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

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The capacity of UL49.5 proteins to inhibit TAP is widely distributed amongst members of the genus *Varicellovirus*

Submitted

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Abstract

The life long infection by varicelloviruses is characterized by a fine balance between the host immune response and immune evasion strategies employed by these viruses. Virus-derived peptides are presented to cytotoxic T lymphocytes by MHC I molecules. The transporter associated with antigen processing (TAP) transports the peptides from the cytosol into the ER, where loading of MHC I molecules occurs. The varicelloviruses bovine herpesvirus 1 (BoHV-1), pseudorabies virus, and equid herpesvirus 1 and 4 have been found to encode a UL49.5 protein that inhibits TAP-mediated peptide transport. To investigate to what extent UL49.5-mediated TAP inhibition is conserved within the family of *Alphaherpesvirinae*, the homologs of another five varicelloviruses, one mardivirus and one iltovirus were studied. The UL49.5 proteins of BoHV-5, bubaline herpesvirus 1, cervid herpesvirus 1 and felid herpesvirus 1 were identified as potent TAP inhibitors. In contrast, simian varicellavirus UL49.5 caused only a slight reduction in peptide transport. VZV infection does not influence TAP function, indicating that the lack of TAP inhibition observed for VZV UL49.5 is not caused by the absence of viral co-factors that might assist in this process. The UL49.5 homologs of the mardivirus Marek's disease virus 1 and the iltovirus infectious laryngotracheitis virus did not block TAP, suggesting that the capacity to inhibit TAP via UL49.5 has been acquired by varicelloviruses only. A phylogenetic analysis of viruses that inhibit TAP though their UL49.5 proteins revealed an interesting hereditary pattern, pointing towards the presence of this capacity in defined clades within the genus *Varicellovirus*.

Introduction

Herpesviruses cause a life long infection in their host. The anti-viral immune response of the host is typically counteracted by immune evasion strategies employed by these viruses. CD8+ cytotoxic T lymphocytes (CTL) play an important role in immunity against viruses, recognizing viral peptides that are presented on major histocompatibility complex class I (MHC I) molecules at the cell surface. The antigenic peptides originate from proteasomal degradation of virus-encoded proteins in the cytosol. These peptides are then transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP). This transporter consists of two subunits, TAP1 and TAP2, forming a pore through which peptides are transported in an ATP-dependent manner (Lehner and Trowsdale, 1998; Amills et al., 1998; Abele and Tampe, 1999). TAP proteins are highly conserved amongst various species: human, porcine, bovine and rodent TAP 1 and TAP2 demonstrate 70-80% amino acid identity (Garcia-Borges et al., 2006; McCluskey et al., 2004). Yet, polymorphisms of TAP1 and TAP2 are found in many species, ranging from single amino acid differences in humans to larger variations in rat that even affect the nature of the peptides presented (McCluskey et al., 2004). Despite the apparent sequence variation in the TAP genes, the transporter is thought to be involved in MHC I-mediated antigen presentation in many different species, including Xenopus (Ohta et al., 2003).

Herpesviruses have developed a number of mechanisms to counteract MHC I presentation. Particularly the inhibition of peptide transport by TAP is often exploited by these viruses to prevent elimination by CTLs. The first identified TAP inhibitor was the alphaherpesvirus herpes simplex virus 1 (HSV-1)-encoded ICP47 protein. ICP47 protein prevents peptide transport by obstructing the peptide binding site of the TAP complex (Ahn et al., 1996; Aisenbrey et al., 2006; Fruh et al., 1995; Hill et al., 1995; Tomazin et al., 1996). The US6 protein of the betaherpesvirus human cytomegalovirus (HCMV) interferes with ATP binding to TAP, thereby limiting its energy supply and, consequently, the transport of peptides (Ahn et al., 1997; Halenius et al., 2006; Hengel et al., 1997; Hewitt et al., 2001; Lehner et al., 1997). The gammaherpesvirus Epstein-Barr virus (EBV) encodes the TAP inhibitor BNLF2a that blocks both the binding of peptides and ATP to TAP (Hislop et al., 2007; Horst et al., 2009). Another gammaherpesvirus protein that affects TAP is mK3 encoded by the murine gammaherpesvirus 68. This protein interferes with antigen presentation by targeting the MHC I heavy chains molecules for degradation and, secondarily, by destabilizing TAP (Stevenson et al., 2000; Boname and Stevenson, 2001; Boname et al., 2005).

A new class of TAP-inhibiting proteins, encoded by the UL49.5 gene, has been identified in varicelloviruses. The UL49.5 homologs encoded by bovine herpesvirus 1 (BoHV-1), equid herpesvirus (EHV) 1 and 4, and suid herpesvirus 1 or pseudorabies virus (PRV) all inhibit TAP function. Homologs of UL49.5 proteins have been found in all

herpesviruses sequenced (McGeoch et al., 2006; Davison et al., 2009). UL49.5 encodes a transmembrane protein that is often N-glycosylated and therefore known as glycoprotein N or gN. In several herpesviruses, UL49.5 has been demonstrated to be involved in virion maturation and infectivity: UL49.5 forms a heterodimeric complex with glycoprotein M (gM) and is necessary for proper glycosylation and maturation of the complex (Fuchs and Mettenleiter, 2005; Klupp et al., 2000; Lipinska et al., 2006; Osterrieder et al., 1996; Wu et al., 1998). Thus, for some viruses, UL49.5 possesses a dual role, functioning both as a molecular chaperone and as an immune evasin.

The family of *Herpesviridae* has been classified into three subfamilies, the *Alpha-*, *Beta-*, and *Gammaherpesvirinae*. Isolated expression of the UL49.5 homologs encoded by members of these subfamilies, including HSV-1 and -2 (α), HCMV (β) and EBV (γ), did not result in reduced TAP function (Koppers-Lalic et al., 2005). So far, the viruses that code for TAP-inhibiting UL49.5 proteins (BoHV-1, EHV-1, EHV-4, and PRV) all belong to the genus *Varicellovirus* of the *Alphaherpesvirinae*. However, VZV and CaHV-1, which are members of the same genus, exhibit no or poor TAP inhibition, respectively. This implies that TAP-inhibiting capacity of UL49.5 proteins has not been acquired by all varicelloviruses, or that some viruses have lost the ability to block TAP through the UL49.5 protein.

Unexpectedly, the precise mechanisms by which UL49.5 homologs inhibit TAP demonstrates remarkable heterogeneity (Koppers-Lalic et al., 2008). The BoHV-1 UL49.5 has been shown to induce degradation of TAP1 and TAP2, in addition to the inhibition of peptide transport (Koppers-Lalic et al., 2005). This was not observed for the UL49.5 proteins of EHV-1 and PRV. In contrast, EHV-1 and EHV-4 UL49.5 prevent ATP binding to TAP (Koppers-Lalic et al., 2008). All varicellovirus-encoded TAP inhibitors studied thus far block conformational changes within the complex that are required for peptide transport (Koppers-Lalic et al., 2008). The UL49.5 of human herpesvirus 3 or varicella-zoster virus (VZV) does not affect TAP function, although the protein does associate with the TAP transporter (Koppers-Lalic et al., 2008). Canid herpesvirus 1 (CaHV-1) UL49.5 has a modest effect on peptide transport in canine cells (Koppers-Lalic et al., 2008).

The *Alphaherpesvirinae* include two other genera, the *Mardivirus* and the *Iltovirus*. Gallid herpesvirus 2 or Marek's disease virus 1 (MDV-1) is a member of the mardiviruses. Serotype 1 of MDV is oncogenic, inducing T cell tumors in infected poultry. MHC I downregulation has been observed on MDV-1 infected chicken cells (Levy et al., 2003; Hunt et al., 2001) and in epithelial and infiltrating cells derived from brain tissue of infected chickens (Gimeno et al., 2001). Additionally, *in vitro* infections with viruses of the non-oncogenic serotypes 2 (MDV-2) and 3 (Turkey herpesvirus or HVT) led to decreased MHC I cell surface expression on the infected cells (Hunt et al., 2001). To date the fate of the MHC I molecules in infected cells is unknown, as well as the responsible viral protein(s) involved. Gallid herpesvirus 1 or infectious laryngotracheitis virus (ILTV) is a member of the

iltoviruses. At present, it is unclear if MHC I-restricted antigen presentation is affected by ILTV. Yet, $CD8^+$ T cells recognizing epitopes encoded by this virus have been detected in tracheal mucosa (Devlin et al., 2010) and in peripheral blood (Pavlova et al., 2009) of ILTV infected chickens.

In this study, TAP inhibition by UL49.5 homologs of various clades within the genus *Varicellovirus* was assessed. The UL49.5 proteins of BoHV-5, water buffalo herpesvirus or bubaline herpesvirus 1 (BuHV-1), red deer herpesvirus or cervid herpesvirus 1 (CvHV-1), and feline rhinotracheitis virus or felid herpesvirus 1 (FeHV-1) were identified as potent TAP inhibitors. The UL49.5 homolog of cercopithecine herpesvirus 9 or simian varicella virus (SVV), which is closely related to VZV, only slightly reduced peptide transport by rhesus macaque TAP. UL49.5 proteins of other alphaherpesviruses, including HSV-1 and HSV-2 (simplexviruses) (Koppers-Lalic et al., 2005), MDV-1 (mardivirus), and ILTV (iltovirus) fail to inhibit TAP. These findings are discussed in the context of the phylogeny of these viruses.

Materials and methods

UL49.5 constructs Purified viral DNA from BoHV-5 strain Evi 88/95 (D'Arce et al., 2002), BuHV-1 strain B6 (kind gift from M.J. Studdert, Faculty of Veterinary Science, University of Melbourne, Australia), CvHV-1 strain 1426 (kindly provided by P. F. Nettleton, Moredun Research Institute, Edinburgh, Great Britain), FeHV-1 strain B927 (kindly provided by R. de Groot, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands), and MDV-1 strain CVI988 (J. van Oirschot, Department of Virology, Central Veterinary Institute, Lelystad, The Netherlands) were used as a template for polymerase chain reaction (PCR) amplification. PCR-reactions were performed using *Pfu* (Invitrogen), Taq (Promega) or KOD DNA polymerase (Novagen-Merck) and specific primers (Table 1) for amplification of the coding sequence of the UL49.5 genes. The sequences of the primers were based on published sequences found in the NCBI database. PCR-generated products were sequenced and inserted into the retroviral expression vectors pLZRS-IRES-GFP, behind the HCMV IE1 promoter and upstream of an internal ribosome entry site (IRES) element, followed by GFP. SVV UL49.5 (strain Delta) in pcDNA3.1 were kindly provided by I. Messaoudi and K. Früh (Vaccine and Gene Therapy Institute, Oregon Health and Science University, Oregon, USA). The SVV insert was amplified by PCR (primers Table 1) and cloned into pLZRS using GATEWAY technology (Invitrogen). ILTV UL49.5 (strain A489) was recloned from a pcDNA3.1 vector (a kind gift from W. Fuchs and T.C. Mettenleiter, Institute of Molecular Biology, Friedrich-Loeffler Institut, Greifswald-Insel Riems, Germany) (Fuchs and Mettenleiter, 2005) into

Table 1: PCR primers

Primer name	Primer sequence [a]
BoHV-5 UL49.5	Fw: 5'-GCC**GGATCC**GCTCCACGACGACCATGTCGCGC-3'
BuHV-1 UL49.5	Fw:5'-GCC**GGATCC**GACGACCATGTCGCGCTCGCT-3'
BoHV-5/BuHV-1 UL49.5	Rev: 5'-GCG**GAATTC**CGCTCAACCCCGCCCCCGCAC-3'
CvHV-1UL49.5	Fw: 5'-GCC**GGATCC**GAGCCGAGCACCATGGCGAGG-3'
CvHV-1UL49.5	Rev: 5'-GCG**GAATTC**CGGTCAGCCCCGCCCCCGCGA-3'
FeHV-1 UL49.5	Fw: 5'-CG**GGATCC**CACCATGGATCGTTTATCC-3'
FeHV-1 UL49.5	Rev: 5'-GCG**GAATTC**TTAGTGTGGCATGC-3'
SVV UL49.5	Fw:5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT**GAATTC**-ACCATGGCTTCAAATTGCTCTT-3'
SVV UL49.5	Rev: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTC**CTCGAG**-TTACCATGTACTACGTAAGACGGATCG-3'
SVV-HA UL49.5	Rev: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTC**CTCGAG**-TTAAGCGTAGTCTGGGACGTCGTATGG-3'
MDV-1 UL49.5	Fw: 5'-CG**GGATCC**CACCATGGGACTCATG-3'
MDV-1 UL49.5	Rev: 5'-CC**GAATTC**CTTACCACTCCTCTTTAAAC-3'
ILTV UL49.5	Fw: 5'-CG**GGATCC**CACCATGAGGCTGC-3'
ILTV UL49.5	Rev: 5'-G**GAATTC**CTACCATCGAGAACTAATGAC-3'

a = restriction sites used in bold

pLZRS (primers Table 1). Information on the pLZRS vector can be obtained at www.stanford.edu/group/nolan/retroviral_systems/retsys.html.

The pDONR209 plasmids containing the UL49.5 constructs of MDV1 strains RB1B and CVI988 were kind gifts from J. Haas (Division of Pathway Medicine, University of Edinburgh, UK). The inserts were cloned into the lentiviral vector pDEST-LV-IRES-GFP (K. Franken, Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, The Netherlands) upstream of an IRES element, which was followed by GFP using the GATEWAY technology. Sequences were of the resulting expression vectors were verified.

Cell lines and recombinant viruses The human melanoma cell line Mel JuSo (MJS), MJS BoHV-1 UL49.5-IRES-GFP (Koppers-Lalic et al., 2005), MJS IRES-GFP, the epithelial cell line Madin-Darby bovine kidney (MDBK) cells (American Type Culture Collection, ATCC; commonly used to propagate BoHV-5, BuHV-1 and CvHV-1) and MDBK UL49.5-IRES-GFP (Koppers-Lalic et al., 2008) were maintained in RPMI-1640 medium; the feline-derived epithelial cell line CRFK (provided by R. de Groot, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands), the

avian hepatoma cell line LMH, and human fibroblast cell line MRC-5 (a gift from G.M. Verjans, Rotterdam Eye Hospital, The Netherlands) were cultured in DMEM medium; the rhesus macaque-derived epithelial cell line LLC-MK2 (kindly provided by A.D. Hislop, School of Cancer Sciences, University of Birmingham, United Kingdom) were cultured in EMEM supplemented with non-essential amino acids. Media were supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine (Invitrogen), 140 IU/ml penicillin and 140 mg/ml streptomycin.

Recombinant viruses were made using the Phoenix amphotropic packaging system as described before (www.stanford.edu/group/nolan/retroviral_systems/retsys.html). The retroviruses were used to transduce target cells, after which GFP positive cells were selected using a FACSAria cell sorter (Becton Dickinson). The following stable cell lines were generated: MJS expressing BoHV-5, BuHV-1, CvHV-1, FeHV-1, SVV, SVV-HA, and ILTV UL49.5; 5; CRFK expressing FeHV-1; LLC-MK2 expressing SVV UL49.5; and LMH expressing ILTV UL49.5. The generation of recombinant retroviruses for MDBK cell line transductions was described before (Koppers-Lalic et al., 2008). GP2-293 pantropic packaging cells were co-transfected with pZLRS-UL49.5-IRES-GFP and pVSV-G construct (envelope vector) to obtain retroviruses used for the establishment of MDBK stably expressing BoHV-5, BuHV-1, and CvHV-1 UL49.

Antibodies The following antibodies were used for flow cytometry: anti-human MHC I complexes mAb W6/32 (Barnstable et al., 1978), anti-human MHC II HLA-DR mAb L243 (ATCC), anti-feline MHC I complexes mAb H58A (VMRD Inc.), anti-FeHV-1 MCA2490 (Serotec), anti-BoHV-1 glycoprotein B mAb 14 (Division of Infection Disease, Lelystad, the Netherlands) anti-chicken MHC I complexes mAb F21-2 (SouthernBiotech), and anti-human transferrin receptor mAb CD71 (Becton Dickinson).

For detection of UL49.5, rabbit polyclonal anti-sera were raised against a synthetic peptide (RLMGASGPNKKESRGRG) derived from the C-terminal domain of BoHV-1 UL49.5 (Lipinska et al., 2006). In addition, we used rabbit anti-ILTV UL49.5 (kindly provided by W. Fuchs and T.C. Mettenleiter) (Fuchs and Mettenleiter, 2005), anti-TAP1 mAb 148.3 (Meyer et al., 1994), anti-TAP2 mAb 435.3 (kind gift from P. van Endert, INSERM, U580, Université Paris Descartes, Paris, France), and rabbit anti-GFP (van den Born et al., 2007). Anti-actin mAb AC-74 (Sigma-Aldrich) was used as a control.

Flow cytometry Surface expression levels of MHC I and MHC II molecules were determined by flow cytometry. Cells were stained with the indicated primary antibodies and, after washing, with the secondary goat anti-mouse allophycocyanin Ab (Leinco Technologies) or goat anti-mouse phycoerythrin Ab (Jackson ImmunoResearch Laboratories) at 4°C. Stained cells were measured using a FACSCalibur (Becton Dickinson) and analyzed using CellQuest (Becton Dickinson) or FlowJo (Tree Star) software. One representative experiment out of two independent experiments is shown.

Peptide transport assay Cells were permeabilized using 2.5 units/ml Streptolysin-O (Murex Diagnostics) at 37°C for 10 min. Permeabilized cells were incubated with 4.5 µM of the fluorescein-conjugated synthetic peptide CVNKTERAY (*N*-core glycosylation site underlined) in the presence of 10 mM ATP or 0.125 M EDTA at 37°C for 10 min. Peptide translocation was terminated by adding 1 ml of ice-cold lysis buffer (1% Triton X-100, 500 nM NaCl, 2 mM $MgCl_2$, 50 mM Tris HCl, pH 8.0). After lysis for 30 minutes at 4°C, cells were centrifuged at 16,000 g for 20 minutes at 4°C in order to obtain post-nuclear lysates. Glycosylated peptides were isolated from these lysates by incubation with concanavalin A-Sepharose beads (GE Healthcare) for 2 h at 4°C. After washing of the beads, glycosylated peptides were eluted from the beads with elution buffer (500 mM mannopyranoside, 10 mM EDTA, 50 mM Tris HCl pH 8.0) during a one hour incubation step at room temperature. Fluorescence was measured using a Mithras LB 940 multilabel reader (Berthold Technologies). Peptide transport is expressed as percentage of translocation, relative to the translocation observed in control cells (set at 100%). One representative experiment out of two independent experiments is shown.

Western blotting Cells were lysed in 1% Nonidet P-40 and proteins were separated by SDS-PAGE and subsequently transferred to PVDF membranes (GE Healthcare). UL49.5 proteins were separated using 16.5%-tricine PAGE. The blots were incubated with the indicated antibodies, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (DAKO and Jackson ImmunoResearch Laboratories). Bound HRP-labeled antibodies were visualized using ECL Plus (GE Healthcare). One representative experiment out of two independent experiments is shown.

Transient expression MJS and LMH cells were transiently transfected using the Amaxa Nucleofector II device according to the manufacturer's instructions. For MJS, program T-020 and solution L were used and for LMH, program T-020 and solution T. Two days post transfection cells were analyzed for MHC I expression.

Viruses and viral infections CRFK cells were infected with FeHV-1 (strain B927) at an m.o.i. of 10 in serum-free DMEM. After one hour of virus adsorption, DMEM containing 10% FBS was added to make the final FBS concentration 5%. After 5 or 12 h cells were harvested. To restrict viral gene expression to immediate early (IE) and early (E) genes, cells were treated with 300 µg/ml phosphonoacetic acid (PAA: Sigma) for 2 h before addition of the virus and during infection. The Dutch BoHV-1.1 field strain Lam was used to infect MRC-5 cells at an m.o.i. of 20. After 2 h of virus adsorption, the inoculum was removed and cells were incubated in the presence of complete RPMI-1640 medium for 3 h.

For VZV infection we used the recombinant viruses VZV pOka expressing ORF66 N-terminally tagged with eGFP (Eisfeld et al., 2007) and VZV-GFP (Li et al., 2006) that contains eGFP inserted between ORF65 and ORF66. Trypsinized VZV-infected MRC-5 cells

were used to infect confluent monolayers of MRC-5 at a 4:1 ratio (uninfected cells to infected cells). This yielded a 100% eGFP-positive population that was harvested 72-88 h post infection. During infection MRC-5 cells were cultured in DMEM supplemented with 2% FBS.

In all experiments, mock-infected cells were treated under the same conditions as infected cells. One representative experiment out of two independent experiments is shown.

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% Nonidet P-40 or in 1% (w/v) digitonin, 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 150 mM NaCl, 5 mM iodoacetamide, and 1 mM AEBSF to preserve the interactions between TAP1, TAP2, and UL49.5. Hydrated C-8 ATP-agarose (Fluka/Sigma) was added to the post-nuclear supernatant and incubated at 4°C o/n. 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 beads were eluted with 500 mM EDTA and SDS-PAGE sample buffer was added to both the supernatant and the pellet. The samples were separated using SDS-PAGE and analyzed by Western blotting. One representative experiment out of two independent experiments is shown.

Results

TAP inhibition by herpesviruses infecting ruminants

The UL49.5 proteins encoded by BoHV-5, BuHV-1, and CvHV-1 are remarkably homologous to the BoHV-1 UL49.5 (Fig. 1). However, despite the fact that quite some heterogeneity occurs amongst these proteins, it remains to be tested whether these proteins inhibit TAP function, like BoHV-1 UL49.5. To evaluate the capacity to inhibit TAP, these UL49.5 proteins were expressed in bovine cells (MDBK) and human (MJS) cells. Expression of the UL49.5 homologs was verified using an antibody specific for BoHV-1 UL49.5, this antibody was capable of detecting the UL49.5 proteins of BoHV-1 and CvHV-1 in MDBK and MJS cells (Fig. 2A). BuHV-1 UL49.5 could not be detected, possibly due to differences within the C-terminal region against which the antibody was raised. In the retroviral vector used to establish the cell lines, the UL49.5 homologs were placed upstream of an internal ribosomal entry site (IRES) that is followed by an GFP gene. Consequently, the two proteins are encoded by one transcript and, therefore, expression of GFP reflects expression of the UL49.5 protein. Detection of GFP in cells transduced with the BuHV-1 UL49.5-IRES GFP retrovirus confirmed expression of the corresponding transcript (Fig. 2A).

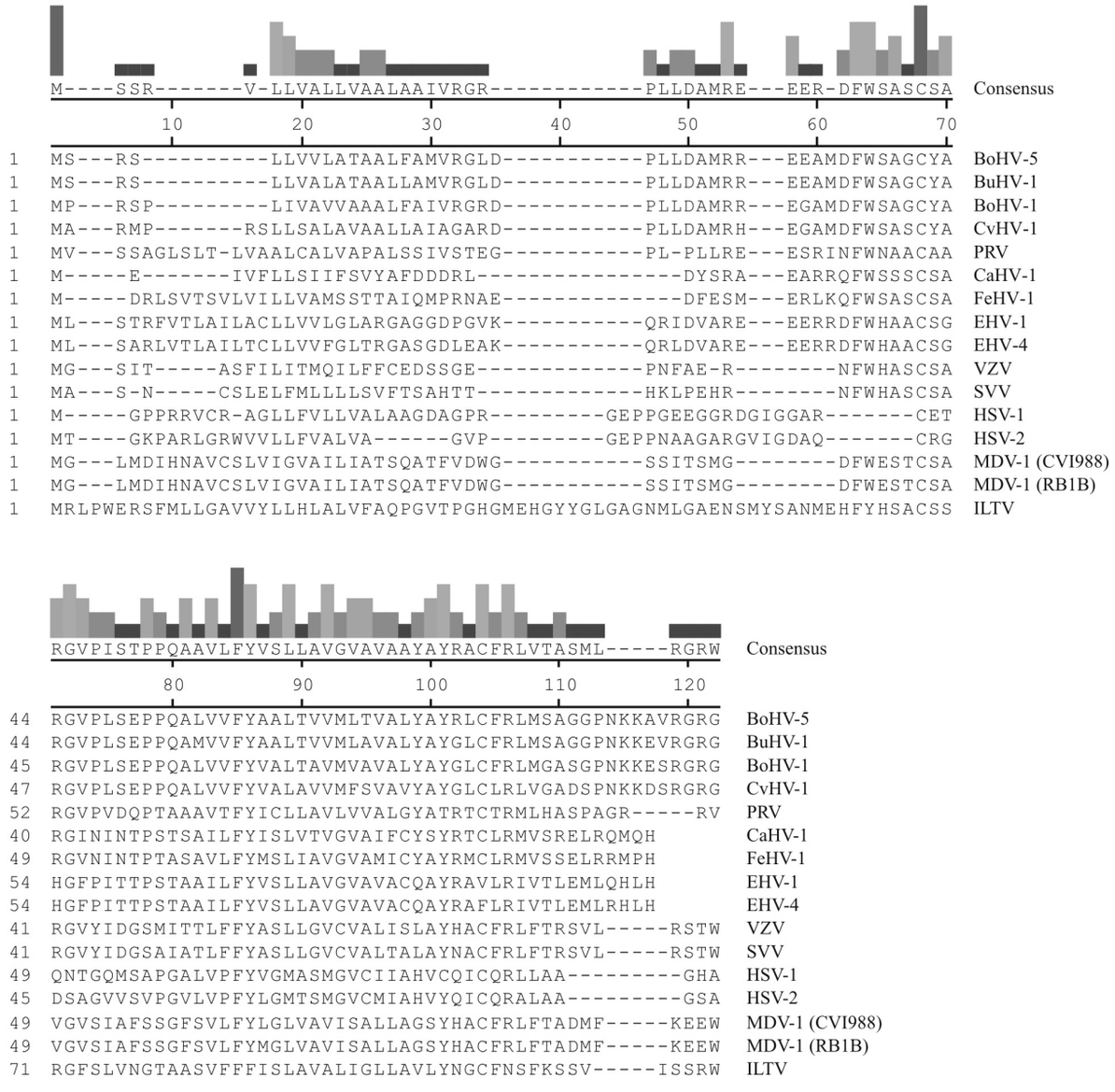
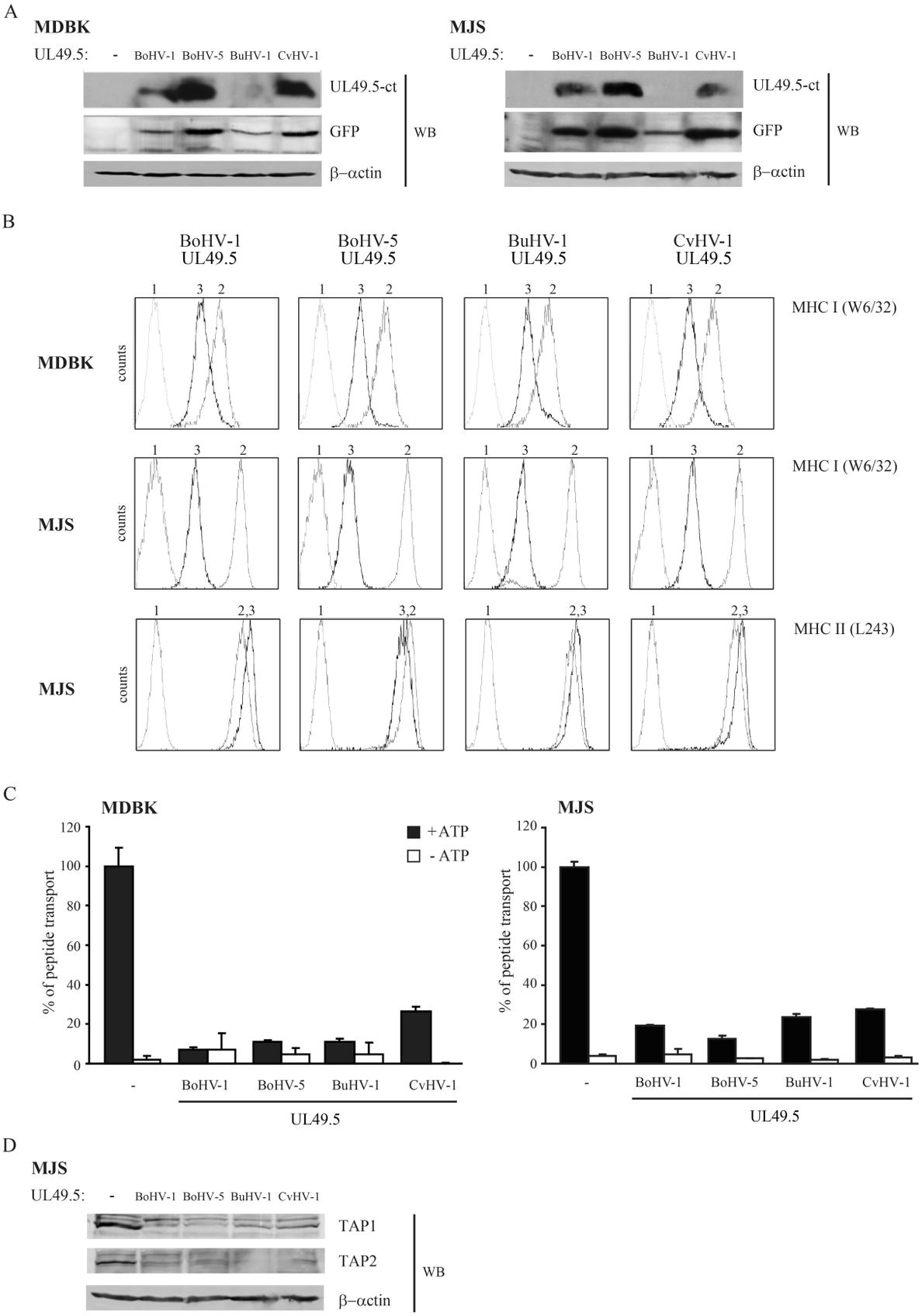


Fig. 1, alignment of the amino acid sequences of a selection of alphaherpesvirus UL49.5 proteins. The amino acid sequence alignment of UL49.5 homologs was performed using the ClustalV of the MegAlign software from DNASTAR. BoHV-5 UL49.5: bovine herpesvirus 5 (accession number NP_954898); BuHV-1 UL49.5: bubaline herpesvirus 1 (F.A.M. Rijsewijk personal communication); BoHV-1 UL49.5: bovine herpesvirus 1 (NP_045309); CvHV-1 UL49.5: ceprid herpesvirus 1 (F.A.M. Rijsewijk personal communication); PRV/SuHV-1 UL49.5: pseudorabies virus (YP_068325); CaHV-1 UL49.5: canid herpesvirus 1 (patent: EPO910406); FeHV-1 UL49.5: felid herpesvirus 1 (YP_003331529); EHV-1 UL49.5: equid herpesvirus 1 (YP_053055); EHV-4 UL49.5: equid herpesvirus 4 (NP_045227); VZV/HHV3 UL49.5: varicella-zoster virus (YP_068406); SVV UL49.5: simian varicella virus (NP_077423); HSV-1 UL49.5: herpes simplex virus 1 (NP_044652); HSV-2 UL49.5: herpes simplex virus 2 (NP_044520); MDV-1/GaHV-2 UL49.5: Marek's disease virus 1 strain CVI988 (NP-057812); MDV-1 strain RB1B (YP_001033979.1) ILTV/GaHV-1 UL49.5: infectious laryngotracheitis virus (YP_182341). The bars at the top of figure are proportional in height to the degree of homology of amino acid conservation among the different viral proteins.


A
MDBK
UL49.5: - BoHV-1 BoHV-5 BuHV-1 CvHV-1
UL49.5-ct
GFP
β–αctin
WB
MJS
UL49.5: - BoHV-1 BoHV-5 BuHV-1 CvHV-1
UL49.5-ct
GFP
β–αctin
WB
B
BoHV-1 UL49.5
BoHV-5 UL49.5
BuHV-1 UL49.5
CvHV-1 UL49.5
MDBK
MJS
MJS
counts
MHC I (W6/32)
MHC I (W6/32)
MHC II (L243)
C
MDBK
MJS
+ATP
- ATP
% of peptide transport
120
100
80
60
40
20
0
-
BoHV-1
BoHV-5
BuHV-1
CvHV-1
UL49.5
D
MJS
UL49.5: - BoHV-1 BoHV-5 BuHV-1 CvHV-1
TAP1
TAP2
β–αctin
WB

Analysis of MHC I cell surface expression revealed that the BoHV-5, BuHV-1, and CvHV-1 UL49.5 proteins caused a strong downregulation on both MDBK and MJS that was comparable to the BoHV-1 UL49.5-induced reduction (Fig. 2B). This downregulation was specific for MHC I, as MHC II expression was unaffected by all UL49.5 homologs. Next, TAP function was assessed in BoHV-5, BuHV-1, and CvHV-1 UL49.5-expressing MDBK and MJS cells. Transport of the reference peptide was reduced by 70 to 90% in both bovine and human cells, indicating that the observed reduction of MHC I results from TAP inhibition (Fig. 2C).

Previously, BoHV-1 UL49.5 was found to induce degradation of human and bovine TAP (Koppers-Lalic et al., 2005; Koppers-Lalic et al., 2008). To evaluate whether BoHV-5, BuHV-1, and CvHV-1 UL49.5 posses a similar capacity, TAP1 and TAP2 levels were analyzed in MJS cells expressing these proteins (Fig. 2D). TAP levels were strongly reduced in the presence of all three UL49.5 homologs tested, suggesting that UL49.5-induced degradation of the TAP complex is conserved for UL49.5 proteins of herpesviruses infecting ruminants.

FeHV-1 UL49.5 inhibits TAP-mediated peptide transport

FeHV-1 has recently been found to reduce MHC I levels on infected cells (Montagnaro et al., 2009). To assess if this downregulation resulted from the inhibition of peptide transport by TAP, TAP activity was analyzed in feline CRFK cells infected with FeHV-1 for 5 and 12 h. To evaluate the contribution of FeHV-1 late proteins to TAP inhibition, the experiment was also performed in the presence of PAA, which inhibits late viral protein synthesis. FeHV-1 infection of the cells was confirmed using an antibody recognizing several FeHV-1 glycoproteins (Fig. 3A, left panel). As expected, MHC I levels were downregulated on FeHV-1-infected cells (Fig. 3A, right panel) and, additionally, TAP function was strongly reduced already at 5 h.p.i. (Fig. 3B). TAP was still inhibited after 12 h, irrespective of the presence of PAA, implying that an immediate early or early gene is responsible for this effect, possibly UL49.5.

Fig. 2 (overleaf), the UL49.5 proteins of BoHV-5, BuHV-1, and CvHV-1 inhibit peptide transport and mediate the degradation of TAP1 and TAP2. (A) Lysates derived from control and UL49.5-expressing MDBK and MJS cells were stained for GFP and UL49.5 by SDS-PAGE and Western blotting (WB) using specific antibodies. The β-actin signal was used as a loading control. (B) Surface expression of MHC I (MDBK and MJS) and MHC II (MJS) molecules was assessed by flow cytometry on untransduced cells (graph 2) and on cells expressing the UL49.5 homologs of BoHV-1, BoHV-5, BuHV-1, and CvHV-1 (graph 3) using the indicated antibodies. Graph 1, background staining in the presence of secondary antibody only. (C) Transport activity of TAP was analyzed in MDBK and MJS expressing the UL49.5 homologs. Peptide transport was evaluated in the presence of ATP (black) or EDTA (white bars). (D) Steady state levels of TAP1 and TAP2 in MJS cells were determined using specific antibodies. The β-actin signal was used as a loading control.

To study if FeHV-1 UL49.5 plays a role in the observed TAP inhibition, this homolog was expressed in CRFK cells and in MJS cells. FeHV-1 UL49.5 induced a strong downregulation of MHC I in CRFK and a slightly weaker downregulation in MJS cells. The inhibition of MHC I expression was specific, as MHC II expression was unaffected (Fig. 4A). Accordingly, TAP function was inhibited in both FeHV-1 UL49.5-expressing cell lines (Fig. 4B). In the absence of FeHV-1 UL49.5-specific antibodies, expression of UL49.5 gene was inferred from the presence of GFP that is encoded by the same transcript (Fig. 4C). The antibody used to detect human TAP1 cross-reacted with feline TAP1 (Fig. 4C). Therefore, this antibody could be used to investigate if FeHV-1 UL49.5 shares the capacity of UL49.5 of ruminant varicelloviruses to degrade TAP. However, in the presence of FeHV-1 UL49.5 no degradation of TAP was observed (Fig. 4C).

Preventing ATP-binding to TAP is a strategy often exploited by TAP inhibitors, including EHV-1 UL49.5 (Koppers-Lalic et al., 2008). To investigate if FeHV-1 UL49.5 uses this strategy, the ATP-binding capacity of TAP was assessed in lysates from untransduced

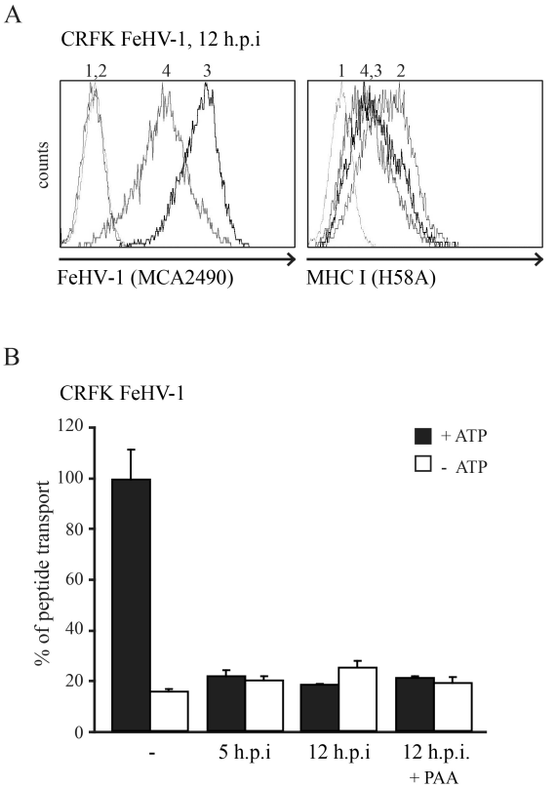


Fig. 3, TAP is inhibited in FeHV-1-infected cells by an immediate early or early protein. (A) CRFK cells were mock-infected (graph 2) or infected with FeHV-1 for 12 hr in the absence (graph 3) or presence (graph 4) of PAA. The expression of FeHV-1 glycoproteins and cell surface expression of MHC I were assessed by flow cytometry using specific antibodies. Graph 1, secondary antibody only. (B) TAP activity was determined in mock- and FeHV-1-infected cells at 5 and 12 h.p.i. (in the absence and presence of PAA). The assay was performed in the presence of ATP (black) or EDTA (white bars).

cells and cells stably expressing FeHV-1 or EHV-1 UL49.5. In control CRFK cells, but also in FeHV-1 UL49.5-expressing CRFK cells, TAP1 was detected in the pellet fraction, showing that TAP1 was bound to ATP (Fig. 4D; lane 1-4). Correspondingly, in MJS cells expressing FeHV-1 UL49.5, TAP1 was found in the pellet fractions (Fig. 4D; lanes 5-8). In contrast, in the presence of EHV-1 UL49.5, very little TAP1 was found in the pellet fraction, confirming

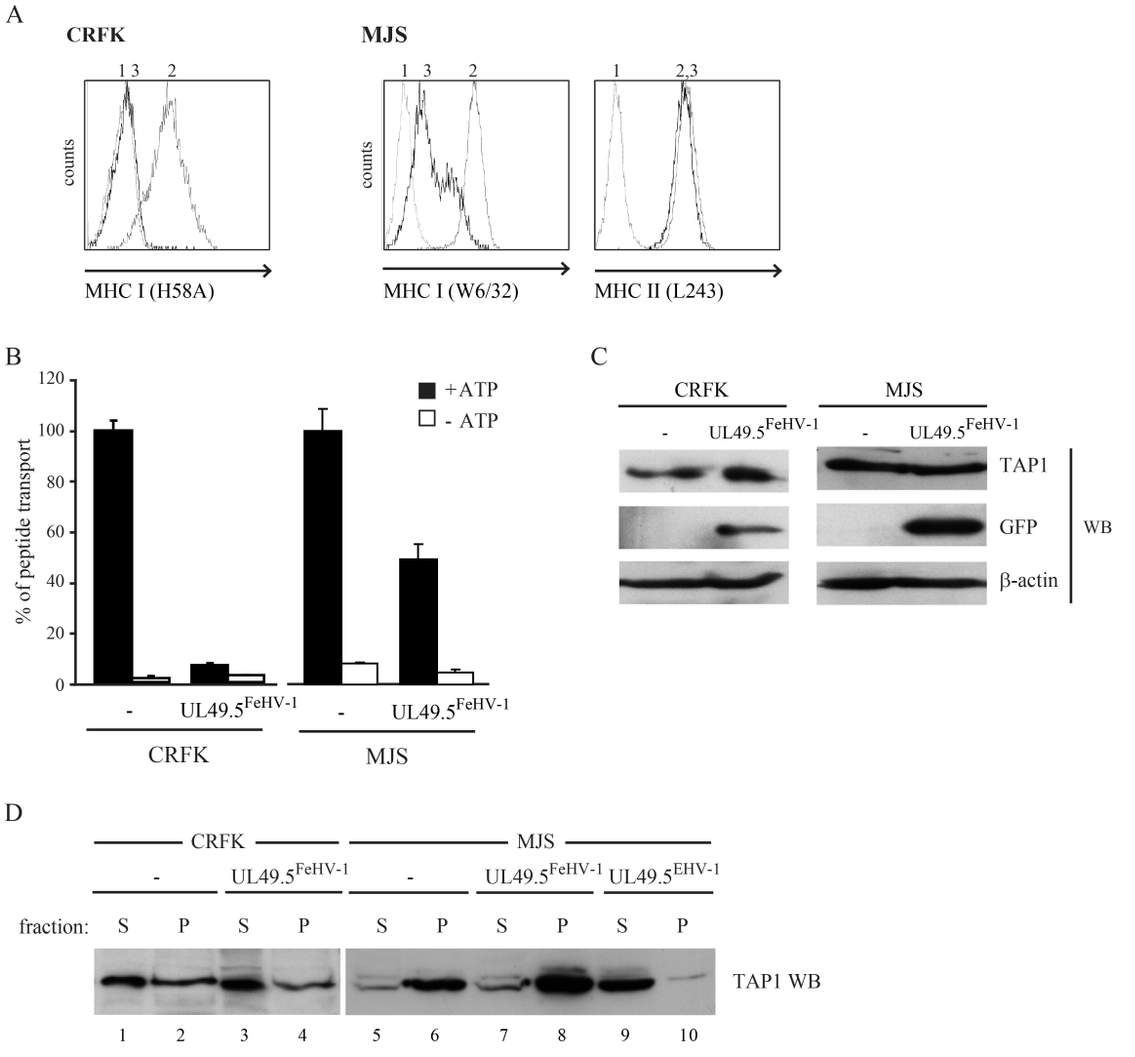


Fig. 4, FeHV-1 UL49.5 downregulates MHCI and strongly inhibits TAP-mediated peptide transport. (A) Surface expression of MHC I (CRFK and MJS) and MHC II (MJS) molecules was assessed by flow cytometry on untransduced cells (graph 2) and on cells expressing FeHV-1 UL49.5 (graph 3) using the indicated antibodies. Graph 1, background staining in the presence of secondary antibody only. (B) Transport activity of TAP was analyzed in CRFK and MJS expressing the FeHV-1 UL49.5. Peptide transport was evaluated in the presence of ATP (black) or EDTA (white bars). (C) The steady state levels of TAP1 and GFP in FeHV-1 UL49.5-expressing cells were determined by SDS-PAGE and Western blotting (WB) using specific antibodies. The β-actin signal was used as a loading control. (D) Western blot analysis of ATP-bound (p) or unbound (s) TAP molecules found in CRFK or MJS cells expressing FeHV-1 UL49.5 or EHV-1 UL49.5 (control).

interference with ATP-binding to TAP by EHV-1 UL49.5 (Fig. 3D; compare lane 9 with 10). These results demonstrate that ATP-binding to TAP is not affected by FeHV-1 UL49.5.

The effect of SVV UL49.5 on TAP-mediated peptide transport

The protein sequences of VZV and SVV UL49.5 display a high degree of homology (Fig. 1). Previously, we have shown that VZV UL49.5 is not interfering with TAP function (Koppers-Lalic et al., 2008). To study whether the UL49.5 protein of SVV blocks TAP, the viral protein was expressed in a rhesus macaque-derived cell line (LLC-MK2) and in MJS cells. On LLC-MK2 cells expressing SVV UL49.5, MHC I expression was slightly reduced (Fig. 5A). This was accompanied by a 15% reduction of peptide transport in those cells (Fig. 5B). SVV UL49.5-induced downregulation of MHC I expression could not be detected on MJS (Fig. 5A). In accordance, TAP inhibition was undetectable in SVV UL49.5-expressing MJS cells (Fig. 5B). GFP was expressed in both cell lines, indicating proper expression of the SVV

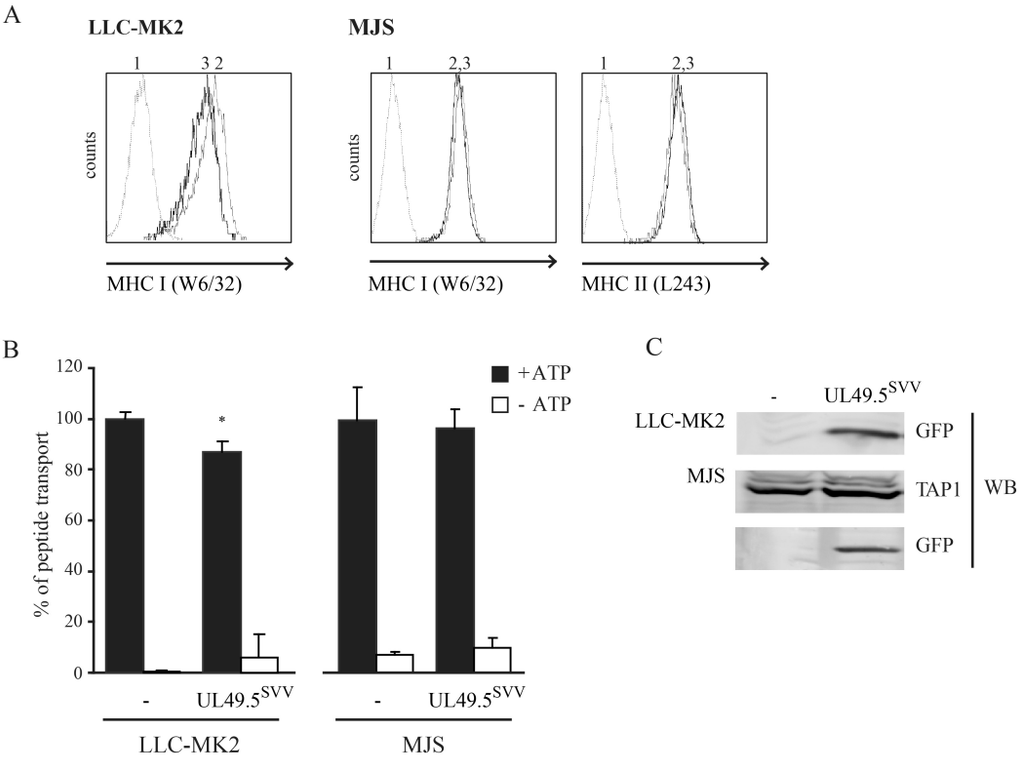


Fig. 5, SVV-encoded UL49.5 does not affect TAP function. Surface expression of MHC I (LLC-MK2 and MJS) and MHC II (MJS) molecules was assessed by flow cytometry on untransduced cells (graph 2) and SVV UL49.5-expressing cells (graph 3) using the indicated antibodies. Graph 1, secondary antibody only. (B) Transport activity of TAP was analyzed in LLC-MK_2 and MJS expressing SVV UL49.5. Peptide transport was evaluated in the presence of ATP (black) or EDTA (white bars). *A difference at $p<0.05$ was considered significant. (C) The steady state levels of TAP1 and GFP in SVV UL49.5-expressing cells were determined by SDS-PAGE and Western blotting (WB) using specific antibodies.

UL49.5-encoding transcript (Fig. 5C). Protein steady state levels of TAP were not affected by SVV UL49.5. All together, these data indicate that SVV UL49.5 has a minimal effect on TAP function in monkey cells.

VZV infection does not result in TAP inhibition

Despite the fact that VZV UL49.5 can be found in association with the TAP complex, TAP inhibition has not been observed. Possibly, VZV UL49.5 requires another VZV gene product to accomplish TAP inhibition. To investigate this possibility, TAP function was assessed in VZV-infected MRC-5 fibroblasts. To this end, we used a recombinant virus in which eGFP was placed between ORF65 and ORF66 (VZV-GFP) (Li et al., 2006). In addition, a VZV recombinant was used in which eGFP was fused to the N-terminus of the ORF66 protein

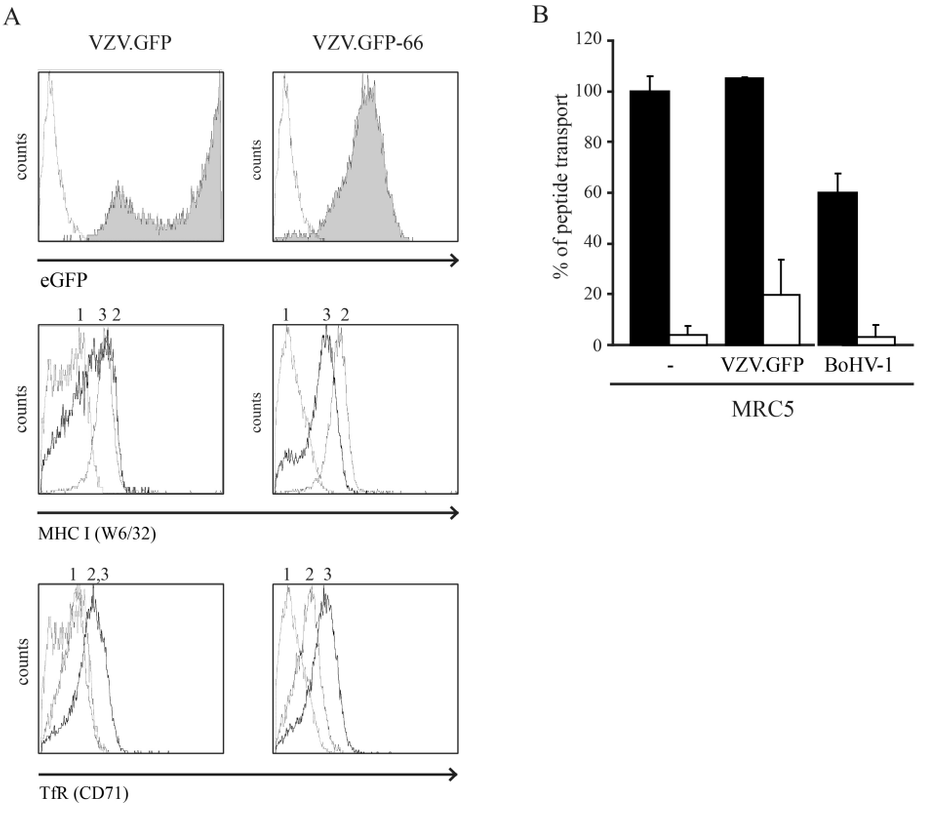


Fig. 6, VZV-infection does not result in reduced TAP function. (A) MRC5 cells were either mock-infected (graph 2), or infected with VZV-GFP or VZV.GFP-66 (graph 3) for 88 hr and analyzed by flow cytometry to determine expression of eGFP and the cell surface expression of MHC I molecules and transferrin receptor (TfR) using the indicated antibodies. Graph 1, secondary antibody only. (B) MRC5 cells were mock- and VZV-GFP-infected for 72 hr, after which peptide transport was assessed. As a control for TAP inhibition cells were infected with BoHV-1 for 3 hr at a m.o.i. of 20. The transport activity of TAP was analyzed in the presence of ATP (black) or EDTA (white bars).

(VZV.GFP-66) (Eisfeld et al., 2007). The use of eGFP-expressing viruses allowed us to determine the efficiency of infection by flow cytometry. MRC-5 cells were infected with VZV-GFP and VZV.GFP-66 at a ratio of 4:1 (uninfected to VZV-infected cells) for 88 h, after which the efficiency of infection was found to be nearly 100% (Fig. 6A; eGFP). Next, we compared the MHC I and transferrin receptor cell surface levels of mock- and VZV-infected MRC-5 cells. A moderate downregulation of MHC I was observed on VZV-infected cells, whereas transferrin receptor levels were upregulated (Fig. 6A). These results are in line with previous observations (Cohen, 1998; Eisfeld et al., 2007).

Next, peptide transport was assessed in MRC-5 cells that were infected with VZV.GFP. As a positive control, MRC-5 cells were infected with BoHV-1. Infection was verified by eGFP expression (VZV) or by cell surface staining using a BoHV-1 gB-specific antibody (data not shown). TAP function was not affected in VZV.GFP-infected MRC-5 cells (Fig. 6B). In contrast, BoHV-1 infection resulted in a 40% reduction of peptide transport (Fig. 6B), which is in accordance with previous observations (Koppers-Lalic et al., 2003). The experiment was repeated and confirmed in MRC-5 cells infected with VZV.GFP-66 (data not shown). Thus, the downregulation of MHC I cell surface expression detected on VZV-infected MRC-5 cells did not result from a block of peptide transport by TAP.

UL49.5 of MDV-1 is not responsible for MHC I downregulation by this virus

MDV-1 is known to reduce MHC I cell surface expression levels on infected cells (Gimeno et al., 2001; Hunt et al., 2001). To assess if MDV-1 UL49.5 is responsible for this phenomenon, we transiently expressed chicken hepatoma cells (LMH) and MJS cells with plasmids coding for the UL49.5 homologs of the MDV-1 strains RB1B and CVI988. BoHV-1 UL49.5 was used as a control. At 48 h post transfection, cell surface expression of MHC I and, for MJS, MHC II were analyzed. None of the UL49.5 proteins was able to reduce MHC I cell surface expression on LMH cells (Fig. 7). In contrast, on MJS cells, BoHV-1 UL49.5 induced a reduction of MHC I that was not observed for either MDV-1 homolog. The reduction by BoHV-1 UL49.5 was specific, as cell surface expression of MHC II was unaffected.

In addition, baculoviruses were used to express BoHV-1 and MDV-1 CVI988 UL49.5 in MJS cells. While BoHV-1 UL49.5-expressing cells displayed evident MHC I downregulation, a reduction of MHC I cell surface levels could not be detected in cells with MDV-1 UL49.5 (Supplementary fig. 1A). Also, TAP function was not affected detectably by MDV-1 UL49.5, whereas BoHV-1 UL49.5 reduced peptide transport by 40% in the same assay (Supplementary Fig. 1B). All together, these experiments indicate that MDV-1 UL49.5 does not affect the transport of peptides via TAP.

TAP function is not affected by ILTV UL49.5

To study if ILTV UL49.5 interferes with peptide transport, the gene was expressed in LMH or MJS cells by retroviral transduction. The analysis of MHC I cell surface levels on both control and UL49.5-expressing cells did not reveal an ILTV UL49.5-induced downregulation (Fig. 8A). In accordance, reduced TAP function was not detected in ILTV UL49.5-expressing cells (Fig. 8B). Proper expression of ILTV UL49.5 was demonstrated using a specific antibody (Fig. 8C). All together, these results indicate that ILTV UL49.5 does not function as a TAP inhibitor.

Discussion

Herpesviruses employ many strategies to avoid elimination by the host immune system (Hansen and Bouvier, 2009). A strategy often exploited by these viruses is the inhibition of TAP-mediated peptide transport, illustrating the key role of this process for immunity against these viruses. UL49.5 proteins of varicelloviruses have been identified as a new

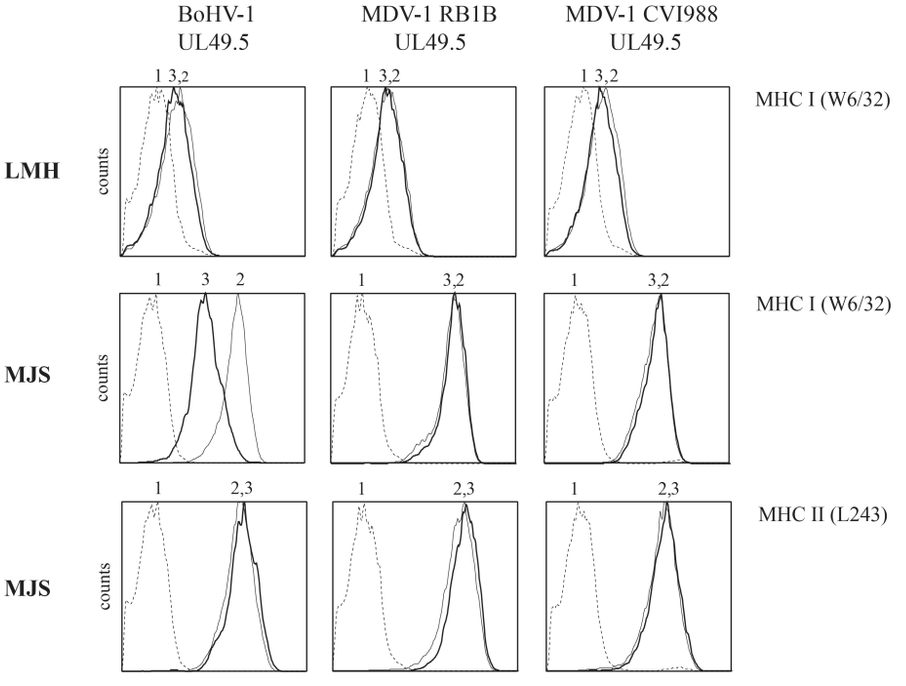


Fig. 7, the UL49.5 proteins of MDV-1 RB1B and MDV-1 CVI988 do not affect MHC I surface expression. BoHV-1, MDV-1 RB1B and MDV-1 CVI988 UL49.5 were transiently expressed in LMH and MJS cells. Cell surface expression levels of MHC I and MHC II molecules was assessed by flow cytometry on cells transfected with a control plasmid (graph 2) or with the UL49.5-expressing plasmids (graph 3) using specific antibodies. Graph 1, secondary antibody only.

class of TAP inhibitors that now includes the UL49.5 homologs encoded by BoHV-1, BoHV-5, BuHV-1, CvHV-1, PRV, EHV-1, EHV-4 and FeHV-1 (Koppers-Lalic et al., 2008). SVV UL49.5 was found to have a minor effect on TAP-mediated peptide transport, whereas VZV UL49.5 did not block TAP at all, despite its observed interaction with the complex (Koppers-Lalic et al., 2008). No TAP inhibition was found after infection with VZV, suggesting that no other VZV proteins contribute to TAP inhibition by VZV UL49.5 or exhibit this capacity themselves. Expression of the UL49.5 proteins encoded by alphaherpesviruses belonging to the genera *Mardivirus* (MDV-1) and *Iltovirus* (ILTV) did not result in reduced MHC I expression or inhibition of TAP function, suggesting that TAP inhibition is a unique property of UL49.5 proteins encoded by varicelloviruses.

Within the *Varicellovirus* genus, clusters of closely related herpesviruses can be identified (Fig. 9). Varicelloviruses infecting ruminants, including BoHV-1, BoHV-5, BuHV-1, and CvHV-1, evolved in close proximity (Thiry et al., 2006) and share a high

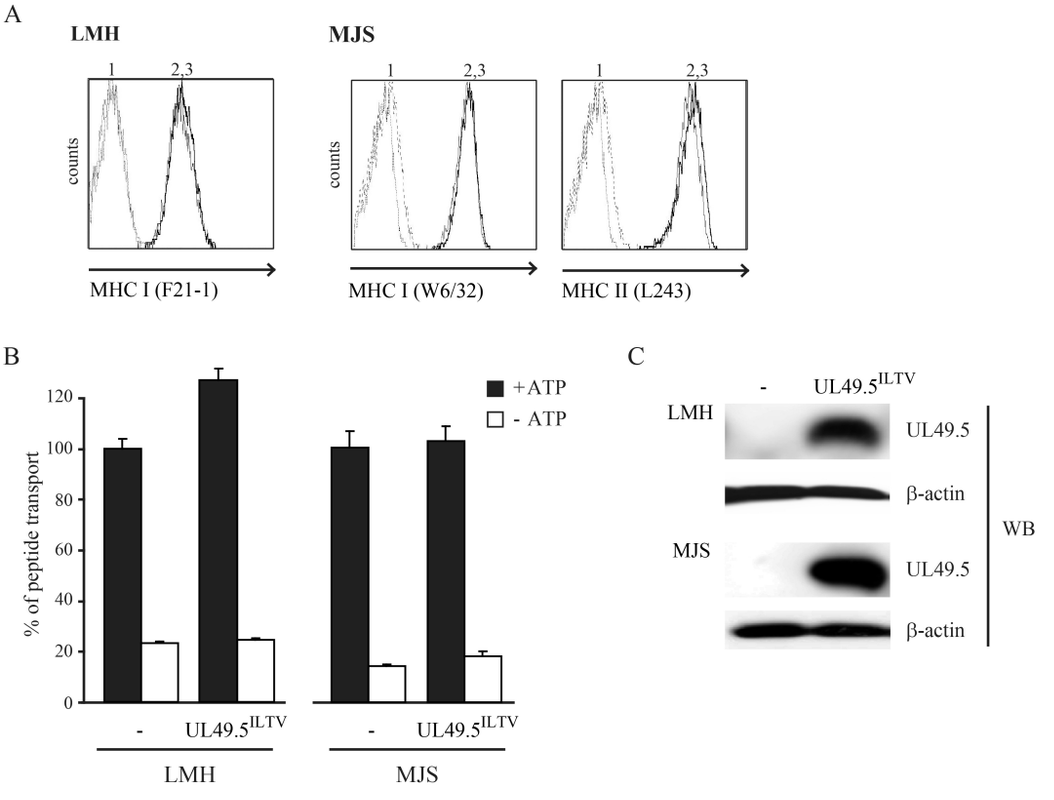


Fig. 8, ILTV-encoded UL49.5 does not affect TAP function. (A) Surface expression of MHC I (LMH and MJS) and MHC II (MJS) molecules was assessed by flow cytometry on untransduced cells (graph 2) and ILTV UL49.5-expressing cells (graph 3) using the indicated antibodies. Graph 1, secondary antibody only. (B) Transport activity of TAP was analyzed in LMH and MJS expressing ILTV UL49.5. Peptide transport was evaluated in the presence of ATP (black) or EDTA (white bars). (C) The steady state levels of UL49.5 (MJS and LMH) and TAP1 (MJS) were determined using SDS-PAGE and Western blotting (WB) using the indicated antibodies. The β-actin signal was used as a loading control.

degree of sequence homology (Fig. 1). Here we show that the UL49.5 proteins of these viruses affect TAP to the same extent and, most probably, via the same mechanism. BoHV-1 UL49.5 renders the transporter in a translocation incompetent state and induces the degradation of both TAP subunits. Correspondingly, the UL49.5 proteins of BoHV-5, BuHV-1, and CvHV-1 also induce the degradation of TAP. Thus, the strategy to inhibit TAP seems conserved in the cluster of varicelloviruses of ruminants.

As displayed in figure 1, regions of homology between all UL49.5 proteins are limited and scattered throughout the sequence. This makes it difficult to locate domains or individual amino acid residues that might be responsible for TAP inhibition. So far, the only exception is the UL49.5-mediated degradation of TAP, that appears to be related to defined amino acid residues within the cytoplasmic tail of the protein (Koppers-Lalic et al., 2005).

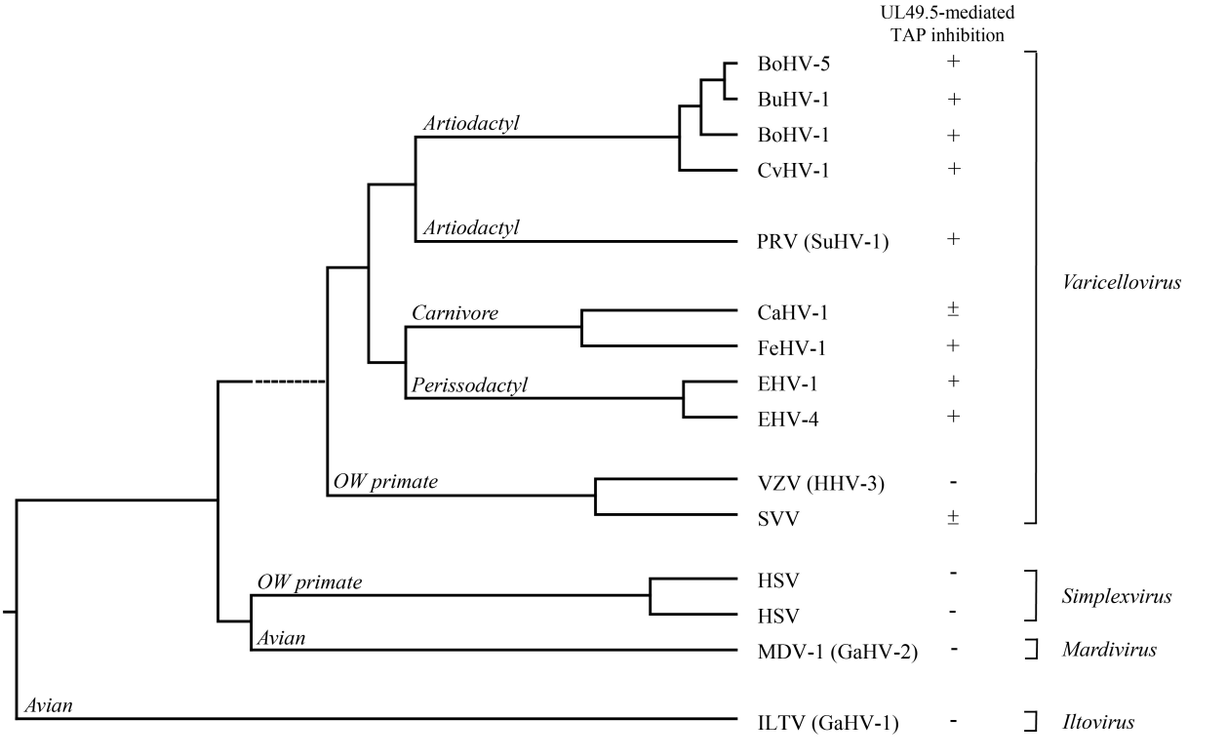


Fig. 9, phylogenetic tree of a selection of alphaherpesviruses based on gB sequences. The tree has been constructed using the Clustal W (slow/accurate, Gonnet) method of the alignment program MegAlignTM 500 of the sequence analysis software of DNASTAR Inc. BoHV-5 gB: bovine herpesvirus 5 (AAD46112.2); BuHV-1 gB: Bubaline herpesvirus 1 (AAL88794.1); BoHV-1 gB: bovine herpesvirus 1 (P12640.1); CvHV-1 gB: ceprid herpesvirus 1 (AAD46115.2); PRV/SuHV-1 gB: pseudorabies virus (ACT78489.1); CaHV-1 gB: canid herpesvirus 1 (AAK51052.1); FeHV-1 gB: felid herpesvirus 1 (YP_003331552.2); EHV-1 gB: equid herpesvirus 1 (YP_053078.1); EHV-4 gB: equid herpesvirus 4 (P17472.1); VZV/HHV3 gB: varicella-zoster virus (AAP32845.1); SVV gB: simian varicella virus (NC_002686.2); HSV-1 gB: herpes simplex virus 1 (ABM66851.1); HSV-2 gB: herpes simplex virus 2 (AAA60540.1); MDV-1/GaHV-2 gB: Marek's disease virus 1 (BAA02866); ILTV/GaHV-1 gB: infectious laryngotracheitis virus (CAA39573.1). OW: old world.

Preliminary data suggest that the conserved RGRG sequence at the C-terminus of BoHV-1, BoHV-5, BuHV-1, and CvHV-1 UL49.5 is involved in the degradation process (Verweij, Wiertz et al., unpublished observations).

CaHV-1 and FeHV-1, both infecting carnivores, are genetically and serologically related (Gaskell and Willoughby, 1999). As depicted in Fig. 9, these viruses are thought to have a common ancestor. Earlier, we showed that CaHV-1 UL49.5 has a minor effect on TAP function in canine cells (Koppers-Lalic et al., 2008). Surprisingly, FeHV-1 UL49.5 was identified as a potent inhibitor of feline TAP and, to a lesser extent, human TAP. FeHV-1 and CaHV-1 are related to herpesviruses infecting odd-toed ungulates or *Perrisodactyla* (EHV-1 and EHV-4) (Fig. 9). The UL49.5 homologs encoded by these viruses were previously shown to strongly inhibit TAP (Koppers-Lalic et al., 2008). Hence, in this cluster of related varicelloviruses, CaHV-1 appears to be the odd one out, coding for a UL49.5 protein that only moderately affects TAP. This UL49.5 homolog might have lost the capacity to strongly inhibit TAP.

Previously, it has been shown that ATP-binding to TAP was prevented by EHV-1 UL49.5 in an indirect manner. An EHV-1 UL49.5Δtail recombinant was still able to block ATP binding (Koppers-Lalic et al., 2008), indicating that the protein is not directly obstructing the ATP binding site of TAP. Yet, the protein arrests the transporter in a translocation-incompetent state, incompatible with ATP-binding. In the presence of FeHV-1 UL49.5, TAP was still capable of binding ATP. Thus, the mechanism through which FeHV-1 UL49.5 inhibits TAP differs from that utilized by the related EHV-1 and EHV-4 UL49.5 proteins. Similar to FeHV-1 UL49.5, BoHV-1 and PRV UL49.5 do not interfere with ATP-binding to TAP (Koppers-Lalic et al., 2008). Presumably, differences in the capacity to inhibit ATP-binding result from slightly different conformational changes in TAP that are induced by these proteins.

VZV and SVV belong to the evolutionary cluster of varicelloviruses that infect Old World primates (Fig. 9). Despite an observed interaction between TAP and VZV UL49.5, this protein does not inhibit TAP (Koppers-Lalic et al., 2008). SVV UL49.5 moderately affect peptide transport in rhesus macaque cells. Possibly, the UL49.5 proteins of these viruses have lost the capacity to (strongly) interfere with TAP function or, alternatively, have never (fully) acquired this property. This somewhat unique situation amongst varicelloviruses might be related to the evolutionary position of these viruses, which seem to have separated from the other varicelloviruses at a relatively early stage (Fig. 9).

Cells infected with VZV display reduced expression of MHC I at the cell surface (Eisfeld et al., 2007; Abendroth et al., 2001; Cohen, 1998). The VZV ORF66-encoded serine-threonine protein kinase has been shown to contribute to the observed downregulation (Abendroth et al., 2001; Eisfeld et al., 2007), but additional proteins appear to be involved (Eisfeld et al., 2007). We did not observe reduced TAP function in VZV-infected cells,

indicating that VZV does not code for a TAP inhibitor. This finding is rather surprising, as most of the alphaherpesviruses encode a TAP inhibitor (Hansen and Bouvier, 2009).

All MDV serotypes, including MDV-1, MDV-2, and meleagrid herpesvirus 1 or turkey herpesvirus (HVT) reduce MHC I expression on infected cells (Hunt et al., 2001). Highly virulent strains of MDV-1, such as RB1B, cause a stronger MHC I downregulation than less virulent or vaccine strains, like CVI988 (Gimeno et al., 2001). Nevertheless, downregulation of MHC I was not detectable in chicken and human cells expressing the UL49.5 proteins of RB1B and CVI988. In addition, MDV-1 CVI988 UL49.5 protein did not detectably affect TAP-mediated peptide transport in human cells. Thus, MHC I downregulation observed on MDV-1-infected chicken cells is not mediated by UL49.5 alone. The action of one or more other MDV-1 proteins might be required for TAP-inhibition by MDV-1 UL49.5. However, such co-operation has not been observed for any of the other TAP-inhibiting UL49.5 proteins.

The UL49.5 protein of ILTV did not reduce TAP-mediated peptide transport. ILTV and the recently identified psittacid herpesvirus 1 (PsHV-1) are the only known iltoviruses (McGeoch et al., 2006). These viruses have been classified as members of the *Alphaherpesvirinae* (Davison et al., 2009), but are distantly related to all other members (Fig. 9). Similarly, MDV-1 branched away from the varicelloviruses and simplexviruses relatively early in evolution (Fig. 9). Given their distant relateness, ILTV and MDV-1 probably evolved genes other than UL49.5 to circumvent MHC I-mediated antigen presentation.

In conclusion, TAP-inhibiting UL49.5 proteins have been identified uniquely in varicelloviruses, now including the proteins of BoHV-1, BoHV-5, BuHV-1, CvHV-1, PRV, EHV-1, EHV-4, and FeHV-1. These UL49.5 homologs appear to have acquired a function in immune evasion, in addition to their common function as a molecular chaperone for gM that seems to be conserved among all herpesviruses. Possibly, this dual function relies on a hitherto unidentified structural similarity between TAP and gM. The UL49.5 homologs of the simplexviruses HSV-1 and HSV-2, the mardivirus MDV-1, the iltovirus ILTV, the betaherpesvirus HCMV, and the gammaherpesvirus EHV have no effect on TAP function (Koppers-Lalic et al., 2005). However, within all three subfamilies other potent TAP inhibitors are identified (e.g. ICP47, US6, and BNLF2a), making TAP inhibition by herpesviruses a striking example of functional convergent evolution.

Acknowledgements

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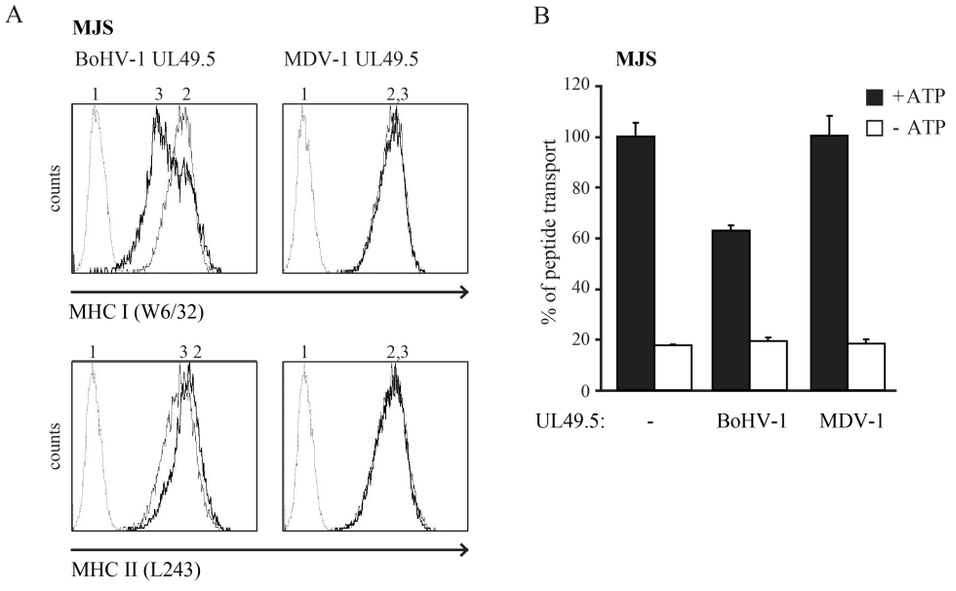
Supplementary materials and methods

UL49.5 constructs For the baculovirus vector the MDV-1 UL49.5 gene (strain CVI988) was isolated from purified viral MDV-1 DNA using specific primers (Table 1) and inserted into the pFastBac-Dual (pFBD, Invitrogen) expression vector in which the polyhedrin promoter was replaced by the HCMV immediate-early promoter/enhancer (described in Grabowska et al., 2009). pFBD and pFBD BoHV-1 UL49.5 (containing BoHV-1 UL49.5 that was subcloned from pLZRS UL49.5-IRES-GFP) were used as controls.

Recombinant viruses Recombinant baculoviruses were generated using the Bac-to-Bac system according to the manufacturer's instructions (Invitrogen). Baculoviruses were used to infect MJS cells that were seeded onto 6-well plates and cultured overnight. Before transduction cells were washed 3 times with D-PBS (phosphate-buffered saline containing Ca^{2+} and Mg^{2+}, Sigma), after which baculovirus inocula (in D-PBS) were added at an m.o.i. of 50. After incubation for 2 h at 27°C and one hour at 37°C, the cells were washed and cultured for 48 h in fresh complete medium containing 10 mM sodium butyrate (Sigma). Mock-infected cells were treated under the same conditions as infected cells.

Supplementary figure



Supplementary fig. 1, MDV-1 CVI988-encoded UL49.5 does not affect TAP function. BoHV-1 and MDV-1 UL49.5 were expressed in MJS cells using baculovirus expression vectors. (A) Cell surface expression levels of MHC I and MHC II molecules was assessed by flow cytometry on cells transduced with a control baculovirus (graph 2) or with the UL49.5-expressing baculoviruses (graph 3) using specific antibodies. Graph 1, secondary antibody only. (B) Transport activity of TAP was evaluated in the presence of ATP (black) or EDTA (white bars).

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