The UL41-encoded virion host shutoff (vhs) protein and vhs-independent mechanisms are responsible for down-regulation of MHC class I molecules by bovine herpesvirus 1

Danijela Koppers-Lalic,1 Frans A. M. Rijsewijk,2 Sylvia B. E. Verschuren,2 Jacqueline A. M. van Gaans-van den Brink,3 Anne Neisig,4 Maaike E. Ressing,1 Jacques Neefjes4 and Emmanuel J. H. J. Wiertz1

1 Department of Medical Microbiology, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands
2 Division of Infectious Diseases and Food Chain Quality, ID-Lelystad, PO Box 65, 8200 AB Lelystad, The Netherlands
3 Laboratory for Vaccine Research, RIVM, PO Box 1, 3720 BA Bilthoven, The Netherlands
4 Division of Tumor Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

The virion host shutoff (vhs) protein of alphaherpesviruses causes a rapid shutoff of host cell protein synthesis. We constructed a bovine herpesvirus 1 (BHV1) deletion mutant in which the putative vhs gene, UL41, has been disrupted. Whereas protein synthesis is inhibited within 3 h after infection with wild-type BHV1, no inhibition was observed after infection with the BHV1\textsuperscript{vhs}\textsuperscript{−} deletion mutant. These results indicate that the BHV1 UL41 gene product is both necessary and sufficient for shutoff of host cell protein synthesis at early times post-infection. Using the vhs deletion mutant, we investigated the mechanism of BHV1-induced down-regulation of MHC class I cell surface expression. In contrast to BHV1 wild-type infection, the BHV1\textsuperscript{vhs}\textsuperscript{−} mutant allows detection of MHC class I molecules at much later time-points after infection. This illustrates the role the vhs protein plays in MHC class I down-regulation. However, even after infection with BHV1\textsuperscript{vhs}\textsuperscript{−}, MHC class I cell surface expression is impaired. In BHV1\textsuperscript{vhs}\textsuperscript{−}-infected cells, MHC class I molecules are retained within the endoplasmic reticulum (ER). Moreover, the transporter associated with antigen presentation (TAP) is still blocked. Temporal control of viral protein expression using chemical inhibitors shows that viral protein(s) expressed within the early phase of BHV1 infection are responsible for ER retention of MHC class I molecules. These results indicate that multiple mechanisms are responsible for down-regulation of MHC class I molecules in BHV1-infected cells.

Introduction

Alphaherpesviruses carry a structural protein, the virion host shutoff (vhs) protein, that inhibits host cell protein synthesis by initiating degradation of cellular mRNAs (Elgadi et al., 1999; Hinkley et al., 2000; Jones et al., 1995a; Oroskar et al., 1987; Read et al., 1993; Smibert et al., 1992). For herpes simplex virus 1 (HSV1) and HSV2, the vhs protein was shown to be encoded by UL41, a well conserved gene among alphaherpesviruses such as varicella-zoster virus (VZV), bovine herpesvirus 1 (BHV1) and pseudorabies virus (PrV). The effect of the vhs protein hampers biochemical studies addressing the biology of virus–host cell interactions. For HSV1, vhs deletion mutants have been constructed that allowed detailed investigation of the function of the vhs protein (Jones et al., 1995a; Oroskar et al., 1987; Read et al., 1993). Moreover, HSV1\textsuperscript{vhs}\textsuperscript{−} mutants were instrumental in many studies addressing cell biological and immunological aspects of HSV1 infection (Geiss et al., 2000; Smith et al., 2000; Tigges et al., 1996; York et al., 1994; Strelow & Leib, 1995).

Here, we report on the construction of a vhs deletion mutant of BHV1 (BHV1\textsuperscript{vhs}\textsuperscript{−}). BHV1 is a member of the genus Varicellovirus and is the aetiological agent of infectious bovine rhinotracheitis and infectious pustular vulvovaginitis in cattle. Although the presence of a vhs gene in BHV1 has been
documented (Schwyzer et al., 1996), the function of the putative BHV1 vhs gene product has not been examined. Based on the high degree of homology of the BHV1 UL41 gene with the vhs-encoding UL41 of HSV1 and HSV2, we decided to construct a UL41 deletion mutant of BHV1. Biochemical experiments described in this study indicate that, contrary to wild-type BHV1, the BHV1 vhs− mutant does not interfere with host cell protein synthesis at early times post-infection. This indicates that UL41 indeed encodes the BHV1 vhs protein.

We used the BHV1 vhs− mutant to examine the mechanisms by which BHV1 evades host immune responses. Like other herpesviruses, BHV1 has the capacity to establish latency and may be periodically reactivated during the lifetime of the host, despite a state of antiviral immunity (Denis et al., 1993; Lawman et al., 1987; Rock, 1994). The fact that BHV1 is capable of establishing a life-long infection in a fully immunocompetent host indicates that this virus is capable of escaping from detection by the immune system. Cytotoxic T cells (CTLs) play an important role in the elimination of virus-infected cells (Townsend & Bodmer, 1989). CTLs recognize antigenic peptides in the context of MHC class I molecules (Townsend & Bodmer, 1989; Yewdell & Bennink, 1992). Highly effective strategies that specifically interfere with MHC class I-restricted activation of CTLs have been found for all herpesviruses scrutinized for such properties. Each step within the MHC class I antigen presentation pathway forms a potential target for virus evasion strategies (Brodney et al., 1999; Fruh et al., 1999; Plough, 1998; Wiertz et al., 1997).

At present, viral gene products that subvert the MHC class I-restricted antigen presentation pathway have been identified for HSV1 and -2 (York et al., 1994), murine (MCMV; Reusch et al., 1999) and human cytomegalovirus (HCMV; Ahn et al., 1996, 1997; Hengel et al., 1997; Jones et al., 1995b, 1996; Wiertz et al., 1996a, b), human herpesvirus-8 (Coscos & Ganem, 2000; Ishido et al., 2000), Epstein–Barr virus (Levitskaya et al., 1995) and gammaherpesviruses-68 (Stevenson et al., 2000). The varicelloviruses VZV (Cohen, 1998), BHV1 (Hariharan et al., 1993) and PrV (Sparks-Thissen & Enquist, 1999) have also been found to down-regulate MHC class I expression, but the viral gene products involved remain to be identified. None of these varicelloviruses code for proteins homologous to the presently known gene products from other (herpes) viruses that interfere with MHC class I antigen presentation.

In the current study, we show that infection of MDBK cells with BHV1 vhs− does not affect MHC class I synthesis during early stages of infection, which indicates that the UL41 gene product plays a role in MHC class I down-regulation. In addition to the vhs protein activity, other BHV1 gene products may interfere with MHC class I expression in a more specific fashion. Previous studies addressing this possibility have been hampered by the vhs effect, which precludes biochemical studies beyond 3 h of infection. The BHV1 vhs− mutant provides us with a unique opportunity to investigate specific interference with the MHC class I pathway of antigen presentation.

### Methods

- **Cells and viruses.** Madin–Darby bovine kidney (MDBK) cells (ATCC) were maintained in RPMI-1040 medium, supplemented with 25 mM HEPES buffer, 2 mM l-glutamine, 10% foetal bovine serum, 140 IU/ml benzylpenicillin and 140 µg/ml streptomycin. The Dutch BHV1.1 field strain Lam (wild-type BHV1), isolated in 1972 from one of the first cases of infectious bovine rhinotracheitis in The Netherlands, was used as parental strain to generate a vhs deletion mutant.

- **Construction of the BHV1 vhs− mutant.** To introduce a deletion in the coding region of the UL41 gene of the parental (Lam) strain, both an upstream region and a downstream region of the UL41 gene were sub-cloned into pUC18. Both regions were isolated from pBR322 clone p115, which contains the 10.7 kb EcoRI–HindIII fragment that harbours the UL41 gene and neighbouring genes from the parent strain (Rijsewijk et al., 1999). The downstream fragment was the 1040 bp BstZ17I–SalI fragment that starts in the open reading frame of UL41 at amino acid residue 336 and ends in UL40. This fragment has been cloned into the HindIII site of pUC18. The upstream fragment was the 5665 bp Apol–Apol fragment that starts at the UL44 (glycoprotein C) gene and ends in the open reading frame of UL41, including the first 150 amino acids of the encoded vhs protein. This fragment has been cloned into the EcoRI site of pUC18, upstream of the downstream fragment. The resulting construct had a unique Xhol site in between the upstream and the downstream fragments. The Xhol site was made blunt and used to insert the expression cassette of pcDNA3 (Invitrogen) carrying the hGFP gene. This cassette is located on the 2.2 kb NrdI–PvuII fragment of pcDNA3-hGFP and contains the HCMV ie1 promoter, the green fluorescent protein gene hGFP and the bovine growth hormone terminator sequence. The resulting recombination plasmid was named pS297. It has a 562 bp deletion in the central part of the open reading frame of the UL41 gene, resulting in the removal of amino acids 151–336 and the loss of expression of the amino acids downstream of residue 336 (Pak et al., 1995; Strolev & Leib, 1996). This deletion includes the most conserved part of the vhs protein, which shows homology to the fen-1 family of nucleases (Doherty et al., 1998). To propagate a UL41 deletion mutant, a 6 kb Xhol fragment was liberated from plasmid pS297 (Fig. 1A) and cotransfected with genomic DNA of the parental strain Lam into embryonic bovine trachea cells according to the method of Graham & Van der Eb (1973). Mutants expressing GFP in the UL41 locus were selected under the UV microscope and were plaque purified three times. Restriction enzyme analysis of the BHV1 vhs− mutant showed no other gross genomic rearrangements than the intended deletion/insertion. The mutant virus can be grown to the same titres as the parent strain (10⁷ TCID₅₀/ml). The viruses were propagated on MDBK cells to obtain stocks with titres of 10⁸ TCID₅₀/ml, which were stored at −80 °C until use.

- **Antibodies.** Bovine MHC class I molecules were immuno-precipitated using the monoclonal antibody (MAb) W6/32, which is specific for a conformation-dependent epitope on the MHC class I heavy chain (Parham et al., 1979). A rabbit polyclonal antiserum recognizing the cytoplasmic tail of MHC class I heavy chains was kindly provided by H. L. Plough (Harvard Medical School, Boston, MA, USA). Although originally raised against human MHC class I molecules, both antibodies cross-react with bovine MHC class I molecules. The monoclonal antibodies IL-A19, recognizing bovine MHC class I complexes, and IL-A165, directed against the bovine transferrin receptor, were a kind gift...
BHV1vhs interferes with MHC class I function

Fig. 1. (A) Construction of a BHV1vhs\(^{-}\) deletion mutant. (I) Structure of the BHV1 genome with its large (L) and short (S) segments. The S segment is bordered by an inverted repeat (hatched boxes). (II) The XhoI fragment harbours the vhs gene and some neighbouring genes, indicated by boxes. The arrows depicted in the genes indicate the transcription direction. The Apol and BstZ17I sites have been used to delete 562 bp in the middle of the vhs open reading frame. (III) The deleted region has been replaced by an expression cassette of the GFP gene. hCMV is the HCMV IE 1 promoter and BGH is the bovine growth hormone terminator sequence. (B) The BHV1 vhs protein blocks host protein synthesis at early times of infection. MDBK cells were mock-infected or infected at an m.o.i. of 10 with wild-type BHV1 or BHV1vhs\(^{-}\) for 2 h. Two or 4 h after infection, the cells were depleted of methionine and cysteine. After 1 h, the cells were metabolically labelled with \(^{35}\text{S}\)methionine/cysteine for 30 min. Cells were lysed in the presence of NP-40 and the proteins indicated in the figure were isolated by immunoprecipitation and separated by 10% SDS–PAGE.

from J. Naessens (ILRAD, Nairobi, Kenya). Bovine immune serum specific for BHV1 was purchased from DAKO. Monoclonal antibodies against BHV1 glycoprotein B (MAb 14) and glycoprotein C (MAb 71) were obtained from ID-Lelystad (Lelystad, The Netherlands). A rabbit polyclonal antiserum specific for ERp57 was a kind gift from T. Wileman (Division of Immunology, Pirbright Laboratory, UK). The mouse MAb 7A11, recognizing a 25 kDa subunit of the proteasome, was purchased from ICN Pharmaceuticals.

Infection of cells and temporal control of viral protein expression. Confluent MDBK cells were washed once with PBS and infected with either wild-type BHV1 or BHV1vhs\(^{-}\) at an m.o.i. of 10 in serum-free RPMI-1640 medium for 2 h at 37 °C, followed by addition of
complete RPMI-1640 medium. In all experiments, mock-infected cells were treated under the same conditions as infected cells. To restrict viral gene expression to immediate-early (IE) or early genes, the protein synthesis inhibitor cycloheximide (50 μg/ml; ICN Pharmaceuticals) was added 15 min before infection and maintained until the metabolic labelling period, when it was removed or replaced by the transcription inhibitor actinomycin D (10 μg/ml; Boehringer Mannheim), which was present throughout the chase. Late viral gene expression was prevented by infecting MDBK cells in the presence of the viral DNA synthesis inhibitor phosphonoacetic acid (PAA; ICN Pharmaceuticals) at a concentration of 300 μg/ml.

Flow cytometry. MDBK cells were infected with either wild-type BHV1 or BHV1vhs− at an m.o.i. of 10 for 2 h and incubated for 10 h. Cells were trypsinized and resuspended in PBS containing 1% BSA. The cells were incubated with IL-A19 or anti-glycoprotein B antibody no. 14 on ice for 1 h. After washing, the cells were incubated with phycoerythrin (PE)-conjugated anti-mouse antibody for 45 min. Stained cells were analysed by flow cytometry (FACScan, Becton Dickinson).

Biochemical experiments. BHV1-infected and mock-infected MDBK cells were incubated for 1 h in RPMI-1640 medium lacking methionine and cysteine, followed by a 30 min pulse with 200 μCi/ml of [35S]methionine/cysteine (Redivue PRO-MIX, Amersham Life Science). Cells were lysed in NP-40 lysis mix containing 50 mM Tris–HCl, pH 7.4, 5 mM MgCl₂ and 0.5% NP-40, supplemented with 1 mM AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride), 1 mM leupeptin and 20 μM Cbz-L3 (carbobenzyloxy-l-leucyl-l-leucyl-l-leucinal-H; Peptides International). Samples were kept on ice throughout the experiment. The post-nuclear supernatant was precleared twice with normal mouse serum, normal rabbit serum and protein G–Sepharose beads (Amersham Pharmacia Biotech). Proteins of interest were extracted from the lysates by 1 h incubation with specific antibodies and 30 min with protein G–Sepharose beads. Immunoprecipitates were washed in 1 × NET buffer (0.5% NP-40, 50 mM Tris–HCl, 150 mM NaCl, 0.1% SDS, 5 mM EDTA, pH 7.4). The pellets were resuspended in SDS sample buffer and boiled at 95 °C for 5 min. Eluted proteins were separated on 10% SDS–polyacrylamide gels and were visualized by autoradiography on Kodak XAR film. Analysis and quantification of radiolabelled proteins were performed using Quantity One quantification software (BIO-RAD Laboratories).

For pulse–chase analysis, cells were labelled for 30 min with 200 μCi/ml of [35S]methionine/cysteine at 37 °C and were either lysed (chase time 0) or incubated in complete medium, supplemented with cold methionine and cysteine at a final concentration of 1 mM for the chase times indicated.

Endoglycosidase H (EndoH) digestion was performed following the instructions of the manufacturer (New England Biolabs). Mock-treated samples served as reaction controls.

Peptide transport assay. BHV1-infected and mock-infected MDBK cells were washed twice with incubation buffer (130 mM KCl, 10 mM NaCl, 1 mM CaCl₂, 2 mM EGTA, 2 mM MgCl₂, 5 mM HEPES, pH 7.3) at 4 °C. The cells were permeabilized in incubation buffer containing 2 IU/ml of Streptolysin O (Murex Diagnostics) for 10 min at 37 °C. Permeabilization was assessed using trypan blue staining. Permeabilized cells (3 × 10⁵ cells per sample) were incubated with 10 μl (∼100 ng) of a radioiodinated model peptide 417 (TVNKTERAY) in the presence or absence of ATP (10 μM final concentration) in a total volume of 100 μl at 37 °C for 10 min (Neefjes et al., 1993). Peptide translocation was terminated by adding 1 ml of ice-cold lysis buffer (1% Triton X-100, 500 mM NaCl, 5 mM MgCl₂, 50 mM Tris–HCl, pH 7.5) and samples were left on ice for 20 min. After centrifugation at 12,000 g supernatants were collected and incubated with 100 μl of concanavalin A–Sepharose to isolate the glycosylated peptides (Amersham Pharmacia Biotech) at 4 °C for 1 h. The beads were washed four times with lysis buffer and the amount of radioiodinated peptide associated was quantified by gamma counting.

Results

BHV1vhs− does not interfere with host cell protein synthesis during early stages of infection

The construction of a vhs deletion mutant of BHV1 is shown in Fig. 1(A). The effects of the vhs deletion were assessed by comparing steady state levels of several cellular proteins immunoprecipitated from MDBK cells either upon infection with wild-type BHV1 or the BHV1vhs− mutant. In wild-type BHV1-infected cells, reduced synthesis of MHC class I heavy chains, proteasome subunits and the endoplasmic reticulum (ER)-resident chaperone ERP57 occurred as early as 3 h post-infection (Fig. 1B, compare lanes 1 and 2). The expression level of these cellular proteins continued to decrease as infection progressed (5 h post-infection; Fig. 1B, compare lanes 4 and 5) and dropped to undetectable levels by 8 h after infection (data not shown). Concomitantly, the expression of viral proteins increased over time, as was apparent from immunoprecipitations performed with a BHV1 immune serum. The reduction in MHC class I expression was observed after immunoprecipitation with both a conformation-dependent MAb, W6/32, and with conformation-independent antibodies directed against the cytoplasmic tail of MHC class I heavy chains. This indicates a virus-induced reduction in the synthesis of MHC class I molecules rather than interference with the formation of MHC class I heavy chain–β₂m–peptide complexes.

In contrast, BHV1vhs− infection of MDBK cells did not influence the synthesis of host cell proteins at early stages of infection. At 3 and 5 h after infection with BHV1vhs−, cellular proteins were synthesized at levels comparable to those in uninected cells (Fig. 1B, compare lanes 1 and 3 with lanes 4 and 6). These results indicate that the BHV1 vhs protein is both necessary and sufficient to reduce de novo synthesis of host cell proteins at these time-points. Therefore, the BHV1vhs− mutant allows us to perform biochemical studies at time-points at which the wild-type virus blocks protein synthesis. We have used the new BHV1vhs− mutant to investigate the mechanisms underlying the down-regulation of MHC class I expression in BHV1-infected cells.

BHV1vhs− affects intracellular trafficking of newly synthesized MHC class I molecules

Using flow cytometry, BHV1 has been shown to down-regulate cell surface expression of MHC class I molecules (Nataraj et al., 1997). From the experiments performed, however, it cannot be concluded whether this down-regulation is the result of a general effect of BHV1 infection, e.g. is caused by the vhs function, or whether the virus specifically interferes
with the surface expression of MHC class I molecules. The BHV1vhs− mutant allows us for the first time to distinguish between these possibilities.

Cell surface expression of MHC class I molecules on wild-type BHV1- and BHV1vhs−-infected cells was examined by flow cytometry (Fig. 2A–D). All cells were infected, as shown by expression of the viral glycoprotein gB (Fig. 2B, D). This experiment indicates that even when the vhs gene is deleted, MHC class I surface expression is still impaired (Fig. 2A, C). It is unlikely that the observed down-regulation is caused by expression of the GFP gene carried within BHV1vhs−, since uninfected, GFP-transfected MDBK cells show no reduction in MHC class I cell surface expression (data not shown). For control proteins, such as the transferrin receptor or MHC class II molecules, no down-regulation was observed (data not shown).

Fig. 2. (A)–(D) Cell surface expression of MHC class I molecules is reduced by infection with BHV1vhs−. MDBK cells were infected with wild-type BHV1 (A and B, dark lines) or BHV1vhs− (C and D, dark lines) or mock-infected (light lines). At 12 h post-infection, MHC class I molecules were stained using IL-A19 (A and C) and BHV1 glycoprotein B was stained using MAb 14 (B and D). Surface expression was measured by flow cytometry. Background levels (PE-conjugated anti-mouse antibody alone) are shown (dotted line). (E) Maturation of MHC class I molecules is impaired in BHV1vhs−-infected cells. MDBK cells were mock-infected or infected with BHV1vhs− at an m.o.i. of 10. At 4–5 h post-infection, cells were metabolically labelled for 30 min (chase point 0) and chased for 60 and 180 min. After lysis of the cells, MHC class I molecules were isolated using the MAb W6/32. Immunoprecipitates were mock-treated (−) or treated (+) with EndoH. Indicated are MHC class I heavy chain molecules resistant (R) and susceptible (S) to EndoH.

Fig. 3. Both wild-type BHV1 and BHV1vhs− inhibit ATP-dependent peptide transport by TAP. MDBK cells were mock-infected or infected with wild-type BHV1 or BHV1vhs− at an m.o.i. of 10. At 4 h post-infection, cells were permeabilized with Streptolysin O and translocation of the iodinated peptide TVNKTERAY into the ER of the cells was measured. Transport rates in the presence (+) and absence (−) of ATP are indicated. The bars represent means of triplicate values, expressed as counts per minute.
D. Koppers-Lalic and others

Fig. 4. BHV1 late proteins are not responsible for inhibition of MHC class I maturation. MDBK cells were mock-infected or infected with BHV1vhs− (m.o.i. of 10) in the absence or presence of PAA. At 8-5 h post-infection the cells were pulse-labelled. PAA remained present throughout the infection, metabolic labelling and chase. (A) MHC class I molecules were immunoprecipitated from the lysates with MAb W6/32. Bovine transferrin receptor (TfR) was isolated using MAb IL-A165. The immunoprecipitates were mock-treated (−) or treated (+) with EndoH. (B) BHV1 glycoprotein B, an early gene product, was isolated from the time-point 0 sample using MAb 14. Glycoprotein C, a late BHV1 gene product, was recovered from the same sample using MAb 71. The autoradiograms on the left part of (A) are shorter exposures of the same experiment.

shown). Thus, in addition to the effect of the vhs protein, post-translational mechanism(s) are responsible for down-regulation of MHC class I surface expression by BHV1.

Biosynthesis and post-translational processing of MHC class I molecules was monitored in BHV1vhs−-infected cells by pulse–chase analysis. MHC class I molecules isolated at different time-points were subjected to digestion with EndoH. In mock-infected MDBK cells, the majority of MHC class I molecules showed conversion to EndoH-resistant forms in the course of the chase, indicating migration of these glycoproteins from the ER–cis-Golgi network to the medial Golgi (Fig. 2E, left panel). Strikingly, MHC class I heavy chains synthesized 4-5 h after infection with BHV1vhs− remained EndoH sensitive, even after 180 min of chase (Fig. 2E, right panel). The fact that the lack of glycan maturation, which presumably reflects ER retention of MHC class I molecules, occurs in BHV1vhs−-infected cells indicates that BHV1 gene products other than the vhs protein are responsible for this effect.

**TAP activity is inhibited in MDBK cells infected with BHV1vhs−**

In addition to being retained in an ER/cis-Golgi compartment, MHC class I molecules isolated from BHV1vhs−-infected cells are unstable at 37 °C (data not shown). This is indicative of a lack of peptides that stabilize MHC class I heavy chain–β2m complexes (Ljunggren et al., 1990). The absence of peptide within MHC class I complexes may be the consequence of inhibited peptide translocation from the cytosol into the ER by the transporter associated with antigen presentation (TAP). HSV1, HSV2 and HCMV encode proteins which act as efficient inhibitors of TAP (Fruh et al., 1995; Hill et al., 1995; Tomazin et al., 1996; York et al., 1994; Ahn et al., 1997; Hengel et al., 1997; Lehner et al., 1997). PrV (Ambagala et al., 2000) and BHV1 (Hinkley et al., 1998) also inhibit transport of peptides by TAP. However, no homologues of viral proteins known to interfere with TAP transport have been found in these viruses. It is possible that the inhibition of TAP in BHV1-infected cells is due to an indirect effect of the vhs protein. To investigate this possibility, we compared TAP activity in mock-infected MDBK cells and in cells infected with either wild-type BHV1 or BHV1vhs−.

At 4 h post-infection, peptide translocation was inhibited in cells infected with wild-type BHV1 (Fig. 3). Similar results were obtained with the BHV1vhs− mutant, which indicates that the vhs protein is not responsible for inhibition of TAP. This also implies that BHV1 encodes a protein that directly interferes with peptide transport by TAP.

**BHV1 early protein(s) is (are) responsible for impaired transport of MHC class I molecules**

The BHV1 genome encodes about 70 proteins, which are expressed in a cascade fashion with three main phases, IE, early and late. As a first step towards the identification of the gene(s) responsible for interference with MHC class I assembly, we employed chemical inhibitors to arrest viral gene expression at each of these phases (Ludwig & Letchworth, 1987). First, the effect of late BHV1 gene products on maturation of MHC class I molecules was examined using PAA. PAA inhibits late gene expression without influencing IE and early gene expression. Two viral glycoproteins were used as a control for PAA-induced inhibition of late viral gene expression: glycoprotein C, a late viral protein, and glycoprotein B, an early viral protein. As expected, expression of glycoprotein C was reduced in the presence of PAA, whereas the synthesis of glycoprotein B was not affected (Fig. 4B).

PAA treatment of uninfected cells had no effect on post-
BHV1-vhs interferes with MHC class I function

Fig. 5. Intracellular transport of MHC class I molecules during the IE and early phases of viral gene expression. MDBK cells were pretreated with cycloheximide (Chx) (□). The cells were mock-infected or infected with BHV1-vhs− (■) in the presence of cycloheximide. After a brief incubation in complete medium supplemented with cycloheximide (□), cells were starved in methionine/cysteine-deficient medium (■) and metabolically labelled (■). The cells were chased in the presence of excess cold methionine/cysteine (□). Actinomycin D (Act D) was added with the starvation medium (A) or with the chase medium (B). MHC class I molecules were isolated from the lysates with W632 and subjected to EndoH treatment. To monitor BHV1 infection, lysates from mock-infected and BHV1-vhs−-infected cells were subjected to immunoprecipitation with bovine BHV1 immune serum (A, right panel). BHV1 early gene expression was monitored using an antibody recognizing glycoprotein B, a prototypical early protein (B, right panel).

Translational processing of MHC class I molecules (Fig. 4A, compare lanes 1 and 2 with 5 and 6). In BHV1-vhs−-infected cells, with or without PAA treatment, MHC class I molecules failed to acquire EndoH resistance (Fig. 4A, compare lanes 7 and 8 with lanes 11 and 12). Based on these data, we can exclude late BHV1 proteins as a cause of impaired maturation of MHC class I molecules.

Maturation of bovine transferrin receptor was not influenced by BHV1 infection or PAA treatment, as this molecule acquired resistance to EndoH within 30 min of chase in BHV1-vhs−-infected, PAA-treated cells (Fig. 4A, top panel).

In the following series of experiments, viral gene expression was limited to IE genes. Cells were infected with BHV1-vhs− in the presence of the protein synthesis inhibitor cycloheximide, which prevents synthesis of viral transactivators of early viral genes. The protocol used in this experiment is summarized in Fig. 5. Cycloheximide treatment not only inhibits protein synthesis but also enhances transcription of IE genes. Prior to metabolic labelling, cycloheximide was replaced by actinomycin D, which inhibits RNA transcription. IE mRNAs are then translated, whereas transcription of early genes is blocked, which leads to enhanced and selective synthesis of IE proteins.
Under conditions of selective IE expression, no difference in MHC class I maturation was observed between mock-infected and BHV1\textsuperscript{vhs−}-infected cells (Fig. 5A). These data indicate that not IE but rather early viral proteins are involved in MHC class I down-regulation. In addition, we can exclude involvement of viral tegument proteins that are released into the cell upon virus entry.

To prove that BHV1 early genes are responsible for the observed ER retention of MHC class I molecules, actinomycin D was not added immediately after removing cycloheximide, but 75 min later. Thus, a time window was created in which the IE proteins that were synthesized could transactivate transcription of early genes. The duration of early gene transcription is defined by the time at which actinomycin D is added. Maturation and intracellular trafficking of MHC class I molecules were monitored by pulse–chase analysis and EndoH treatment of immunoprecipitates (Fig. 5B). In mock-infected cells, the majority of the MHC class I heavy chains acquired EndoH resistance during 120 min of chase. Sequential presence of the transcriptional and translational inhibitors had no effect on expression of MHC class I molecules in control cells. However, more than half of the MHC class I molecules isolated from BHV1\textsuperscript{vhs−}-infected cells still exhibited sensitivity to EndoH treatment after 120 min of chase. The ratio of EndoH-sensitive versus EndoH-resistant material was 25/75 in mock-infected cells and 73/27 in BHV1\textsuperscript{vhs−}-infected cells. In conclusion, chemical control of the virus replication cycle strongly suggests that BHV1 early protein(s) specifically interfere with the maturation of MHC class I molecules.

### Discussion

Infection of MDBK cells with wild-type BHV1 inhibits synthesis of host cell proteins as early as 3 h post-infection. Experiments with the BHV1\textsuperscript{vhs−} deletion mutant constructed in this study indicate that this effect can be attributed to the product of the UL41 gene. Infection of cells with the BHV1\textsuperscript{vhs−} mutant does not influence the synthesis of host cell proteins, including MHC class I molecules, at early time-points after infection. To formally prove that the observed phenotype is due to deletion of the UL41 gene only, we are currently constructing a revertant of the BHV1\textsuperscript{vhs−}.

Whereas the vhs protein of wild-type BHV1 precludes biochemical experiments beyond 3 h after infection, the BHV1\textsuperscript{vhs−} mutant allowed us to monitor the biosynthesis and intracellular trafficking of MHC class I molecules at later stages of infection. Interestingly, the behaviour of MHC class I molecules still remains abnormal in cells infected with the BHV1\textsuperscript{vhs−} mutant. A temporal analysis of intracellular transport of MHC class I molecules by pulse–chase analysis revealed that in BHV1\textsuperscript{vhs−}-infected cells the conversion of mannose-rich glycans of MHC class I molecules into the complex form is inhibited, most likely due to retention of newly synthesized MHC class I proteins in an ER/cis-Golgi compartment (Fig. 2E). BHV1\textsuperscript{vhs−} does not affect intracellular trafficking of other cellular proteins, such as the transferrin receptor (Fig. 4).

The observed ER retention of MHC class I molecules may be related to a lack of peptides, which may be due to inhibition of TAP-mediated peptide transport by BHV1. The experiments shown in Fig. 3 indicate that in cells infected with BHV1\textsuperscript{vhs−}, TAP-dependent peptide translocation is inhibited by 76%. Similar results were obtained with wild-type BHV1, which is in accordance with previous reports (Hinkley et al., 1998). Based on our findings with the BHV1\textsuperscript{vhs−} mutant, we can now conclude that there must be one or more BHV1 gene products that specifically interfere with the function of TAP. Ultimately, MHC class I molecules that have not been loaded with peptides will be degraded in the cytosol (Hughes et al., 1997; Wiertz et al., 1996b).

Inhibition of TAP-dependent peptide transport represents an immune evasion strategy that is often employed by herpesviruses. Three other members of the alphaherpesvirus subfamily, HSV1 (Hill et al., 1995), HSV2 and PrV (Ambagala et al., 2000) also interfere with peptide transport by TAP. In the case of HSV1 and HSV2, inhibition of TAP relies on a cytosolic protein, ICP47. For PrV, the molecule(s) involved in TAP inhibition remain to be identified. Interestingly, HCMV also interferes with TAP-dependent peptide transport, but does so in a completely different fashion. HCMV encodes an ER-resident type 1 membrane glycoprotein, US6, that interacts with luminal domains of the TAP transporter (Ahn et al., 1997; Hengel et al., 1997; Lehner et al., 1997).

Despite the high degree of genomic and biological similarity, searches of genomic and protein databases have not yielded obvious homologues of the closely related HSV1/2 ICP47 or the more distantly related HCMV US6 within the BHV1 genome. Based on our experiments, we cannot conclude whether the BHV1-associated inhibition of TAP involves a soluble or a membrane protein. Permeabilization of BHV1-infected cells and repeated washes prior to the peptide translocation assay did not abrogate the inhibitory effect. A putative cytosolic inhibitor should therefore possess a high binding affinity for TAP, like ICP47 (Tomazin et al., 1996). Alternatively, the TAP inhibitor could be a membrane protein like US6 (Ahn et al., 1997; Hengel et al., 1997), and would thus be resistant to cytosol depletion by washing the permeabilized cells.

The BHV1 genome encodes about 70 proteins, which are expressed during IE, early and late stages of virus infection. To investigate to which sub-group the BHV1 genes that interfere with MHC class I expression belong, we have controlled the viral gene expression cascade using transcription/translation inhibitors and inhibitors of viral DNA synthesis. These experiments indicate that early gene product(s) of BHV1 are responsible for altered intracellular trafficking of MHC class I molecules. In this respect, BHV1 differs from HSV1 and HSV2, which block MHC class I cell surface expression via an IE gene product, ICP47. A recent report on immunoevasive strategies
used by PrV also suggests the involvement of an early gene product in down-regulation of MHC class I surface expression (Ambagala et al., 2000).

At present, it is unclear whether the ER retention of MHC class I molecules in BHV1-infected cells is solely due to a lack of antigenic peptides. The observed retention of MHC class I molecules could involve additional viral protein(s) specifically binding to MHC class I molecules and retaining them in the ER, analogous to the adenovirus E3/19K protein (Burgt & Kvist, 1985), MCMV gp40 (Ziegler et al., 1997, 2000) or HCMV gpUS3 (Jones et al., 1996; Lee et al., 2000), which all prevent egress of MHC class I molecules from the ER. In our biochemical experiments, we did not observe any co-precipitation of viral protein(s) with MHC class I molecules. The association with the viral protein, however, could be weak or transient, as is the case for the MCMV-encoded gp40 (Ziegler et al., 2000).

The use of multiple strategies to subvert antigen presentation is advantageous for viruses. The vhs protein efficiently reduces the synthesis of new MHC class I molecules but has no effect on the existing pool of MHC class I molecules that will continue to present antigenic peptides to CTLs. The effectiveness of multiple independent evasion mechanisms has been clearly illustrated for HSV1 (Tigges et al., 1996). The presence of either the HSV1 vhs protein or ICP47 partially inhibits the lysis of infected fibroblasts by CTLs. When both proteins are present, they act synergistically and almost completely inhibit lysis by specific CTL clones.

In conclusion, we have shown that BHV1 uses at least two different strategies to interfere with the expression of antigen-loaded MHC class I molecules at the cell surface. The BHV1vhs− mutant allowed a biochemical analysis of the integrity of the MHC class I-restricted antigen presentation pathway at time-points at which wild-type BHV1 shuts off host protein synthesis. The BHV1vhs− mutant will facilitate the identification and characterization of the gene product(s) involved in the BHV1-mediated inhibition of MHC class I-restricted antigen presentation.

We would like to thank Stef Letteboer for technical assistance and Drs Caroline Brown, Fimme-Jan van der Wal and Marjolein Kikkert for critically reading the manuscript. We thank Dr J. Naessens (ILRAD, Nairobi) for the monoclonal antibodies IL-A19 and IL-A165, Dr H. L. Ploegh (Harvard Medical School, Boston) for the rabbit anti-heavy chain serum and Dr T. Wileman (Division of Immunology, Purbright Laboratory, UK) for the rabbit polyclonal antisera specific for ERp57.

References


Received 26 March 2001; Accepted 28 May 2001

**BHV1**<sup>vs−</sup> interferes with MHC class I function