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

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Herpesvirus-encoded immune evasion molecules cross species barriers to inhibit mouse MHC I-restricted antigen presentation

Submitted

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Abstract

Herpesviruses escape elimination by cytotoxic T lymphocytes through specific interference with the antigen-presenting function of MHC I molecules. The Transporter associated with Antigen Processing (TAP) forms a bottleneck in the MHC I antigen presentation pathway. The fact that multiple viruses, especially herpesviruses, encode molecules blocking TAP function is a case in point. The action of these viral immuno evasins is usually potent and very specific, making these proteins valuable tools for studying the cell biology of antigen presentation, including alternative antigen processing pathways. Yet, no dedicated TAP inhibitor has been described for any of the mouse herpesviruses.

To permit the use of immuno evasins derived from non-mouse herpesviruses in mouse models, we assessed the cross-species activity of four TAP inhibitors and one tapasin inhibitor–in the context of three different mouse haplotypes, H-2^b, H-2^d, and H-2^k. Two of the four TAP inhibitors, the bovine herpesvirus 1-encoded UL49.5 protein and the human cytomegalovirus (HCMV) US6 protein, potently inhibited mouse TAP. ICP47 and BNLF2a, encoded by herpes simplex virus 1 and Epstein-Barr virus, respectively, failed to inhibit TAP in all mouse cells tested. Previous work, however, demonstrated that US6 did not cross the mouse species barrier. We now show that substitution of the cysteine residue at position 108 was responsible for this lack of activity. The HCMV-encoded tapasin inhibitor US3 efficiently downregulated H-2^d molecules on 3T3 cells, but not in other cell lines tested. Finally, we show that synthetic peptides comprising the functional domain of US6 can be exploited as a versatile TAP inhibitor. In conclusion, a complete overview is presented of the applicability of herpesvirus-encoded TAP and tapasin inhibitors in mouse cells of different genetic background.

Introduction

Major histocompatibility complex class I (MHC I)-mediated antigen presentation to T cell receptors on $CD8^+$ cells is essential for the establishment of a sustained immune response against intracellular pathogens. The antigenic peptides originate from proteasomal degradation of cytosolic proteins, including - in case of a viral infection - virus-encoded proteins. The transporter associated with antigen processing (TAP) translocates the resulting peptides into the endoplasmic reticulum (ER) for loading onto MHC I molecules. TAP occurs in a complex with MHC I heavy chains (HC), β2m, and the ER-resident chaperone molecules tapasin, calreticulin, and ERp57, together constituting the MHC I peptide-loading complex (PLC). While calreticulin and ERp57 stabilize peptide-receptive MHC I complexes, tapasin links the MHC I - β_2m complexes and ERp57 to TAP and facilitates the acquisition of high affinity peptides. This arrangement ascertains quality-controlled peptide-loading of newly synthesized MHC I molecules and, consequently, egress of properly assembled MHC I complexes from the ER for display at the cell surface (Lehner and Trowsdale, 1998; Wright et al., 2004; Cresswell et al., 2005; Peaper and Cresswell, 2008; Rizvi and Raghavan, 2009).

Herpesviruses are intracellular pathogens that have acquired multiple mechanisms to prevent clearance by the immune system (Lilley and Ploegh, 2005; Hansen and Bouvier, 2009). These immune evasion strategies allow the viruses to establish a lifelong infection. Herpesviruses downmodulate antigen presentation and prevent elimination by cytotoxic T lymphocytes by interfering with different stages of the MHC I antigen processing and presentation pathway. Human cytomegalovirus (HCMV) encodes two molecules, US2 and US11, that dislocate MHC I complexes to the cytosol, where they are degraded by proteasomes (Wiertz et al., 1996a; Wiertz et al., 1996b; Furman et al., 2002; Furman et al., 2003). Increased degradation of MHC I complexes is also induced by the mouse gammaherpesvirus 68 mK3 protein, which directs MHC I molecules to the proteasome via ubiquitination of their cytoplasmic tail (Stevenson et al., 2000; Wang et al., 2004; Lybarger et al., 2005; Lybarger et al., 2003). Kaposi's sarcoma herpesvirus-encoded K3 and K5 ubiquitinate MHC I molecules at the cell surface, thereby accelerating their endocytosis (Coscoy and Ganem, 2000; Ishido et al., 2000; Cadwell and Coscoy, 2005; Cadwell and Coscoy, 2008). HCMV US3 targets the MHC I pathway via tapasin. By inhibiting tapasin-facilitated peptide loading, MHC I molecules become unstable, are transported out of the ER and degraded (Jones et al., 1996; Lee et al., 2000; Park et al., 2004). US3-mediated tapasin inhibition only affects tapasin-dependent MHC I alleles.

Peptide transport by TAP is a critical step of the MHC I-antigen presentation pathway and is a frequent target of herpesviruses. Currently, four genes encoding TAP inhibitors have been identified for herpesviruses. The ICP47 protein encoded by herpes simplex virus 1 and 2 prevents peptide translocation by obstructing the peptide binding site of the TAP

transporter (Fruh et al., 1995; Hill et al., 1995; Ahn et al., 1996; Tomazin et al., 1996; Aisenbrey et al., 2006). The bovine herpesvirus 1 (BoHV-1) UL49.5 protein induces a conformational arrest within the TAP complex, preventing structural rearrangements required for peptide transport (Koppers-Lalic et al., 2005; Verweij et al., 2008). In addition, TAP is targeted for proteasomal degradation (Koppers-Lalic et al., 2005). The UL49.5 homologs encoded by pseudorabiesvirus and equine herpesvirus 1 and 4 also inhibit TAP. HCMV and its close relative rhesus cytomegalovirus both code for the TAP inhibitor US6. HCMV US6 prevents ATP-binding to TAP, thereby limiting the energy supply that is necessary for peptide transport (Ahn et al., 1997; Lehner et al., 1997; Hengel et al., 1997; Hewitt et al., 2001; Halenius et al., 2006; Pande et al., 2005). Finally, the BNLF2a proteins of Epstein-Barr virus (EBV) and related gamma 1 herpesviruses infecting Old World primates have also been identified as TAP inhibitors. EBV BNLF2a has been shown to prevent both peptide and ATP binding to TAP (Hislop et al., 2007; Horst et al., 2009). Remarkably, the cowpox virus protein CPX012 was recently found to block TAP as well (Alzhanova et al., 2009; Byun et al., 2009). This is the first TAP-inhibitor outside the herpesvirus family; its mode of action is still elusive and is currently being investigated.

Because of their effectiveness and specificity, viral immuno evasins are very suitable to study and modify both conventional and alternative routes of antigen presentation. For example, the TAP inhibitor HCMV US6 has been a useful tool to elucidate mechanisms involved in cross-presentation of exogenous antigens. Soluble US6, encompassing amino acids 20-146, blocked cross-presentation of endocytosed ovalbumin by MHC I, pointing towards the involvement of TAP in this pathway (Ackerman et al., 2003; Burgdorf et al., 2008). Recently, we have used BoHV-1 UL49.5 to inhibit TAP-dependent antigen presentation in mouse colon carcinoma cells. As a result, recognition of the tumors by conventional tumor-specific T cells was reduced, but presentation of T cell epitopes associated with impaired peptide processing, 'TEIPP' antigens, was enhanced (van Hall et al., 2007). These antigens were originally identified by their enhanced appearance on tumors that have defects in their conventional antigen presentation pathways (van Hall et al., 2006), for example due to reduced TAP expression (Khong and Restifo, 2002; Marincola et al., 2000). Additionally, expression of HCMV US11 reduces rejection of human mesenchymal stem cells transplanted in NK cell-depleted immunocompetent mice (Knaän-Shanzer et al., manuscript in preparation). These examples substantiate the successful application of herpesvirus-encoded immune evasion molecules in unraveling immunological pathways and highlight their potential application in anti-tumor therapy and transplantation studies.

Experimental mouse models have been indispensable for the studies discussed above, yet no dedicated TAP inhibitor is known for any of the mouse herpesviruses at present. A complete and consistent analysis on the efficacy of immuno evasins from non-mouse

viruses in mouse cells is lacking. Hence, the presently described herpesvirus-encoded inhibitors of TAP and tapasin were evaluated for their capacity to interfere with MHC I-restricted antigen presentation in mouse cells of different haplotypes.

Materials and methods

DNA constructs The genes encoding UL49.5, UL49.5$^{KK/AA}$ (described in Verweij et al., 2008) and BNLF2a with a C-terminal HA-tag and 4 methionines (BNLF2aHA4M) were cloned into the pLZRS vector as described (Koppers-Lalic et al., 2005; Hislop et al., 2007). US3 and US6 were isolated from viral DNA (HCMV strain AD169) by PCR amplification. PCR products were cloned into the pLZRS vector. US6$_{Tyr}$ (Hengel et al., 1997) and ICP47 (a kind gift of K. Früh, Vaccine and Gene Therapy Institute, Oregon Health and Science University, Oregon, USA) in pcDNA3.1 were ligated into the pLZRS vector. The pLZRS vector information can be found at www.stanford.edu/group/nolan/retroviral_systems/retsys.html.

Cell lines and recombinant viruses The cell lines NIH/3T3 (H-2^d – BALB/c, American Type Culture Collection, ATCC), L-cells (H-2^k – C3H/Law, ATCC), C26 (a gift from G. Hartmann, Munich, Germany) and HeLa cells (ATCC CCL-2) were maintained in DMEM medium. MC38 (H-2^b – C57BL/6, ATCC) were maintained in IMDM medium, and the human melanoma cellline Mel JuSo (MJS) was cultured in RPMI-1640 medium. Media were supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine (Invitrogen), 140 IU/ml penicillin and 140 µg/ml streptomycin. Where indicated 3T3 cells were cultured in the presence of 20 U/ml of recombinant mouse IFNγ (Pepro Tech) for 48 h.

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 or ΔNGFR positive cells were selected using a FACSVantage cell sorter (Becton Dickinson). The following stable cell lines were generated: MJS expressing UL49.5, BNLF2a, US6$_{Cys}$, US6$_{Tyr}$, ICP47, or US3; L-cells expressing UL49.5$^{KK/AA}$, BNLF2a, US6$_{Cys}$, US6$_{Tyr,}$ ICP47, or US3; 3T3 expressing UL49.5$^{KK/AA}$, BNLF2a, US6$_{Cys}$, US6$_{Tyr,}$ ICP47, or US3; and MC38 expressing UL49.5$^{KK/AA}$, BNLF2a, US6$_{Cys}$, ICP47, or US3.

US6 in p7.5K131 (Hengel et al., 1997) containing a mutation leading to a Cys to Tyr change at position 108 was corrected by site-directed mutagenesis. Recombinant vaccinia viruses (recVACV) expressing US6 Cys108 and US6 Tyr108 were constructed as described before (Halenius et al., 2006). The ICP47 expressing recVACV has been described elsewhere (Banks et al., 1994).

Antibodies The following antibodies were used for flow cytometry: mAb Y3 binding to H-2K^b complexes and H-2^k (ATCC), biotinylated mAb 15-5-5.3 binding to H-2D^k (Abcam), biotinylated mAb 28-14-8S binding to H-2D^b and H-2L^d (Abcam), mAb 34-1-2S binding to

H-2D^d and H-2K^d (BioLegend), biotinylated mAb 34-2-12 binding to H-2D^d (BD Pharmingen) and mAb W6/32 binding to human MHC I complexes (Barnstable et al., 1978). For detection of UL49.5, a rabbit polyclonal anti-serum (H11) was raised using a synthetic peptide derived from the N-terminal domain of BoHV-1 UL49.5 as an antigen (Lipinska et al., 2006). BNLF2a expression was verified using the rat antibody MHV 5B9 (Horst et al., 2009). In addition, we used rabbit anti-HCMV US6 R.US6C, goat anti-mouse TAP1 M18 (Santa Cruz), rabbit anti-mouse TAP2 688 (a kind gift from F. Momburg, Division of Molecular Immunology, German Cancer Research Center (DKFZ), Heidelberg, Germany), and hamster anti-mouse tapasin 5D3 (kindly provided by T.H. Hansen, Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, USA). Anti-β-actin mAb AC-74 (Sigma-Aldrich) was used as a control.

Peptides Synthetic peptides were prepared by normal Fmoc-chemistry using preloaded Tentagel resins, PyBop/NMM for in situ activation and 20% piperidine in NMP for Fmoc removal (Hiemstra et al., 1997). Couplings were performed for 75 min. After final Fmoc removal peptides were cleaved with TFA/H_2O 19/1 containing additional scavengers when C or W residues were present in the peptide sequence. Peptides were isolated by ether/pentane precipitation. Peptides were checked on purity using rpHPLC-MS and on integrity using Maldi-Tof mass spectrometry, showing the expected molecular masses.

Fl-labeled peptides were synthesized as Cys-derivative. This was enabled by the addition of a single cysteine at the N- or C-terminal site of the protein and by replacing the remaining cysteines in US6 by alpha-amino-n-butyric acid (Abu), an isosteric variant of cysteine. Replacing cysteines by Abu did not affect TAP inhibition by the US6 peptide (data not shown). Labeling was performed with 5-(iodoacetamido)fluorescein (Fluka Chemie AG) at pH 7.5 (Na-phosphate in water/acetonitrile 1:1 v/v). The labeled peptides were desalted over Sephadex G-10 and further purified by C18 rp-HPLC. The molecular mass of the labeled peptides was checked using Maldi-Tof mass spectrometry.

Western blotting Cells were dissolved in lysis buffer containing 1% Nonidet P-40 and lysates were separated by SDS-PAGE and transferred to PVDF membranes (GE Healthcare). UL49.5 and BNLF2a were separated using 16.5%-tricine PAGE. The blots were incubated with the indicated antibodies, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (DAKO). Bound HRP-labeled antibodies were visualized using ECL Plus (GE Healthcare).

RNA isolation and RT-PCR Cellular RNA was isolated from cells using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using random nanomer primers and Moloney murine leukemia virus reverse transcriptase (Finnzymes). The absence of genomic DNA was verified by parallel reactions performed in the absence of

reverse transcriptase. To detect ICP47, US3, and GAPDH cDNA, the following primers were used: 5'- GCC GAA TTC CAT GTC GTG GGC CCT GGA A -3' (ICP47, sense); 5'- GTC TCG AGA TTA ACG GGT TAC TGG A -3' (ICP47, anti-sense); 5'-ATT CTC GAG TTA AAT AAA TCG CAG ACG GGC GCT-3' (US3, sense); 5'- TAT GAA TTC CAC CAT GAA GCC GGT GTT GGT GCT-3' (US3, anti-sense).

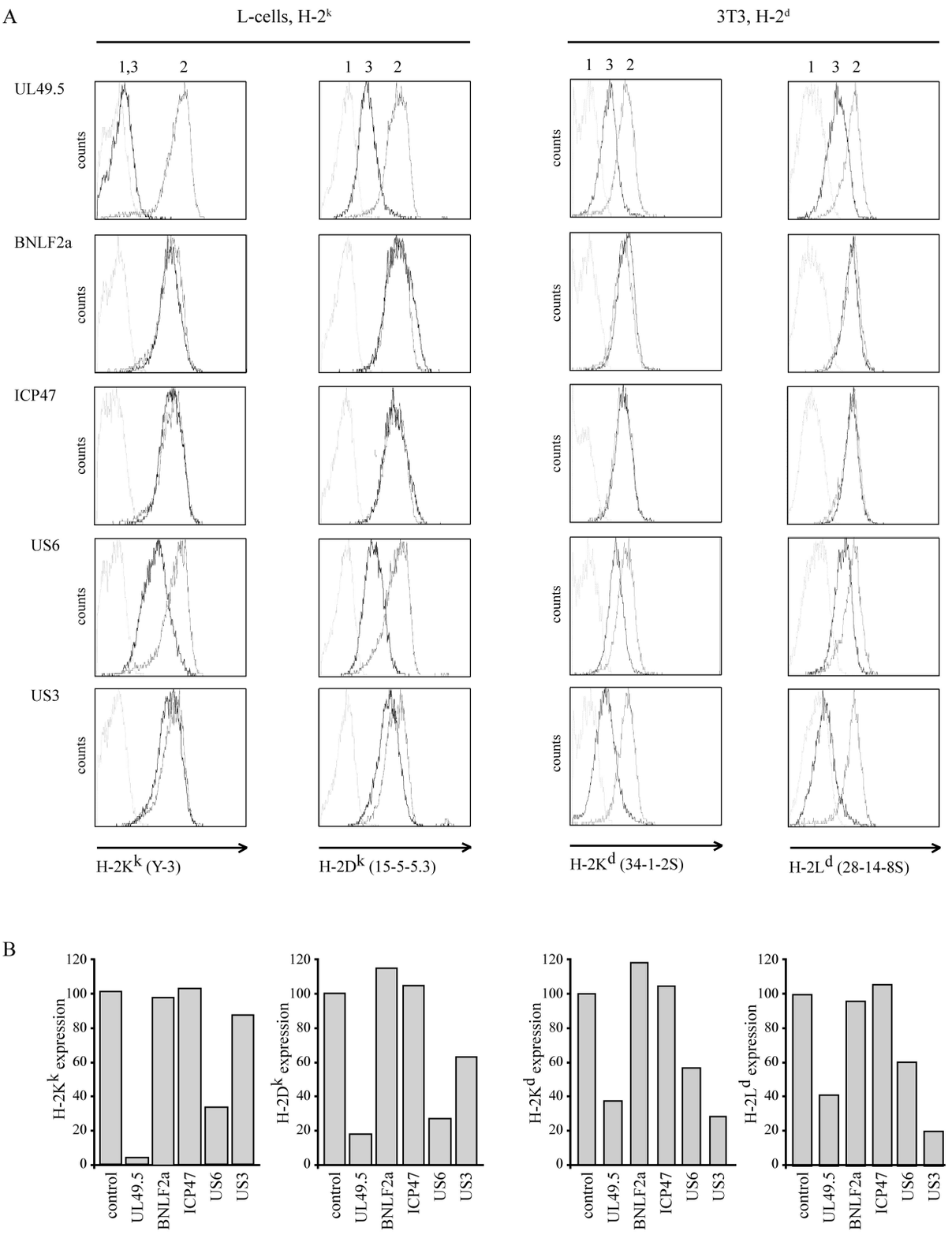
Flow cytometry Surface levels of MHC I molecules were determined by flow cytometry. Cells were stained with the indicated primary antibodies and, after washing, with the secondary goat anti-mouse allophycocyanin Ab (Leinco Technologies) or goat anti-mouse phycoerythrin Ab (Jackson ImmunoResearch Laboratories) at 4°C. Stained cells were measured using a FACSCalibur (Becton Dickinson) and analyzed using CellQuest software.

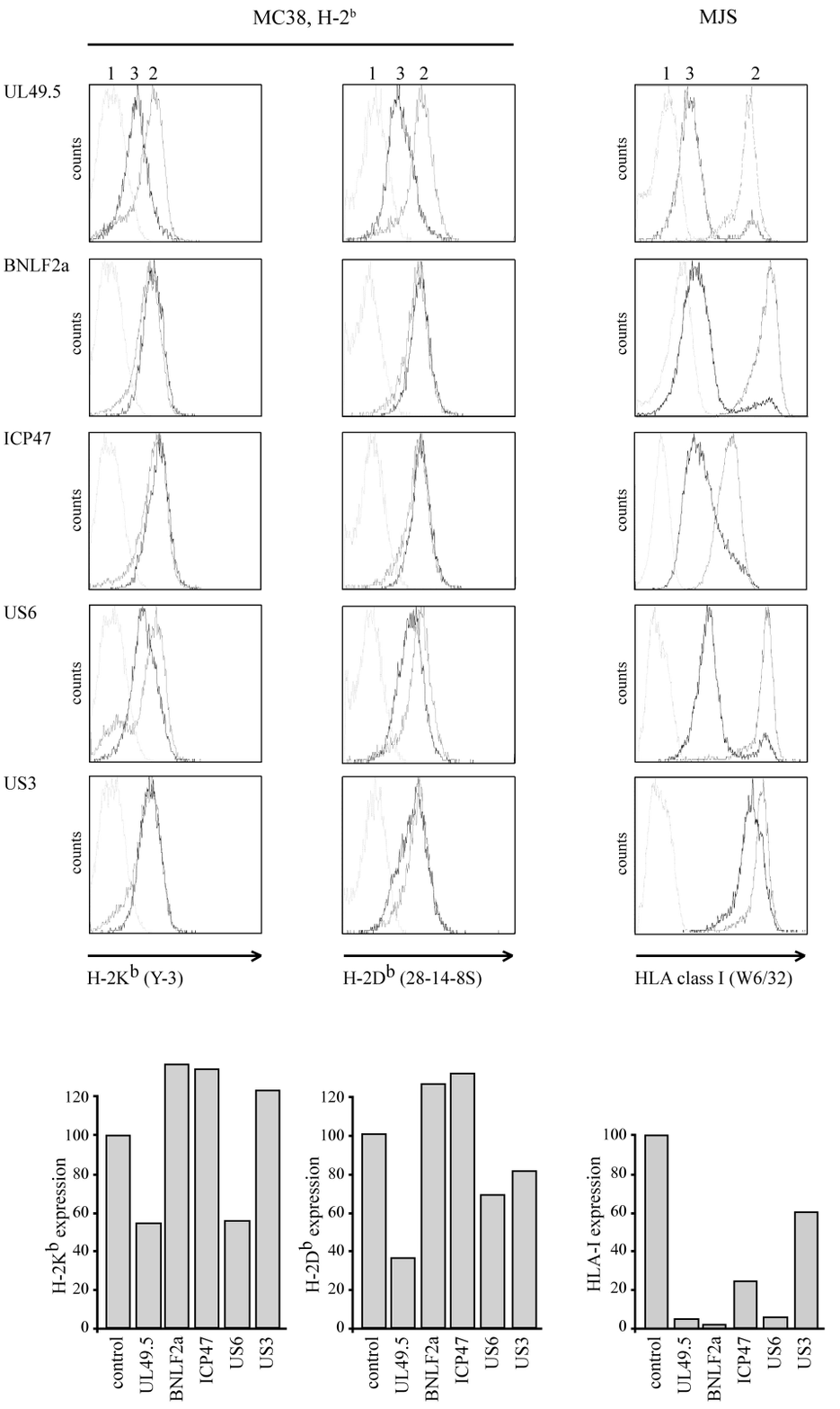
Peptide transport assay Cells were permeabilized using Streptolysin-O (Murex Diagnostics) at 37°C for 10 min. Permeabilized cells were incubated with 4.5 µM of the fluorescein-conjugated synthetic peptide CVNKTERAY (*N*-core glycosylation site underlined) in the presence of 10 mM ATP or 0.125 M EDTA at 37°C for 10 min. The inhibiting $US6_{81-115}$ peptide-variants were added 3 minutes before CVNKTERAY-incubation. In case of $guUS6_{81-115}$-C^{FL}, permeabilized cells were incubated with the indicated concentrations of this peptide and CVNKTERAY was left out. 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 with 1st (3 times) and 2nd (1 time) wash buffer (0.1% Triton X-100, 500 mM NaCl, 2 mM MgCl2, 50 mM Tris HCl pH 8.0 and 500 mM NaCl, 50 mM Tris HCl pH 8.0, respectively), 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).

Results

Mouse MHC I haplotypes are differentially affected by herpesvirus immuno evasins

In this study, we evaluated the effect of the TAP-inhibitors UL49.5 (encoded by BoHV-1), BNLF2a, US6, and ICP47, and the tapasin-inhibitor US3 on MHC I-mediated antigen presentation in mouse cells. A panel of mouse cells was retrovirally transduced to stably express the viral inhibitors. L-cells (H-2^k), 3T3 cells (H-2^d), and MC38 (H-2^b) were included in this study to investigate the extent to which different mouse MHC I locus products are


A
L-cells, H-2k
3T3, H-2d
1,3
2
1
3
2
UL49.5
BNLF2a
ICP47
US6
US3
counts
H-2Kk (Y-3)
H-2Dk (15-5-5.3)
H-2Kd (34-1-2S)
H-2Ld (28-14-8S)
B
H-2Kk expression
H-2Dk expression
H-2Kd expression
H-2Ld expression
0
20
40
60
80
100
120
control
UL49.5
BNLF2a
ICP47
US6
US3


MC38, H-2b
MJS
1 3 2
UL49.5
BNLF2a
ICP47
US6
US3
counts
H-2Kb (Y-3)
H-2Db (28-14-8S)
HLA class I (W6/32)
H-2Kb expression
H-2Db expression
HLA-I expression
0
20
40
60
80
100
120
control
UL49.5
BNLF2a
ICP47
US6
US3

affected by these viral immuno evasins. MHC I cell surface expression, evaluated using flow cytometry, was reduced in all cell lines expressing UL49.5 and US6 (Fig. 1A and B). In contrast, MHC I levels were not affected on mouse cells expressing BNLF2a and ICP47, whereas these inhibitors were functional in the human MJS cells (Fig. 1A and B).

In all cell lines tested, the effect on surface MHC I expression was stronger for UL49.5 than for US6, indicating that UL49.5 is the most potent viral TAP inhibitor in these mouse cells. Interestingly, differences were apparent between the three haplotypes. MHC I downregulation was most prominent in L-cells (H-2^k) and less in MC38 (H-2^b), reflecting differences in TAP-dependency for peptide loading. Also, the extent to which H-2K and H-2D molecules were downregulated by UL49.5 on both cell lines varied. For the detection of H-$2K^k$ and H-$2K^b$, the same conformation-dependent monoclonal antibody was used (Y3), indicating that the haplotype differences were based on TAP-dependency and not on variation in antibody affinities. The tapasin inhibitor US3 caused a surprisingly strong MHC I downregulation of H-2^d alleles on 3T3 cells. A minor reduction of the H-$2K^k$ and H-$2D^k$ proteins was observed on L-cells, comparable to the HLA I reduction at the surface of the human MJS cells (Fig. 1A and B). In contrast, the H-2^b proteins on MC38 cells were insensitive to US3-induced downregulation.

Proper expression of UL49.5, BNLF2a, and US6 proteins was visualized by Western blotting (Fig. 2A). For ICP47 and US3, mRNA-levels were determined using RT-PCR (Fig. 2A). Equal loading of the samples was confirmed by staining for β-actin and by determining GAPDH mRNA levels (data not shown). The results presented in Fig. 2A confirm proper expression of UL49.5, BNLF2a, US6, ICP47, and US3 in the mouse cell lines, although some differences were observed. The UL49.5 expression levels were considerably lower in 3T3 cells, compared to L-cells and MC38 cells, but this was not reflected in MHC I phenotype, as UL49.5 downregulated H-2^d alleles on 3T3 to the same extent as H-2^b alleles on MC38 cells (Fig. 1A and B). Thus, although variations were observed in expression levels of the inhibitors, this was not reflected by their phenotypes.

To investigate whether the expression levels of TAP1, TAP2, and tapasin influenced the efficiency of MHC I downregulation, steady state levels of these proteins were determined using Western blotting (Fig. 2B). TAP1, TAP2, and tapasin levels were consistently lower in 3T3 cells, compared to L-cells and MC38 cells. Yet, these lower expression levels of the PLC members did affect the intensity of MHC I downregulation, as the TAP inhibitors UL49.5

Fig. 1 (overleaf), UL49.5, US6, and US3 downmodulate MHC I molecules on mouse cells. (A) Surface expression of MHC I molecules was assessed on untransduced L-cells, 3T3, and MC38 cells (graph 2) and cells expressing UL49.5, BNLF2a, ICP47, US6, or US3 (graph 3). Cells were stained using the allele-specific antibodies indicated. Graph 1, background staining in the presence of secondary antibody only. (B) MHC I expression levels on inhibitor-expressing cells, expressed relative to the levels of MHC I molecules on control (-) cells (set at 100 %). One representative experiment out of two independent experiments is shown.

and US6 caused the strongest inhibition in L-cells, in which TAP levels are relatively high (Fig. 1A and B). However, the low tapasin levels in 3T3 cells might contribute to the strong US3-mediated inhibition of MHC I expression observed in these cells.

Taken together, our data indicate that the TAP inhibitors UL49.5 and US6, and the tapasin inhibitor US3 cross species barriers to interfere with MHC I-mediated antigen presentation in mouse cells.

The effect of US3, but not UL49.5, on MHC I expression can be overruled by IFNγ treatment

To investigate if US3-induced MHC I downregulation is indeed affected by expression levels of PLC components, 3T3 cells expressing US3 were treated with IFNγ. UL49.5-expressing cells were taken along as a control, as the effect of this inhibitor did not vary between cells with differing PLC expression levels (Fig. 1 and Fig. 2B). IFNγ-treatment enhanced expression of TAP1, TAP2, and tapasin, as confirmed by Western blot analysis (Fig. 3A). Flow cytometric analysis revealed that IFNγ treatment largely reversed the MHC I downregulation induced by US3 in 3T3 cells (Fig. 3B). As expected, incubation with IFNγ did not alter UL49.5-mediated reduction of MHC I expression. These results suggest that the effect of US3 on MHC I is influenced by the steady state levels of the constituents of the PLC, including tapasin, and can be overruled by IFNγ-treatment. In contrast, UL49.5 represents a very potent TAP inhibitor that induces MHC I downregulation, irrespective of IFNγ-enhanced TAP levels.

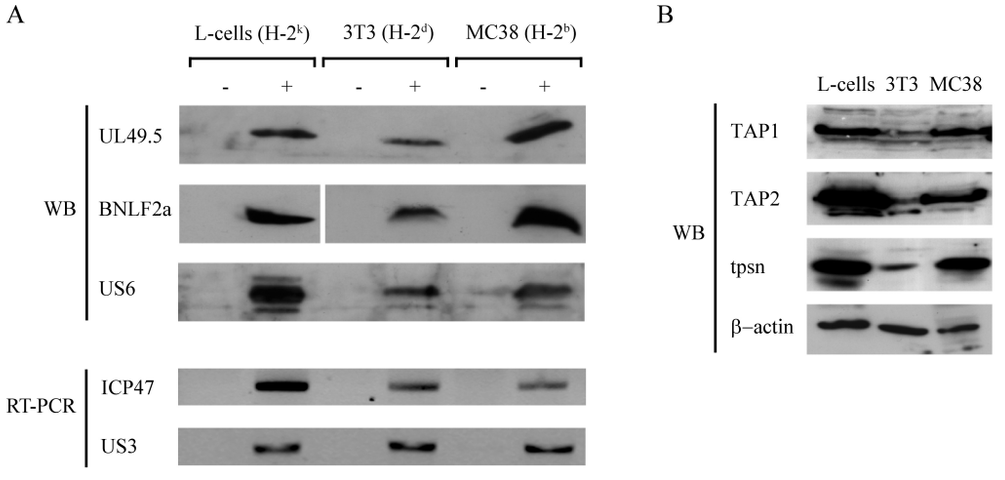


Fig. 2, expression levels of the viral inhibitors in mouse cells. (A) Expression of UL49.5, BNLF2a, and US6 in L-cells, 3T3, and MC38 cells was evaluated by SDS-PAGE and Western blotting (WB) using the antibodies indicated. After isolating total RNA from L-cells, 3T3, and MC38 cells, the expressing of ICP47 was verified by RT-PCR using ICP47-specific primers. - = control cells, + = cells expressing inhibitors. (B) Steady state levels of TAP1, TAP2, and tapasin expressed in the mouse cell lines were determined by SDS-PAGE and Western blotting (WB).

UL49.5 and US6 inhibit peptide transport by mouse TAP

Next, we evaluated to what extent mouse TAP function was inhibited by the herpesvirus molecules. TAP-mediated peptide transport was analyzed in Streptolysin-O (SLO)-permeabilized cells using a reporter peptide carrying a glycosylation consensus sequence and a fluorescent moiety. UL49.5 and US6 were found to strongly reduce peptide transport in L-cells, 3T3 and MC38 (Fig. 4A-C). In contrast, expression of the TAP inhibitors BNLF2a or ICP47 in mouse cells did not alter peptide transport, whereas their functionality was demonstrated in human cells (Fig. 4D). Overall, these data are consistent with downregulation of mouse MHC I caused by the TAP-inhibitors in the various cell lines (Fig. 1).

The efficiency of MHC I downregulation, however, is not necessarily reflected by the extent to which TAP is inhibited. UL49.5- and US6-mediated reduction of H-2K^k expression, as detected by the conformation-dependent Y-3 antibody, is more efficient than that of H-2K^b, detected by the same antibody. These findings cannot be explained by differences in expression levels of the TAP-inhibitors, nor expression of the PLC-constituents in these cells (Fig. 2A and B). In addition, TAP function was reduced to the same extent in all cell lines expressing UL49.5 and US6, respectively. Thus, these findings indicate that the H-2K^k haplotype is more sensitive to TAP-inhibition, and therefore more dependent on proper peptide-loading, than H-2K^b. The combined data indicate that both US6 and UL49.5 can be utilized to block TAP in mouse cells, however, UL49.5 is most effective in inhibiting MHC I surface expression (Fig. 1 and 3).

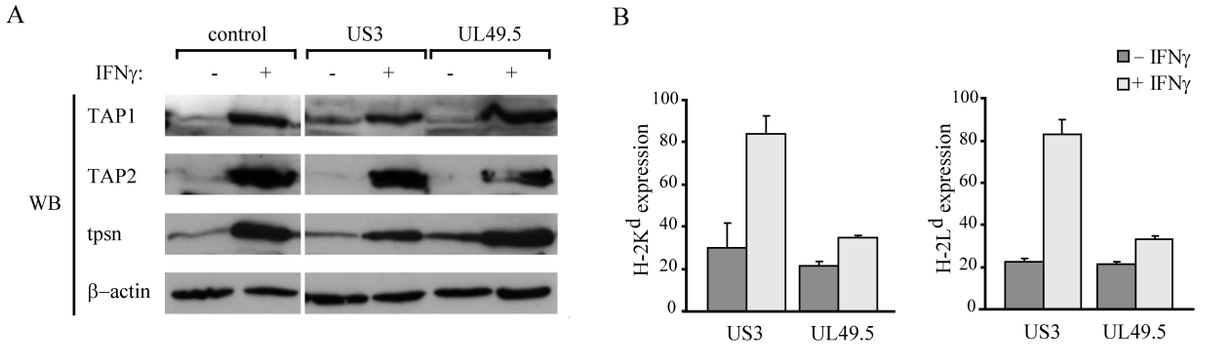


Fig. 3, tapasin levels influence US3-induced downregulation. Control 3T3 cells (-) and US3- and UL49.5-expressing 3T3 cells were treated with mouse IFNγ for 48 h (+), after which TAP1, TAP2, and tapasin levels were determined by SDS-PAGE and Western blotting. (B) Surface MHC I expression on untreated (dark gray bars) and IFNγ-treated (light gray bars) cells was assessed after staining with the H-2K^d- and H-2L^d-specific antibodies. The MHC I expression levels on inhibitor-expressing 3T3 cells was expressed relative to the expression levels of MHC I molecules on control cells (set at 100%). The average of two independent experiments is shown.

Cysteine residue at position 108 of US6 influences species-specificity of US6

Our finding that US6 inhibits mouse TAP is in contradiction with earlier observations, showing that US6 does not block mouse TAP in 3T3 (H-2^d) and 1T (H-2^k) cells (Halenius et al., 2006). This discrepancy prompted us to compare the sequences of the US6 constructs used in both studies. Intriguingly, this revealed one nucleotide difference, resulting in a tyrosine residue at position 108 of the construct of Halenius et al., instead of the cysteine residue in the US6 protein used in the present study so far.

To investigate if the species specificity of US6 is influenced by the substitution of cysteine 108 for tyrosine, we compared the capacity of $US6_{Cys}$ and $US6_{Tyr}$ to inhibit TAP. At first, expression and oligomerization of the US6 proteins were verified by Western blotting (Fig. 5A). Previously, US6 has been shown to assemble into homodimers and -multimers (Halenius et al., 2006; Dugan and Hewitt, 2008), which was speculated to be necessary for

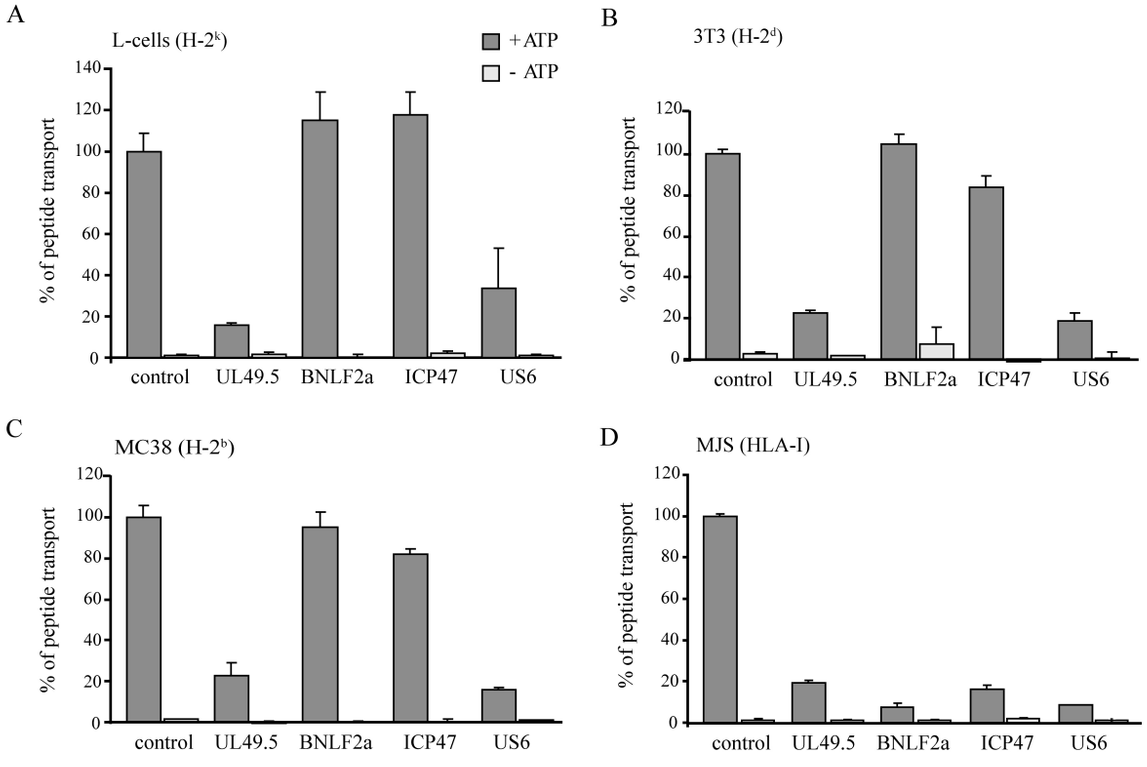


Fig. 4, UL49.5 and US6 efficiently inhibit mouse TAP. Transport activity of TAP was analyzed in L-cells (A), 3T3 cells (B), and MC38 cells (C) expressing herpesvirus-encoded inhibitors of TAP and tapasin. Translocation of the peptide CVNKTERAY, of which the cysteine was labeled with fluorescein, was evaluated in the presence of 10 mM ATP (dark gray bars) or EDTA (light gray bars). Peptide transport is expressed as percentage of translocation, relative to the translocation observed in control cells (set at 100%). The functionality of the viral immune evasion proteins was verified by analyzing the inhibition of peptide transport in the human melanoma cell line MJS (D).

efficient inhibition of the TAP transporter. However, both proteins displayed similar patterns of oligomerization, when visualized under non-reducing conditions (Fig. 5A). This observation indicates that Cys108 is not essential for intermolecular disulfide bonds. $US6_{Tyr}$ was found to efficiently block peptide transport in MJS cells (Fig. 5B), confirming that substituting the cysteine did not affect inhibition of human TAP. Subsequently, peptide transport was analyzed in a direct comparison using L-cells and 3T3 cells expressing either $US6_{Cys}$ or $US6_{Tyr}$ (Fig. 5C). Interestingly, peptide transport was strongly reduced in $US6_{Cys}$-expressing cells, but only moderate inhibition was observed in the presence of $US6_{Tyr}$ (Fig. 5C), indicating that $US6_{Cys}$ efficiently blocks both human and mouse TAP, but the substitution of Cys108 for tyrosine selectively abolished US6 interference with peptide translocation in mouse cells.

TAP inhibition by a synthetic peptide derived from the ER-luminal domain of US6

A recombinant soluble form of US6 has been shown to block antigen cross-presentation, i.e., MHC I-restricted presentation of exogenously derived antigenic peptides (Ackerman et

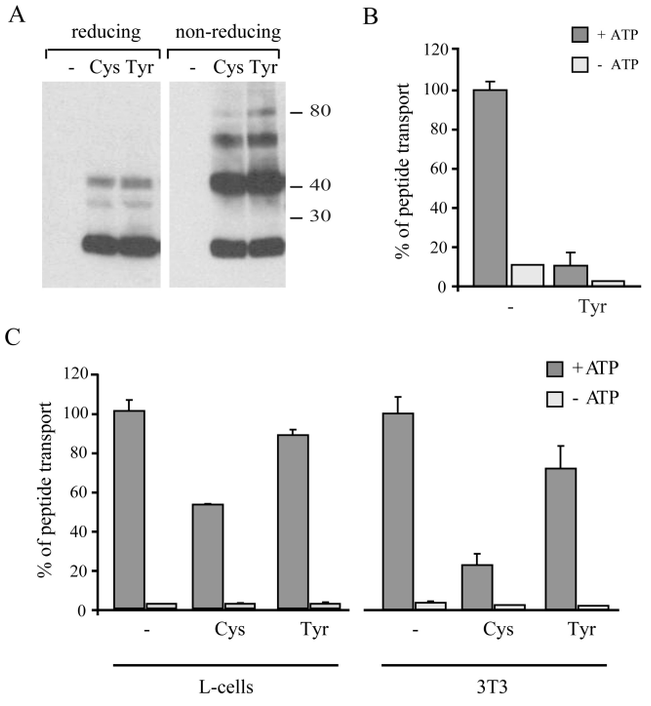


Fig. 5, $US6_{Tyr}$ inhibits human TAP, but is unable to block mouse TAP. (A) $US6_{Cys}$ and $US6_{Tyr}$ and ICP47 as a control (-) were expressed by recVACV in HeLa cells at an MOI of 5. At 15 hrs post-infection, cell lysates were prepared and resolved under reducing or non-reducing conditions by SDS-PAGE. US6 was stained by a specific antibody. Transport activity of TAP was assessed in MJS cells expressing $US6_{Tyr}$ (B), and L-cells and 3T3 cells expressing either $US6_{Cys}$ or $US6_{Tyr}$ (C) using the fluorescein-labeled reference peptide CVNKTERAY. The assay was performed in the presence of 10 mM ATP (dark gray bars) or EDTA (light gray bars). Peptide transport is expressed as percentage of translocation, relative to the translocation observed in control cells (set as 100%).

al., 2003; Burgdorf et al., 2008). Residues 89 to 108 of the US6 protein have been suggested to represent the minimal amino acid sequence required to block TAP-mediated peptide translocation, as deduced form a panel of N- and C-terminally truncated US6 proteins (Dugan and Hewitt, 2008). Here, we tested if a synthetic peptide comprising the putative minimal functional entity of US6, $US6_{81\text{-}115}$, possessed the capacity to inhibit TAP. Peptide transport was measured in SLO-permeabilized cells that were incubated with the $US6_{81\text{-}115}$ peptide for 3 minutes before addition of the fluorescein-conjugated reference peptide. Incubation with $US6_{81\text{-}115}$ caused a dose-dependent inhibition of TAP function, reducing translocation of the reference peptide by 70% at the highest concentration tested (Fig. 6A). A control peptide carrying the US6 sequence in reversed order did not block TAP (Fig. 6A; $US6_{rev}$). These data indicate that a synthetic peptide comprising residues 81 to 115 of US6 is sufficient to block TAP-mediated peptide transport when added exogenously.

This finding was unexpected, since endogenously expressed US6 acts on ER-luminal domains of TAP (Ahn et al., 1997), whereas our exogenous peptide is delivered into the

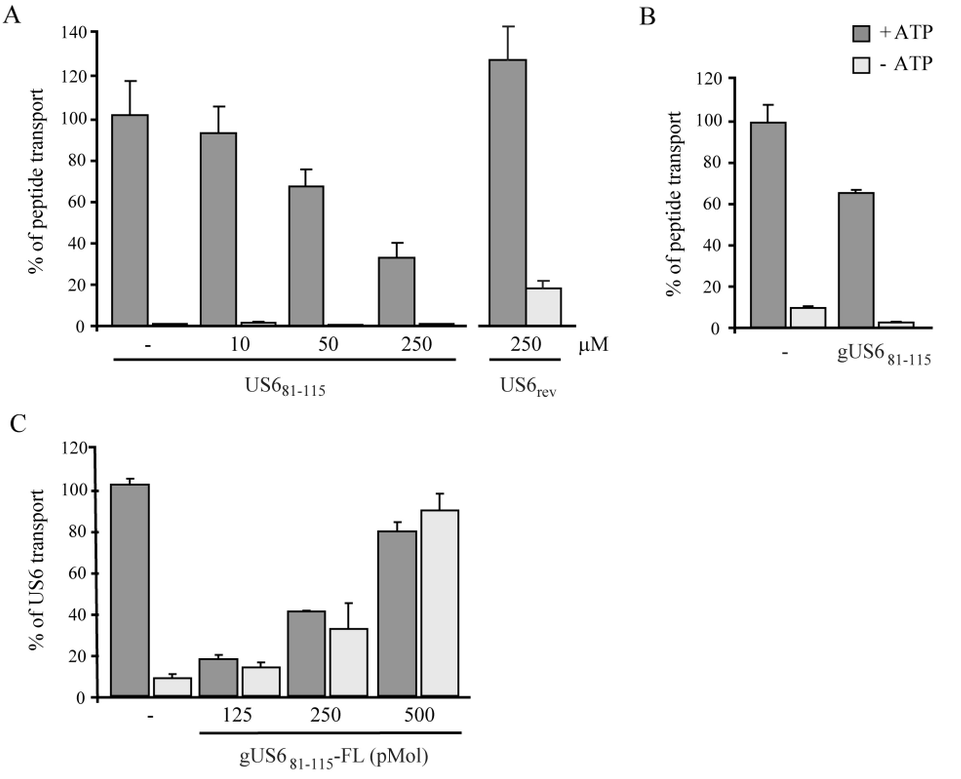


Fig. 6, a US6-derived synthetic peptide inhibits TAP. Permeabilized MJS were incubated with increasing concentrations of the $US6_{81\text{-}115}$ peptide or a control peptide with the inverted amino acid sequence of US6 (A) or with 50 µM of $gUS6_{81\text{-}115}$ (B). Translocation of the reference peptide was evaluated in the presence of 10 mM ATP (dark gray bars) or EDTA (light gray bars). Peptide transport is expressed as percentage of translocation, relative to the translocation observed in control cells (set as 100%). (C) Permeabilized MJS were incubated with increasing amounts of fluorescein-conjugated $gUS6_{81\text{-}115}$. Peptide transport is expressed as percentage of translocation, relative to the translocation observed in cells incubated with 125 pmol of the reference peptide (set as 100%).

cytosol of SLO-permeabilized cells. To determine if $US6_{81-115}$ enters the ER lumen, a reporter was introduced into the peptide sequence. To this end, a glycosylation consensus sequence was incorporated into the peptide, permitting Con-A precipitation via its N-linked glycan introduced upon entry into the ER (Table I; $gUS6_{81-115}$). In addition, a fluorescein moiety was added at the N-terminus of the peptide to allow detection after precipitation ($gUS6_{81-115}$-C^{FL}). The presence of a glycosylation consensus sequence ($gUS6_{81-115}$) did not alter TAP inhibition by the US6 peptide (compare Fig. 6A and 6B; 50 μM). SLO-permeabilized cells were incubated with either the reference peptide or the modified $US6_{81-115}$ peptide ($gUS6_{81-115}$-C^{FL}), after which transport into the ER lumen was assessed (Fig. 6C). Interestingly, $gUS6_{81-115}$-C^{FL} peptide was also detected in the ER following exogenous addition, but ATP-depletion did not block ER entry of $gUS6_{81-115}$-C^{FL}. In contrast, the reference peptide was translocated into the ER in an ATP-dependent manner, which points towards TAP-mediated transport. Two additional $gUS6_{81-115}$-C^{FL} peptides, bearing glycosylation consensus sequences at different locations (Table I; peptides #2 and #3), similarly reached the ER in an ATP-independent manner (data not shown). This indicates that the US6 inhibitory peptide entered the ER in a TAP-independent fashion and was able to inhibit TAP-mediated transport from the ER-luminal side.

In summary, the results presented create the opportunity of using US6-based peptides as versatile tools to specifically inhibit TAP function.

Discussion

In this study, we presented a systematic analysis of the applicability of endogenously expressed herpesvirus-encoded immune evasion molecules in mouse cells of different haplotypes. BoHV-1 UL49.5 and HCMV US6 cross species barriers to induce a strong downregulation of MHC I expression in mouse cells of the H-2^k, H-2^d and H-2^b haplotype, with the H-2^k allele appearing most TAP-dependent. The observed downregulation reflects the UL49.5- and US6-induced reduction in TAP function. Dissimilarities observed amongst US6 studies in mouse cells appeared to arise from an amino acid substitution at position 108: the replacement of the cysteine by a tyrosine completely abolished interference with mouse TAP. The HCMV-encoded tapasin inhibitor US3 was shown to block mouse tapasin, with most prominent MHC I downregulation observed for H-2^d alleles. Amongst the viral immune evasion proteins tested, UL49.5 was found to be most potent with respect to MHC I downregulation. Finally, a synthetic US6 peptide was shown to block peptide transport when exogenously added to semi-permeabilized cells. The comparative analysis of UL49.5-, US6- and US3-mediated inhibition of mouse TAP illustrates the possibilities to exploit herpesvirus-encoded inhibitors in antigen presentation studies in mouse cells of different haplotypes.

US3 has been described to efficiently interfere with the function of mouse tapasin expressed in human cells (Park et al., 2004), but efficacy in mouse cells had not been investigated so far. Here, we show that US3 efficiently downregulates MHC I expression on 3T3 cells (H-2^d), but not on L-cells (H-2^k) or MC38 cells (H-2^b). The prominent US3-induced downregulation of MHC I on 3T3 cells is in agreement with the observation that maturation of H-2^d proteins strongly depends on tapasin (Turnquist et al., 2001; Myers et al., 2000; Prasanna and Nandi, 2004). H-2^b proteins were also proven to be tapasin-dependent (Myers et al., 2000; Grandea, III et al., 2000), however, US3 was unable to reduce MHC I expression on MC38 cells. Analysis of the steady state levels of PLC components revealed that 3T3 cells contain low levels of the PLC components compared to MC38 cells, including tapasin. The differences in tapasin expression levels between the cell lines might explain why US3 strongly reduces MHC I expression in 3T3, but not in MC38 cells. Indeed, enhancement of expression of the PLC components via IFNγ-treatment reduced US3-mediated MHC I downregulation in 3T3 cells. Apparently, both the expression levels of tapasin, and possibly other components of the PLC, as well as the tapasin-dependency of the MHC I locus products influence the level of US3-induced downregulation of MHC I observed in the mouse cells.

The inhibition of mouse TAP by UL49.5 and US6 affected the expression of H-2^k molecules more strongly than molecules of the H-2^d and H-2^b haplotypes. This implies that H-2^k is relatively TAP-dependent, which is in line with previous observations (Prasanna and Nandi, 2004). Our finding that US6 inhibits TAP in all mouse cell lines tested seemed in discrepancy with a recent study describing US6 not to block mouse TAP (Halenius et al., 2006). This paradoxical situation appears to results from the use of $US6_{Tyr}$, which is incapable of inhibiting mouse TAP, while retaining its potential to block human TAP (Fig. 5).

Cys108 has been suggested to form an intramolecular disulfide bridge with Cys129 (Kyritsis et al., 2001). Hence, substitution of Cys108 may influence the folding of US6. Apparently, the resulting structural alteration did not reduce the ability of the protein to block human TAP, while mouse TAP was no longer inhibited. Interestingly, $US6_{Tyr}$ was found unable to bind TAP in cells of rat origin (Halenius et al., 2006). It is conceivable that this holds true for the interaction of $US6_{Tyr}$ with mouse TAP as well, implying that the Cys108 allows US6 to recognize functionally relevant, but structurally different determinants on human and mouse TAP.

The viral inhibitor-induced downregulation of MHC I was much more pronounced on human cells than on mouse cells. In contract, the intensity of TAP inhibition induced by UL49.5 and US6 was highly comparable between cells of both species. The MJS cells used in this study are homozygous for HLA-A1. This allele has been shown to be extremely TAP dependent (Luckey et al., 2001), which might explain the relatively strong downregulation

of MHC I expression. Moreover, the immune evasion molecules encoded by the human herpesviruses may be adapted to their natural target to achieve optimal inhibition of TAP function.

The exogenous addition of the synthetic peptide $US6_{81-115}$ inhibits TAP function in SLO-permeabilized cells, suggesting that the peptide is transported from the cytosol into the ER-lumen. Since TAP has been shown to translocate peptides of up to 40 amino acid residues (Momburg et al., 1994; Koopmann et al., 1996), $US6_{81-115}$ could be transported by TAP itself. However, $US6_{81-115}$ was found to enter the ER in an ATP-independent manner, which implies that TAP is not involved. A study by Ackerman et al. reported that endocytosed β2m, and by analogy soluble US6 are able to enter the ER (Ackerman et al., 2005). The authors hypothesized that endocytosed proteins are transported through phagosome-ER intermediate compartments or retrograde vesicular trafficking to finally reach the ER. However, in our experimental system, the $US6_{81-115}$ peptide is added to semi-permeabilized cells, making the involvement of vesicular transport unlikely. Thus, the known trafficking routes of proteins to and from the ER are unlikely to contribute to $US6_{81-115}$ entry. Further studies are needed to elucidate the mechanisms involved in ATP-independent translocation of peptides into the ER lumen.

The results presented in this study designate UL49.5, $US6_{(Cys)}$ and US3 as inhibitors of the antigen presentation pathway in mouse cells. Moreover, US6 can be utilized to inhibit TAP as synthetic peptide added to semi-permeabilized cells. Synthetic peptides can be easily prepared and modified to promote uptake and to be targeted to particular intracellular compartments. The latter may be achieved through introduction of specific targeting structures, such as TLR ligands or antibody epitopes (Khan et al., 2007; Schuurhuis et al., 2002; van Montfoort et al., 2009). In this way, inhibitor-derived peptides can be used to study novel antigen-presentation routes, including cross-presentation. The identification of the functional entity within UL49.5 and US3 will be instrumental for the design of comparable peptides.

Together, these data demonstrate cross-species activity for several herpesvirus immuno evasins and facilitate their selection for specific inhibition at defined stages of the MHC I antigen presentation pathway in the mouse. Additionally, our results illustrate the applicability of herpesvirus-encoded immune evasion proteins, and peptides derived thereof, as powerful immune modulators with potential applications in the treatment of cancer and autoimmune diseases.

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