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

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CHAPTER 9

Summary and Discussion

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Identification of herpesviruses-encoded TAP inhibitors

One of the first herpesvirus encoded immunoevasins identified to be associated with the inhibition of TAP was the ICP47 protein (1-9). This immediate early (US12) gene product of herpes simplex virus 1 and 2 (HSV-1 & 2), is both necessary and sufficient to block the transport of MHC class I molecules to the cell surface and to inhibit lysis by $CD8^+$ CTLs. ICP47 inhibits peptide transport into the ER by stable interaction with the cytosolic face of TAP. Thus, through TAP inhibition, ICP47 indirectly affects MHC class I export from the ER. The discovery of ICP47 was followed by the identification of two more viral TAP blockers. A member of the *Betaherpesvirinae* subfamily, HCMV, expresses the US6 protein that interferes with the peptide translocation function of TAP (10-12). Some years later, the MHV-68-encoded mK3 protein was also identified as an inhibitor of TAP activity (13-16). MHV-68 belongs to the subfamily of *Gammaherpesvirinae*. Although ICP47, US6 and mK3 all target TAP, they are very different proteins with distinctive TAP inhibiting properties.

HSV-1 and 2 (genus *Simplexvirus*) are not the only *Alphaherpesvirinae* to specifically down-regulate the expression of MHC class I molecules. Several other members of this subfamily also have been found to down-regulate MHC class I molecules at the cell surface of infected cells. This group includes pseudorabies virus (PRV; genus *Varicellovirus*; 17), bovine herpesvirus type 1 (BHV-1; genus *Varicellovirus*; 18, 19), varicella-zoster virus (VZV; genus *Varicellovirus*; 20), Marek's disease virus 1 (MDV-1; genus *Mardivirus*; 21) and equine herpesvirus type 1 (EHV-1; genus *Varicellovirus*; 22). Like HSV-1-mediated MHC class I down-regulation, BHV-1, PRV and EHV-1-mediated MHC class I down-regulation could be attributed to the observed inhibition of TAP activity in cells infected by these viruses (23-25).

This thesis is dedicated to the discovery of another herpesvirus immuno-evasin, the varicelloviruses-encoded TAP inhibitor UL49.5. Possible ways of manipulating antigen presentation by the use of viral immunoevasins will be addressed, as will the application in vaccine development of viral mutants lacking genes encoding immuno-evasive proteins.

In search for a BHV-1 encoded TAP inhibitor

The first step towards the identification of relevant varicelloviruses-encoded proteins that might block TAP and consequently counteract host immune functions was to compare their viral sequences with published sequences encoding known TAP inhibitors and to search for homologies. Analyses of the coding sequences of BHV-1, PRV and EHV-1 failed to identify homologs of any of the previously identified immuno-evasive proteins known to target TAP (10-13).

The genome of BHV-1 comprises about 70 open reading frames (ORFs) (26). Although most of the BHV-1 genes are homologous to HSV-1 genes, an US12 homolog is missing and the precise functional role of many of the BHV-1 genes in the context of a viral infection is, in most cases, still unknown. The BHV-1 genome also contains four genes that have no homolog within the genome of HSV-1. These genes are designated *circ*, UL0.7, UL0.5, and US1.67. In contrast to HSV-1 ICP47, when expressed individually in cells, none of their gene products influenced the expression of MHC class I molecules at the cell surface (Heidt S. & D. Koppers-Lalić and Marks H. & F. Rijsewijk, unpublished observations). Thus, these four genes of BHV-

1 do not seem to play a role in MHC class I down-regulation. From these observations, it was concluded that a BHV-1-encoded TAP inhibitor could be a homologous protein for BHV-1 and HSV-1. It seemed likely that the protein in question would possess a dual function or that its function would be distinct from its counterpart in HSV-1.

BHV-1 employs at least two strategies to prevent MHC class I antigen presentation

As with in other herpesviruses, BHV-1 gene expression occurs in a cascade fashion through immediate-early (IE), early (E) and late (L) phases. The three phases can be controlled through the chemical treatment of infected cells. As described in chapter 2, this approach led to the conclusion that viral genes expressed during the early phase of BHV-1 infection are responsible for down-regulation of bovine MHC class I molecules. A similar conclusion was drawn from the experiments on the temporal control of PRV gene expression (24). In addition, BHV-1 and PRV-mediated inhibition of TAP appeared to be specific, since the general effect of host protein synthesis shut-off mechanisms employed by these viruses could be ruled out (27, 28). Although not directly involved in TAP inhibition, vhs (virion host shut-off) proteins partially contribute to the down-regulation of MHC class I molecules at the very beginning of a viral infection. The importance of shutting down antigen presentation early in infection is apparently related to “peak-time” synthesis of viral proteins that leads to an increase in the endogenous pool of antigenic peptides. According to a study by Reits et al. (29), the main substrates for TAP are derived from newly synthesized proteins. The MHC class I molecules will therefore be rapidly exposed to viral peptides, ensuring prompt presentation to the surveying immune system. Thus, it appears that BHV-1 uses a synergy of two strategies to prevent MHC class I antigen presentation. How much the vhs effect contributes to the down-regulation of MHC class I antigen presentation remains to be evaluated.

The BHV-1-encoded TAP inhibitor UL49.5 acts across species barriers

Since co-immunoprecipitation experiments with known TAP inhibitors, ICP47 and US6, showed that both proteins associated with TAP, it was assumed that the BHV-1 TAP inhibiting protein could also directly associate with components of the peptide-loading complex and that this viral protein could be extracted and identified through a co-precipitation method. However, one of the experimental disadvantages of studying BHV-1-mediated TAP inhibition in bovine cells was the unavailability of reagents specific for the components of the bovine MHC class I processing pathway. Therefore, the ability of BHV-1 to infect human cells and to cause inhibition of human TAP was evaluated (chapter 3). Although BHV-1 is species-specific, under laboratory conditions BHV-1 was able to productively infect human melanoma cell line (Mel JuSo; further referred to as MJS). Importantly, TAP function in MJS cells appeared to be inhibited throughout the course of BHV-1 infection. The BHV-1-mediated TAP inhibition phenotype in bovine and human cells was clearly comparable. Thus, the human cell line MJS and immunological reagents specific for the components of the human MHC class I processing pathway, proved suitable tools for the identification of a BHV-1 gene product that inhibits TAP activity (chapter 3).

Interestingly, co-immunoprecipitation experiments performed in human (MJS TAP1-GFP) cells infected with BHV-1, revealed the specific association of the viral protein UL49.5 with the

human peptide-loading complex (chapter 4). Stable expression of BHV-1 UL49.5 protein in human cells resulted in a strong inhibition of peptide transport by TAP. Thus, BHV-1 encodes a potent inhibitor of TAP that not only blocks the activity of the bovine peptide transporter but also its human counterpart (chapter 5).

Likewise, the HSV-1 ICP47 protein is able to interact with TAP transporters of other host species, as it blocks bovine, porcine, and canine TAP (30). It has, however, little or no effect on small rodents TAP (31). BHV-1 infection of murine cells results in down-regulation of murine class I molecules at the cell surface (18). The expression of the BHV-1 TAP inhibitor UL49.5 in murine cells resulted in inhibition of TAP (32) (chapter 8). Interestingly, BHV-1 UL49.5 has the ability to inhibit the function of TAP from more species than ICP47 as UL49.5 blocks besides the bovine, human, and porcine TAP (Koppers-Lalić, unpublished observation) also the murine and rat TAP (Koppers-Lalić, unpublished observation). These results qualify BHV-1 UL49.5 as an almost universal TAP inhibitor, which can be widely applied in antigen presentation studies (e.g. from mouse models to applications in humans). Regarding UL49.5-TAP interaction, it is likely that BHV-1 UL49.5 targets common domains among TAP proteins or TAP associated proteins in different species.

BHV-1 UL49.5 association with the peptide-loading complex (what binds to what?)

The close interaction between peptide-loading complex components makes it difficult to determine the individual target proteins for UL49.5. At present, the interaction of UL49.5 and TAP can only be detected through lysis of the cells under mild detergent conditions (1% digitonin) that also preserve intermolecular interactions between the components of the peptide-loading complex. Co-immunoprecipitation experiments performed on MJS TAP1-GFP cells expressing BHV-1 UL49.5 show that all the components of the peptide-loading complex appear to be present within the complex together with UL49.5. Thus, it remains unclear whether UL49.5 directly associates with TAP to fulfill its inhibitory function or whether this phenotype requires the presence and/or interaction of UL49.5 with the other components of the peptide-loading complex. However, when the UL49.5 protein is expressed in a TAP-deficient cell line (human cell line T2; TAP1^{-}/2^{-}) containing all the other components of the peptide-loading complex, e.g. tapasin and MHC class I molecules, the UL49.5 was not found to bind those proteins. Thus, it is unlikely that UL49.5 interacts directly with tapasin or MHC class I molecules. However, failure to detect the interaction between tapasin and UL49.5 could be due to the absence of TAP, thus, UL49.5 can still physically interact with tapasin if TAP is present. In addition, the interaction of UL49.5 with tapasin might be transient or weak and thus hard to detect in our system. Additionally, the stability of UL49.5 in the absence of TAP could be compromised.

Based on the results from T2 cells, it is most likely that UL49.5 directly binds to TAP. In agreement with this, the expression of UL49.5 in tapasin-deficient human B cell line 721.220 resulted in the inhibition of TAP-dependent peptide transport (Koppers-Lalić, unpublished observations), thus suggesting that UL49.5 probably associates with the TAP transporter in order to block its function, independently of the presence of tapasin. However, these results remain inconclusive, since the observed UL49.5-mediated inhibition of TAP might involve the truncated tapasin product (missing the first 55 amino acids of the ER luminal part) present in 721.220 cells that still binds to TAP (33). As the truncated tapasin construct is still expressed in very low amounts, 721.220 is not a truly tapasin-deficient cell line. Although 721.220 cells are not the most reliable system to study UL49.5-TAP interaction in the absence of tapasin, the results obtained at least indicate that UL49.5 does not require full-length tapasin to block

TAP.

Since BHV-1 UL49.5 also blocks mouse TAP (32) (chapter 8), the alternative is to use cells isolated from tapasin-deficient mice to study the UL49.5-TAP interaction in the absence of tapasin ($Tps^{-/-}$) (34).

TAP is a heterodimer comprised of two subunits, TAP1 and TAP2. Thus, the UL49.5 association with the peptide-loading complex could occur through binding either to TAP1 or to TAP2. Alternatively, heterodimerization of the two subunits could be a requirement for UL49.5 to engage TAP. The studies addressing UL49.5 association with TAP2 proved to be difficult since TAP2 expressed as a single subunit is degraded rapidly, as its structural stability is dependent on the presence of TAP1 and tapasin (33, 34, 35). On the other hand, TAP1 levels were not obviously affected by the absence of TAP2, thus to study UL49.5-TAP1 interaction appears to be more feasible. In T2 cells that stably express the TAP1 subunit, UL49.5 was not found to co-precipitate with the TAP1/tapasin/MHC class I complex (Koppers-Lalić, unpublished observations). However, the association of UL49.5 and TAP1 could be weak or transient and thus difficult to detect. For example, the HCMV-encoded TAP inhibitor US6 binds to both TAP1 and to TAP2, but it can only be detected in association with either of them if both subunits are present (36, 37). Apparently, US6-TAP interaction is dependent on structural rearrangements within TAP1 and TAP2 due to their heterodimerization. This could also be the requirement for UL49.5-TAP association. Since the TAP1 and TAP2 domains that play a role in US6 binding are known (37) it would be interesting to investigate whether there is an overlap of UL49.5 and US6 TAP1/2 interaction sites (e.g. US6 and UL49.5 could be mutually exclusive).

Thus far, it can be concluded that UL49.5 association with TAP requires both subunits to be assembled in the presence of tapasin. However, a direct interaction between UL49.5 and TAP has yet to be demonstrated.

The mechanism(s) of BHV-1 UL49.5-mediated TAP inhibition

The expression of UL49.5 in human cells (MJS) mediated the proteasome-dependent degradation of both TAP subunits (chapter 4). However, strongly reduced TAP levels would make it impossible to identify UL49.5 in association with the peptide-loading complex. Interestingly, the initial experiments were performed in BHV-1-infected MJS cells expressing a TAP1-GFP fusion protein (the c-terminus of TAP1 was fused to GFP), and these experiments revealed a specific association of UL49.5 with the peptide-loading complex. Apparently, the GFP fusion to TAP impedes the UL49.5-mediated degradation of the TAP complex. Thus, the presence of the TAP1-GFP fusion protein within the TAP complex was critical for the identification of UL49.5. MJS TAP1-GFP cells still express the endogenous TAP1 that appears to be strongly reduced in the presence of UL49.5. Apparently, the GFP fusion to c-terminus of TAP1 has a stabilizing effect and furthermore implies that the cytosolic c-terminal tail of TAP1 might be a target for the proteasomal-degradation machinery. Although the presence of GFP at the tail of TAP1 does not interfere with UL49.5-TAP interaction, it is possible that GFP renders TAP1 unapproachable for degradation-associated auxiliary proteins.

The expression level of TAP1 controls the amount of stable heterodimeric TAP (38, 35) since the TAP1 level regulates the escape of newly synthesized TAP2 from ER-associated degradation. The formation of functional transporters requires the assembly of pre-existing TAP1 with newly synthesized TAP2, but not vice versa (35). In UL49.5-expressing MJS TAP1-GFP cells, TAP2 levels appear not to be affected by UL49.5. Since TAP1-GFP appears to have a stabilizing effect on TAP2, the data from the MJS cell line expressing UL49.5 in the absence of TAP1-GFP indicate that the decrease in TAP2 levels could be a consequence of

TAP1 degradation rather than TAP2 being the direct target for UL49.5-mediated degradation.

Whereas TAP2 stability relies exclusively on TAP1, TAP1 steady-state levels depend on its interaction with tapasin (39, 40). The stabilization of the TAP1 subunit occurs via tapasin-binding to the core TM domain. Truncated rat TAP1 (missing the first 4 transmembrane (TM) domains) retains the full ability to interact with tapasin and MHC class I, indicating that the singly expressed TAP1 TM core domain acts as an exclusive tapasin-docking site.

Based on experiments with rat TAP mutants, it has been proposed that the heterodimeric assembly of the TAP subunits is accompanied by a displacement of tapasin from the core TM domains to the N-terminal domain in TAP1 (41). The assembly of pre-existing TAP1 chains with newly synthesized TAP2 might control the relocation of tapasin binding within TAP1.

Although, the results from the study on TAP1-GFP/TAP2-UL49.5 interaction suggest that TAP1 is the primary target for UL49.5-mediated degradation, failure to detect UL49.5-TAP1 association or UL49.5-mediated degradation of TAP1 in T2 cells not expressing TAP2 would seem to contradict this. However, this could be related to the following; in T2 cells, due to the absence of TAP2, the binding site for UL49.5 to TAP1 could be shielded by tapasin. Therefore, UL49.5-TAP1 complexes could be difficult to detect in those cells. It seems that the presence of TAP2 is compulsory for UL49.5-TAP1 interaction to occur. Heterodimerization of TAP subunits could lead to conformational changes and exposition of the core TM domains required for UL49.5 interaction.

TAP2 interacts with tapasin exclusively via its N-terminal domain. Interestingly, the inability of tapasin to associate with truncated TAP2 (TAP2 missing its N domain), seems to disturb the association of the peptide-loading complex chaperones (calnexin, calreticulin and ERp57). The N domains of TAP1 and TAP2 apparently have distinct requirements for the stable recruitment of ERp57, calreticulin and calnexin and the N domain of TAP2 (the tapasin-docking site) plays a key role in the functional integrity of the peptide-loading complex (41). To investigate the possibility that UL49.5-TAP interactions occur via the N-terminal domain of TAP2, it is of interest to examine whether UL49.5 alters the recruitment of ERp57, calreticulin and calnexin into the peptide-loading complex.

BHV-1 UL49.5 employs a two-step strategy to inhibit TAP function

Steady-state protein levels of TAP1-GFP and TAP2 appear not to be notably altered in the presence of UL49.5. However, the presence of UL49.5 still strongly impairs TAP activity.

Apparently, TAP inhibition and TAP degradation by UL49.5 are separable events. Experimental data from mutagenesis of the BHV-1 UL49.5 gene showed that its cytoplasmic domain is essential for the degradation activity of UL49.5 (chapter 4). The tailless mutant retained its ability to interact with the TAP transporter and to block its function despite the loss of features important for degradation.

One of the requirements for proteasome-dependent degradation is the attachment of ubiquitin moieties to lysines residues usually exposed at the cytoplasmic domains of target molecules (42-44). As a double lysine motif (KK) sequence is present in the cytoplasmic tail of BHV-1 UL49.5, it was of interest to investigate whether this motif is responsible for mediating TAP degradation. However, mutagenesis of these two residues into alanines (AA) could not rescue TAP1 and TAP2 proteins from degradation, implying that the KK-motif does not play a role in UL49.5-mediated degradation of TAP (chapter 5). At present, the existence of a direct

interaction between the UL49.5 cytoplasmic tail and the cytosolic domains of TAP1 and TAP2 cannot be ruled out. This interaction may very well exist and may be important for the UL49.5-mediated breakdown of TAP. For example, the cytoplasmic tail of UL49.5 could play a role in recruiting accessory proteins involved in the proteasome-dependent degradation pathway. Additionally, it would be interesting to design chimerical constructs where an irrelevant protein is tagged to the cytoplasmic tail of UL49.5, to test if the tail itself is able to promote degradation of those constructs.

Interestingly, BHV-1 UL49.5 itself is also degraded by proteasomes. Pulse-chase experiments revealed that the degradation of the BHV-1UL49.5$^{KK/AA}$ mutant was considerably delayed when compared to wild type UL49.5, suggesting a role for the KK-motif in the degradation of UL49.5 itself (Koppers-Lalić et al. unpublished data).

Since a UL49.5 mutant lacking the cytoplasmic tail still blocks peptide transport by TAP, it is likely that the binding domain is located within the transmembrane domain and/or the ER luminal domain of UL49.5. Whether the UL49.5 ER luminal domain can bind to TAP on its own is not known, but it fails to promote TAP inhibition when expressed alone (without the transmembrane anchor; D. Koppers-Lalić, unpublished observation). In contrast, the ectodomain of US6 (HCMV) alone is still able to bind and inactivate TAP (10, 36, 37, 45). Taken together, it appears that the transmembrane domain of UL49.5 is crucial for UL49.5-mediated TAP inhibition. However, its role in UL49.5-TAP binding and/or blocking still remains to be elucidated.

BHV-1 UL49.5-induced conformational arrest of the TAP complex

A number of mechanisms have been implicated in the ER degradation of misfolded or short-lived proteins (43, 44). It appears that UL49.5 induces the degradation of molecules that have already been properly folded, as UL49.5 targets fully assembled peptide-loading complexes. Interestingly, the UL49.5 tailless mutant does not target TAP for degradation, but still inhibits peptide transport. Thus, its effect on the conformation of TAP does not appear to make the transporter behave as a misfolded protein, despite the fact that it still blocks peptide transport. However, it is conceivable that such an interaction will still induce aberrant structural changes in TAP since this mutant is still able to block TAP. Indirect evidence that the UL49.5-TAP interaction influences the structural conformation of the transporter was obtained from the study of the lateral mobility of TAP in vivo (in living MJS TAP1-GFP cells). The translocation cycle is associated with conformational changes within the TAP complex. These changes are reflected by alterations in the lateral diffusion rate of TAP within the ER membrane. According to a study by Reits et al. (29), 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. Instead of fast lateral mobility as a result of UL49.5 inactivation of the TAP function, TAP exhibited a somewhat slower diffusion rate in the presence of UL49.5. This could be due to the fact that UL49.5 association with TAP affects the conformation of the transporter, or, simply that the addition of another transmembrane domain (UL49.5 TM) physically slows down TAP movements. It would be interesting to investigate how many UL49.5 molecules are bound to the TAP complex and whether additional transmembrane region(s) could slow down TAP mobility at the rate observed in those experiments.

Conformational changes within the TAP complex that alter its lateral mobility (e.g. the opening of the translocation pore) can be stimulated through the binding of long side chain peptides to TAP. The mobility of TAP in the presence of these peptides is considerably lower when compared to conventional peptides, as TAP appears to be trapped in an “open”

conformation and diffuses slowly through the ER. In UL49.5-expressing cells, long side chain peptides were unable to stimulate an “open” conformation of TAP. As peptides can bind to TAP regardless of UL49.5 presence, the failure of long-side-chain peptides to induce translocation pore opening could suggest that UL49.5 targets TAP core domains. UL49.5-induced conformational changes in TAP might change or obstruct the rotation and tilting of the transmembrane segments in the lipid bilayer necessary for translocation pore opening and closure. As the UL49.5-TAP interaction domains are unknown, this remains speculative. However, the possibility that UL49.5 binds to the TAP1 TM core domain (tapasin-binding site) could be a likely explanation for the inhibition of translocation pore opening. It has been proposed that the migration of tapasin from the TAP1 TM core domains upon TAP1 and TAP2 dimerization is a necessary event enabling the TM core domains to move freely during the peptide translocation process. Regardless of this apparently aberrant structural conformation of the transporter, the binding of ATP and peptides to TAP were not affected. Apparently, UL49.5-induced conformational changes in TAP appear not to be as strong as US6 conformational alterations of TAP. The binding of the US6 ectodomain to the ER luminal side of TAP, not only blocks the opening of the translocation pore, it also renders the TAP conformation incapable of binding ATP (29, 36).

Based on the data discussed above, at least two hypothetical models for UL49.5 interaction with TAP can be proposed.

First model: UL49.5 binds to the TM core domains of TAP1, possibly in the region of the alternative binding site for tapasin proposed on the basis of experiments with rat TAP mutants (41). The interaction between UL49.5 and the core TM domains of TAP1 are apparently dependent on heterodimerization of TAP subunits. When TAP1 is expressed as a single subunit in T2 cells, tapasin binds only at the TAP1 core TM domain, thus occupying a potential UL49.5 binding site. This could explain the inability to detect UL49.5-TAP1 association in T2 cells. Furthermore, distortion of the peptide-translocation step indicates that UL49.5 binds to the TAP1 core domain (translocation pore) when TAP1 is assembled with TAP2. Thus, UL49.5-binding to the TAP1 TM core domain physically obstructs the rotation and tilting of the transmembrane segments necessary for the peptide translocation process.

Second model: UL49.5 binds to both TAP1 and TAP2, where the ER luminal domains of each UL49.5 interact with the core domain of the transporter (translocation pore) as is the case with US6 (which forms oligomers). UL49.5 “crossed-links” both subunits of TAP, thus physically obstructing the pore and preventing the release of the peptide into the ER lumen.

The ability to block TAP is not a conserved feature of all UL49.5 proteins

Sequence comparison results showed that BHV-1 UL49.5 is encoded by a gene, which is present throughout the family *Herpesviridae* (46, 47). Among the first to be tested for TAP inhibiting properties were UL49.5 homologs encoded by PRV (UL49.5/gN) and EHV-1(gene 10), as it was known that both viruses strongly inhibit the TAP transporter during infection. Indeed, both homologs were able to promote TAP inhibition (chapter 5). Another UL49.5 homolog encoded by EHV-4 also exhibited TAP-inhibition properties although this virus had not previously been tested for such properties. The infection of natural host cells with UL49.5 mutant viruses of BHV-1, PRV and EHV-1 exhibited a loss of TAP inhibition phenotype. This observation confirmed that UL49.5 of these viruses is responsible for most of this phenotype

in the natural context of a viral infection.

However, not all UL49.5 homologs encoded by the various herpesviruses have the ability to block TAP. When UL49.5 counterparts encoded by HSV-1 and - 2 (UL49A), and HCMV (UL73/gN) were evaluated for TAP inhibition properties, the negative results were not surprising. All three viruses already encode potent TAP inhibitors (ICP47 for HSV-1 and -2; US6 for HCMV) and when ICP47 or US6 encoding genes are removed from their viral genomes (3, 10) leaving UL49.5 homologous genes intact, these viruses were no longer able to block TAP. The UL49.5 homolog encoded by the EBV BLRF1 gene also did not block TAP, although EBV infection mediates TAP inhibition (48).

Apparently, only a subset of UL49.5 homologs possesses the ability to block TAP (figure 1, tested homologs of UL49.5 are indicated). Apart from BHV-1, PRV, EHV-1 and -4, the UL49.5 homologs encoded by other members of the genus *Varicellovirus* could also fall into the category of TAP inhibitors (figure 1). However, two members of this genus (CHV and VZV) did not exhibit inhibitory properties. The UL49.5 homolog encoded by canine herpesvirus (CHV) was unable to induce TAP inhibition either in human cells or in its natural host cells. So far, it is not known whether CHV down-regulates MHC class I presentation in infected cells.

Although VZV infection strongly influenced MHC class I expression at the cell membrane, the inhibition of TAP could not be held responsible for this phenomenon. In fact, the TAP function has not been examined in the context of VZV infection. However, data published by Abendroth et al. (49) suggests that MHC class I molecules are not retained within the ER, but are further transported to the Golgi compartments where they are retained. This argues in favor of a rather different mechanism than TAP inhibition (76). This is further supported by our findings that the UL49.5 homolog encoded by VZV gene 9A is not able to block TAP (chapter 5).

It is possible that, apart from TAP inhibition, BHV-1 also possesses alternative strategies to prevent MHC class I cell surface display similar to VZV. Until the genes that encode the two known immunoevasins of BHV-1 (UL41 and UL49.5) are both removed from the genome and the resulting viral mutant is re-tested for interference with MHC class I presentation, this possibility remains to be confirmed. A similar approach could be applied to PRV and EHV-1.

Although, UL49.5 proteins encoded by different varicelloviruses are homologous, it remains puzzling why some of them block TAP and others do not. Apparently, the UL49.5 homologs that do not block TAP lack essential residues required for TAP inhibition. Testing of all available alphaherpesviruses-encoded UL49.5 proteins for TAP inhibition properties, followed by their sequence comparison and site directed mutagenesis, could lead to the identification of such residues.

Varicelloviruses-encoded TAP inhibitors: similarities and differences

Despite their close evolutionary relatedness and their common target, the UL49.5 proteins of BHV-1, PRV, and EHV-1 exhibit differences in the mechanism of human TAP inhibition. Contrary to BHV-1 UL49.5, the PRV and EHV-1 encoded UL49.5 homologs are not able to reduce steady-state levels of human TAP. The PRV encoded homolog in human cells does not prevent the TAP transporter to bind peptides or to acquire ATP (chapter 5). It is likely that PRV UL49.5 employs a similar mechanism in blocking TAP as the BHV-1 UL49.5 tailless mutant, e.g. arrest of the transporter in the translocation-incompetent state. Surprisingly, the mechanism by which the EHV-1 UL49.5 homolog blocks human TAP differs from the other two homologs. When expressed in human cells, EHV-1 UL49.5 prevents TAP accessibility to ATP

(chapter 5). The observed differences are remarkable since the proteins are evolutionarily closely related. The predicted structural properties of all three proteins appear to be similar, and they all share at least 40% amino acid homology. The highest similarity among all three proteins is within their ER luminal and transmembrane domains, whereas the cytoplasmic tails share the least homology (figure 1).

Thus far, the cytoplasmic tail of BHV-1 UL49.5 is the only one that can mediate the degradation of TAP. Although EHV-1 UL49.5 is different from the other two with regard to its inhibitory properties, the cytoplasmic tail of this protein is not required for its phenotype (chapter 5). It remains puzzling why, unlike the EHV-1 homolog, BHV-1 and PRV UL49.5 proteins are unable to block ATP binding to human TAP. It is possible that EHV-1 UL49.5-TAP interaction induces stronger structural alterations to the TAP transporter than the other two homologs or that this interaction occurs at a different TAP domain.

Although the transmembrane (TM) domains of TAP-inhibiting UL49.5 homologs are similar, they vary in the content of polar and charged amino acids. This could represent an interesting feature in the context of UL49.5-TAP interaction, since hydrogen bonding between polar amino acids can mediate strong interactions between alpha-helices within the lipid bilayer (50). Polar or charged amino acid side chains in the single TM domain of a membrane protein are likely to form favorable contacts with residues of other proteins within the cellular lipid bilayer and thereby facilitating the assembly of protein complexes (51). EHV-1 and EHV-4 UL49.5 predicted TM domains contain the polar amino acids serine (S; position 71) glutamine (Q; position 82) and arginine (R; position 85). Interestingly, glutamine residues apparently play a role in interhelical hydrogen bonding that strengthens TM domain association (50). Thus, it would be interesting to examine whether the glutamine residues within the TM domains of EHV-1 and 4 UL49.5 are involved in targeting TAP. These glutamine residues could form stringent associations between the TM domains of the EHV-1 and 4 homologs and TAP, which would cause displacement of TAP TM domains and the regions important for the binding of ATP. However, the domains and amino acids that interact between TAP subunits and UL49.5 have yet to be elucidated.

The diversity of TAP-inhibition mechanisms among UL49.5 homologs

Up to this point, the mechanistic aspects of UL49.5-mediated TAP inhibition were studied using the human cell line Mel JuSo. It is not known if the homologs induce a similar phenotype in host species-derived cells.

BHV-1 UL49.5 blocks human and bovine TAP to a similar extent (chapter 5). The inhibition of human and equine TAP by EHV-1 UL49.5 also appears comparable. Unfortunately, immunological tools for studying bovine, porcine and equine TAP in the presence of UL49.5 are not yet available. Furthermore, the sequences of equine TAP1 and 2 are currently unknown. Thus, one can only speculate that the mechanisms employed are similar across species barriers.

Interestingly, the PRV UL49.5 homolog, though acting as an inhibitor of both human and porcine TAP, exhibits obvious differences in TAP-blocking abilities (chapter 5). The expression of PRV UL49.5 in porcine cells (natural host) induces a strong inhibition of TAP, whereas in human cells the blocking of TAP activity is not complete (chapter 5; figure 3). Thus, it is possible that PRV UL49.5 acts differently when expressed in cell lines other than its natural host. Intriguingly, when PRV UL49.5 was tested in bovine cells, it did not induce bovine TAP

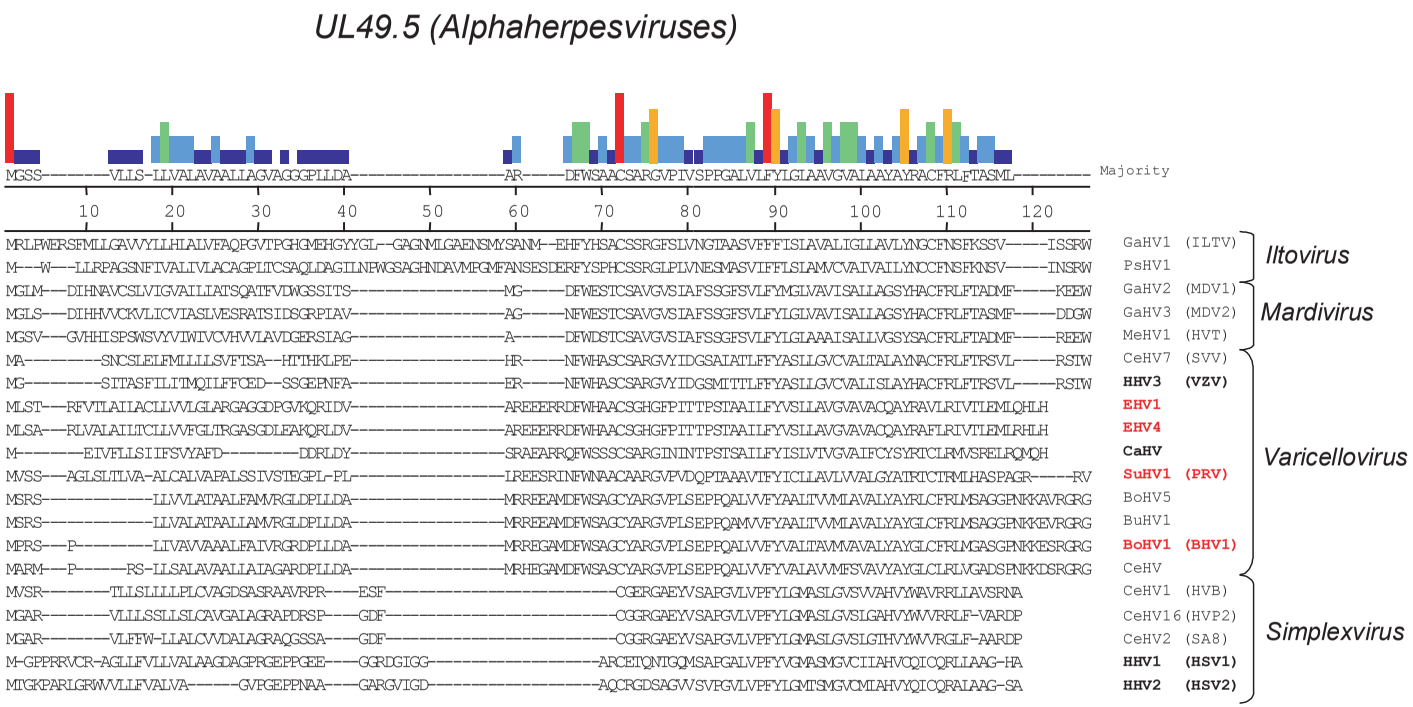

Fig. 1. Alignment of the amino acid sequences of UL49.5 homologs in alphaherpesviruses. Amino acid sequence alignment analysis of UL49.5 homologs (GaHV1 [YP_182341]; PsHV1 [AAQ73691]; GaHV2 [NP_057812]; GaHV3 [NP_066882]; MeHV1 [AAG30090]; CeHV7 [NP_077423]; HHV3 [YP_068406]; EHV1 [AAT67267]; EHV4 [CAA35670]; CaHV [patent EPO910406]; SuHV1 [YP_068325]; BoHV5 [NP_954898]; BuHV1 (F.A.M. Rijsewijk pers. comm.); BoHV1 [NP_045309]; CeHV (F.A.M. Rijsewijk pers. comm.); CeHV1 [AAP41468]; CeHV16 [YP_443897]; CeHV2 [AAU88116]; HHV1 [NP_044652]; HHV2 [NP_044520]) was performed by using ClustalW method (http://www.ebi.ac.uk/clustalw/). In bold: UL49.5 homologs tested for MHC class I down-regulation properties; bold red: UL49.5 homologs that block TAP.

inhibition (Koppers-Lalić, unpublished observation). This is in accordance with a previous study of Ambagala et al. (24) in which it was shown that an otherwise productive PRV infection of bovine cells could not induce bovine TAP inhibition. In contrast, BHV-1 UL49.5 strongly inhibits porcine TAP activity (Koppers-Lalić, unpublished observation). Until the TAP domains and/or the domains of TAP associated proteins that are targeted by UL49.5 homologs are precisely identified, the differences in cross-species activity cannot be fully addressed.

Regarding the UL49.5-TAP interaction, it is likely that BHV-1 UL49.5 targets a common domain or domains among TAP proteins from different species. Since human, porcine and bovine TAP1 and TAP2 demonstrate substantial homogeneity (around 80% identity; 52), the inability of PRV UL49.5 to block bovine TAP may be related to sequence differences within domains critically involved in PRV UL49.5 TAP interaction.

Dual function of UL49.5

In the context of a viral infection, all UL49.5 homologs investigated so far form a complex with the transmembrane protein, glycoprotein M (gM) (53-56). Generally, this is regarded as their common characteristic. The gN/gM complex is implicated in the maturation of virions and control of membrane fusion (57, 58). There is evidence that UL49.5 is covalently linked to glycoprotein M (gM) through the formation of a disulfide bond (59, 60). This bond is established through a cysteine residue that is highly conserved among the UL49.5 homologs and occurs in the extracellular domain of UL49.5. Despite complex formation with gM, the UL49.5 homologs of BHV-1, PRV and EHV-1 also associate with TAP.

In the study reported in chapter 6 it was investigated whether the UL49.5 protein can combine the interactions with gM and the TAP complex. During a BHV-1 infection, the UL49.5 protein is expressed at early and late stages of infection, whereas gM is detected only at a late stage. This difference in UL49.5 and gM expression kinetics permits UL49.5 to act as a TAP inhibitor at early times of infection without the interference of gM. At late stages of infection, there is an abundance of non-gM-bound UL49.5 which can continue TAP inhibition. Thus, the gM expression and gN/gM complex formation appear not to interfere with UL49.5-mediated TAP inhibition.

Interestingly, in BHV-1 infected cells, gM could not be found associated with the peptide loading complex, suggesting that the binding of UL49.5 to the TAP complex and to gM are mutually exclusive events. Co-expression of gM and UL49.5 in a cell line that is stably expressing both proteins abrogates UL49.5-mediated TAP inhibition (chapter 6). It seems that UL49.5- binding to gM is a preferential interaction that dominates outside the context of a viral infection.

In infected cells, UL49.5 can be found in different forms: as a monomer, a disulfide-linked homodimer and a disulfide-linked heterodimer to gM. In BHV-1 infected MJS cells, there are indications that a monomeric form of UL49.5 preferentially binds gM (59, 61 and chapter 6), whereas UL49.5 disulfide-linked homodimers (60) can be detected free from interaction to gM (61 and chapter 6).

Applications for immuno-evasive proteins; the future of UL49.5

UL49.5 as a tool for studying and manipulating antigen presentation A TAP-inhibited Antigen-Presenting Cell (APC) that has been exogenously loaded with peptides retains the capacity to stimulate cytotoxic T lymphocytes (CTLs) specific for the added peptide, without adventitious co-stimulation of CTLs specific for other endogenous peptides. For example, CTL recognition of APCs transduced with UL49.5 can be fully restored by the addition of exogenous peptide (chapter 7). Thus, CTL responses can be specifically directed towards a desired target sequence. This approach proved to be useful for generating minor Histocompatibility antigens (mHags)-specific CTLs in vitro that are potent reagents for adoptive immunotherapy of leukemia after allogeneic stem cell transplantation (62). Furthermore, HLA-A2^{pos} APCs, transduced with BHV-1 UL49.5 and pulsed with mHag peptides, can indeed elicit mHag-specific but not alloHLA-specific CTL responses. Such APCs are suitable antigen-specific stimulators for the induction of mHag-specific alloHLA-A2-restricted T cells. These CTLs may serve as reagents for the treatment of relapsed leukemia after HLA-mismatched stem cell transplantation.

However, the generation of mHag-specific alloHLA-A2-restricted CTLs is hampered by adventitious expansion of broad alloHLA-A2-specific T cells present in the T cell repertoire of HLA-A2^{neg} individuals (63). Such alloreactivities are directed against a variety of peptides ubiquitously presented by alloHLA molecules and are potentially harmful to the patient (64). Minimizing the number of peptides presented by the APC's alloHLA molecules could reduce the induction of undesired alloHLA-reactivities. Since UL49.5 also reduces alloHLA-reactivity and facilitates preferential presentation of a target sequence even in an HLA-mismatched setting, it provides an excellent tool to further study fundamental aspects of alloHLA-reactivity.

Impairment of the TAP function, which leads to the reduction of antigen-presentation, is frequently observed in human tumors (65). Tumors may thus escape from immune surveillance by CTLs (66, 67). An alternative repertoire of peptide epitopes emerging at the surface of murine cells with impaired function of TAP has recently been described (68). These peptides may act as immunogenic neo-antigens and can be exploited as targets for immunotherapy against TAP-deficient tumors.

Since BHV-1 UL49.5 protein inactivates TAP function in mouse cells, its expression in mouse colon carcinomas reduced surface display of MHC class I and allowed these tumors to avoid recognition by conventional tumor-specific CTL. The TAP-inhibitory effect of UL49.5 resulted in alterations of the peptide repertoire by strongly promoting presentation of TEIPP (T cell Epitopes associated with Impaired Peptide Processing) antigens (32, 68) (Chapter 8).

Since TAP-deficiency leads to the presentation of CTL epitopes that are not normally displayed at the cell surface, this suggests that CTL with this novel specificity might be recruited for the immune control of tumors or viruses that employ immune evasion strategies.

Vaccine strains without immune evasion genes are anticipated to be more efficacious Prophylactic vaccination envisages to protect the host against disease evoked by an infection and, if possible, to control or even eradicate the infectious agent. Good vaccines confront the host with specific antigens and evoke appropriate B-cell and T-cell responses that eventually lead to memory B-cells and T-cells that can readily reactivate upon infection (69).

The discovery of immune evasion genes within viral genomes has initiated a new wave in vaccine improvement. Ferko et al. (70) obtained an influenza mutant with a deletion in the dsRNA binding region of the NS1 protein. This mutant was deficient in binding dsRNA, hence unable to block the signal transduction of Toll-like receptor 3 (TLR-3) and the downstream

induction of IFNα/β. Consequently, it evoked markedly higher levels of IFNα/β in serum than in wild type virus. As a result of strong antiviral response, the NS1 deletion mutant was unable to efficiently replicate in the host. Despite the lack of replication in the host, the NS1 mutant induced qualitatively better B-cell and T-cell responses than the wild type virus. Mice immunized with this deletion mutant were completely protected against a challenge infection (70). This example shows two simultaneous vaccine improvements: knocking out an immune evasion gene leads to a more immunogenic vaccine and it makes the live vaccine less virulent and, therefore, safer.

Compared to RNA viruses, herpesviruses harbor many more immune evasion genes, both counteracting the innate (71) and the acquired immune responses. There are indications that even knocking out one of these immune evasion genes may produce a more immunogenic and safer virus. For example, after an intranasal infection in mice with K3-deficient viruses, these viruses were not cleared from the lung more rapidly, but the number of latently infected spleen cells was reduced and the frequency of virus-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) was increased (72).

Before the role of UL49.5 in TAP inhibition was uncovered, an interesting experiment was reported by Liang et al. (73) who used a BHV-1 deletion mutant encompassing the UL50, UL49.5 and UL49 genes in a vaccination / challenge experiment in calves. First of all, the BHV-1 mutant of Liang et al. had a strongly reduced virulence and could hardly be recovered after intranasal infection, reminiscent of the results with the influenza NS1 mutant (70). Secondly, this BHV-1 mutant protected the vaccinated calves against secretion of challenge virus better than any other live BHV-1 vaccine. Commonly, live BHV-1 vaccines are able to reduce the titers of the wild type challenge virus about 100-fold, but the UL50/UL49.5/UL49 deletion mutant reduced the challenge virus at least 10,000 times, indicating the induction of a superior immune response. The most straightforward explanation for the improved immune response is the induction of a stronger anti-BHV-1 T-cell response, due to the improved MHC class I presentation. This model implicates that wild type BHV-1 virus is not able to escape from T-cell presentation. Indeed, after wild type BHV-1 infection, despite the TAP inhibiting function of UL49.5, specific anti-BHV-1 cytotoxic T-cells can be detected (74, 75) Theoretically, the results obtained with the BHV-1 mutant of Liang *et al*. may be (partly) due to the deletion of the neighbouring UL50 and/or UL49 genes. Evaluation of the immunogenicity of a BHV-1 UL49.5 deletion mutant with intact UL50 and UL49 genes *in vivo* and detailed analysis of the T-cell responses after infection and subsequent challenge will lead to a better understanding of the influence of UL49.5 on the pathogenesis of and immunity against BHV-1 and related viruses encoding UL49.5 proteins with TAP-inhibiting properties.

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