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

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CHAPTER 5

Manuscript in Preparation

Varicellovirus UL49.5-encoded TAP inhibitors: one family, one target, but diverse mechanisms

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The Transporter associated with Antigen Processing (TAP) plays a crucial role in MHC class I-restricted antigen presentation, thus representing an attractive target for viruses aiming to escape from immunosurveillance. Several members of the subfamily *Alphaherpesvirinae*, genus *Varicellovirus*, specifically block TAP-dependent peptide transport within a few hours after infection. Previously, we have shown that bovine herpesvirus 1 (BHV-1), a member of the genus *Varicellovirus*, encodes a potent inhibitor of TAP, the UL49.5 protein. In this study, we characterize UL49.5 homologs encoded by other varicelloviruses with respect to TAP inhibition. The UL49.5 proteins of BHV-1, pseudorabies virus (PRV), and equine herpesvirus 1 and 4 (EHV-1 & 4) inhibit TAP-mediated peptide transport. Corresponding mutant viruses, from which the UL49.5-gene has been deleted, no longer blocked TAP, indicating that UL49.5 is necessary and sufficient to abolish TAP function during productive virus infection. When studying the mechanisms of action of these different TAP-inhibiting proteins, we surprisingly found each viral homolog to act via a different mechanism. BHV-1 UL49.5 destabilizes TAP, whereas PRV UL49.5 and EHV-1 UL49.5 do not. Furthermore, EHV-1 UL49.5 blocks ATP-binding to TAP while UL49.5 homologs of PRV and BHV-1 do not. Thus, these results classify a number of varicellovirus-encoded UL49.5 homologs as members of a novel family of TAP inhibiting proteins acting through a variety of mechanisms.

Evolving under the selective pressure of the host immune system, viruses have developed countermeasures to prevent the recognition by cytotoxic $CD8^+$ T lymphocytes (CTLs). CTLs recognize viral antigens presented as peptides bound to major histocompatibility complex (MHC) class I molecules at the surface of infected cells. In particular, herpesviruses have acquired diverse mechanisms to escape from the cellular immune response by inhibiting antigen presentation in the context of MHC class I molecules (1, 2).

Most peptide antigens presented by MHC class I molecules are transported across the endoplasmic reticulum (ER) membrane by the transporter associated with antigen processing (TAP). TAP is a heterodimer composed of TAP1 and TAP2 subunits and belongs to the ATP-binding cassette (ABC) family of transporters. TAP is highly conserved and most of our present knowledge is based on studies on human TAP (3), but other mammalian TAP homologs are believed to function in much the same way (4). TAP can translocate a broad spectrum of peptides across the ER membrane and requires hydrolysis of ATP for this purpose. TAP is part

of the MHC class I peptide-loading complex that also contains tapasin, MHC class I heavy and light chains, and several auxiliary proteins including calreticulin and ERp57 (3-5, 53).

Several herpesviruses have acquired mechanisms to interfere with TAP function. Interestingly, inhibition of TAP transport is achieved through different mechanisms, exerted by different gene products. ICP47, encoded by the alphaherpesviruses herpes simplex virus type 1 and 2 (HSV1 and 2), acts as a high-affinity competitor for peptide binding to cytosolic domains of TAP (6-15). US6, encoded by the betaherpesvirus human cytomegalovirus (HCMV), inhibits the interaction of ATP with TAP (16-21). The mK3 protein encoded by the murine gammaherpesvirus-68 (MHV-68), acts as a ubiquitin ligase linking MHC class I molecules and TAP to the ubiquitin/proteasome degradation pathway (22-31).

Within the subfamily of *Alphaherpesvirinae*, members of the genus *Varicellovirus* also cause down-regulation of MHC class I molecules at the cell surface. Previous studies have shown that bovine herpesvirus type 1 (BHV-1), pseudorabies virus (PRV), and equine herpesvirus type 1 (EHV-1) strongly affect TAP-dependent peptide transport (32-37) and as a result of this virus-induced TAP inhibition, MHC class I molecules are retained in the ER between 3-5 hours after infection (32, 33, 36).

We have recently shown that the BHV-1-encoded UL49.5 protein effectively inactivates TAP (35, 37). The mechanism of TAP inhibition appears to be unique compared to that of other TAP-inhibiting proteins. BHV-1 UL49.5-mediated inactivation of TAP involves two events: an arrest of the peptide transporter in a translocation-incompetent state and proteasomal degradation of both subunits of TAP (35).

Bovine UL49.5 encodes a type I transmembrane protein of 96 amino acids residues. It has an N-terminal signal peptide, which is cleaved off (35), a predicted ER-luminal domain of 32 amino acids, a transmembrane region of 25 amino acids, and a cytoplasmic tail of 17 amino acids (38). UL49.5 homologs have been found in every alpha-, beta- and gammaherpesvirus sequenced to date (39-41), and the interaction with the viral membrane glycoprotein M (gM) appears to be a common property of all UL49.5 homologs (37, 42-44). UL49.5, also known as glycoprotein N (gN), forms a complex with gM. gN/gM complexes are implicated in virion maturation and membrane fusion processes (45, 46). Despite its interaction with gM, BHV-1 UL49.5 is still capable of blocking TAP in the context of BHV-1 infection (37). Expression of the early protein UL49.5 precedes that of the late gene product gM. In addition to UL49.5-gM heterodimers, UL49.5 exists as a homodimer in which the subunits are connected through disulfide bridges. The presence of a single cysteine residue in the ER-luminal/extracellular domain is a conserved property of all UL49.5 proteins (39). Whereas this cysteine residue is essential for the interaction of UL49.5 with gM, it is not involved in the binding of UL49.5 to TAP (35).

The capacity to inhibit peptide transport by TAP has only been established for BHV-1 UL49.5, but not for its homologs encoded by other varicelloviruses. The UL49.5 gene products of HSV1 (an alphaherpesviruses), HCMV (a betaherpesvirus) and EBV (a gammaherpesvirus) fail to block TAP, indicating that not all UL49.5 homologs act as inhibitors of TAP (8, 10, 35). In this study, the effects on TAP-function were assessed in more detail for UL49.5 homologs encoded by various members of the genus *Varicellovirus*. The UL49.5 homologs from PRV, EHV-1 and EHV-4 were found to down-regulate MHC class I surface expression through TAP inhibition in their natural host and in human cells. Using recombinant deletion mutants of BHV-1, PRV and EHV-1, we have shown that the UL49.5 proteins of these viruses are necessary and sufficient for the inhibition of TAP-dependent peptide transport. These data indicate that these proteins are indeed responsible for the previously observed TAP inhibition and MHC class I downregulation during BHV-1, PRV and EHV1 infection (32, 33, 36).

The expression of PRV and EHV-1 UL49.5 homologs in human cells did not diminish the steady state levels of TAP1 and TAP2, as previously observed for BHV-1 UL49.5 (35).

Interestingly, the EHV-1 UL49.5 homolog interferes with TAP function through the inhibition of ATP-binding by TAP, a function that was not influenced by BHV-1 UL49.5 (35). Thus, although BHV-1, PRV, and EHV-1-encoded UL49.5 proteins all induce a similar phenotype ultimately, i.e. inhibition of peptide transport, they act via different mechanisms.

Materials and Methods

UL49.5 constructs Purified viral DNA from BHV-1 strain Lam and CHV strain Eva (Animal Sciences Group, Lelystad, The Netherlands), PRV strain Kaplan (Ka) (42), EHV-1 strain Ab-4 (kindly provided by J.Rola; National Veterinary Research Institute, Pulawy, Poland) and EHV-4 (kindly provided by R. de Groot; Dept. of Infectious Diseases and Immunology, Utrecht University, The Netherlands), and VZV (viral DNA extracted from patient material; kindly provided by E. Klaas, LUMC, Leiden, The Netherlands) were used as a template for polymerase chain reaction (PCR) amplification. PCR-reactions were performed under standard conditions using *Pfu* DNA polymerase (Invitrogen) and specific primers (see Table 1) for amplification of the full length coding sequence of the UL49.5 genes of BHV-1 (35), PRV, EHV-1, EHV-4, CHV and VZV UL49.5. The sequences of the primers are based on published sequences found in the NCBI database, except for the sequence of the CHV primers (Haanes, E. and Rexann, F. ´Recombinant canine herpesviruses´, patent number EPO910406, publication date 1997-08-21). To obtain tailless versions of the EHV-1 UL49.5 gene, the specific primer (table 1) was used to generate PCR product lacking the last 45 nucleotides (15 amino acids from the C-terminus). In the BHV-1 UL49.5$^{KK/AA}$ mutant, the two lysine residues in the cytoplasmic tail of UL49.5 of BHV-1 were replaced using specific primers. PCR-generated products were inserted into pLZRS upstream of the internal ribosome entry site (IRES) element and GFP (pLZRS-IRES-GFP; information can be obtained at www.stanford.edu/group/nolan/retroviral_systems/retsys.html).

Table 1

Primer name	Primer sequence (restriction enzymes used in bold)	
BHV-1UL49.5$^{KK/AA}$	Fw:	5'-CA**GAATTC**ACCATGCCGCGGTCG-3'
BHV-1UL49.5$^{KK/AA}$	Rev:	5'-GGG**GAATTC**TTTCAGCCCCGCCCCCGCGACTCCGCGGCATTGGGC-3'
PRV UL49.5	Fw:	5'-CGC**GGATCC**GACACACCAGGATGGTC-3'
PRV UL49.5	Rev:	5'-GCG**GAATTC**GGATCGCTCTTTATACGC-3'
EHV-1UL49.5	Fw:	5'-GCCGCCACCATGCTGTCCACGAGATTC-3'
EHV-1UL49.5	Rev:	5'-TTGTCAATGCAGGTGTTGCAACATCTC-3'
EHV-1UL49.5Δtail	Fw:	5'-GA**GAATTC**AGGACCATGCTGTCCACGAGATTC-3'
EHV-1UL49.5Δtail	Rev:	5'-CG**CTCGAG**CTTCAGCGGTATGCCTG-3'
EHV-4 UL49.5	Fw:	5'-GCCGCCACCATGTTGTCAGCGAGATTAG-3'
EHV-4 UL49.5	Rev:	5'-TGCTCAGTGTAGGTGTCGCAAATC-3'
CHV UL49.5	Fw:	5' -GCCGCCACCATGGAGATAGTATTTTTAC-3'
CHV UL49.5	Rev:	5' -CCATTAGTGTTGCATCTGACGAAGTTC-3'
VZV UL49.5	Fw:	5'-GCC**GGATCC**AAGATGGGATCAATTAC-3'
VZV UL49.5	Rev:	5'-CCG**GAATTC**CGGTTACCACGTGCTGCG-3'

Cell Lines and Retroviral Transduction The human melanoma cell line Mel JuSo (MJS) and Madin-Darby bovine kidney (MDBK) cells (American Type Culture Collection, ATCC) were maintained in RPMI-1640 medium; porcine kidney (PK15) cells, Madin-Darby canine kidney I (MDCK I) cells, and the equine epithelial cell line E.derm were maintained in DMEM medium. Media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (with the exception of E.derm cells that required 20%), 2 mM L-glutamine (Invitrogen), 140 IU/ml penicillin and 140 µg/ml streptomycin.

Recombinant retroviruses were obtained in the Phoenix amphotropic packaging system as described previously (www.stanford.edu/group/nolan/retroviral_systems/retsys.html). For MDBK cell transductions, the GP2-293 packaging cell line was used as described (BD Bioscience Clontech; www.bdbiosciences.com). Cells were cotransfected with retroviral expression vector and pVSV-G construct (envelope vector) for retrovirus production. Retrovirus containing medium was collected 48 hours post transfection. MJS, MDCKI, PK15, and E.derm cells were transduced with recombinant retroviruses to generate the following stable cell lines: MJS, MDCKI, PK15, and E.derm controls (containing BHV-1 UL49.5 in anti-sense orientation, GFP^+); MJS UL49.5^{VZV} (containing VZV UL49.5 in sense orientation, GFP^+); MJS UL49.5$^{BHV\text{-}1}$ (containing BHV-1 UL49.5 in sense orientation, GFP^+); MJS UL49.5^{CHV} and MDCKI UL49.5^{CHV} (containing CHV UL49.5 in sense orientation, GFP^+); MJS UL49.5^{PRV} and PK15 UL49.5^{PRV} (containing PRV UL49.5 in sense orientation, GFP^+); MJS UL49.5^{EHV1} and E.derm UL49.5^{EHV1} (containing EHV-1 UL49.5 in sense orientation, GFP^+); MJS UL49.5$^{EHV1\Delta tail}$ (containing EHV-1 UL49.5Δtail in sense orientation, GFP^+); MJS UL49.5^{EHV4} and E.derm UL49.5^{EHV4} (containing EHV-4 UL49.5 in sense orientation, GFP^+). MDBK cells were transduced four times with VSV-G containing recombinant retroviruses to generate the following stable cell lines: MDBK control (containing BHV-1 UL49.5 in anti-sense orientation, GFP^+) and MDBK UL49.5$^{BHV\text{-}1}$ (containing BHV-1 UL49.5 in sense orientation, GFP^+). Cells were selected for GFP expression using a FACSVantage cell sorter (Becton Dickinson). To obtain MJS cells stably expressing the HCMV-encoded US6 (MJS US6), MJS cells were transfected with pCDNA3-US6-IRES-NLS-GFP and selected for neomycin resistance (36).

Viruses and Virus Infections The wild type viruses used in this study are: BHV-1 strain Lam, PRV strain Kaplan (Ka) and EHV-1 strain RacL11. The UL49.5 deletion mutants of PRV and EHV-1 used in this study have been described before (42, 43). Infections with wild type and mutant herpesviruses were performed with the following cell lines: MDBK cells for BHV-1; PK15 cells for PRV and E.derm cells for EHV-1. The cells were washed once with PBS and infected with BHV-1 or with PRV at an m.o.i. of 10, and with EHV-1 at an m.o.i. of 5 at 37°C in serum-free medium. After 2 hrs, medium containing 10% FBS was added. Mock-infected cells were treated under the same conditions as infected cells. After 5 hours of infection, cells were collected and prepared for the peptide translocation assay.

Construction BHV-1 UL49.5 deletion mutant The BHV-1 UL49.5 mutant was generated by homologous recombination. The parent strain was BHV-1 strain Lam. The recombination region upstream of the UL49.5 gene was a 1.4 kb fragment running from nucleotide residue 7670 to 9061 (residue numbers based on the complete BHV-1 genome with NCBI accession number NC_001847, updated 30 March 2006). This fragment starts at a BstXI site 1.3 kb upstream of the start codon of the UL49.5 open reading frame and ends at its amino acid residue 31. The recombination region downstream of the UL49.5 gene was a 1.9 kb fragment that runs from nucleotide residue 9075 to 10972. This fragment starts at amino acid residue 36 of UL49.5 and ends at an FspI site 1.7 kb downstream of the UL49.5 open reading frame. In between the two UL49.5 recombination fragments, a 2.2 kb NruI – PvuII fragment was cloned that carries the hGFP gene in the expression cassette of pCDNA3 (Invitrogen). The complete recombination fragment (5.5 kb) was co-transfected with purified BHV-1 Lam DNA into EBTr

cells using the method of Graham and van der Eb (1973). After plating the supernatant of freeze/thawed transfected cells, a green plaque was found that, following three rounds of plaque purification, failed to react with anti-UL49.5 serum. This BHV-1-UL49.5 mutant could be grown to a titer of $10^{7.0}$ $TCID_{50}$/ml and was capable of penetrating bovine cells with the same kinetics as the wild type Lam strain (data not shown).

Reagents The antibodies used in this study were: anti-transferrin receptor (TfR) monoclonal antibody (mAb) 66Ig10, anti-TfR mAb H68.4 (Roche), anti-human MHC class I complexes mAb W6/32, anti-human MHC class I heavy chain mAb HC-10 (kindly provided by H. Ploegh, Whitehead Institute, MIT, Boston, Massachusetts, USA), anti-human class II HLA-DR mAb Tü36 (kindly provided by A. Ziegler, Institute for Immunogenetics, Universitätsklinikum Charité, Berlin, Germany), anti-TAP1 mAb 143.5 (kindly provided by R. Tampe, Institute of Biochemistry, Johann Wolfgang Goethe University, Frankfurt, Germany), anti-TAP2 mAb 435.3 (a kind gift from P. van Endert, INSERM U25, Institut Necker, Paris, France), monoclonal antibody IL-A19 directed against bovine MHC class I molecules, (a gift from Dr. J. Naessens, ILRAD, Nairobi, Kenya). The anti-equine and anti-canine MHC class I complexes mAb H58A and anti-porcine MHC class I mAb PT85A were purchased from VMRD Inc., Pullman, WA, U.S.A. Mouse anti-BHV-1 UL49.5 serum was kindly provided by G.J. Letchworth, University of Wisconsin, Madison, Wisconsin, USA. Polyclonal rabbit anti-BHV-1 UL49.5 serum H11 was raised against a synthetic peptide representing the N-terminal sequence of BHV-1 UL49.5 and has been described (37), as was the anti-EHV-1 pUL49.5 rabbit serum (43). The rabbit antiserum raised against PRV-gN was previously described (47).

Flow Cytometry Cells were trypsinized and resuspended in phosphate-buffered saline (PBS) containing 1% BSA and 0.05% sodium azide. The cells were incubated with specific antibodies on ice for one hour. After washing, the cells were incubated with PE-conjugated anti-mouse antibody for 45 min. Stained cells were analyzed by flow cytometry on a FACS Calibur flow cytometer (Becton Dickinson). To exclude dead cells, 7-aminoactinomycin D (7-AAD, Sigma-Aldrich) was added at a concentration of 0.5 µg/ml to all samples before analysis. Cells were analyzed using CellQuest software (Becton Dickinson).

Immunoblotting Cells were lysed in NP-40 lysis mix containing 50 mM Tris-HCL, pH 7.4, 5 mM $MgCl_2$ and 0.5% NP-40, supplemented with 1 mM AEBSF (4-(2-Aminoethyl)-benzenesulfonyl fluoride), 1 mM leupeptin and 20 µM Cbz-L3 (Carbobenzoxy-1-Leucyl-1-Leucyl-1-Leucinal-H; Peptides International, Inc). The samples were kept on ice throughout the experiment. Protein complexes were denatured in reducing sample buffer (2% SDS, 50 mM Tris pH 8.0, 10% glycerol, 5% β-ME, 0.05% bromophenol blue) for 5 min at 96°C. Immunoblot (IB) analysis was performed on denatured cell lysates separated by SDS-PAGE and blotted onto PVDF membranes. The blots were stained with the antibodies indicated, followed by horseradish peroxidase-conjugated goat-anti-mouse or swine-anti-rabbit Igs (Jackson Laboratories), and visualized by ECLplus (Amersham).

Peptide Transport Assay The fluorescence-based peptide transport assay was performed as previously described (35). In brief, MJS cells were permeabilized with Streptolysin O (Murex Diagnostics Ltd.) at 37°C, followed by incubation with the fluorescein-conjugated synthetic peptide CVNKTERAY in the presence or absence of ATP. Peptide translocation was terminated by adding ice-cold lysis buffer containing 1% Triton X-100. After lysis, cell debris was removed by centrifugation, and supernatants were collected and incubated with Concanavalin A (ConA)-Sepharose (Amersham). After extensive washing of the beads, the peptides were eluted with elution buffer (500 mM mannopyranoside, 10 mM EDTA, 50 mM Tris-HCl pH 8.0), by vigorous shaking and further separated from ConA by centrifugation

at 12,000 x g. The fluorescence intensity was measured using a fluorescence plate reader (CytoFluor, PerSeptive Biosystems; excitation 485 nm/ emission 530 nm).

ATP-Agarose Binding Assay TAP binding to ATP-agarose was assayed as described (35). In brief, cells were solubilized in 1% (w/v) digitonin, 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 150 mM NaCl, 5 mM IAA (iodoacatamide) and 1 mM AEBSF. Hydrated C-8 ATP-agarose (Fluka/Sigma) was added to the post nuclear supernatant and incubated by rotation at 4°C. After 2 hours, the supernatant was separated from the ATP-agarose pellet after centrifugation and the resulting pellet washed three times with 0.1% (w/v) digitonin, 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ and 150 mM NaCl. Proteins bound to ATP-agarose were eluted with 500 mM EDTA and SDS sample buffer was added to both the supernatant and the pellet. The samples were heated at 65°C for 10 min and analyzed by SDS-PAGE and immunoblotting.

Results

UL49.5 proteins of several varicelloviruses block TAP Homologs of the UL49.5 gene have been found in every herpesvirus sequenced to date (40). Although the UL49.5 proteins encoded by these viruses have a similar overall structure, their amino acid sequence demonstrates considerable heterogeneity, even amongst the varicellovirus UL49.5 proteins (Fig. 1a). Thus, at this moment, no predictions can be made on the basis of amino acid sequence as to which homologs are capable of inhibiting TAP. In this study, we have examined the TAP-inhibiting capacity of the UL49.5 proteins encoded by the varicelloviruses PRV, EHV-1, EHV-4, CHV and VZV.

Downregulation of class I expression by UL49.5 homologs was investigated using host cell lines transduced with recombinant retroviruses carrying the UL49.5 genes upstream of an IRES element and GFP. In the presence of the PRV, EHV-1 and EHV-4-encoded UL49.5 proteins, class I expression was reduced by 78%, 97% and 96%, respectively. The UL49.5 proteins encoded by CHV and VZV failed to down-regulate MHC class I surface expression (data not shown). These results indicate that the PRV, EHV-1 and EHV-4 UL49.5 gene products (in addition to BHV-1 UL49.5) interfere with antigen presentation via MHC class I molecules.

To investigate whether the observed down-regulation of MHC class I cell surface expression relied on inhibition of TAP, cell lines stably expressing these UL49.5 homologs were evaluated for TAP-dependent peptide transport. The UL49.5 proteins of BHV-1, PRV, EHV-1 (Fig. 1b) and EHV-4 (data not shown) were found to strongly inhibit TAP activity. Thus, although the identity at the amino acid level is limited (less then 40% identity) among the BHV-1, PRV and EHV1-encoded UL49.5 proteins (Fig. 1a), the ability to inhibit TAP is a common property of these varicellovirus gene products.

UL49.5 is responsible for TAP transport inhibition in virus-infected cells Infection of cells with BHV-1, PRV and EHV-1 results in a strong inhibition of TAP-dependent peptide transport (32, 33, 48). To confirm that the UL49.5 protein is responsible for TAP inhibition during viral infection, we assayed peptide transport activity in the natural host cells infected with wild type BHV-1, PRV and EHV-1 or with the corresponding mutant viruses lacking a functional UL49.5 gene (42, 43). The strong reduction of peptide transport observed in cells infected with wild-type viruses did not occur in the presence of the viral mutants lacking the UL49.5 protein (Fig. 2). Therefore, the UL49.5 gene products of BHV-1, PRV and EHV-1 are both necessary and sufficient for the inhibition of peptide translocation during productive virus infection.

A

Alphaherpesvirinae — *MHC class I downregulation*

Simplexvirus

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HSV1------MGPPRRVCRAGLLFVLLVALAAGDAGPRGEPPGEEGGRDGIGGARCETQNTGQMSAPGALVPFYVGMASMGVCIIAHVCQICQRLLAAGHA---------  -
HSV2-----MTGKPARLGR--WVVLLFVALVAGVPG---EPPNAAGARGVIGDAQCRGDSAGVVSVPGVLVPFYLGMTSMGVCMIAHVYQICQRALAAGSA---------  -
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Varicellovirus

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BHV1---------MPRSPLIVAVVAAALFAIVRGRDPLLDAMR-REGAMDFWSAGCYARGVPLSEPPQALVVFYVALTAVMVAVALYAYGLCFRLMGASGPNKKESRGRG  +
PRV  --MVSSAGLSLTLVAALCALVAPALSSIVSTEGPLPLLR-EESRINFWNAACAARGVPVDQPTAAAVTFYICLLAVLVVALGYATRTCTRMLHASPAGRRV-----  +
EHV1MLSTRFVTLAILACLLVVLGLARGAGGDPGVKQRIDVAR-EEERRDFWHAACSGHGFPITTPSTAAILFYVSLLAVGVAVACQAYRAVLRIVTLEMLQHLH-----  +
EHV4MLSARLVTLAILTCLLVVFGLTRGASGDLEAKQRLDVAR-EEERRDFWHAACSGHGFPITTPSTAAILFYVSLLAVGVAVACQAYRAFLRIVTLEMLRHLH-----  +
CHV  --------------MEIVFLLSIIFSVYAFDDDRLDYSR-AEARRQFWSSSCSARGININTPSTSAILFYISLVTVGVAIFCYSYRTCIRMVSRELRQMQH-----  -
VZV  -----------MGSITASFILITMQILFFCEDS---SGEPNFAERNFWHASCSARGVYIDGSMITTLFFYASLLGVCVALISLAYHACFRLFTRSVLRSTW-----  -
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Conserved amino acids among UL49.5 proteins of several members of the subfamily *Alphaherpesvirinae*

Conserved amino acids among UL49.5 proteins of several members of the genus *Varicellovirus*

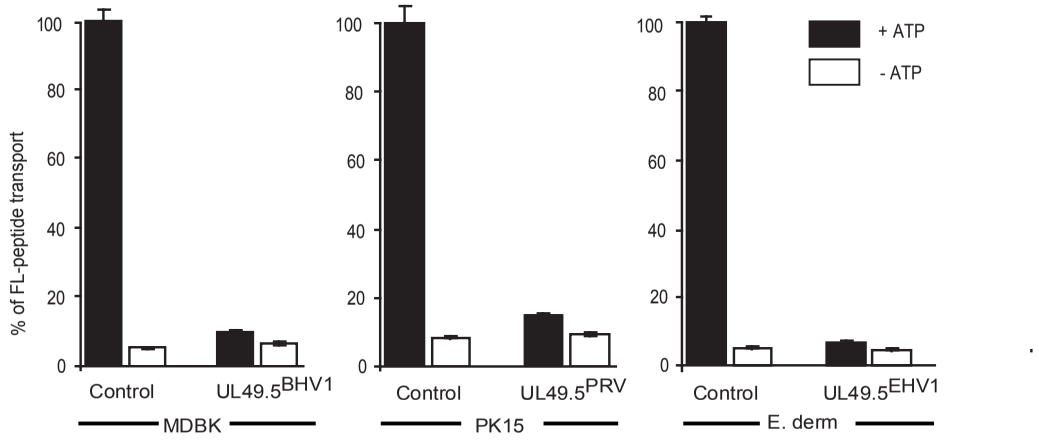

Fig. 1. TAP inhibition by varicellovirus-encoded UL49.5 homologs. (A) Alignment of the amino acid sequences of UL49.5 homologs in alphaherpesviruses (HSV-1 [NP_044652], HSV-2 [NP_044520], BHV-1 [NP_045309], PRV [YP_068325], EHV1 [AAT67267], EHV4 [CAA35670], CHV [patent EPO910406], VZV [YP_068406]). Hydrophobic residues indicative of N-terminal signal sequences (dashed line) and membrane anchor regions (bold line) are indicated. Amino acid sequence alignment analysis of UL49.5 homologs was performed by using VectorNTI software (Invitrogen). Downregulation of MHC class I surface expression is indicated. (B) TAP-dependent peptide transport is inhibited in natural host cells expressing the UL49.5 homologs of BHV-1, PRV, and EHV-1. Cell lines used are specified. Peptide transport is expressed as percentage of translocation, relative to the translocation observed in control cells (defined as 100%).

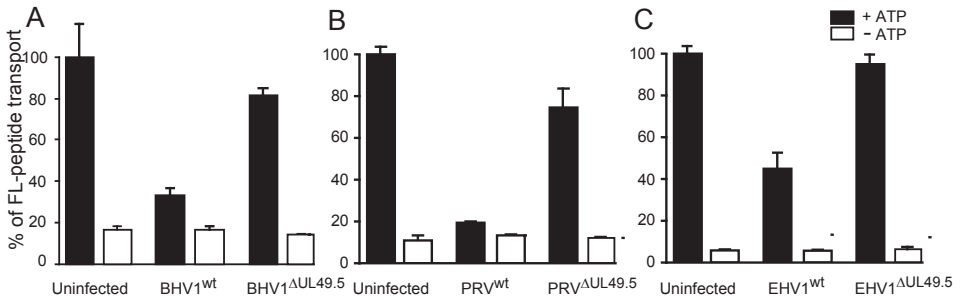

Fig. 2. UL49.5 is responsible for inhibition of TAP in virus-infected cells. (A) Bovine MDBK cells were infected with wild-type BHV-1 or with a UL49.5 deletion mutant. (B) Porcine PK15 cells were infected with wild-type PRV or with a UL49.5 deletion mutant. (C) Equine E.derm cells were infected with wild-type EHV-1 or with a UL49.5 deletion mutant. In all experiments, mock-treated (uninfected) natural host cells were used as a control. Peptide transport is assessed at 5 hrs post-infection and the data are expressed as percentage of translocation, relative to the translocation observed in control cells (defined as 100%).

The influence of UL49.5 homologs on TAP1 and TAP2 steady-state levels The UL49.5 protein homologs of BHV-1, PRV, and EHV-1 strongly inhibit TAP activity in the corresponding host cell lines. However, due to the lack of host-specific immunological reagents, the molecular basis of UL49.5-mediated TAP inhibition could not be further addressed in the animal cells used. Therefore, the BHV-1, PRV, and EHV-1 UL49.5 homologs were stably expressed in human melanoma (MJS) cells and evaluated for TAP-inhibiting properties. The results show a reduction of peptide transport by 98% for BHV-1 UL49.5, 78% for PRV UL49.5 and 95% for EHV-1 UL49.5 (Fig. 3a). Thus, the UL49.5 homologs exhibit the ability to target human TAP, in addition to the TAP molecules produced in the natural host cells.

We previously demonstrated that the expression of BHV-1-encoded UL49.5 in human cells strongly reduces steady state levels of TAP1 and TAP2, a phenomenon that relies on

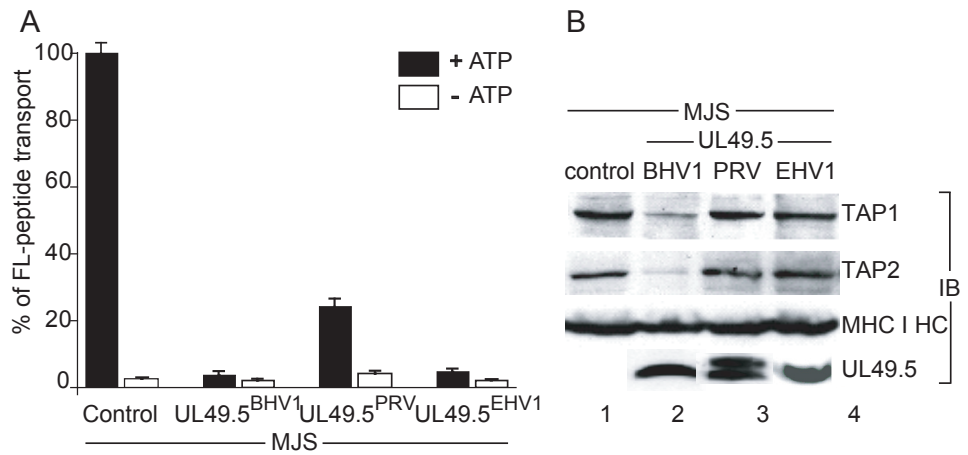

Fig. 3. UL49.5 homologs of PRV and EHV-1 have no effect on steady-state protein levels of TAP1 and TAP2. (A) TAP-dependent peptide transport is inhibited in human melanoma cells (MJS) expressing the UL49.5 homologs of BHV-1, PRV, and EHV-1. Peptide transport is expressed as percentage of translocation, relative to the translocation observed in control cells (defined as 100%). (B) MJS cells transduced with retrovirus encoding BHV-1 UL49.5, PRV UL49.5 and EHV-1 UL49.5 were solubilized in NP-40 and proteins present in post-nuclear supernatants were separated using SDS-PAGE. The proteins detected by immunoblotting are indicated (MHC I HC, MHC class I heavy chain; IB, immunoblot).

proteasomal degradation of both TAP subunits (35). To investigate the molecular basis for inhibition of TAP function by other UL49.5 homologs, we first evaluated the integrity of the peptide-loading complex in MJS cells stably expressing the UL49.5 molecules. TAP1, TAP2, and class I heavy chain protein levels in MJS cells were determined by immunoblotting. The expression of BHV-1 UL49.5 in MJS cells promotes degradation of TAP1 and TAP2 which is in accordance with previous observations (Fig. 3b, compare lanes 1 and 2) (35). In contrast, the expression of the UL49.5 homologs of PRV and EHV-1 did not affect TAP1 and TAP2 protein levels (Fig. 3b, lanes 3 and 4). Steady state levels of class I heavy chains are similar in control and all UL49.5 cells. Thus, contrary to BHV1-encoded UL49.5, expression of the PRV and EHV-1 homologs had no effect on the integrity of the peptide-loading complex in MJS cells. Apparently, the UL49.5 homologs of PRV and EHV-1 inhibit peptide transport by TAP through a mechanism different from the reduction of TAP1/TAP2 protein expression levels.

BHV-1 UL49.5 does not require cytosolic tail lysines to mediate degradation of TAP
We have shown that the cytoplasmic tail of the BHV-1-encoded UL49.5 is essential for TAP degradation (35). Two adjacent lysine residues are present within the cytoplasmic tail of BHV-1 UL49.5 but not within the cytoplasmic tails of the PRV and EHV-1 homologs (Fig. 1a). Lysine residues are potential targets for the attachment of ubiquitin chains, which serve as recognition signals for proteasomes (49). This could explain the ability of BHV-1 UL49.5, but not the other two homologs, to destabilize TAP proteins and direct them for proteasomal degradation. To investigate whether the decrease in TAP1 and TAP2 steady state protein levels is a result of proteasomal degradation signaled by the KK-motif present within in the tail of BHV-1 UL49.5, the two lysine (KK) residues were replaced by alanines (AA). The BHV-1 UL49.5$^{KK/AA}$ mutant was introduced in MJS cells by retroviral transduction. When TAP-dependent peptide transport was compared in MJS cells expressing the wild type UL49.5 and the UL49.$^{5KK/AA}$ mutant, both molecules downregulated TAP transport to a similar degree (Fig. 4a). Both molecules were able to decrease steady state levels of TAP1 and TAP2 proteins, implying that the KK-motif does not play a role in UL49.5-mediated degradation of TAP (Fig. 4b).

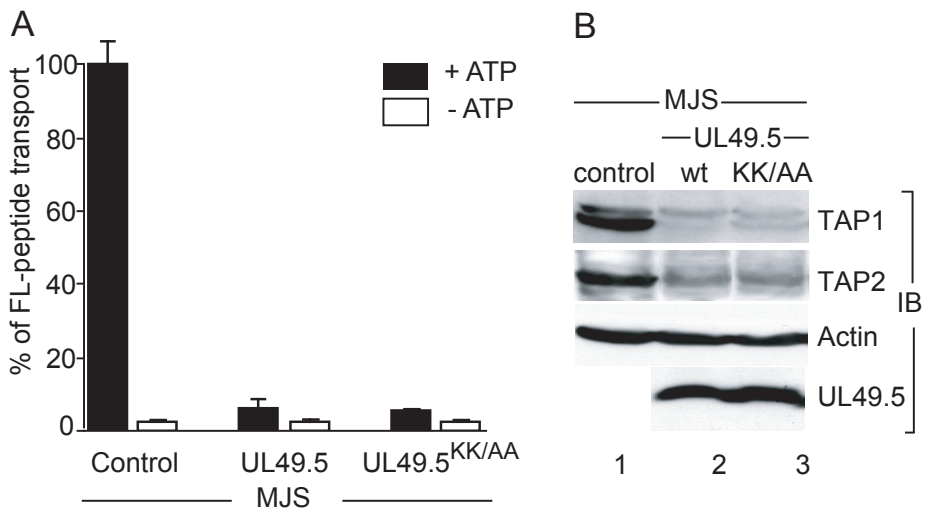

Fig. 4. Peptide transport inhibition and degradation of TAP by BHV-1 UL49.5 is not dependant on the di-lysine motif. (A) TAP transport of MJS cells transduced with BHV-1 UL49.5 or BHV-1 UL49.5$^{KK/AA}$ retroviruses. Peptide transport is expressed as percentage of translocation, relative to the translocation observed in control cells (defined as 100%). (B) Immunoblot analysis of lysates from MJS cells transduced with retrovirus encoding BHV-1 UL49.5 or BHV-1 UL49.5$^{KK/AA}$. Cell lysates were analyzed using SDS-PAGE and immunoblotting with antibodies against the proteins indicated (IB, immunoblot).

EHV-1 UL49.5 homolog blocks ATP-binding by TAP The UL49.5 homologs of PRV and EHV-1 mediate inhibition of TAP-dependent peptide transport, but the molecular basis of this inhibition is unknown. Since ATP-binding and hydrolysis are required to energize peptide translocation by TAP, we investigated whether the expression of these two homologs affected binding of ATP to TAP. Previous experiments indicated that the BHV-1 UL49.5 protein did not influence the interaction of ATP with TAP (35). The ATP-binding capacity of TAP in lysates from MJS cells was compared with those from MJS cells stably expressing PRV UL49.5, EHV-1 UL49.5 or the HCMV-encoded US6 protein. US6 is known to inhibit ATP-binding to TAP and thereby peptide transport. Cell lysates prepared in the presence of the mild detergent digitonin were incubated with ATP-agarose. Proteins bound to ATP-agarose (Fig. 5, pellet "*P*") were eluted with EDTA and displayed next to the proteins in the unbound supernatant fraction (Fig. 5, "*S*").

In lysates from PRV UL49.5-expressing cells, TAP1 and TAP2 were found to bind to the ATP-agarose to a similar extent as in control cells (Fig. 5a, compare lanes 2 and 4). The expression of US6 completely abolished the interaction of ATP with TAP (lane 6). These data show that TAP retains the capacity to bind ATP in the presence of PRV UL49.5.

Interestingly, in EHV-1 UL49.5-expressing cells, no TAP1 and TAP2 could be detected in the ATP-agarose fraction (Fig. 5b, compare lanes 2 and 4). Since the C-terminus of UL49.5 is exposed in the cytosol, this domain might be responsible for the inhibition of ATP to the

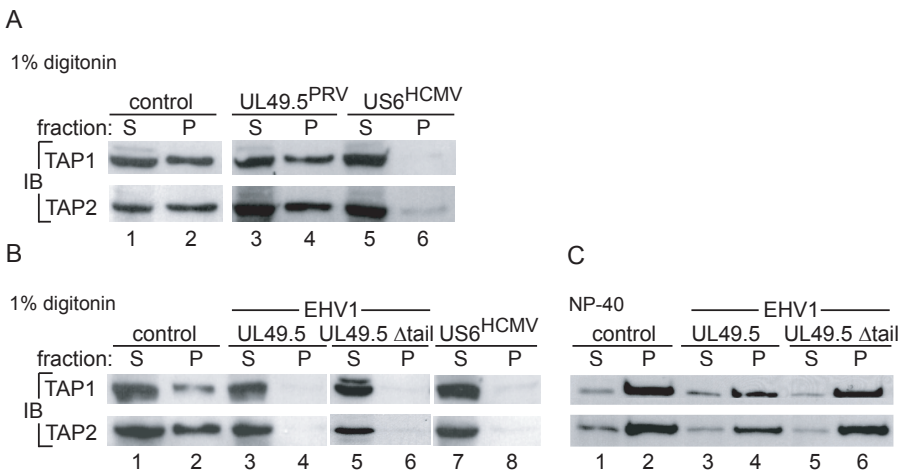

Fig. 5. The UL49.5 homolog of EHV-1 blocks ATP binding in human cells. Immunoblot analysis of MJS cell lysates stably expressing, PRV UL49.5 (A), EHV-1 UL49.5 and EHV-1 UL49.5 tailless (UL49.5Δtail) (B and C), and HCMV US6 (A and B). The cell lysates were incubated with ATP-agarose. The pellet (P) that contains all ATP-binding proteins was separated from supernatant (S; contains all cellular proteins depleted of the ATP-binding proteins) by centrifugation. Cells were solubilized with 1% digitonin (A and B) or with NP-40 (C). Agarose bound (P) and unbound (S) fractions were analyzed using SDS-PAGE and immunoblotting with antibodies against the proteins indicated.

nucleotide-binding domains of TAP. To test this hypothesis, a deletion mutant of EHV-1 UL49.5 was constructed and expressed in MJS cells through retroviral transduction. Figure 5b shows that the removal of the cytoplasmic tail of EHV-1 UL49.5 did not restore the capacity of TAP to bind ATP (lane 6). Nevertheless, when the EHV-1 UL49.5 association with TAP was disrupted by lysis in NP-40, the ability of TAP to bind to the ATP-agarose was restored (Fig.5c, compare lanes 2, 4 and 6). Our results suggest that the ER luminal portion of EHV-1 UL49.5 together with the transmembrane domain are sufficient to block the recruitment of ATP by TAP.

Discussion

Although UL49.5 homologs are encoded by all members of the *Herpesviridae* presently analysed (40), the TAP-blocking function of UL49.5 homologs appears to be restricted to certain members of the genus *Varicellovirus*. Varicelloviruses coevolved with their hosts and this has been especially well established for four Eutherian orders: 1) the even-toed ungulates or *Artiodactyla*, like bovines and pigs with BHV-1 and PRV, 2) the odd-toed ungulates or *Perrisodactyla*, like horses with EHV-1 and EHV-4, 3) the carnivores, like cats and dogs with FHV-1 and CHV and 4) Old World primates like humans with VZV (41). To establish whether all the currently distinguished clades of the genus *Varicellovirus* code for a UL49.5 homolog that can block the TAP transporter, the UL49.5 proteins of BHV-1, PRV, EHV-1, EHV-4, CHV and VZV were studied. The identification of the UL49.5 homologs of PRV and EHV-1 as members of the UL49.5 family of TAP inhibitors suggest that more members of this family may be found in the even- and odd-toed ungulate hosts.

The absence of TAP inhibition for CHV UL49.5 is remarkable because (herpesviruses of) carnivores share much of their evolution with (herpesviruses of) odd-toed ungulates. It will therefore be interesting to study the UL49.5 homologs of other carnivore varicelloviruses, like FHV-1. The observation that VZV UL49.5 does not block TAP is in agreement with previous results of Abendroth *et al*. (50) who did not observe inhibition of TAP by VZV. VZV occupies a somewhat isolated position in the phylogenetic tree of varicelloviruses. Whether VZV and CHV UL49.5 can interact with TAP still has to be established.

The molecular details of the interaction between BHV-1 UL49.5 and bovine TAP are unknown. Although the interaction with bovine TAP is more authentic than the interaction between BHV-1 UL49.5 and human TAP, studying the interaction with human TAP is at present technically more feasible. Interestingly, PRV UL49.5 and EHV-1 UL49.5 are also able to block human TAP. It is likely that all three proteins interact with part(s) of TAP that are conserved between human, swine, bovine and equine TAP. The homology between human, bovine and swine TAP1 and TAP2 is approximately 80% (51). No sequence data are yet available on equine TAP1 or TAP2.

An unexpected observation is that the UL49.5 homologs studied exhibit differences in their mechanisms of TAP inhibition, despite their close evolutionary relatedness and their common target, TAP. BHV-1 UL49.5 destabilizes TAP, whereas the PRV and EHV-1 homologs do not. It is also remarkable that the EHV-1UL49.5 appears to be more distinct from the other two homologs since it is the only UL49.5 of these three able to block the binding of ATP to TAP. This may be related to the fact that both BHV-1 and PRV belong to the clade of herpesviruses that infect even-toed ungulates while EHV-1 belongs to the odd-toed ungulate clade.

Interestingly, the removal of the cytoplasmic tail of EHV-1 UL49.5 did not restore the ability of TAP to bind ATP. Thus, direct interaction of EHV-1 UL49.5 with the ATP-binding sites at the cytosolic side of TAP is unlikely. This reflects, to some extent, the type of structural change caused by the HCMV US6 gene product (17). The US6 protein, a type I transmembrane protein, interacts with the luminal side of the TAP transporter and blocks ATP binding by inducing conformational changes within TAP. The inability of BHV-1UL49.5 and PRV UL49.5 to block ATP-binding may be due to a more limited conformational change induced by these proteins.

In the case of BHV-1 UL49.5, the cytoplasmic tail is essential to the degradation of TAP, a phenomenon not observed for any of the other UL49.5 proteins. The cytoplasmic tail of BHV-1 UL49.5 contains two lysine residues that are not present in the cytoplasmic domains

of the PRV or EHV-1 encoded homologs. These cytoplasmic lysine residues could serve as acceptors for ubiquitin moieties. However, the experiments with the UL49.5$^{KK/AA}$ mutant indicated that these residues are not required to direct TAP for proteasomal degradation. Thus, other amino acid residues within the cytoplasmic domain of UL49.5 mediate the degradation of TAP. Recently, also cysteine, threonine and serine residues have been found to serve as acceptors for ubiquitin moieties (52, 54). The cytoplasmic domain of BHV-1 UL49.5 uniquely carries a serine residue that may fulfill such function.

In summary, three additional members of the UL49.5 family of new TAP inhibiting proteins have been identified. Although closely related, they block TAP through different mechanisms. Most likely, these seemingly different mechanisms are functionally related and it is hypothesized that the various UL49.5 proteins cause different degrees of conformational changes within the TAP complex.

Acknowledgements

We thank P. Admiraal for technical assistance, Dr. R. de Groot (Dept. of Infectious Diseases and Immunology, Utrecht University, The Netherlands) for providing equine epithelial cells and EHV-4 viral DNA, Dr. W. E. Benckhuijsen for peptide synthesis and purification, and Dr. J. von Einem for providing reagents. We also thank M. van der Keur and R. van der Linden at the Flowcytometry Research Unit (LUMC) for their technical assistance and support. This work was supported by the Dutch Diabetes Research Foundation (to D.K.L.) and a FEBS Collaborative Experimental Scholarship for Central and Eastern Europe (to A. D. L.).

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