Unmasking the masters of evasion : TAP inhibition by varicellovirus UL49.5 proteins

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Varicellovirus UL 49.5 proteins differentially affect the function of the transporter associated with antigen processing, TAP

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Abstract

Cytotoxic T lymphocytes play an important role in the protection against viral infections, which they detect through the recognition of virus-derived peptides presented in the context of MHC I molecules at the surface of the infected cell. The transporter associated with antigen processing (TAP) plays an essential role in MHC I-restricted antigen presentation, as TAP imports peptides into the ER, where peptide loading of MHC I molecules takes place. In this study, the UL49.5 proteins of the varicelloviruses bovine herpesvirus 1 (BoHV-1), pseudorabies virus (PRV), and equid herpesvirus 1 and 4 (EHV-1 and EHV-4) are characterized as members of a novel class of viral immune evasion proteins. These UL49.5 proteins interfere with MHC I antigen presentation by blocking the supply of antigenic peptides through inhibition of TAP. BoHV-1, PRV, and EHV-1 recombinant viruses lacking UL49.5 no longer interfere with peptide transport. Combined with the observation that the individually expressed UL49.5 proteins block TAP as well, these data indicate that UL49.5 is the viral factor that is both necessary and sufficient to abolish TAP function during productive infection by these viruses. The mechanisms through which the UL49.5 proteins of BoHV-1, PRV, EHV-1 and EHV-4 block TAP exhibit surprising diversity. BoHV-1 UL49.5 targets TAP for proteasomal degradation, whereas EHV-1 and EHV-4 UL49.5 interfere with the binding of ATP to TAP. In contrast, TAP stability and ATP recruitment are not affected by PRV UL49.5, although it has the capacity to arrest the peptide transporter in a translocation-incompetent state, a property shared with the BoHV-1 and EHV-1 UL49.5. Taken together, these results classify the UL49.5 gene products of BoHV-1, PRV, EHV-1, and EHV-4 as members of a novel family of viral immune evasion proteins, inhibiting TAP through a variety of mechanisms.

Introduction

Evolving under the selective pressure of the host immune system, herpesviruses have developed countermeasures to prevent recognition of infected cells by cytotoxic CD8$^+$ T lymphocytes (CTL). CTLs recognize viral antigens presented as peptides bound to major histocompatibility complex class I (MHC I) molecules at the surface of infected cells. Herpesviruses in particular have acquired diverse mechanisms to inhibit antigen presentation in the context of MHC I molecules, thereby escaping from elimination by CTLs (Yewdell and Hill, 2002; Lilley and Ploegh, 2005; Vossen et al., 2002; Abele and Tampe, 2006).

Most peptides presented by MHC I molecules are transported into the ER lumen 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 family of transporters (Abele and Tampe, 2004; van Endert et al., 2002). TAP translocates peptides across the ER membrane via a conformational transition that is energized by the hydrolysis of ATP. TAP is part of the MHC I peptide-loading complex that also contains tapasin, MHC I heavy and light chains, and several auxiliary proteins including calreticulin and ERp57 (Abele and Tampe, 2004; Garbi et al., 2005; Garbi et al., 2007; Peaper et al., 2005; Koch and Tampe, 2006).

Several herpesviruses have acquired mechanisms to interfere with TAP function. Interestingly, inhibition of TAP transport is achieved through different strategies, exerted by unique gene products. Although the varicellovirus bovine herpesvirus 1 (BoHV-1) and the simplexviruses herpes simplex virus (HSV) type 1 and 2 all belong to the subfamily of alphaherpesviruses, they block TAP through proteins that have an entirely different structure and mode of action. The inhibition of TAP by BoHV-1 relies on the UL49.5 gene product, a type I transmembrane protein of 75 amino acids (Koppers-Lalic et al., 2005). Inactivation of TAP by UL49.5 involves two events; the arrest of the peptide transporter in a translocation-incompetent state and the proteasomal degradation of both subunits of TAP (Koppers-Lalic et al., 2005). In contrast, the ICP47 proteins of HSV-1 and -2 are soluble cytosolic proteins acting as high-affinity competitors for peptide binding to TAP (Hill et al., 1995; Fruh et al., 1995; Ahn et al., 1996; Tomazin et al., 1996; Galocha et al., 1997; Neumann et al., 1997; Aisenbrey et al., 2006). Within the subfamily of betaherpesviruses, human cytomegalovirus (HCMV) was found to encode a protein, US6, that inhibits TAP function by reducing the interaction of ATP with TAP (Ahn et al., 1997; Hengel et al., 1997; Lehner et al., 1997; Hewitt et al., 2001; Kyritsis et al., 2001; Halenius et al., 2006). The murine gammaherpesvirus-68 encodes the mK3 protein that acts as a ubiquitin ligase linking MHC I molecules and TAP to the ubiquitin/proteasome degradation pathway (Stevenson et al., 2000; Boname and Stevenson, 2001; Stevenson et al., 2002; Yu et al., 2002; Lybarger et al., 2003; Boname et al., 2004; Wang et al., 2004; Boname et al., 2005;

Wang et al., 2005; Wang et al., 2006; Wang et al., 2007). Recently, the BNLF2a protein of Epstein-Barr virus (EBV) and of related primate gamma-1 herpesviruses has been characterized as a potent TAP inhibitor, preventing the binding of both peptides and ATP to TAP (Hislop et al., 2007).

Homologs of UL49.5 (commonly known as glycoprotein N or gN) are encoded by every alpha-, beta- and gammaherpesvirus sequenced to date (Barnett et al., 1992; Davison, 2002; McGeoch et al., 2006). The UL49.5 genes are all predicted to encode a type I membrane protein with a putative cleavable signal sequence. The UL49.5 proteins interact with glycoprotein M (gM), with which they form a disulfide-linked heterodimer through a conserved cysteine residue within their ER-luminal/extracellular domain (Barnett et al., 1992; Jons et al., 1998; Lipinska et al., 2006; Rudolph et al., 2002; Wu et al., 1998; Mach et al., 2007). Nevertheless, the amino acid sequences of UL49.5 proteins demonstrate considerable heterogeneity, even among varicellovirus UL49.5 proteins (Fig. 1). The only exceptions are EHV-1 and EHV-4 UL49.5, which differ by only seven amino acid residues. Thus, at this moment, it is impossible to predict on the basis of amino acid sequence whether any of these proteins have the same capacity to inhibit TAP that was found for BoHV-1 UL49.5. The UL49.5 gene products of HSV-1, HSV-2, HCMV, and EBV fail to block TAP, indicating that not all UL49.5 molecules act as inhibitors of TAP (Barnett et al., 1992; Koppers-Lalic et al., 2005).

In this study, the effects on TAP function were assessed in more detail for UL49.5 encoded by various members of the genus *Varicellovirus*. The UL49.5 proteins of BoHV-1, PRV, EHV-1, and EHV-4 were found to downregulate MHC I cell surface expression through TAP inhibition. Their ability to block TAP was observed in cells of the relevant host species, as well as in human cells. Using UL49.5 deletion mutants of BoHV-1, PRV and EHV-1, it was

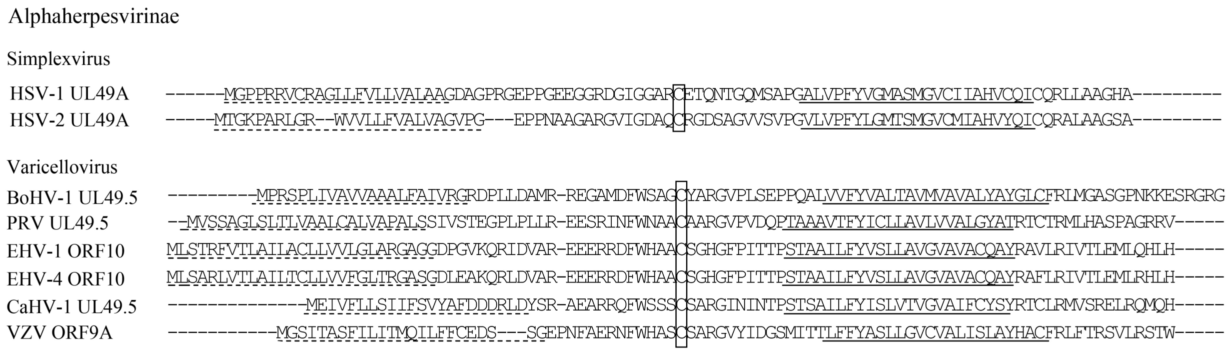

Fig. 1, alignment of the amino acid sequences of UL49.5 homologs of a selection of alphaherpesviruses. Hydrophobic residues indicative of N-terminal signal sequences (dashed line) and membrane anchor regions (bold line) are indicated. The conserved cysteine residue involved in disulfide bond formation with the viral glycoprotein M is indicated. The amino acid sequence alignment of UL49.5 homologs was performed using Vector*NTI* software (Invitrogen). The NCBI accession numbers are summarized in the materials and methods section.

shown that the UL49.5 proteins of these viruses are responsible for the inhibition of TAP-dependent peptide transport. The UL49.5 homologs of CaHV-1 and VZV did not affect MHC I surface expression. BoHV-1 UL49.5 strongly reduces the steady state protein levels of TAP in both bovine and human cells, whereas the UL49.5 proteins of EHV-1, EHV-4 or PRV were not observed to have this capacity. Interestingly, the EHV-1 and EHV-4 UL49.5 homologs interfere with the binding of ATP to TAP, a function that is not influenced by BoHV-1 or PRV UL49.5. The UL49.5 proteins of PRV and EHV-1 arrest TAP in a translocation-incompetent state, a property that is shared with BoHV-1 UL49.5. Thus, the BoHV-1, PRV, EHV-1 and EHV-4-encoded UL49.5 proteins all induce a similar phenotype, i.e., inhibition of peptide transport, but their modes of action demonstrate a surprising diversity.

Materials and methods

UL49.5 constructs Purified viral DNA from BoHV-1 strain Lam and CaHV-1 strain Eva (Animal Sciences Group, Lelystad, The Netherlands), PRV strain Kaplan (Jons et al., 1998), EHV-1 strain Ab-4 (kindly provided by J. Rola; National Veterinary Research Institute, Pulawy, Poland), 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, Leiden University Medical Center, 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 (Table 1) for amplification of the full length coding sequence of the UL49.5 genes of BoHV-1 (Koppers-Lalic et al., 2005), PRV, EHV-1, EHV-4, CaHV-1 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 CaHV-1 primers (Haanes, E. and Rexann, F. ´Recombinant canine herpesviruses´, patent number EPO910406, publication date 1997-08-21). To generate the EHV-1 UL49.5Δtail construct, primers (Table 1) were used to obtain a PCR product lacking 3’-terminal 45 nucleotides, thereby deleting the 15 carboxy-terminal amino acids. PCR-generated products were sequenced and inserted into the retroviral expression vectors pLZRS-IRES-GFP or pLZRS-IRES-ΔNGFR, upstream of the internal ribosome entry site (IRES) element. pLZRS vector information can be obtained at www.stanford.edu/group/nolan/retroviral_systems/ retsys.html).

Cell lines and retroviral transduction The human melanoma cell line Mel JuSo (MJS), MJS TAP1-GFP (Reits et al., 2000) and Madin-Darby bovine kidney (MDBK) cells (American Type Culture Collection, ATCC) were maintained in RPMI-1640 medium; GP2-293 cells, porcine kidney (PK15) cells, the embryonic bovine trachea (EBTr) cell line, Madin-Darby canine kidney I (MDCK I) cells, and the equid epithelial cell line E.derm were maintained in DMEM medium. Media were supplemented with 10% heat-inactivated fetal bovine serum

Table 1, PCR primers

Primer name	Primer sequence [a]
PRV UL49.5	Fw: 5’-CGC**GGATCC**GACACACCAGGATGGTC-3’
PRV UL49.5	Rev: 5’-GCG**GAATTC**GGATCGCTCTTTATACGC-3’
EHV-1 UL49.5	Fw: 5’-GCCGCCACCATGCTGTCCACGAGATTC-3’
EHV-1 UL49.5	Rev: 5’-TTGTCAATGCAGGTGTTGCAACATCTC-3’
EHV-1 UL49.5Δtail	Fw: 5’-GA**GAATTC**AGGACCATGCTGTCCACGAGATTC-3’
EHV-1 UL49.5Δtail	Rev: 5’-CG**CTCGAG**CTTCAGCGGTATGCCTG-3’
EHV-4 UL49.5	Fw: 5’-GCCGCCACCATGTTGTCAGCGAGATTAG-3’
EHV-4 UL49.5	Rev: 5’-TGCTCAGTGTAGGTGTCGCAAATC-3’
CaHV-1 UL49.5	Fw: 5’ -GCCGCCACCATGGAGATAGTATTTTTAC-3’
CaHV-1 UL49.5	Rev: 5’ -CCATTAGTGTTGCATCTGACGAAGTTC-3’
VZV UL49.5	Fw: 5’-GCC**GGATCC**AAGATGGGATCAATTAC-3’
VZV UL49.5	Rev: 5’-CCG**GAATTC**CGGTTACCACGTGCTGCG-3’

a = restriction enzymes used in bold

(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. PHA-treated T cell blasts positive for HLA-A1 and HLA-A2 were generated from PBMCs by stimulation with 0.8 μg/ml PHA and were subsequently cultured in IMDM supplemented with 100 IU/ml IL-2 and 10% FBS. The HLA-A2-expressing melanoma cell line 518, Mel518 (a kind gift from E. Verdegaal, department of Clinical Oncology, Leiden University Medical Center, Leiden, The Netherlands) was maintained in DMEM containing 4.5 mM glucose, supplemented with 8% FBS, 2 mM L-glutamine (Invitrogen), 140 IU/ml penicillin and 140 μg/ml streptomycin.

Recombinant retroviruses were prepared using the Phoenix amphotropic packaging system as described previously (www.stanford.edu/group/nolan/retroviral_systems/retsys.html). MJS, MDCK I, PK15, and E.derm cells were transduced with recombinant retroviruses to generate the following stable cell lines: MJS, MDCK I, PK15, and E.derm controls (containing BoHV-1 UL49.5 in the anti-sense orientation, GFP$^+$); MJS UL49.5$^{\text{BoHV-1}}$, PHA T cell blast UL49.5$^{\text{BoHV-1}}$ and Mel518 UL49.5$^{\text{BoHV-1}}$ (containing BoHV-1 UL49.5 in the sense orientation (SO), GFP$^+$); MJS UL49.5$^{\text{VZV}}$, PHA T cell blast UL49.5$^{\text{VZV}}$ and Mel518 UL49.5$^{\text{VZV}}$ (containing VZV UL49.5 SO, GFP$^+$); MJS UL49.5$^{\text{CaHV-1}}$ and MDCK I UL49.5$^{\text{CaHV-1}}$ (containing CaHV-1 UL49.5 SO, GFP$^+$); MJS UL49.5$^{\text{PRV}}$ and PK15 UL49.5$^{\text{PRV}}$ (containing PRV UL49.5 SO, GFP$^+$); MJS UL49.5$^{\text{EHV-1}}$ and E.derm UL49.5$^{\text{EHV-1}}$ (containing EHV-1 UL49.5 SO, GFP$^+$); MJS UL49.5$^{\text{EHV-1}\Delta\text{tail}}$ (containing tail-less EHV-1 UL49.5 SO, GFP$^+$); MJS UL49.5$^{\text{EHV-4}}$ and E.derm UL49.5$^{\text{EHV-4}}$ (containing EHV-4 UL49.5 SO, GFP$^+$). In addition, MJS TAP1-GFP cells were transduced with recombinant retrovirus to generate MJS TAP1-GFP control (containing the empty pLZRS construct, ΔNGFR$^+$); MJS TAP1-GFP UL49.5^{BoHV-1} (containing BoHV-1 UL49.5 SO, ΔNGFR$^+$); MJS TAP1-GFP UL49.5^{PRV} (containing PRV UL49.5 SO, ΔNGFR$^+$) and MJS TAP1-GFP UL49.5^{EHV-1} (containing EHV-1 UL49.5 SO, ΔNGFR$^+$). To generate recombinant retroviruses for MDBK cell line transductions, the GP2-293 pantropic packaging cell line was used according to the protocol obtained from BD Bioscience Clontech (www.bdbiosciences.com). In brief, 1x10^6 of GP2-293 cells were co-transfected with retroviral expression vector (pZLRS-IRES-GFP containing the BoHV-1 UL49.5 gene in anti-sense or in the sense orientation) and pVSV-G construct (envelope vector) for retrovirus production. Retrovirus-containing medium was collected 48 h post-transfection. MDBK cells were transduced four times with VSV-G containing recombinant retroviruses to generate the following stable cell lines: MDBK control (containing BoHV-1 UL49.5 in anti-sense orientation, GFP$^+$) and MDBK UL49.5^{BoHV-1} (containing BoHV-1 UL49.5 SO, GFP$^+$). All cell lines generated in this study were selected for GFP or ΔNGFR 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 (Koppers-Lalic et al., 2003).

Reagents The following antibodies were used in this study: anti-transferrin receptor (TfR) monoclonal antibody (mAb) 66Ig10, anti-TfR mAb H68.4 (Roche), anti-human MHC I complexes mAb W6/32, anti-human MHC I heavy chain mAb HC-10 (kindly provided by H. Ploegh, Whitehead Institute, Cambridge, Massachusetts, USA), anti-human class II HLA-DR mAb Tü36 (kindly provided by A. Ziegler, Institute for Immunogenetics, Universitäts-klinikum Charité, Berlin, Germany), anti-TAP1 mAb 148.3 (Meyer et al., 1994) and anti-TAP2 mAb 435.3 (kind gift from P. van Endert, Institut National de la Santé et de la Recherche Médicale, Paris, France). For the detection of equid TAP2, the polyclonal antibody anti-rat TAP2 Mac394 was used (kindly provided by M. Knittler, Institute of Immunology, Friedrich-Loeffler-Institute, Tübingen, Germany). For preparation of bovine TAP1 specific antibody, the bovine TAP1 ORF sequence encoding amino acid residues 117 to 167 were amplified from bovine genomic DNA and cloned into pGEX-4T-2 (GE Healthcare). The TAP1 polypeptide encompassing residues aa117-167 was purified as described previously (Chowdhury et al., 1999). The monoclonal antibody IL-A19 directed against bovine MHC I molecules (a kind gift from Dr. J. Naessens, ILRAD, Nairobi, Kenya). The anti-equid and anti-canine MHC I complexes mAb H58A and anti-porcine MHC I mAb PT85A were purchased from VMRD Inc.

Mouse anti-BoHV-1 UL49.5 serum was kindly provided by G.J. Letchworth (University of Wisconsin, Madison, Wisconsin, USA). Polyclonal rabbit anti-BoHV-1 UL49.5 serum H11 was raised against a synthetic peptide representing the N-terminal sequence of BoHV-1 UL49.5 and has been previously described (Lipinska et al., 2006). In fig. 5, a different polyclonal rabbit anti-BoHV-1 UL49.5 was used, obtained using a synthetic peptide corresponding to amino acid residues 27-41 of UL49.5 ([H] DAMRREGAMDFWSAGC*-[OH]). To facilitate conjugation to keyhole limpet hemocyanin, an additional irrelevant cysteine was added at the C-terminus of the peptide (indicated by an asterisk). Rabbits were immunized as described earlier (Chowdhury et al., 2000). The rabbit antiserum raised against PRV UL49.5 (gN) has been described (Jons et al., 1996), as was the anti-EHV-1 UL49.5 rabbit serum (Rudolph et al., 2002). The VZV UL49.5-specific antibody was raised against two synthetic peptides: the N-terminal peptide EPNFAERNFWHASCSARGVYI DGSMITTLFKK and the C-terminal peptide RLFTRSVLRSTW. Both peptides were conjugated to glutathione S-transferase (GST) according to the methods described in (Lipinska et al., 2006). The peptide-GST conjugates were mixed at a 1:1 ratio and emulsified in Freund's complete adjuvant for the first immunization and Freund's incomplete for the following immunizations. At 3-weeks intervals, the rabbit received four additional subcutaneous immunizations with the conjugates.

Flow cytometry Cells were trypsinized and resuspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.05% sodium azide. Cells were incubated with specific antibodies on ice for one hour. After washing, the cells were incubated with phycoerytrin(PE)-conjugated anti-mouse antibody for 45 min. Stained cells were analyzed by flow cytometry on a FACSCalibur (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).

Peptide transport assay The fluorescence-based peptide transport assay was performed as previously described (Koppers-Lalic et al., 2005; Blevitt et al., 1999). 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 (N-core glycosylation site underlined) 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 for 2 minutes. The fluorescence intensity was measured using a fluorescence plate reader (CytoFluor, PerSeptive Biosystems; excitation 485 nm/emission 530 nm). The data were analyzed using the unpaired t-test. Statistical significance was set at $p < 0.05$.

Immunoprecipitations, Western blotting and radiolabeling Cells were lysed in a buffer containing 1% (w/v) digitonin, 50 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 150 mM NaCl, 1 mM leupeptin, and 1 mM AEBSF (4-(2-Aminoethyl)-benzenesulfonyl fluoride), and subjected to immune precipitations using anti-TAP1 mAb 148.3 o/n. To determine steady state protein levels, 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, 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. Western blotting (WB) analysis was performed on denatured cell lysates separated by SDS-PAGE and blotted onto PVDF membranes. Blots were incubated with the antibodies as indicated, followed by horseradish peroxidase-conjugated goat-anti-mouse or swine-anti-rabbit Igs (DAKO and Jackson Laboratories), and visualized by ECLplus (Amersham).

Steady-state labeling of MDBK cells with [^{35}S]-methionine/cysteine and subsequent immunoprecipitations with rabbit BoHV-1 UL49.5-specific antibody were performed as described (Al-Mubarak et al., 2004). Western blotting procedures with rabbit anti-bovine TAP1 and rabbit anti-α-tubulin have been described (Chowdhury et al., 1999).

Cytotoxicity assay A total of 1,000 ^{51}Cr-labeled target cells were incubated with different $CD8^+$ CTL clones at various effector to target ratios. The HY-A1 clone recognizes an HY epitope in the context of HLA-A1, and the HA2.27 clone recognizes the histocompatibility antigen HA-2 in the context of HLA-A2. After 4 h of incubation at 37°C, ^{51}Cr release into the supernatant was measured using standard methods. The mean percentage of triplicate wells was calculated as follows: % specific lysis = (experimental release – spontaneous release)/(maximal release – spontaneous release) x 100. For analysis of IFNγ production, 20,000 T cells were co-cultured with 10,000 target cells. After 24 h, the supernatant was harvested and the concentration of IFNγ was measured by standard ELISA (Sanquin, Amsterdam, The Netherlands).

Viruses and virus infections The wild-type viruses used in this study were: BoHV-1 strain Lam, BoHV-1 strain Cooper (Fig. 5), PRV strain Kaplan and EHV-1 strain RacL1. The UL49.5 deletion mutant of PRV used in this study has been described before (Jons et al., 1998; Rudolph et al., 2002). The UL49.5 deletion mutant of EHV-1 (strain RacL11) was a gift from J. von Einem (College of Veterinary Medicine, Cornell University, Ithaca, NY, USA). Infections with wild-type and mutant herpesviruses were carried out on the following cell lines: MDBK cells for BoHV-1; PK15 cells for PRV and E.derm cells for EHV-1. The cells were washed once with PBS and infected with BoHV-1 and 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 h, medium containing 10% FBS was added. Mock-infected cells were treated under the same conditions as infected cells. After 5 h of infection, cells were collected and prepared for the peptide translocation assay. For Western blotting and metabolic labeling experiments, MDBK cells were infected with BoHV-1 wild-type and UL49.5Δtail viruses for 12 h.

Construction of BoHV-1 UL49.5 recombinant viruses The BoHV-1 UL49.5 mutant was generated by homologous recombination, using BoHV-1 strain Lam as parent strain. 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 BoHV-1 genome with NCBI accession number NC_001847, updated 30 March 2006). This fragment starts at a *Bst*XI 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 provided by a 1.9 kb fragment from nucleotide residue 9075 to 10972. This fragment starts at amino acid residue 36 of UL49.5 and ends at an *Fsp*I site 1.7 kb downstream of the UL49.5 open reading frame. A 2.2 kb *Nru*I – *Pvu*II fragment was cloned between the two UL49.5 recombination fragments that carries the hGFP gene in the expression cassette of pcDNA3 (Invitrogen). The complete recombination fragment (5.5 kb) was co-transfected with purified BoHV-1 Lam DNA into EBTr cells using a calcium phosphate-based transfection method. 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-BoHV-1 UL49.5 serum. The BoHV-1-UL49.5 mutant could be grown to a titer of 10^7 $TCID_{50}$ /ml and was capable of penetrating bovine cells with the same kinetics as the wild-type Lam strain.

The BoHV-1 recombinant virus gN Am80, expressing a form of UL49.5 lacking its cytoplasmic domain, was constructed by introducing an amber mutation at gN residue 80R (AGG to TAG) by using a BoHV-1 BAC clone (Liu and Chowdhury, manuscript in preparation).

Peptide binding assay Cellular microsomes were prepared as described (Schrodt et al., 2006). Microsomes isolated from 7 x 10^6 homogenized cells were pre-incubated in 50 μl of AP buffer (5 mM $MgCl_2$ in phosphate-buffered saline, pH 7.0) on ice for 45 min in the absence or presence of a 200-fold molar excess of the non-labeled TAP-specific viral inhibitor ICP47 (Aisenbrey et al., 2006). Different concentrations of radiolabeled peptide (RR[^{125}I]YQKSTEL) were added equally to the samples with or without ICP47 and incubated on ice (Koch et al., 2004). Non-bound peptides were removed by washing the membranes with 400 μl of AP buffer and subsequent centrifugation at 20,000 g for 8 min. The amount of radioactivity bound to the membranes was quantified by γ-counting and corrected for the signal obtained in the presence of ICP47. All experiments were performed in triplicate.

ATP-agarose binding assay TAP binding to ATP-agarose was assayed as described (Koppers-Lalic et al., 2005). 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 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 h, the supernatant was separated from the ATP-agarose pellet by 5 minutes centrifugation. The resulting pellet was 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 the ATP-agarose were eluted with 500 mM EDTA and SDS sample buffer was added to both the supernatant and the pellet. The samples were separated using SDS-PAGE and analyzed by Western blotting.

FRAP Confocal microscopy and fluorescence recovery after photobleaching (FRAP) assays were performed as described (Koppers-Lalic et al., 2005; Reits et al., 2000). In short, a circular spot in the ER was bleached at full intensity, and an attenuated laser beam was used to monitor recovery of fluorescence. The half-time for recovery was calculated from each recovery curve after correction for loss of fluorescence caused by imaging (usually <4%). The diffusion coefficient D was determined from at least seven cells measured in different experiments.

Accession numbers UL49.5 sequence data used to generate the alignment shown in Fig.1 and the phylogenetic tree shown in Fig.7, have been obtained from the NCBI (www.ncbi.nlm.nih.gov) database with the accession numbers: bovine herpesvirus 1 (BoHV-1) [NP_045309], bovine herpesvirus 5 (BoHV-5) [NP_954898], cercopithecine herpesvirus 1 (CeHV-1) [AAP41468], cercopithecine herpesvirus 9 (CeHV-9) [NP_077423], cercopithecine herpesvirus 16 (CeHV-16) [YP_443897], equid herpesvirus 1 (EHV-1) [AAT67267], equid herpesvirus 4 (EHV-4) [CAA35670], gallid herpesvirus 1 (GaHV-1) [YP_182341], gallid herpesvirus 2 (GaHV-2) [NP_057812], gallid herpesvirus 3 (GaHV-3) [NP_066882], human herpesvirus 1 (HSV-1) [NP_044652], human herpesvirus 2 (HSV-2) [NP_044520], human herpesvirus 3 (VZV) [YP_068406], meleagrid herpesvirus 1 (MeHV-1) [AAG30090], psittacid herpesvirus 1 (PsHV-1) [AAQ73691], suid herpesvirus 1 (PRV) [YP_068325], transporter 1 ATP-binding cassette sub-family B [*Bos taurus*] [AAY34698]. Not obtained from the NCBI database are: bubaline herpesvirus 1 (BuHV-1) [MSRSLLVALATAALLAMVRGLDPLLDAMRREEAMDFWSAGCYARGVPLSEPPQAMVVFYAALT VVMLAVALYAYGLCFRLMSAGGPNKKEVRGRG; FAMR, unpublished], canid herpesvirus (CaHV-1) [patent EP0910406 http://ep.espacenet.com], and cervid herpesvirus 1 (CvHV-1) [MARMPRSLLSALAVAALLAIAGARDPLLDAMRHEGAMDFWSASCYARGVPLSEPPQ ALVVFYVALAVVMFSVAVYAYGLCLRLVGADSPNKKDSRGRG; FAMR, unpublished].

Results

Inhibition of TAP by UL49.5 proteins of varicelloviruses

To evaluate the TAP-inhibiting capacity of the UL49.5 proteins encoded by the varicelloviruses PRV, EHV-1, EHV-4, CaHV-1 and VZV, cell lines of the relevant host species were transduced using a retrovirus-based gene delivery system to express the corresponding UL49.5 proteins. Downregulation of MHC I expression by the UL49.5 gene products was evaluated using flow cytometry. In cells expressing UL49.5 of BoHV-1, PRV, EHV-1 and EHV-4, MHC I surface expression was reduced (Fig. 2A). The UL49.5 proteins of CaHV-1 and VZV failed to downregulate MHC I surface expression. These results indicate that UL49.5 of BoHV-1, PRV, EHV-1 and EHV-4 interfere with MHC I-restricted antigen presentation.

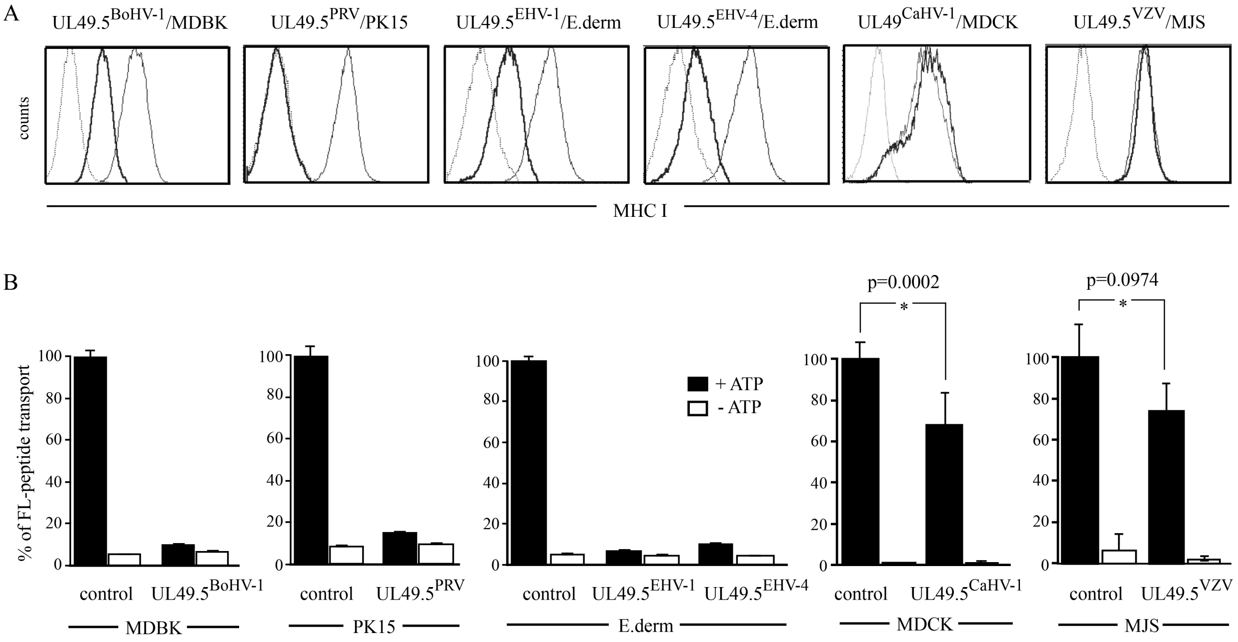

Fig. 2, TAP inhibition by Varicellovirus-encoded UL49.5 homologs in natural host cells. (A) MHC I expression in cells from the relevant host species stably expressing the UL49.5 homologs of BoHV-1, PRV, EHV-1, EHV-4, CaHV-1 and VZV. Cells of bovine (MDBK), porcine (PK15), equid (E.derm), canine (MDCK), and human origin (MJS) were transduced with retroviruses expressing the respective UL49.5 proteins. Surface MHC I molecules were stained with specific antibodies and analyzed using flow cytometry. Shown are the MHC I levels on cells without UL49.5 (thin line) and with UL49.5 (boldface line). Dashed line, goat anti-mouse phycoerythrin control. (B) TAP-dependent peptide transport is inhibited in cells from the relevant host species expressing BoHV-1, PRV, EHV-1, EHV-4 and CaHV-1 UL49.5, but not in MJS cells expressing VZV UL49.5. Peptide transport was assessed in the presence and absence of ATP (black and open bars, respectively). Peptide transport is expressed as percentage of translocation, relative to the translocation observed in control cells (defined as 100%). The standard deviations are represented by the error bars. A difference at $p<0.05$ was considered significant.

To investigate whether the observed downregulation of MHC I cell surface expression relies on the inhibition of TAP, species-specific cell lines stably expressing these UL49.5 homologs were evaluated for TAP-dependent peptide transport. The UL49.5 proteins of BoHV-1, PRV, EHV-1 and EHV-4 strongly inhibited TAP activity in the corresponding natural host cell lines (Fig. 2B). Despite the absence of a detectable reduction in cell surface MHC I levels (Fig. 2A), some inhibition of TAP-dependent peptide transport was observed in canine cells expressing the CaHV-1 UL49.5 protein (Fig. 2B). Apparently, the inhibition of TAP by CaHV-1 UL49.5 was insufficient to observe MHC I downregulation at the cell surface. VZV UL49.5 had no significant effect on TAP activity. Thus, although the amino acid sequences of the UL49.5 proteins of BoHV-1, PRV, and EHV-1/EHV-4 demonstrate considerable variation (Fig. 1), their ability to inhibit TAP was found to be a common property of these varicellovirus proteins.

The interaction of VZV UL49.5 with the peptide-loading complex has no functional consequences for MHC I-restricted antigen presentation

VZV infection has been shown to cause downregulation of MHC I expression at the cell surface (Cohen, 1998; Abendroth et al., 2001; Eisfeld et al., 2007). This phenotype could not be reproduced by the VZV-encoded UL49.5 protein when expressed individually (Fig. 2A). To examine whether the absence of MHC I downregulation by VZV UL49.5 is due to a loss of the interaction of the viral protein with the TAP complex, TAP was immunoprecipitated from VZV UL49.5-expressing MJS cells that were solubilized in the presence of the mild detergent digitonin. The resulting protein complexes were separated by SDS PAGE and analyzed for the presence of UL49.5 by Western blotting. Surprisingly, VZV UL49.5 was found to interact with the TAP complex (Fig. 3A).

Although VZV UL49.5 associates with TAP, this appears to be insufficient to effectively inhibit peptide transport (Fig. 2A and B). VZV UL49.5 could, however, interfere with peptide-loading and MHC I-restricted antigen presentation in a different way. For instance, the US3 protein encoded by human cytomegalovirus binds both tapasin and TAP, without having an effect on TAP function. Instead, US3 impairs tapasin-dependent peptide loading and optimization of the MHC I peptide cargo (Park et al., 2004; Park et al., 2006). To investigate whether VZV UL49.5 inhibits MHC I-mediated antigen presentation via a mechanism similar to that of US3, functional T cell assays were performed using a panel of HLA-A1 and HLA-A2-restricted CTL clones. It is known that especially HLA-A1-restricted peptide presentation strongly depends on the function of tapasin. Antigen-presenting PHA-treated T cell blasts and the melanoma cell-line Mel518 were transduced to express the UL49.5 proteins of VZV and BoHV-1. While the presence of BoHV-1 UL49.5 greatly reduced specific lysis of the PHA-blasts by CTLs, the expression of VZV UL49.5 had no detectable effect (Fig. 3B). This was observed for HLA-A1 and HLA-A2-restricted CTL clones. VZV UL49.5-expressing and control target cells induced IFNγ production by the CTLs, while reduced IFNγ production was observed when BoHV-1 UL49.5 was expressed by the target

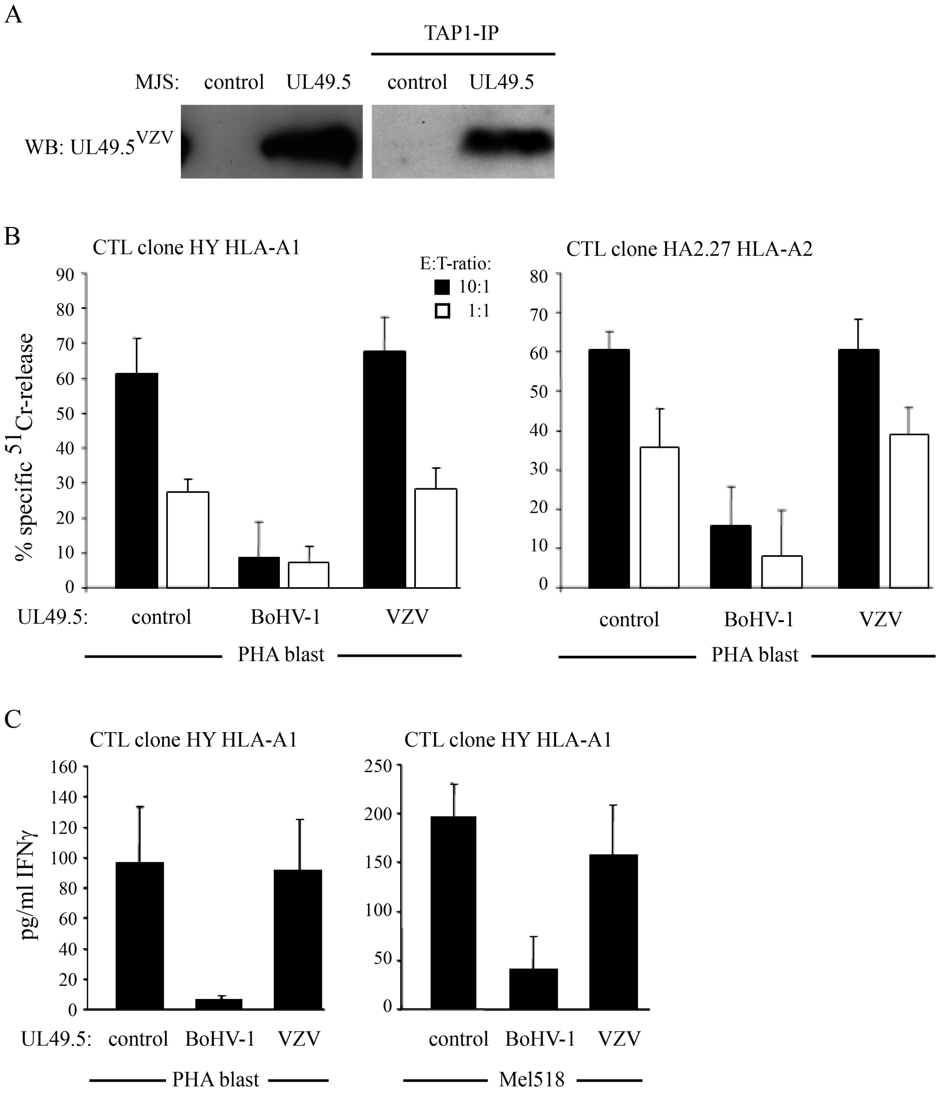

Fig. 3, VZV UL49.5 interacts with the peptide-loading complex, but does not inhibit MHC I-restricted T cell recognition. (A) TAP1 was immunoprecipitated (IP) from MJS cells expressing EHV-1 or VZV UL49.5 proteins. Co-precipitating UL49.5 proteins were analyzed by Western blotting (WB) using antibodies against EHV-1 or VZV UL49.5. Left panel: cell lysates were loaded on SDS-PAGE directly and stained by Western blotting. (B) BoHV-1 or VZV UL49.5-expressing PHA-treated T-cell blasts were labeled with ^{51}Cr and used as target cells for CTL clones specific for the minor histocompatibility antigens HY and HA-2, recognized in the context of HLA-A1 and HLA-A2, respectively. Specific lysis was determined by measuring ^{51}Cr release from the target cells after 4 hrs. Effector to target (E:T) ratios are indicated. (C) PHA-treated T cell blasts or a melanoma cell line (Mel518) carrying the HY antigen were incubated with the HY-specific CTL clone HY HLA-A1. IFNγ levels released by the CTL clones were determined from the supernatants of the co-cultures after 24 hrs.

cells (Fig. 3C). This reflects effective inhibition of CTL recognition by BoHV-1 but not VZV UL49.5. Thus, despite the interaction of the VZV UL49.5 protein with the peptide-loading complex, no interference with MHC I-restricted antigen presentation could be detected.

UL49.5 is responsible for the inhibition of TAP in virus-infected cells

Having observed that the UL49.5 proteins of BoHV-1, PRV, EHV-1, and EHV-4 interfere with MHC I-restricted antigen presentation when expressed individually, we next investigated whether the various UL49.5 proteins are responsible for TAP inhibition during infection with BoHV-1, PRV, or EHV-1. Peptide transport activity was examined in natural host cells infected with wild-type viruses or with the corresponding recombinant viruses lacking a functional UL49.5 gene (Jons et al., 1998; Rudolph et al., 2002). Whereas the wild-type viruses effectively blocked peptide transport, this inhibition was not observed in cells infected with the mutant viruses lacking UL49.5 (Fig. 4). These findings indicate that during infection with BoHV-1, PRV and EHV-1, the UL49.5 gene products of these viruses are responsible for the inhibition of peptide translocation by TAP which was previously observed in virus-infected cells (Ambagala et al., 2004; Ambagala et al., 2000).

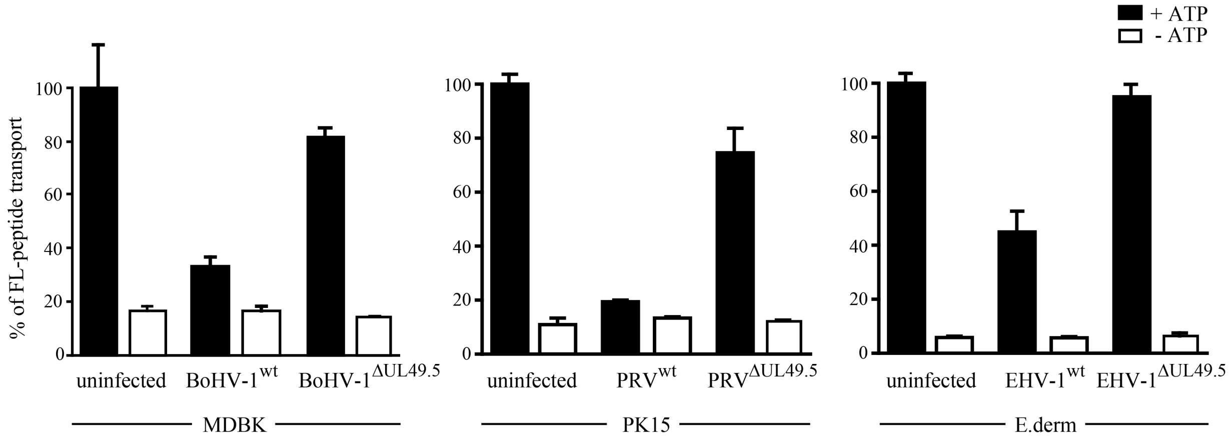

Fig. 4, UL49.5 is responsible for TAP-inhibition in virus-infected natural host cells. Bovine cells (MDBK), porcine cells (PK15), and equid cells (E.derm) were infected with wild-type BoHV-1, PRV, or EHV-1, respectively, or with the corresponding UL49.5-negative recombinant viruses. In all experiments, mock-treated (uninfected) cells from the relevant host species were used as a control. Peptide transport was assessed at 5 hrs post-infection in the presence and absence of ATP (black and open bars, respectively). The data are expressed as percentage of translocation, relative to the translocation observed in control cells (defined as 100%).

UL49.5 protein of BoHV-1 but not of PRV or EHV-1 reduces TAP1 and TAP2 steady state levels

Next, the mechanism of TAP inhibition by the various UL49.5 proteins was investigated. Expression of BoHV-1 UL49.5 strongly reduced TAP1 and TAP2 protein levels in human MJS cells (Koppers-Lalic et al., 2005). It was shown that the cytoplasmic domain of UL49.5 is required for mediating proteasome-dependent degradation of TAP. To investigate whether BoHV-1 UL49.5 has a similar mode of action in natural host cells, bovine MDBK cells were infected with wild-type BoHV-1 or a recombinant virus expressing a UL49.5 protein that lacks its cytoplasmic domain (UL49.5Δtail). Steady state protein levels of bovine TAP were evaluated by Western blotting. Whereas bovine TAP was readily detectable in uninfected MDBK cells, it was no longer observed in cell lysates from wild-type BoHV-1 infected cells (Fig. 5, upper panel, compare lanes 1 and 2). Interestingly, in cells infected with the recombinant virus expressing the UL49.5Δtail mutant, TAP1 steady state levels were not affected (compare lanes 2 and 3). As a control, α-tubulin was consistently detected in all samples (Fig. 5, middle panel). Immunoprecipitation of UL49.5 from the infected cells confirmed the expression of the wild-type and recombinant proteins (Fig. 5, lower panel). These findings indicate that the degradation of TAP by UL49.5 previously observed in human cells also occurs in bovine cells. In addition, like in human cells, the cytoplasmic domain of UL49.5 is critical to TAP degradation in the natural host cells.

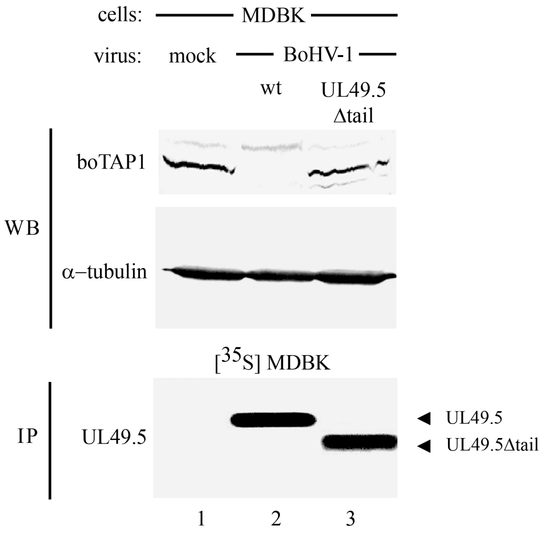

Fig. 5, degradation of bovine TAP is mediated by BoHV-1UL49.5 through its cytoplasmic tail. MDBK cells were mock-infected or infected with wild-type BoHV-1 or BoHV-1 expressing UL49.5Δtail for 12 h. The levels of bovine TAP1 and, as a control, α-tubulin were assessed in lysates of infected cells by Western blotting (WB). BoHV-1 UL49.5 was immunoprecipitated from lysates of metabolically labeled cells (IP).

To further address the molecular basis of TAP inhibition mediated by PRV, EHV-1, EHV-4, and CaHV-1 UL49.5, these proteins were stably expressed in human melanoma (MJS) cells. Like BoHV-1 UL49.5, the PRV and EHV-1 UL49.5 proteins were capable of blocking human TAP (Fig. 6A). CaHV-1 UL49.5 did not inhibit peptide transport in human cells, while in canine cells some reduction in TAP activity was observed without a reduction of MHC I surface expression (Fig. 2A and B). Expression of BoHV-1 UL49.5 in MJS cells resulted in reduced TAP1 and TAP2 protein levels, which is in accordance with previous observations (Fig. 6B, compare lanes 1 and 2) (Koppers-Lalic et al., 2005). In contrast, expression of the UL49.5 homologs of PRV and EHV-1 did not affect TAP1 and TAP2 steady state levels in MJS cells (Fig. 6B, lanes 3 and 4). These findings indicate that the UL49.5 homologs of PRV and EHV-1 inhibit peptide transport by TAP through a different mechanism than by mediating degradation of TAP.

Peptide binding to TAP is not affected by EHV-1 and PRV UL49.5 proteins

The translocation of peptides into the ER lumen is initiated by the association of peptides with the peptide-binding site of TAP (Uebel et al., 1995). To investigate whether the inhibition of peptide transport by PRV and EHV-1 UL49.5 involves blocking of peptide binding to TAP, microsomes were isolated from MJS cells expressing PRV and EHV-1 UL49.5. Microsomes were incubated with a ^{125}I-labeled reporter peptide (Fig. 7). At all

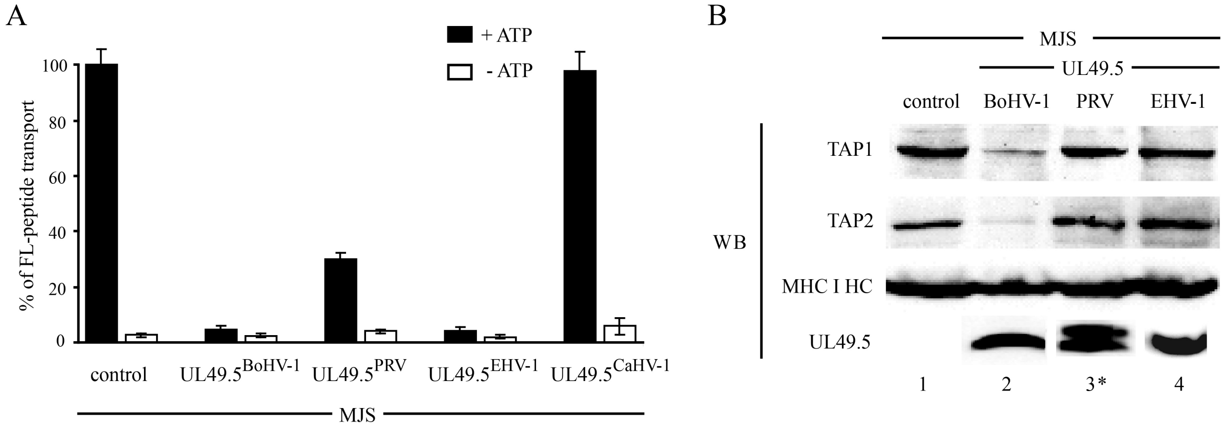

Fig. 6, UL49.5 of BoHV-1 but not of PRV or EHV-1 reduces TAP1 and TAP2 steady state levels in human cells. (A) TAP-dependent peptide transport is inhibited in human melanoma cells (MJS) stably expressing the UL49.5 homologs of BoHV-1, PRV and EHV-1, but not CaHV-1. Peptide transport is depicted as percentage of translocation, relative to the translocation observed in control cells (defined as 100%). (B) Steady state protein levels of TAP1, TAP2, MHC I heavy chains (MHC I HC), and UL49.5 in control cells and cell lines expressing UL49.5 of BoHV-1, PRV and EHV-1. Proteins present in post-nuclear supernatants were separated using SDS-PAGE and detected by Western blotting (WB) using antibodies specific for TAP1, TAP2, MHC I heavy chains, and the UL49.5 proteins of BoHV-1, PRV and EHV-1. *The doublet of PRV UL49.5 is probably related to differential glycosylation.

concentrations tested, the peptide-binding capacity (Bmax) was similar for microsomes prepared from control cells and from cells expressing the PRV or EHV-1 UL49.5 proteins. Most importantly, the binding affinity (K_d) for the peptides was not changed by the viral inhibitors, demonstrating preservation of the peptide binding site of the TAP complex. This has also been observed for BoHV-1 UL49.5 (Koppers-Lalic et al., 2005) and indicates that inhibition of TAP-mediated peptide transport by the BoHV-1, PRV, and EHV-1 UL49.5 proteins does not rely on interference with peptide binding.

EHV-1 UL49.5 inhibits ATP-binding by TAP

Since ATP-binding and hydrolysis are required to energize peptide translocation by TAP (Meyer et al., 1994; Neefjes et al., 1993; Gorbulev et al., 2001), it was investigated whether the expression of the PRV, EHV-1, and EHV-4 UL49.5 proteins affected binding of ATP to TAP. Previous experiments indicated that the BoHV-1 UL49.5 protein did not influence the interaction of ATP with TAP (Koppers-Lalic et al., 2005). The ATP-binding capacity of TAP in lysates from MJS cells (control) was compared to the binding in lysates from MJS cells stably expressing UL49.5 of PRV, EHV-1 or EHV-4, or the HCMV-encoded US6 protein. US6 is known to strongly inhibit ATP-binding to TAP (Hewitt et al., 2001; Kyritsis et al., 2001).

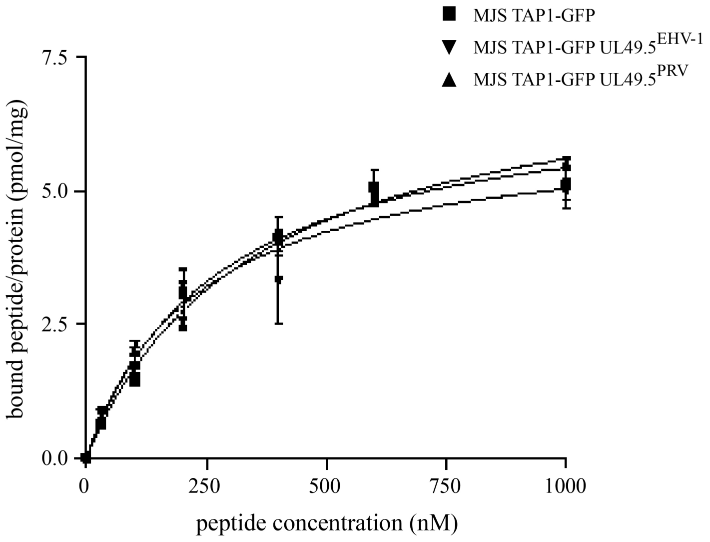

Fig. 7, UL49.5 of PRV and EHV-1 do not interfere with peptide binding to TAP. To evaluate peptide binding to the TAP complex, microsomal membranes from MJS TAP1-GFP cells (control; ■), and MJS TAP1-GFP cells expressing UL49.5 of PRV (▲) or EHV-1 (▼) were incubated with increasing concentrations of the radiolabeled peptide (RR[^{125}I]YQKSTEL). Unspecific binding was determined in the presence of 200-fold excess of ICP47 (data not shown). The amount of specifically bound peptide per amount of microsomal protein is plotted against the peptide concentration. K_d values for control MJS: 277 ± 58 nM, for MJS UL49.5^{PRV}: 351 ± 42 nM and for MJS UL49.5$^{EHV\text{-}1}$: 236 ± 71 nM.

Cell lysates prepared in the presence of the mild detergent digitonin were incubated with ATP-agarose beads. Proteins bound to the ATP-agarose (Fig. 8, pellet "P") were eluted from the beads with EDTA and displayed next to the unbound supernatant fractions (Fig. 8, "S"). TAP1 and TAP2 were detected by Western blotting.

PRV UL49.5 did not alter the binding of ATP to TAP1 or TAP2 (Fig. 8A, compare lanes 2 and 4). As expected, the expression of US6 completely abolished the interaction of ATP

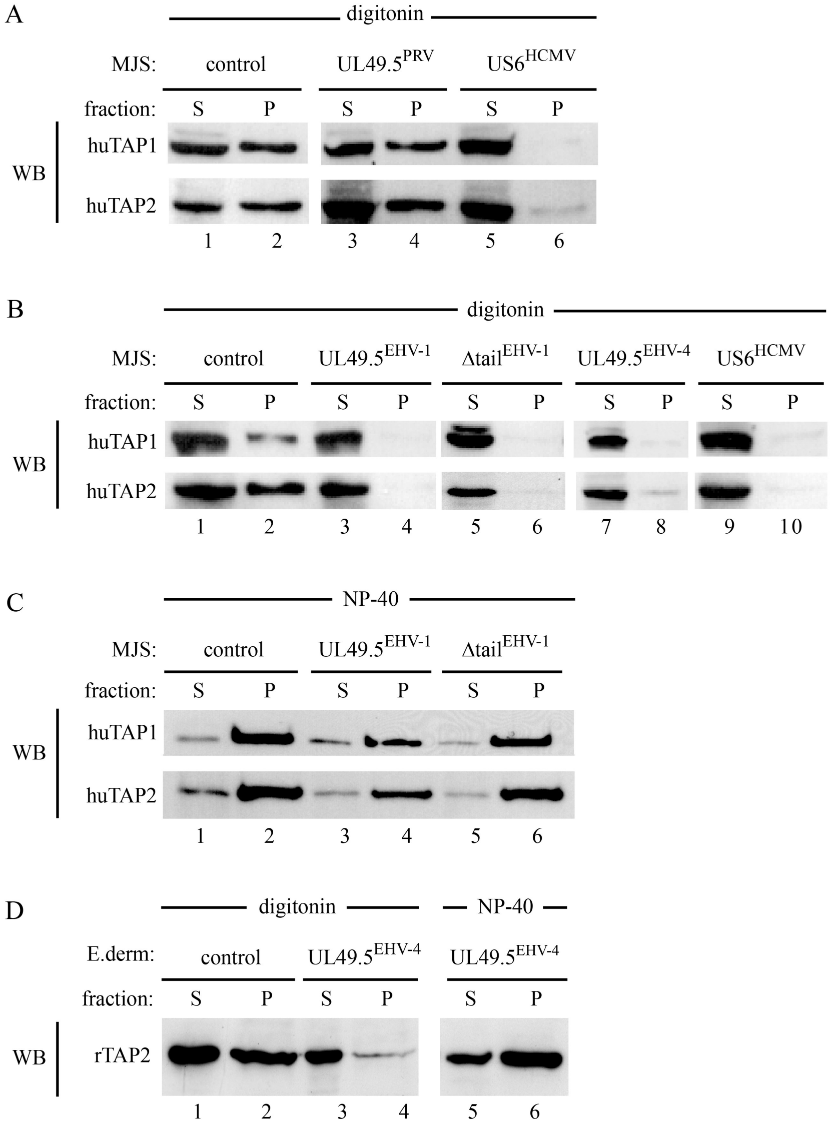

Fig. 8, UL49.5 of EHV-1 and EHV-4 block ATP binding to human and equid TAP. (A-C) MJS cells expressing the UL49.5 proteins or the HCMV-derived TAP-inhibitor US6 and (D) E.derm cells expressing EHV-4 UL49.5 were lysed using digitonin or NP-40 as indicated. Post nuclear lysates were incubated with ATP-agarose. The pellet (P) contains the ATP-binding proteins. The supernatant (S) contains proteins incapable of binding ATP. ATP-bound (ATP-agarose beads; pellet) and unbound (soluble; supernatant) fractions were separated by centrifugation and analyzed using SDS-PAGE and Western blotting (WB) with antibodies against the proteins indicated.

with TAP (Fig. 8A, lane 6). These data show that TAP retains the capacity to bind ATP in the presence of PRV UL49.5.

In EHV-1 and EHV-4 UL49.5-expressing cells, neither TAP1 nor TAP2 could be detected in the ATP-agarose fraction (Fig. 8B, compare lane 2 with lanes 4 and 8). Since the C-terminus of UL49.5 is exposed in the cytosol, this domain might be responsible for the inhibition of ATP-binding to the nucleotide-binding domains of TAP. To evaluate whether the C-terminus of UL49.5 blocks ATP-binding to TAP, a truncated form of EHV-1 UL49.5 lacking the cytoplasmic domain was constructed and expressed in MJS cells. The EHV-1 UL49.5Δtail recombinant still interfered with ATP-binding to TAP (Fig. 8B, lane 6). When the association of wild-type or mutant EHV-1 UL49.5 with TAP was disrupted by lysis of the cells in NP-40, the ability of TAP1 and TAP2 to bind to the ATP-agarose was restored (Fig. 8C, lanes 4 and 6; also compare with Fig. 8B lanes 4 and 6, respectively). These results indicate that the EHV-1 UL49.5 protein is capable of interfering with the recruitment of ATP by human TAP independent of the cytoplasmic domain of UL49.5.

The ability of EHV-1 and EHV-4 UL49.5 to interfere with the binding of ATP to equid TAP was assessed in E.derm cells (data shown for EHV-4). Like human TAP (Fig. 8B, lane 8), equid TAP2 was not able to bind ATP-agarose in the presence of EHV-4 UL49.5 (Fig. 8D, compare lanes 2 and 4). When the experiment was performed in the presence of NP-40, the ability of equid TAP2 to bind ATP was restored (Fig. 8D, compare lanes 4 and 6). These results indicate that the UL49.5 proteins of EHV-1 and EHV-4 inhibit human and equid TAP through similar mechanisms, rendering both human and equid TAP molecules incapable of recruiting ATP.

UL49.5 proteins arrest TAP in a translocation-incompetent state

To obtain further insight into the strategies used by EHV-1 and PRV UL49.5 to block TAP transport, FRAP assays were performed. With this technique, conformational changes of TAP that occur during peptide translocation can be indirectly visualized by measuring the lateral mobility of GFP-tagged TAP within the ER membrane. It has been shown that the lateral mobility of TAP is inversely proportional to its activity, as peptide-transporting TAP molecules diffuse at a slower rate than inactive, closed TAP complexes (Reits et al., 2000). In the absence of ATP, the translocation cycle cannot be initiated and consequently TAP will have a closed, more compact conformation. In agreement with this, depletion of ATP results in increased mobility of TAP in the ER membrane (Fig. 9, control samples; compare black and gray bars). The complex can be trapped in the active conformation by adding long side chain peptides (l.s.c.p.). These peptides bind to TAP, but cannot be translocated over the ER membrane, which results in a retained open conformation and therefore a slow diffusion rate of TAP in the ER membrane (Reits et al., 2000) (Fig. 9, control samples; white bars).

Expression of EHV-1 and PRV UL49.5 results in a decreased mobility of TAP (Fig. 9, untreated samples; black bars). Whereas the diffusion rate of TAP increased considerably in ATP-depleted control cells, only a slight increase in TAP mobility was detected upon ATP depletion in the UL49.5-expressing cells (Fig. 9, gray bars). The failure of TAP to respond to ATP depletion in the EHV-1 UL49.5 cells is in agreement with the observation that this protein interferes with ATP binding to TAP (Fig. 8B). Although ATP can still bind to TAP in the presence of PRV UL49.5 (Fig. 8A), ATP depletion induces only a minor change in TAP mobility in the PRV UL49.5 cells (Fig. 9). Apparently, the presence of PRV UL49.5 prohibits conformational transitions that normally follow ATP-binding.

In the presence of the UL49.5 proteins, l.s.c.p. were also unable to induce conformational changes within the TAP complex (Fig. 9). Since peptides can still bind to TAP in the presence of EHV-1 and PRV UL49.5 (Fig. 7), the failure of l.s.c.p. to induce conformational changes again suggests that the UL49.5 proteins arrest TAP in a translocation-incompetent state.

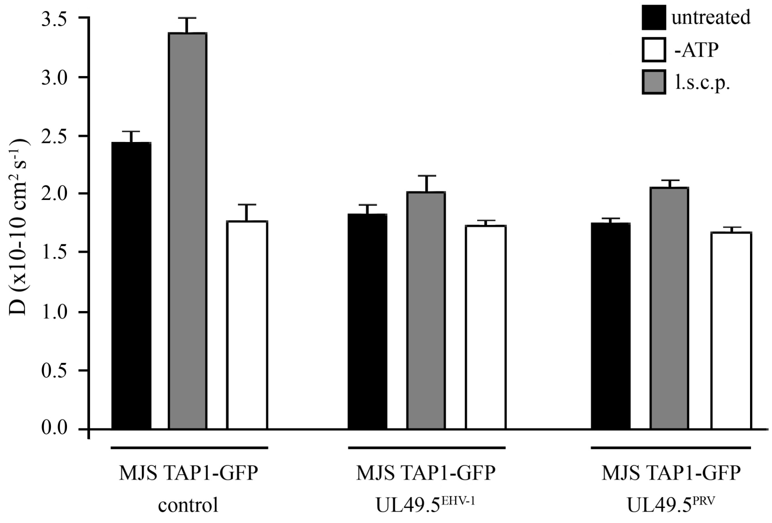

Fig. 9, UL49.5 homologs arrest TAP in a translocation-incompetent state. The lateral mobility of the TAP complex was analyzed in MJS TAP1-GFP cells using confocal microscopy and FRAP. A circular spot in the ER was bleached and recovery of fluorescence was monitored. The half-time for recovery was determined and used to calculate the diffusion coefficient ('D'). Where indicated, ATP was depleted (-ATP) or saturating amounts of substrate peptides (long side chain peptides, l.s.c.p.) were micro-injected into the cells.

Discussion

This study identifies the UL49.5 proteins of BoHV-1, PRV, EHV-1, and EHV-4 as members of a novel class of viral immune evasion proteins. The UL49.5 gene products interfere with MHC I antigen presentation by blocking the supply of antigenic peptides in the ER lumen through inhibition of TAP. Within the UL49.5 family of TAP inhibitors, heterogeneity is observed with respect to the mechanisms that underlie TAP inhibition. Whereas BoHV-1 UL49.5 targets TAP for proteasomal degradation (Koppers-Lalic et al., 2005), PRV and EHV-1 UL49.5 do not diminish the steady state levels of TAP1 or TAP2. Interestingly, EHV-1 and EHV-4 UL49.5 interfere with the binding of ATP to TAP, a function that is not

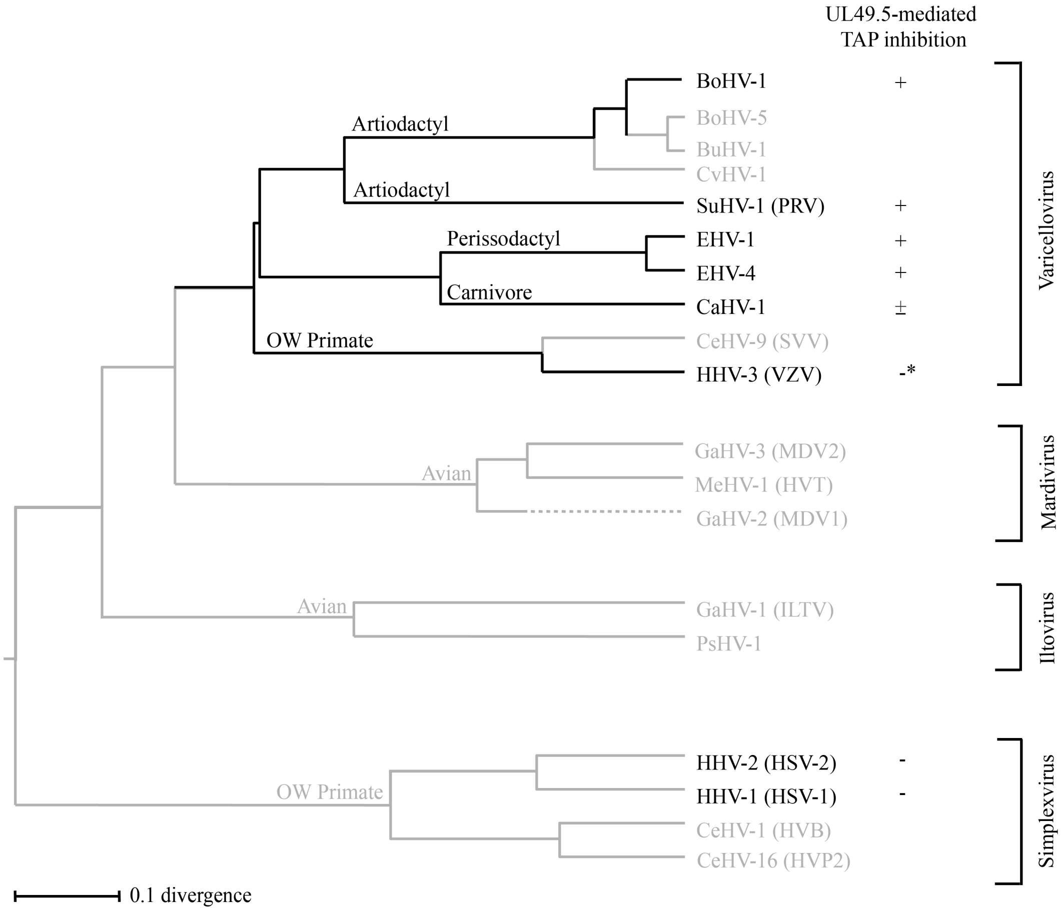

Fig. 10, phylogenetic tree of alphaherpesvirus UL49.5 proteins. The tree was constructed on the basis of all presently known UL49.5 amino acid sequences of *Alphaherpesvirinae* using ClustalV. The ClustalV (PAM250) method was used under the default settings of the alignment program MegAlignTM 500 of the sequence analysis software DNA* of DNASTAR Inc. The UL49.5 proteins for which TAP inhibition has been tested are shown in bold. *The HHV-3 (VZV) UL49.5 protein binds TAP, but does not block peptide transport. The NCBI accession numbers are provided in the materials and methods section.

influenced by BoHV-1 or PRV UL49.5. All TAP-inhibiting UL49.5 proteins arrest the transporter complex in a translocation-incompetent state.

UL49.5 homologs are encoded by all *Herpesviridae* analyzed to date (Davison, 2002). However, the TAP-inhibiting capacities of these proteins appear to be restricted to certain members of the genus *Varicellovirus*. Members of this virus genus have co-evolved with their respective host species (McGeoch et al., 2006). Viruses of even-toed ungulates or *Artiodactyla* like BoHV-1 and PRV co-evolved with cattle and pigs; viruses of odd-toed ungulates or *Perissodactyla* (EHV-1 and EHV-4) with horses; the carnivore viruses FeHV-1 and CaHV-1 with cats and dogs, and the Old World primate virus VZV with humans (McGeoch et al., 2006) (Fig. 10). The identification of the UL49.5 proteins encoded by BoHV-1, PRV, EHV-1, and EHV-4 as members of the UL49.5 family of TAP inhibitors suggests that more UL49.5 proteins with this property may be found in varicelloviruses of even- and odd-toed ungulate hosts. Considering the shared evolution of (herpesviruses from) carnivores and (herpesviruses from) odd-toed ungulates (McGeoch et al., 2006), CaHV-1 UL49.5 was expected to inhibit TAP as effectively as EHV-1 and EHV-4 UL49.5. However, the reduction of TAP-dependent peptide transport caused by CaHV-1 UL49.5 was very moderate compared to the inhibition by the other TAP-inhibiting UL49.5 proteins. The identification of the UL49.5 domains contributing to TAP inhibition will provide more insights into these differences.

VZV infection of human cells results in reduced expression of MHC I at the cells surface (Cohen, 1998; Abendroth et al., 2001; Eisfeld et al., 2007). The VZV ORF66-encoded serine-threonine protein kinase has been shown to be one of the VZV proteins contributing to MHC I downregulation in VZV-infected cells (Eisfeld et al., 2007). However, a VZV recombinant lacking a functional ORF66 product still causes downregulation of MHC I surface expression, indicating that additional modulators of MHC I-restricted antigen presentation are encoded by VZV. The observed downregulation of MHC I surface expression on VZV-infected cells is not induced by UL49.5 when expressed individually. Despite the observed interaction between VZV UL49.5 and the peptide-loading complex, this protein alone did not block peptide transport by TAP and it had no effect on antigen recognition by HLA-A1 and HLA-A2-restricted CTL clones. As VZV occupies a somewhat isolated position in the phylogenetic tree of varicelloviruses (Fig. 10), it seems likely that evolutionary divergence has influenced VZV to acquire a separate mechanism to interfere with MHC I-restricted antigen presentation. Alternatively, UL49.5 might co-operate with another unidentified VZV-encoded protein in order to reduce antigen presentation by MHC I molecules. During virus infection, UL49.5 can be found in a complex with glycoprotein M (gM). However, the co-expression of VZV UL49.5 and glycoprotein M has no effect on the expression of MHC I molecules at the cell surface (Eisfeld et al., 2007), indicating that gM does not act as a modulator of UL49.5 with respect to TAP inhibition.

Interaction with the conserved viral membrane glycoprotein gM appears to be a common property of all UL49.5 homologs, as is the presence of a single cysteine residue in their ER-luminal/extracellular domain (Jons et al., 1998; Wu et al., 1998; Rudolph et al., 2002; Lipinska et al., 2006). This cysteine residue is involved in the interaction of UL49.5 with gM, with which it forms a disulfide-linked heterodimer (Barnett et al., 1992; Mach et al., 2007). The complex of UL49.5 and gM is implicated in virion maturation and membrane fusion processes (Klupp et al., 2000; Osterrieder et al., 1996). Interestingly, the interaction of BoHV-1 UL49.5 with gM interferes with its capacity to block TAP (Lipinska et al., 2006). Nevertheless, UL49.5 blocks peptide transport by TAP in BoHV-1-infected cells. This may be explained by the fact that UL49.5 is expressed prior to and in excess of the early-late gM (Lipinska et al., 2006).

Interference with TAP-mediated peptide transport is an effective way of reducing CTL recognition and is used by several other herpesviruses, including HSV-1 and -2, HCMV, MHV-68, and EBV. Compared to the other herpesvirus-encoded TAP inhibitors, the cross-species activity of UL49.5 proteins is remarkable. Except for CaHV-1 UL49.5, the UL49.5 proteins of BoHV-1, PRV, EHV-1, and EHV-4 all exhibit the ability to target human TAP. In addition, BoHV-1 UL49.5 inhibits peptide transport by murine (van Hall et al., 2007), rat, equid, and porcine TAP (D.K.L. and M.C.V., unpublished observations). Human, porcine, bovine, and rodent TAP1 and TAP2 demonstrate a substantial degree of amino acid identity (70-80%) (Garcia-Borges et al., 2006). Thus, the ability of UL49.5 proteins to act across species barriers most likely relies on structural homology within the TAP domains critically involved in UL49.5-TAP interaction. Apparently, this is less so for the domains within TAP that are targeted by US6, mK3 and BNLF2a, whose actions seem to be restricted largely to the natural host species. BoHV-1 UL49.5 reduces TAP protein levels in bovine, human, and murine cells, and also mediates degradation of human TAP in insect cells when co-expressed with UL49.5 (Loch et al., 2008; Koppers-Lalic et al., 2005; van Hall et al., 2007), indicating conservation of the pathway involved in this degradation process.

The UL49.5 proteins exhibit unexpected differences in their mechanisms of TAP inhibition, despite their close evolutionary relatedness. The cytoplasmic domain of BoHV-1 UL49.5 is essential for mediating degradation of both human and bovine TAP. EHV-1 and PRV UL49.5 have no influence on the stability of TAP. Apparently, degradation of TAP is facilitated by a yet unknown signal within the C-terminal domain of BoHV-1 UL49.5, which is not present in the other homologs. Studies to identify the nature of this sequence motif are in progress.

The interaction of EHV-1 and EHV-4 UL49.5 with TAP blocks ATP binding to TAP. This feature distinguishes EHV UL49.5 from the other homologs studied. Interestingly, removal of the cytoplasmic domain of the EHV-1 UL49.5 protein did not restore the ability of TAP to bind ATP. Therefore, a direct interaction of EHV-1 UL49.5 with the cytosolic nucleotide binding domains of TAP is unlikely. Instead, the viral protein appears to arrest TAP in a translocation-incompetent state, incompatible with ATP-binding. This may resemble the type of structural change caused by HCMV US6 (Halenius et al., 2006). US6, which is a type I transmembrane protein, interacts with the luminal side of the TAP transporter and blocks ATP-binding by prohibiting essential conformational rearrangements within TAP. The inability of the BoHV-1 and PRV UL49.5 homologs to interfere with ATP-binding could be due to a slightly different conformational change induced by these proteins.

Based on the results presented in this study, the UL49.5 proteins encoded by BoHV-1, PRV, EHV-1, and EHV-4 can be classified as a new family of TAP-inhibiting proteins. These proteins share the ability of inducing a conformational arrest of TAP, which results in impaired peptide transport and inhibition of MHC I-restricted antigen presentation. In view of these joint features it is likely that the TAP inhibiting UL49.5 proteins originate from a common ancestral protein, which acquired this capacity earlier during evolution. The VZV UL49.5 protein may be a rudimentary form with respect to TAP inhibition, or it may have lost its TAP inhibitory capacity later on. Alternatively, it may require additional VZV proteins for the inhibition of TAP.

This study has revealed unexpected variation among UL49.5 proteins of varicelloviruses with respect to their mechanisms of TAP inhibition. These differences can be related to distinct evolutionary pathways of these varicelloviruses. The UL49.5 family of TAP-inhibiting proteins does not demonstrate any structural or functional similarity to TAP-inhibiting proteins encoded by other herpesviruses, for instance ICP47, US6, mK3, or BNLF2a. This diversity of TAP-inhibiting proteins acquired by distantly related members of the subgroups of alpha-, beta-, and gammaherpesviruses is remarkable and presents a striking example of functional convergent evolution. At the same time, this identifies TAP as an Achilles' heel of the MHC I antigen presentation pathway. Inhibition of TAP has apparently provided a strong advantage to these herpesviruses during co-evolution with their hosts.

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