chain molecules (approximately 45 kDa) are translated into the ER and glycosylated co-translationally. Addition of the single N-linked glycan on class I heavy-chain molecules serves as a point of recognition for the ER membrane chaperone calnexin (the ER-resident lectin) (33). Together with calnexin, the immunoglobulin-binding protein (BiP) interacts transiently with the newly synthesized heavy chain. Both chaperones promote folding and assembly of the class I heavy-chain with soluble β2m (12 kDa). Calnexin is replaced by the ER-resident lectin calreticulin (34) after the heterodimer of heavy chain and β2m has achieved its proper conformation (figure 1).

Another ER chaperone, the thiol-oxidoreductase ERp57, was found to be involved in the maturation of class I heavy-chain/β2m molecules. ERp57 assists with the disulfide bond formation probably at the early stage of class I heavy-chain folding (34). At the later stages of assembly, MHC class I-associated ERp57 can be found as part of the peptide-loading complex where ERp57 is covalently bound to tapasin. The ERp57-tapasin conjugate is dependent on the presence of MHC class I (35, 36).

Peptide loading of class I molecules does not proceed efficiently unless the calreticulin/class I heavy-chain/β2m/ERp57 complex binds to tapasin (figure 2), an MHC class I chaperone

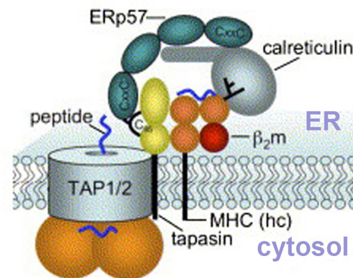

Fig. 2. Model of peptide-loading complex (PLC) (see text for further description). *Adapted from Abele & Tampe FEBS Lett. 2006 580:1156-1163*

required for the association of the complex with TAP (37-40). Tapasin was found to be in concurrent association with the class I heavy-chain and both subunits of TAP (TAP1 and 2). This multi-component association results in the formation of the MHC class I peptide-loading complex (PLC; figure 2) that facilitates the binding of the antigenic peptide within the peptide-binding groove of the heavy chain. Stable MHC class I/β2m/peptide complexes are formed and released from the loading complex to be further translocated via the Golgi apparatus to the cell surface (figure 1) (41, 42).

Key players of peptide-loading complex assembly: tapasin and TAP

Essential building elements of the peptide-loading complex are represented by two TAP subunits (TAP1 and 2) associated with four tapasin and four MHC class I molecules (43).

Tapasin

Tapasin, an ER-resident type I membrane glycoprotein, is the central stabilizer of the peptide-loading complex and interacts with almost all proteins involved in the formation of this multimeric structure (44). The N-terminal domain of tapasin (the ER luminal part) is necessary for the interaction with MHC class I, calreticulin and ERp57, whereas the C-terminal domain (mainly the transmembrane region) of tapasin is sufficient for the interaction with TAP (45). By having discrete binding sites at opposite ends of the molecule, tapasin bridges TAP and class I molecules, thereby facilitating an increase of the local concentration of peptides for MHC class

I loading (46, 47). The MHC class I molecules deprived of peptides will remain associated with tapasin, which retains unloaded molecules within the ER (48). In the TAP-deficient human cell line T2, the association of tapasin with class I molecules appears to be independent of tapasin/TAP interaction. Furthermore, the MHC class I molecules expressed in the tapasin-deficient human cell line 721.220 (37, 38) or in tapasin knockout mice (49, 50) do not interact with TAP. In these cell lines, there is a reduced surface expression of peptide-loaded MHC class I (51) as well as a decrease in cell surface stability of peptide-bound class I molecules (38, 52). These findings signify an important role of tapasin in MHC class I-peptide assembly (53). Surprisingly, the binding affinities of the MHC class I-associated peptides are not altered in the presence or absence of tapasin (46), but the variety of bound peptides is increased when tapasin is associated with MHC class I molecules. Thus, cells containing functional tapasin exhibit improved MHC class I-peptide complex formation and stability, which in its turn provides enhanced levels of MHC class I complexes at the cell surface.

Transporter associated with Antigen Processing (TAP)

TAP belongs to the large family of ABC transporters (54, 55). The family of ABC transporters is defined by their homology within the ATP-binding cassette (ABC) region (56). This region (also known as the nucleotide-binding domain) contains three highly conserved motifs called Walker A and B motifs (figure 3B) as well as the C loop (also known as the ABC signature motif). The Walker A and B motifs are present in many ATP-binding proteins, whereas the C loop is characteristic for ABC transporters. Generally, all ABC transporters consist of a four-domain structure with two hydrophobic transmembrane domains (TMDs) and two hydrophilic nucleotide-binding domains (NBDs). The two NBDs located in the cytoplasm are involved in ATP binding and/or hydrolysis. The NBDs act as “motor domains”, since they convert the chemical energy of ATP hydrolysis into mechanical work. This process induces conformational changes within the TMDs to enable the passage of substrates.

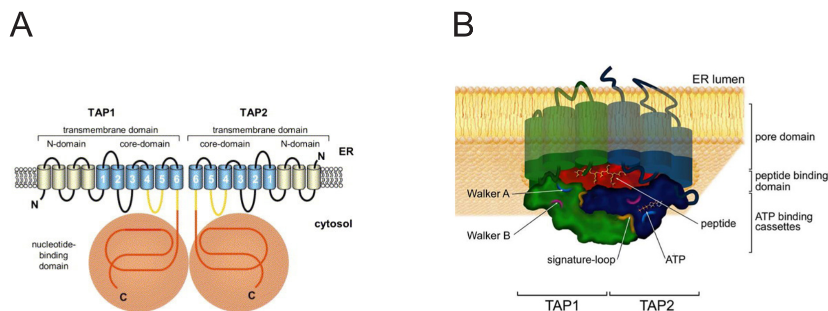

Fig. 3. Model of transporter associated with antigen processing TAP. A Schematic model of the TAP complex. TAP forms a heterodimer composed of TAP1 and TAP2. Each subunit comprises an N-terminal transmembrane domain and a C-terminal, cytosolic NBD (red). The transmembrane domain can be subdivided into a six helices containing core domain and an NH2-terminal extensions (tapasin-binding domains) of four and three helices for TAP1 and TAP2, respectively. Beside the translocation pathway, the TMDs also form the peptide-binding region (orange). *Adapted from Abele & Tampe FEBS Lett. 2006 580:1156-1163.* B TAP forms a transmembrane pore in the ER. The pore is followed by a peptide binding domain located at the cytoplasmic side. The structure is concluded by the two ATP-binding domains (see text for further description). *Adapted from Reits et al. Immunol Today 2000 21:518-600.*

Structural organization of the TAP complex

The peptide transporter TAP is a heterodimer composed of TAP1 (human ABCB2; calculated mol. mass 81 kDa) and TAP2 (human ABCB3; calculated mol. mass 75 kDa), each consisting of an N-terminal transmembrane domain (TMD) and a C-terminal, cytosolic nucleotide-binding domain (NBD) (figure 3A) (57, 58). Both subunits are essential and sufficient for antigen processing (59, 60). TAP is localized in the ER and *cis*-Golgi (61). Neither an N-terminal signal peptide for ER import nor a retention signal for any subunit is known as yet.

The topology of TAP is still under study. Hydrophobicity analysis and sequence comparison with members of the ABC-B subfamily imply that TAP1 and TAP2 contain 10 and 9 transmembrane segments (TMs), respectively (figure 3A). Only the last six transmembrane segments of both subunits show sequence homology to other ABC transporters. Interestingly, a peptide transporter missing the N-terminal domains of four and three putative transmembrane segments of TAP1 and TAP2, respectively, is still active with respect to peptide binding and transport (62). The N-terminal extensions of both subunits are required for the interaction with the transmembrane domain of tapasin (44, 63). However, the precise binding site(s) for tapasin is (are) still unknown (58).

Tapasin is usually present within a functional TAP1 and TAP2 complex and plays an important role in the assembly and stability of TAP. However, it has also been found to bind TAP1 or TAP2 subunits individually. Generally, tapasin stabilizes the TAP1 protein, resulting in a higher steady-state protein expression level of TAP (64). In tapasin-deficient cells or cells expressing tapasin mutants, which are unable to bind to TAP, the steady-state level of TAP is decreased up to 100-fold (44). Therefore, by promoting the TAP heterodimer assembly and stabilizing the peptide-binding site of TAP, tapasin enhances the peptide translocation rate acting as a “chaperone” for TAP (44, 45, 51, 64, 65).

The peptide-binding region of TAP is localized in the last cytosolic loop and a 15-amino acid extension of the last putative transmembrane segments of TAP1 and TAP2 (66). The NBDs of TAP convert the energy of ATP hydrolysis to peptide transport. For such multi-domain machinery, a multi-step process is plausible, where peptide and ATP binding, ATP hydrolysis, and peptide transport occur in an ordered manner. As known from biochemical studies on isolated NBDs, ATP-binding induces a conformational change of the NBD, enabling the dimerization of the catalytic domain (figure 3B), which is a prerequisite for ATP hydrolysis and substrate transport (67-69).

TAP also fulfils the function of a chaperone for the other members of the loading complex. The assembly of class I molecules, ERp57, calreticulin, and tapasin is less efficient in the absence of TAP than in its presence (70). Moreover, class I adopts a different conformation in the absence of TAP than in its presence (71). TAP may also serve as a relay to transmit a signal for dissociation of peptide-loaded MHC class I complexes (72).

How does TAP translocate peptides across the ER membrane?

As already stated, TAP is composed of two subunits, TAP1 and TAP2. Both subunits contain a multi-membrane spanning domain forming the actual pore and also encompassing the peptide-binding site. In addition, TAP1 and TAP2 each carry a hydrophilic nucleotide-binding domain (figure 3B) (reviewed in 73-75). The actual position of TAP1 and TAP2 regarding their orientation within the functional heterodimer still remains questionable, although a head-head/tail-tail orientation has been proposed (58, 76). Studies on tapasin interaction with TAP indicated that the first four and three TMDs of TAP1 and TAP2, respectively, are not essential

for the formation of the functional peptide translocation pore and most likely provide two separate platforms for tapasin/class I association (63).

Although the TAP transporter has been the subject of numerous studies, it has proved very difficult to assess the sequence of events during the peptide transport cycle. The following model for peptide transport by TAP could be proposed (figure 4 modified after 74; reviewed in 73 - 75): Peptide and ATP bind independently to TAP (step 1). ATP binding enables TAP1 and TAP2 NBDs to dimerize. It has been suggested that the binding of ATP first occurs at the TAP2 NBD site (NBD2) which is also the first to be hydrolyzed. Also, ATP hydrolysis in NBD2 is sufficient for peptide transport by weakening the dimer interface. However, ATP hydrolysis in TAP1 NBD (NBD1) alone does not interrupt the dimer interface. ATP can only be hydrolyzed at NBD1 after ATP hydrolysis has occurred at NBD2. Peptide binding (step 2) induces

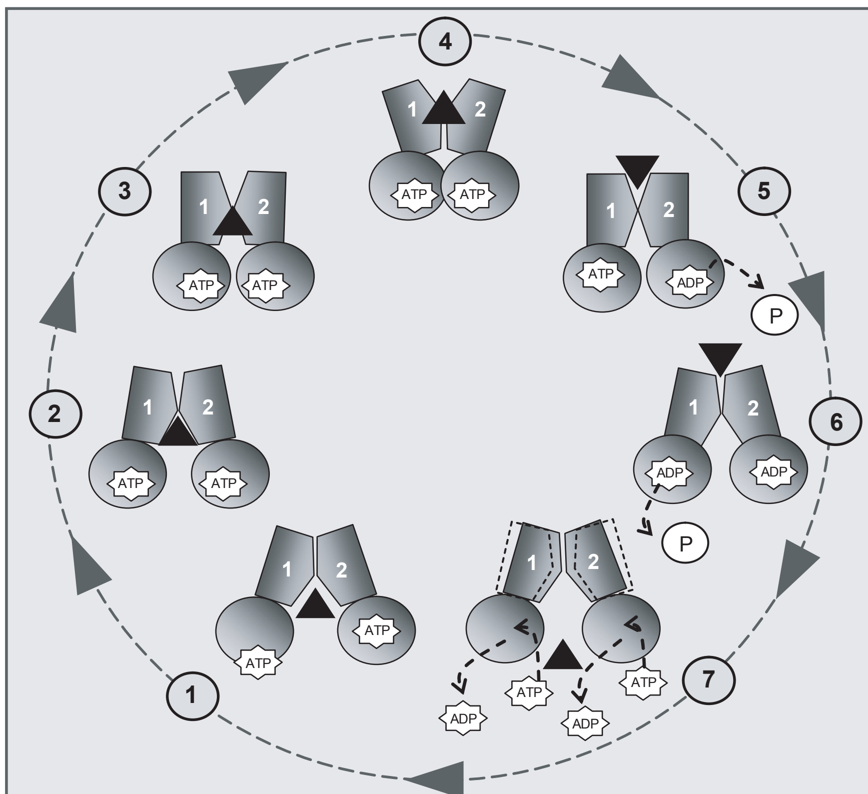

Fig. 4. Proposed peptide transport cycle of TAP. See text for description of each step of the peptide transportation cycle. P-phosphate. *Modified after Abele and Tampe Physiology 2004 19:216-224.*

structural rearrangements in the TMDs of both subunits (step 3), which weakens rigid interactions within NBDs. As a result, ATP-loaded NBDs form a dimer, which subsequently induces peptide release into the ER lumen (step 4). The peptide is released from the altered peptide-binding site, and subsequent translocation through the opened pore (TMDs) into the ER lumen occurs. ATP hydrolysis and phosphate release, first at NBD2 (step 5) and then at

NBD1 (step 6) weaken the interaction of the NBDs. ATP hydrolysis at NBD1 may be required to close the pore, restore the peptide-binding domain and complete the translocation cycle. After the dimer dissociation (step 7), ADP is released and replaced by ATP.

Even though the transport activity of TAP can be directly measured with model substrates (77), conformational changes that occur during transport are difficult to study. However, the conformational changes associated with activity of TAP can, to a certain extent, be visualized in living cells by measuring the lateral mobility of fluorescent GFP-tagged TAP over the ER membrane (78). For this purpose, fluorescence recovery after photo-bleaching (FRAP) technique was used to visualize the lateral mobility and dynamics of TAP complexes in living cells (79). By using this technique, it has been shown that the lateral mobility of TAP molecules is inversely proportional to TAP activity, as peptide transporting TAP molecules diffuse at a slower rate than inactive, closed TAP complexes. Thus, the mobility of inactive TAP is higher when compared to TAP actively pumping peptides.

Apparently, the decrease in mobility is likely due to conformational changes within the TAP complex during pore opening and peptide transfer. In addition, the effect of chemical and viral TAP inhibitors can be visualized through confocal microscopy, the FRAP technique and the measurement of lateral mobility of TAP, provided they affect the conformational changes within the TAP complex (78, 80).

Herpesviruses: masters of disguise

To establish a lifelong infection in the presence of an active (immunocompetent) host immune system, herpesviruses have acquired an impressive array of immune modulatory strategies that contribute to their successful long-term persistence. The success of these strategies relies on virus-encoded proteins that either mimic specific components of the host's immune system or target a variety of host immune surveillance mechanisms. The effectiveness of these viral proteins in escaping from host immune surveillance, ultimately contributes to lifelong infection and pathogenesis of herpesviruses. The number of identified virus-encoded proteins with immune evasion properties has increased dramatically in recent years. In this thesis, only a subset of immune evasion molecules will be covered with the accent on proteins encoded by herpesviruses that target the MHC class I antigen presentation pathway (summarized in table 1).

The virion host shut-off (vhs) mechanism

The shut-off of cellular protein synthesis by the virion host shut-off (vhs) protein is a characteristic feature of alphaherpesviruses. The vhs protein of herpes simplex virus-1 (HSV-1), the prototype alphaherpesvirus, is the best characterized vhs protein. Vhs homologs have been identified in the genome of all the alphaherpesviruses sequenced thus far. However, only a few have been analyzed for host protein synthesis shut-off properties (81-83). The vhs protein, encoded by the HSV-1 UL41 gene, is a tegument protein, which enters the host cell as a component of the infectious virion. Upon entry, the virion vhs protein (but also the vhs proteins that is expressed during the early stage of infection) accelerate the degradation of mRNA, contributing to an overall decrease in host protein synthesis (84-86). It has been shown that during the early stages of BHV-1 infection, the BHV-1 UL41 gene product also strongly affects the synthesis of host proteins, among which the MHC class I molecules. These findings indicate that the BHV-1 vhs protein plays a role in MHC class I down-regulation. In addition to the vhs protein activity, other BHV-1 gene products specifically down-regulate MHC class I

molecules. Thus, the suppression of host gene expression seems to support immune evasion in the early stage of infection (81, 87-89). Apparently, most herpesviruses do not concentrate on a single immuno-evasion strategy but utilize several strategies in parallel.

Table 1

Alphaherpesviruses	Protein	Target	Mechanism (References)
HSV-1& -2	ICP47	TAP	Inhibition of peptide binding (137-138)
BHV-1	UL49.5	TAP	Inhibition and degradation of the PLC (80, 168)
PRV	UL49.5(gN)	TAP	Inhibition of peptide transport (80)
EHV-1& -4	UL49.5	TAP	Inhibition of ATP binding (80, *)
VZV	ORF66	MHC I	Retention in Golgi (159, 170)
Betaherpesviruses			
HCMV	US2	MHC I	Dislocation and proteasomal degradation (91-95)
	US3	MHC I/tapasin	ER retention/ Inhibition of peptide loading (101-105)
	US6	TAP	Inhibition of ATP binding (143-148)
	US10	MHC I	Retention in the ER (106)
	US11	MHC I	Dislocation and proteasomal degradation (91-95)
MCMV	m04	MHC I	Binding to MHC/display at cell surface (110-111)
	m06	MHC I	Degradation in lysosomes (112)
	m152	MHC I	Retention in the ER (108-109)
Gammaherpesviruses			
EBV	EBNA-1	Proteasome	Prevention of proteasomal degradation (156,157)
	vIL-10	TAP	Inhibition of TAP1 expression (154)
	BNLF2a	TAP	Inhibition of peptide transport (155; Hislop, Ressing, Rickinson, Wiertz et al., Pers. comm.)
HHV-7	U21	MHC I	Degradation in lysosomes (127,128)
KSHV	K3	MHC I	Ubiquitinylation/endocytosis in lysosomes (114-119)
	K5	MHC I	Ubiquitinylation/endocytosis in lysosomes (114,115)
MHV-68	mK3	MHC I	Ubiquitinylation/proteasomal degradation (121-126, 149-151)
		TAP/tapasin	Proteasomal degradation (122,149-151)

* Chapter 5 of this thesis

Targeting of the MHC class I complex by viral proteins

The use of multiple strategies to subvert antigen presentation has been clearly illustrated for human cytomegalovirus (HCMV) (90). The US2-US11 (US, unique short region) of HCMV potentially encodes 8-10 membrane glycoproteins, four of which, US2, US3, US10 and US11, are known to target MHC class I molecules and one, US6, specifically inhibits TAP-mediated peptide transport.

US2 and US11, both type-I membrane glycoproteins, induce rapid proteasome- dependent degradation of nascent MHC class I molecules. Located in the ER, they both bind directly to MHC class I heavy chains and cause them to be removed from the ER back to the cytosol through a process termed “dislocation” (retro-translocation) (91-95). It is known that dislocation is a central component of ER associated protein degradation (ERAD), an important mechanism used by cells to degrade improperly assembled or misfolded ER proteins (96). Whereas the ER-luminal domain of US11 is involved in the binding of MHC class I heavy chains, its transmembrane domain is essential for their dislocation (97). The transmembrane domain of US11 mediates the recruitment of MHC class I molecules to Derlin-1, a protein essential for the degradation of misfolded ER-luminal proteins. US11 bridges Derlin-1 with MHC class I, probably delivering MHC class I molecules to the dislocation pore (98, 99). Interestingly, Derlin-1 is not required for US2-mediated degradation of MHC class I molecules, indicating different mechanisms of action (98). Recently, signal peptide peptidase (SPP) has been identified as a partner for the active form of US2, implicating SPP in the US2-mediated MHC class I degradation pathway. Apparently, a decrease in SPP levels inhibits heavy-chain dislocation by US2 but not by US11 (100).

The HCMV-encoded US3 and US10 proteins retain MHC complexes in the ER. US3, the only immediate early US gene, interacts transiently with MHC molecules and prevents their egress to the cell surface (101-103). The transmembrane and the ER luminal domain of US3 are both required for the retention of MHC class I molecules in the ER (102, 104). However, substantial amounts of MHC class I molecules still escape US3-mediated ER arrest. Apparently, US3 down-regulates the cell surface expression of MHC class I molecules in an allele-specific manner. US3 directly binds tapasin, thereby affecting only MHC class I alleles that are dependent on tapasin for their surface expression. Through its interactions, US3 reduces the association between TAP and tapasin. While US3 binds to both tapasin and TAP, it was evident that US3 did not affect TAP function for peptide transport (105).

Like US3, US10 also inhibits the export of MHC class I out of the ER (106). US10 is an ER-localized type I membrane glycoprotein that delays but does not block the transport of MHC class I molecules from the ER. However, the mechanism of interaction between US10 and class I heavy chains remains to be determined.

Thus, HCMV encodes at least 5 proteins with specific tasks to obstruct MHC class I assembly and presentation. Interestingly, two of these, US2 and US3, appear to be multifunctional as they both also target MHC class II molecules, preventing their egress to the cell surface (107).

Murine cytomegalovirus (MCMV) carries three genes encoding type-I membrane glycoproteins known to interfere with the intracellular trafficking of MHC class I molecules: m152/gp40, m04/gp34 and m06/gp48. These viral proteins are expressed sequentially during viral infection and they act synergistically. Whereas gp40 can be detected within a few hours after infection, gp34 and gp48 are expressed later. Although the direct interaction of gp40 with MHC class I could not be detected, the expression of gp40 protein triggers the retention of MHC class I complexes in the ER-Golgi intermediate compartment (ERGIC)/*cis*-Golgi (108, 109). These MHC class I complexes are stable, whereas gp40 is re-directed and degraded in endosomal/ lysosomal compartments (109). In contrast to gp40, gp34 and gp48 are both found

in direct association with MHC class I molecules. gp34 does not alter MHC class I surface expression, but it is found in association with MHC class I molecules at the cell surface (110, 111), whereas gp48-MHC class I complexes are diverted to late endosomes and lysosomes for rapid proteolysis. The lysosomal targeting of gp48-bound MHC class I molecules is mediated by the cytosolic domain of gp48 that contains a di-leucine-motif (LL) (112).

Herpesvirus 8 or Kaposi's sarcoma associated virus (KSHV) encodes two proteins, K3 and K5, which are, until now, unique for KSHV. K3 and K5 are expressed during the early lytic cycle of viral replication and they exhibit 40% of amino acid sequence identity to each other. K3 and K5 are also known as modulator of immune recognition 1 and 2 (MIR1 and MIR2) respectively (113). In their presence, MHC class I molecules exit the ER and reach the cell surface, but are then rapidly endocytosed and degraded in lysosomal compartments (114, 115). K3 and K5 are predominantly associated with the ER. They are type III membrane proteins containing two transmembrane helices. The N-terminus and C-terminus of K3 and K5 are placed in the cytosol. K3 and K5 have an N-terminal RING finger motif that shares structural and sequence homology with the RING finger domain that is found in a subset of E3 ubiquitin ligases. Thus, both proteins belong to the family of type 3 (E3) ubiquitin ligases (116, 117) and can regulate the last step of the ubiquitination cascade (114). The N-terminal domain and the central sorting motif are involved in triggering the internalization of the MHC class I molecules and in re-routing them to the trans-Golgi network (TGN). The C-terminal cluster is engaged in targeting MHC class I molecules to lysosomal compartments (118). K3-mediated ubiquitination of MHC class I molecules signals for both internalization and sorting to late endosomes (119, 120). K3 and K5 both down-regulate MHC class I molecules in a similar way, but they differ in their specificity for MHC class I alleles (114, 115).

Murine γ-herpesvirus 68 (MHV-68) is a γ2-herpesvirus of small rodents that has a homology to the Kaposi's sarcoma-associated herpesvirus (KSHV) in humans. MHV-68 encodes K3 protein (mK3), a zinc binding RING finger protein, that down-regulates MHC class I molecules expression. mK3 is a type III membrane protein localized in the ER membrane where it binds to the cytoplasmic tail of newly synthesized MHC class I molecules. mK3 catalyses ubiquitination of MHC class I through its N-terminal RING-CH domain and causes their rapid, proteasome-dependent degradation (121-126, 150, 169).

A human herpesvirus-7 (HHV-7)-encoded type I membrane glycoprotein, U21, employs a different strategy to stop MHC class I molecules on their journey to the cell surface membrane. U21 binds to MHC class I shortly after its synthesis in the ER and targets MHC class I complexes to lysosomes for degradation (127). Apparently, the ER-luminal domain of U21 is sufficient for the induction of lysosomal degradation (128). U21 shares no significant sequence homology with any other presently known protein, except for U21 of HHV-6.

TAP under attack

Viral proteins can prevent MHC class I presentation at the cell surface by targeting MHC class I molecules directly or through inhibiting TAP-mediated peptide transport. The blocking of MHC class I antigen presentation is a strategy employed by different herpesviruses, and several of them have chosen TAP as the target (figure 5). Peptide transport into the ER can be impaired by different mechanisms, including down-regulation of TAP expression, proteasomal degradation of TAP mediated by viral proteins, and blocking peptide transport by direct interaction of viral proteins with TAP complex (reviewed in 14, 58, 129 and 130).

TAP is a target of ICP47 (Infected Cell Protein 47) of HSV-1 and -2. Although the ICP47

protein (encoded by immediate-early gene US12) of both serotypes share an overall sequence identity of only 42%, they do not differ significantly in their effect on TAP (131). HSV-1 and 2 encoded ICP47s share no relevant sequence homology with other proteins, except with homologues from simian agent 8 (SA8; 132), monkey B virus (Cercopithecine herpesvirus 1; CeHV-1), and baboon herpesvirus 2 (herpesvirus papio 2; HVP-2). ICP47 is expressed as cytosolic protein, of which its N-terminal domain of 32 residues has been identified as the minimal active region required for TAP inhibition (133, 134). This domain appears to be unstructured in an aqueous solution, but an alpha-helical structure is induced

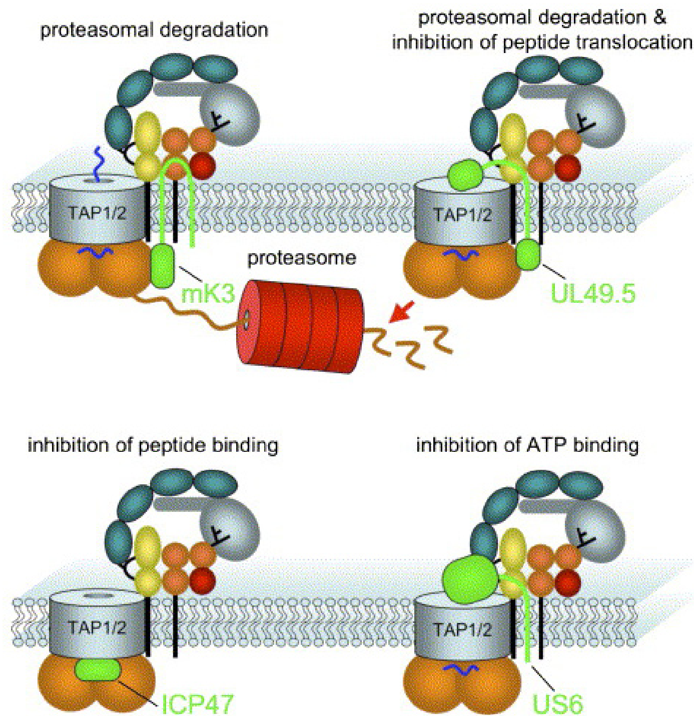

Fig. 5. Modulation of TAP functions by viral factors. The mK3 protein of MHV-68 binds directly to TAP and induces polyubiquitination and proteasomal degradation. The interaction of UL49.5 of BHV-1 arrests TAP in a transport-incompetent conformation, in which binding of ATP and peptides is not affected. Ultimately, UL49.5 mediates the degradation of TAP by proteasomes. ICP47 of the HSV inhibits peptide binding from the cytosolic side of TAP. The association of US6 of the HCMV to the ER-luminal transmembrane core of TAP blocks ATP binding to the cytosolic NBDs. (for the description of the peptide-loading complex components see figure 2) *Adapted from Abele & Tampe FEBS Lett. 2006 580:1156-1163*

at a membrane interface (135, 136). By binding to TAP from its cytosolic side, ICP47 blocks peptide-binding. Although it does not affect ATP binding, the subsequent ATP hydrolysis is abolished (137-140). Additionally, binding of ICP47 seems to disturb stabilization of the TAP1 and TAP2 complex (137). ICP47 is partially species-specific, since it inhibits human, monkey, canine and bovine TAP function, whereas TAP of small rodents is not affected (131, 137, 141). At present, it is unknown which regions of ICP47 and TAP specifically interact with each other (142).

The unique short region of the HCMV genome encodes yet another immuno-evasion protein called US6. This type I transmembrane glycoprotein blocks TAP-dependent peptide transport. The ER luminal domain of US6 has been shown to be essential and sufficient for TAP inhibition (143, 144). Interestingly, US6 inhibits ATP binding and hydrolysis of TAP at its cytosolic side through interaction with TAP at the ER luminal side. This interaction does not affect peptide binding or the stability of TAP complex (143-147). Although US6 binds to TAP1 and TAP2 independently, the US6-mediated inactivation of the transporter requires simultaneous interaction with conformation-dependent ER-luminal loops of both transporter subunits. The US6 luminal domain is enriched with cysteine residues (8 cysteines) that are likely to form a complex intra- and intermolecular network of disulfide bridges. Indeed, US6 forms oligomers that are apparently involved in the bridging of TAP1 and 2 subunits (148). It is likely that such a compact multimeric formation is crucial for inducing an aberrant structural conformation of the transporter, which in its turn prevents acquisition of ATP.

The murine γ-herpesvirus 68 (MHV-68)-encoded mK3 protein is another example of a viral protein with the ability to interfere with the function of peptide-loading complex. mK3 interacts with MHC class I molecules causing their rapid degradation by proteasomes (121-126, 149, 169, 170). In addition, by association with the peptide-loading complex through its C-terminal tail (150), mK3 initiates the degradation of TAP and tapasin (122, 151). To induce TAP degradation, mK3 requires the transmembrane/cytoplasmic part of tapasin (122). Interestingly, mK3-mediated TAP/tapasin degradation appears to be restricted to certain cell types (lymphoid cell lines). This may have an implication in vivo, as the MHV-68 virus establishes latency in B lymphocytes, macrophages and dendritic cells. Indeed, the major function of mK3 appears to be CTL (cytotoxic T lymphocyte) evasion during viral latency expansion (152).

Epstein-Barr virus (EBV; γ1-herpesvirus) is another gammaherpesvirus with the ability to inhibit antigen presentation at the level of TAP (153). During the late lytic phase, EBV encodes an IL-10 homolog (viral interleukin-10; vIL-10), involved in down-regulation of TAP1 expression in EBV-infected B cells (154). Moreover, during the early lytic phase of EBV gene expression, specific inhibition in TAP-dependent transport has been observed (155). Recently, the product of the early lytic gene BNLF2a has been identified as an inhibitor of peptide transport by TAP (Hislop, Ressing, Rickinson, Wiertz et al., personal communication).

The list of herpesviruses-encoded “immuno-evaders” has increased dramatically in recent years. Yet there are many more to identify and explore. The group of viruses that belong to the subfamily of *Alphaherpesvirinae*, genus *Varicellovirus*, also specifically target MHC class I antigen presentation. Varicella-zoster virus (VZV), bovine herpesvirus 1 (BHV-1), pseudorabies virus (PRV), equine herpesvirus 1 and 4 (EHV-1 and 4) and many others are members of the genus *Varicellovirus*. For a few members their immuno-evasive strategies have been elucidated only recently (80). The strategies of other members of this genus remain to be further analyzed.

VZV interferes with MHC class I presentation (158) by retaining MHC class I molecules in Golgi compartments of infected cells. However, the mechanism employed for this strategy is still unknown (159). Marek’s disease virus (MDV; genus *Mardivirus*) of chickens is the only known oncogenic alphaherpesvirus inducing T-cell tumors (160). MDV is recognized by virus-specific CTLs (161); however this response appears to be weak, suggesting potential interference with the MHC class I antigen-presentation pathway. Indeed, MDV shows the ability to down-regulate the surface expression of MHC class I molecules during the early phase of virus replication (162, 163).

The studies reported in this thesis focused on bovine herpesvirus 1, pseudorabies virus, and equine herpesvirus 1, which specifically target TAP function (81, 164-166). The aim was to identify the viral gene(s) responsible for the observed TAP inhibition and to characterize the mechanisms employed.

Outline of this thesis

At the start of this study it was unclear whether the down-regulation of the MHC class I cell surface expression observed after infection by BHV-1 is the result of a general or a specific mechanism. The viral host shut-off (vhs) mechanism largely affects host protein expression, and a BHV-1 vhs deletion mutant was constructed and studied for its ability to down-regulate MHC class I. **Chapter 2** describes that the vhs protein was indeed responsible for part of the down-regulation of the MHC class I cell surface expression. On the other hand, the MHC class I molecules that accumulated in the endoplasmic reticulum (ER) after infection with a BHV-1 vhs deletion mutant did not mature into a stable MHC class I-peptide complexes. This observation led to the hypothesis that BHV-1 could also specifically inhibit peptide transport by TAP, which was shown to be the case.

However, studies aiming at the identification of BHV-1-encoded TAP inhibitor have been hampered by the lack of bovine-specific immunological reagents. The experiments described in **Chapter 3** were performed to investigate whether BHV-1 can infect human cells and interfere with the function of human TAP. Interestingly, a human melanoma cell line appeared to be permissive for BHV-1 infection. BHV-1 mediated inhibition of human TAP and down-regulation of MHC class I molecules at the surface of infected cells. The data indicated that the BHV-1-encoded molecule(s) that block bovine TAP are able to interact with homologous components of the MHC class I peptide-loading complex in human cells.

Indeed, the fact that immune evasion by BHV-1 can be studied in human cells facilitated the identification of a new TAP inhibiting protein, UL49.5, encoded by BHV-1. The results reported in **Chapter 4** establish a novel immune evasion strategy for the inactivation of TAP. The BHV-1 encoded UL49.5 protein blocks TAP through a mechanism that is distinct from those employed by other viral inhibitors described above. The effect of BHV-1 UL49.5 on the TAP complex is striking and relies on two events: inhibition of peptide transport by arresting the TAP complex in a translocation-incompetent state and destabilization of both TAP subunits causing their proteasome-dependent degradation.

Furthermore, a characterization of the UL49.5 gene product encoded by various varicelloviruses as a potent inhibitor of TAP is described in **Chapter 5**. PRV and EHV1-encoded UL49.5 homologs have been identified in association with the peptide-loading complex and are responsible for the inhibition of its function. The UL49.5 proteins of BHV-1, PRV and EHV-1 appear to be relatively homologous based on their amino acid sequences identity and structural organization. Remarkably, experimental data revealed seemingly diverse mechanisms employed by UL49.5 homologs to block TAP. Thus, the results described in chapter 5 classify a number of varicellovirus-encoded UL49.5 homologs as members of a novel family of TAP-inhibiting proteins.

BHV-1 UL49.5, a type-I membrane protein of only 9 kDa, is found as a monomer, homodimer and heterodimer associated with the viral glycoprotein M (gM) in infected cells and virion envelopes. In **Chapter 6** it is shown that UL49.5 can combine the interactions with gM and the TAP complex in the context of natural infection. Although UL49.5/gM association

in isolated system can interfere with the inhibition of TAP, a sufficient amount of non-gM bound UL49.5 protein is available to exceed this interference during productive BHV-1 infection. Thus, BHV-1 UL49.5 inhibits TAP despite complex formation with glycoprotein M.

The results presented above already suggested that the BHV-1 UL49.5 and other varicelloviruses encoded homologs scrutinized for the inhibition of TAP represent potential immune suppressive agents for use in humans. **Chapter 7** shows that the varicellovirus-encoded TAP-inhibitor UL49.5 represents a new tool to study and modulate endogenous antigen-presentation. UL49.5 down-regulates HLA class I expression and inhibits minor (mHag) and major Histocompatibility antigen-specific CTL responses more efficiently than the other two known TAP inhibitors, US6 and ICP47. UL49.5 also reduces alloHLA-reactivity, affecting both peptide-dependent and peptide-independent antigen-presentation. Interestingly, the empty MHC class I molecules that appear at the surface of UL49.5-expressing cells can be loaded with peptides exogenously added to the cells. The resulting MHC class I-peptide complexes are stably expressed and can serve to activate T cells of a desired specificity.

In **Chapter 8**, inhibition of TAP by UL49.5 is described to induce the presentation of a new category of peptides, T cell Epitopes associated with Impaired Peptide Processing (TEIPP) (167). Although these peptides are derived from widely distributed self proteins, they are not presented by normal, antigen processing-proficient cells and therefore the immune system recognizes them as immunogenic neo-antigens. TEIPP have been found to be presented by tumor cells that have antigen processing defects. UL49.5 can be used to specifically induce TEIPP-specific T lymphocytes directed against tumor cells, thereby creating a new opportunity for immunotherapy against cancer. Thus, UL49.5 provides a powerful new tool for the study of fundamental aspects of antigen processing and allows specific modulation of the peptide repertoire presented by MHC class I molecules.

In **Chapter 9** the molecular interaction between the UL49.5 protein and the peptide loading complex are discussed together with the possible therapeutic application of the UL49.5 protein and the use of UL49.5 deletion mutants as vaccines.

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