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Immunology

The human cytomegalovirus US6 glycoprotein inhibits transporter associated with antigen processing-dependent peptide translocation

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ABSTRACT **In its attempt to evade cytotoxic T cell recognition, human cytomegalovirus encodes several genes that target MHC class I molecules at different points in their assembly pathway. We show here that the human cytomegalovirus US6 gene encodes a 22-kDa glycoprotein that binds the transporter-associated with antigen processing (TAP)/class I complex and inhibits translocation of peptide from the cytosol to the endoplasmic reticulum. Major histocompatibility complex class I molecules are therefore unable to load TAP-dependent peptides, resulting in the retention of MHC class I molecules in the endoplasmic reticulum, with a consequent reduction in class I at the cell surface. Interferon-γ treatment of US6 transfected cells overcomes this inhibition of peptide translocation and restores class I at the cell surface to wild type levels. The functional consequence of TAP inhibition is that US6 transfected cells are unable to present endogenous antigen to cytotoxic T lymphocytes and are therefore resistant to cytotoxic T lymphocyte lysis.**

Human cytomegalovirus (HCMV), a β-herpesvirus, is ubiquitous in human populations worldwide, with primary infection being followed by lifelong persistence of the virus in its host. In immunocompetent individuals the vast majority of infections are asymptomatic, but HCMV is an important pathogen in immunocompromised subjects, most notably organ transplant recipients and AIDS patients, as well as infants infected *in utero*. Strong evidence for the importance of the cellular response in controlling HCMV-associated disease comes from experience with both renal and bone marrow transplant recipients where recovery from HCMV infection correlates with detection of HCMV-specific cytotoxic T lymphocytes (CTL) (1). Furthermore, in the murine model, specific major histocompatibility complex (MHC)-restricted CTL have been shown to provide protection against murine CMV (MCMV) infection (2). Such studies have led to the development of an immunotherapy for HCMV based on the adoptive transfer of HCMV-specific CTL (3, 4).

Perhaps in response to host CTL activity, HCMV has evolved an effective mechanism for disrupting the assembly and transport of peptide-loaded class I molecules. It has been shown that HCMV-infected cells show a dramatic decrease in MHC class I expression at the cell surface (5, 6) and resistance to lysis by CTL (7). Several HCMV genes are now known to be involved in the inhibition of class I MHC surface expression. The complete ≈230-kb genome of HCMV (AD169) has been sequenced and shown to encode in the order of 200 open reading frames (8). Experiments using a series of CMV deletion mutants identified a 7-kb region, encoding 10 genes, as being required for class I down-regulation in HCMV-infected cells (9, 10). Three of the genes encoded within this region, US2, US3, and US11, are able to reduce class I expression at the cell surface when expressed individually (11–14). The US2 and 11 gene products decrease surface MHC class I by causing the rapid translocation of class I molecules from the endoplasmic reticulum (ER) back to the cytosol. The US2 protein has been shown to relocate the nascent ER class I chain back to the cytosol through the translocon (sec61 complex) in a reversal of the normal process of cotranslational insertion into the ER membrane (11). US3 expression causes class I molecules to accumulate in the ER, preventing transport of assembled class I to the cell surface (12, 13). Furthermore, the abundant CMV matrix protein pp65 selectively blocks antigen processing and presentation of the principal immediate-early protein, which is abundantly expressed before the onset of the class I blockade (15).

The down regulation of class I presentation in HCMV-infected cells cannot, however, be entirely attributed to US2, US3, and US11. Recently published work has demonstrated that during the early phase of infection, HCMV inhibits the function of the transporter associated with antigen processing (TAP), blocking the translocation of peptides from the cytosol to the ER lumen (10). None of the previously characterized HCMV genes affects TAP function. In this report we identify the HCMV-encoded US6 gene product as the protein responsible for inhibition of TAP-mediated peptide translocation. US6 is a member of the same family and shares homology with US11 (8). The sequence of US6 predicts a type I transmembrane protein and contains a double-arginine motif, a probable ER retention signal in the C-terminal cytoplasmic domain. We show here that the US6 gene product encodes a membrane bound ER glycoprotein which, unlike the other characterized HCMV genes, binds directly to the TAP complex and inhibits peptide translocation.

MATERIALS AND METHODS

Cell Lines and Viruses. The HeLaM cervical carcinoma cell line (16), the Epstein–Barr virus-transformed B-lymphoblastoid cell line Pala, and their stable US6-transfected derivatives were maintained in Iscove's DMEM (GIBCO/BRL) with 5–10% bovine serum (GIBCO) in the presence of 5% CO_2 at 37°C. Influenza A (PR9) virus was a kind gift from Dr. Jonathan Yewdell.

Peptides. The influenza A nucleoprotein-derived HLA-B8 binding peptide N380–88 was used for the CTL experiments

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Abbreviations: CMV, cytomegalovirus, HCMV, human CMV; MCMV, murine CMV; CTL, cytotoxic T lymphocyte; IFN, interferon; ER, endoplasmic reticulum; MHC, major histocompatibility complex; TAP, transporter associated with antigen processing; endo H; endoglycosidase.

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(17). The peptide used for the TAP translocation data was a variant of a histone protein-derived HLA-B27 binding peptide (18) that contained a glycosylation site (RRYQNSTEL; see ref. 19). The peptide was iodinated to a specific activity of 75–90 cpm/fmol, as described using the chloramine T method (19). Both peptides were synthesized by the Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT).

Antibodies. The following antibodies were used: 148.3, an anti-TAP.1 mAb provided by R. Tampe (20); R.RING4C, a rabbit anti-peptide antibody raised to the C-terminal region of TAP.1 (21); w6/32, a β_2-microglobulin-dependent monomorphic mouse anti-human class I murine mAb (22); 3B10.7, a conformation independent rat anti-human class I antibody (23); R.gp48N, a rabbit anti-peptide antibody raised to the N-terminal region of tapasin (24); Clyde, a rabbit anti-peptide antibody raised to the C-terminal region of calnexin (25); MaP.Igκ (an anti-kappa immunoglobulin light chain mAb). A rabbit anti-peptide antibody was raised to a synthetic 17-mer peptide corresponding to the C-terminal region of US6 (R.US6C).

Cloning and Expression of US6 Gene. The open reading frame of US6 was amplified by PCR using the *pfu* DNA polymerase (Stratagene) from a genomic clone *Hin*dIIIx (26). The US6 forward primer was 5′-GGGGTACCGCCACCATGGATCTCTTGATTCGTCTCG-3′. The US6 reverse primer was 5′-GGGAGCTCTCAGGAGCCACAACGTCG-3′. A consensus Kozak sequence was introduced at the 5′ end to ensure optimal expression. The 574-bp amplification product was cloned into the polylinker of the pCR2.1 cloning vector (Invitrogen). The clone was sequenced in both directions and then cloned into the pMCFR-neo vector (a kind gift of Tom Novak, Wyeth–Ayerst, Princeton, N.J.) using the 5′ *Kpn* and 3′ *Sac*I sites.

Generation of Stable Transfectant Cell Lines. Stable HeLaM.US6 clones were generated by transfecting pMCFR-NEO.US6 using the Lipofectin transfection reagent (GIBCO) and selecting in medium containing 1.2 mg/ml G418 (GIBCO). Stable Pala.US6 clones were generated by electroporation of pMCFR-NEO.US6 at 210 V, 960 μF, as described (27) and selection in medium containing 600 mg/ml G418 (GIBCO). All cell lines were grown in Iscove's MDM (GIBCO) with 5% calf serum (HyClone).

Flow Cytometric Analysis. Surface expression of MHC class I complexes on HeLaM, Pala, and their US6 transfectants was detected by flow cytometry as previously described (28).

Immunoprecipitation and Endoglycosidase H Digestion. Cells were extracted for 30 min on ice at 4×10^6 cells/ml in 1% digitonin (Wako Biochemicals, Osaka) or 1% Triton X-100 (Sigma) in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.4), containing 0.5 mM phenylmethylsulfonyl fluoride (Sigma), 0.1 mM *N*-α-tosyl-L-lysyl-chloromethyl ketone (Sigma), and 5.0 mM iodoacetamide (Sigma). The postnuclear supernatant was cleared overnight at 4°C with 5 μl of normal rabbit serum (GIBCO) and 50 μl of Zysorbin (formalin-fixed) *Staphylococcus aureus* (Zymed) per ml of extract. Aliquots were then incubated for either 1 hr at 4°C with w6/32 followed by 30 min at 4°C with protein A-Sepharose or with antibodies that had been conjugated to Biogel A15M beads, washed in 0.05% SDS/0.1% Triton X-100 and equilibrated in Tris-buffered saline. The immunoprecipitates were washed three times in 0.1% Triton X-100 in Tris-buffered saline, separated by SDS/PAGE, dried and processed for fluorography (w6/32 immunoprecipitates), or washed three times in 0.1% digitonin in Tris-buffered saline, eluted in 0.05% SDS/0.1% Triton X-100, and separated by SDS/PAGE (148.3 and anti-kappa chain immunoprecipitates) and processed for immunoblotting. Bands were quantitated with a Bio-Rad GS-250 Molecular Imager. For endoglycosidase (endo H) digestion, either protein A-Sepharose beads, or crude membranes were solubilized in 60 μl of 20 mM $NaPO_4$ (pH 6.5). SDS (0.1%) and the sample divided in two. Recombinant endo H (2 milliunits, Boehringer Mannheim) was added to one of the samples, and both aliquots incubated overnight at 37°C before analysis by SDS/PAGE and (in the case of membranes) Western blot.

Immunoblots. Blots were performed as described (24). In brief, samples were separated by SDS/12.5% PAGE, and electrophoretically transferred to Immobilon-P membranes (Millipore) at 80 mA for 35 min. The membrane was blocked for 1 hr at 25°C in PBS containing 0.05% Tween 20, 5% dehydrated milk, and 5% bovine serum (Blotto), rinsed in PBS and incubated overnight with a 1 μg/ml dilution of purified R.RING4C and R.gp48N, a 1:1 dilution of 3B10.7 hybridoma supernatant in Blotto, a 1:1000 dilution of anti-calnexin antiserum in Blotto, or a 5 μg/ml solution of purified R.US6C in Blotto. Bands were visualized with mouse anti-rabbit or mouse anti-rat horseradish peroxidase secondary reagent (Jackson ImmunoResearch). Reactive bands were detected by chemiluminescence (Pierce).

Peptide Translocation Assay. Peptide translocation was carried out as previously described (19) using Streptolysin-O (Murex, Norcross, GA) to permeabilize cells.

Virus and Peptide Specific Bulk Culture CTL Lines. Virus and peptide-specific CTL lines were generated as described (7). In brief, peripheral blood mononuclear cells (1.2×10^6) from a healthy donor were infected for 30 min with influenza A virus (PR9) and after inactivation of the virus incubated with uninfected cells (1.08×10^7) in RPMI medium 1640 with 10% AB^+ human serum in upright 25-cm^2 tissue culture flasks. After 7 days the cells were transferred to 24-well tissue culture plates and stimulated with autologous, irradiated, peptide-pulsed (N380–88) peripheral blood mononuclear cells and recombinant human IL-2 (10 unit/ml; Boehringer Mannheim). This restimulation procedure was repeated every 7 days, and the cells tested for cytotoxicity between 4 and 7 days after stimulation.

Cytotoxicity Assays. Target cells (US6 or vector-transfected Pala cells) were labeled and used in cytotoxicity assays as described (28).

RESULTS

Expression of US6 Decreases MHC Class I Expression at the Cell Surface and Prevents Presentation of Endogenous Antigen to CTL. The hydropathy profile of US6 suggests a 184-amino acid, type I transmembrane protein. The predicted protein product contains two hydrophobic regions, at the N-terminal end a region likely to be signal sequence, and a longer 21-amino acid region at the C-terminal end corresponding to a potential transmembrane segment, which precedes the cytoplasmic tail. To determine whether the US6 gene product affects MHC class I expression, we established stable transfectants in the HeLaM cervical carcinoma cell line and the Pala lymphoblastoid cell line. MHC class I expression was assessed by flow cytometry with the conformationally sensitive w6/32 antibody. A dramatic (up to 20-fold) reduction in cell surface class I staining was seen in 12/15 US6-transfected HeLaM cell clones and 8/10 US6-transfected Pala cell clones (Fig. 1 *A* and *B*). The effect of US6 was specific for MHC class I molecules, as cell surface expression of the transferrin receptor was not altered (data not shown).

Since the number of cell surface, peptide loaded class I molecules required for CTL recognition is very low (30), it was important to determine whether the decrease in class I at the cell surface was sufficient to prevent presentation of endogenous antigen. We therefore examined the effect of US6 expression on antigen presentation using influenza A-specific CTL. Influenza A was chosen as a model because peptide-specific CTL for this virus have been well characterized. Most importantly, the influenza system allows the processing and

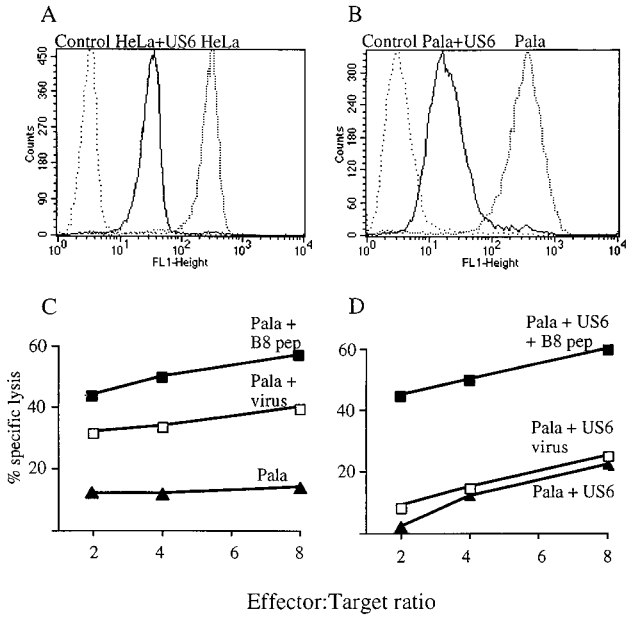

FIG. 1. Expression of the US6 gene decreases cell surface expression of MHC class I molecules and prevents CTL recognition of virus-infected cells. US6 and control-transfected HeLaM cells (*A*) and PaLa cells (*B*) were analyzed for class I surface expression by flow cytometry using the mAb w6/32 and fluorescein-conjugated rabbit anti-mouse IgG. The control was a nonspecific isotype-matched monoclonal antibody. Peripheral blood mononuclear cells from an HLA-B8 positive donor were incubated *in vitro* with influenza A-infected cells and pulsed on day 8 with N380–88 peptide and feeder cells. On day 12 of bulk culture, CTL lysis was tested in a ^{51}Cr release assay against Pala control (*C*) and Pala.US6-transfected (*D*) target cells, which were infected with influenza virus, pulsed with N380–88 peptide (10 μM), or untreated.

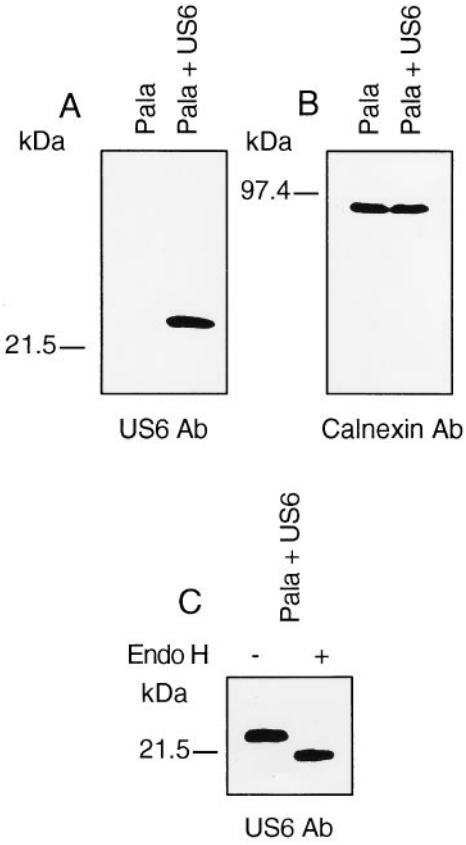

FIG. 2. The US6 gene encodes a 22-kDa glycoprotein. US6 and control-transfected Pala cells were extracted in 1% Triton X-100 and lysates separated by SDS/12.5% PAGE, transferred to Immobilon-P membranes, and probed with anti-US6 (*A*) and anti-calnexin (*B*) antibodies. (*C*) Crude membrane extracts from US6 and control-transfected cells were prepared by freeze thawing. Membranes were extracted in endo H buffer, subjected to endo H or mock digestion, separated by SDS/PAGE, and after transfer to Immobilon-P membranes probed with antibody specific for US6.

presentation of whole virus to be compared with peptide-sensitization at the cell surface. Influenza A-specific, HLA-B8(N380–88)-restricted CTL bulk culture lines were generated from peripheral blood mononuclear cells from an HLA-B8 positive subject, as described (28). These CTL showed specific killing of both N380–388 peptide pulsed, and influenza A-infected Pala target cells (Fig. 1*C*). Lysis of virus-infected cells was less than that of peptide-sensitized cells, as has been observed previously, probably due to efficient binding of the optimal 9-mer peptide. In contrast, influenza A-infected, US6-transfected Pala cells showed no increase in CTL lysis over noninfected cells, but were effectively lysed when sensitized with exogenous peptide (Fig. 1*D*). Both US6 and control-transfected Pala cells showed equal levels of viral infection, as assessed by direct immunofluorescence using influenza-specific antisera (data not shown), so the decreased CTL lysis of US6-transfected cells cannot be explained by a difference in infection. These results indicate that in US6-transfected cells, class I expression was sufficiently inhibited to prevent formation of the relatively low number of class I ligands required for CTL recognition.

To further characterize the US6 gene product, a rabbit antiserum was generated to a synthetic peptide corresponding to the C-terminal 17 amino acids of US6, the putative cytoplasmic tail. Western blot analysis from total cell lysates identified a protein with an apparent molecular mass of 22 kDa in US6 expressing cells, consistent with the US6 protein, which was not apparent in an equal number of control cells, as shown by the presence of comparable levels of calnexin (Fig. 2). To determine whether US6 utilizes its single potential glycosylation site, we examined the sensitivity of the US6 protein to endo H digestion. Crude membrane extracts from US6-transfected Pala cells were treated overnight with endo H and analyzed by Western blot. As shown in Fig. 2*C*, the single N-linked glycan of the US6 protein remains endo H-sensitive. The C terminus of US6 contains two adjacent arginine residues (IRRCGS), and a similar motif is found in the ER retention signal in the cytoplasmic tail of calnexin (ERRRCV). These observations are consistent with the product of the US6 gene being an ER-resident glycoprotein.

MHC Class I Molecules Are Retained in the ER/cis Golgi in US6-Transfected cells. As a first step in determining how US6 disrupts class I assembly, a pulse–chase analysis with endo H digestion was performed on metabolically labeled US6-transfected and control cells (Fig. 3). In vector-transfected HeLaM cells, >60% of class I molecules have acquired endo H resistance after 30 min of chase, and by the 90-min chase point all the class I molecules are endo H-resistant. In contrast, in US6-transfected cells the class I molecules remain endo H-sensitive up to the 2-hr chase point. The failure of MHC class I molecules to acquire endo H resistance in US6-transfected cells implies that the class I molecules are retained in the ER/cis Golgi, and are unable to reach the cell surface. These findings differ from the rapid degradation of MHC class I molecules seen in cell lines expressing HCMV-derived US2 and US11 genes and are consistent with either retention of class I heavy chains in the ER, as seen with the adenovirus E3/19K protein (31) and HCMV US3 protein (12, 13), or incomplete assembly of class I due to a deficiency of peptide in the ER lumen. This latter phenotype is observed in TAP-deficient mutant cell lines (32) and was recently observed in

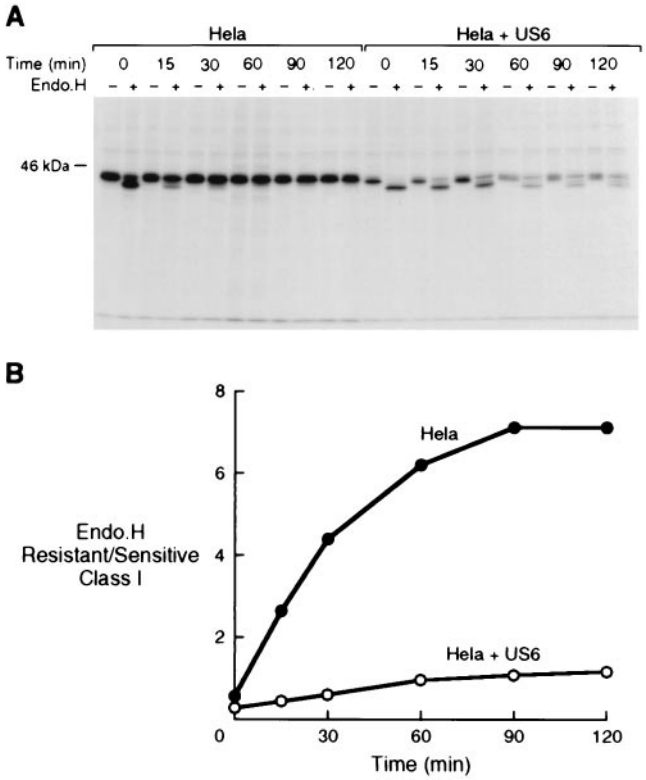

FIG. 3. Transport of class I heavy chains is inhibited in US6-transfected cells. US6 and vector control-transfected HeLaM cells were radiolabeled with [^{35}S]methionine and [^{35}S]cysteine for 15 min and chased for the indicated time periods. Triton X-100 lysates (1%) were immunoprecipitated with the mAb w6/32, digested or mock-digested with endo H, and subjected to 10% SDS/PAGE (*A*), and the ratio of endo H-resistant vs. endo H-sensitive class I heavy chains present at each time point quantitated by image analysis (*B*).

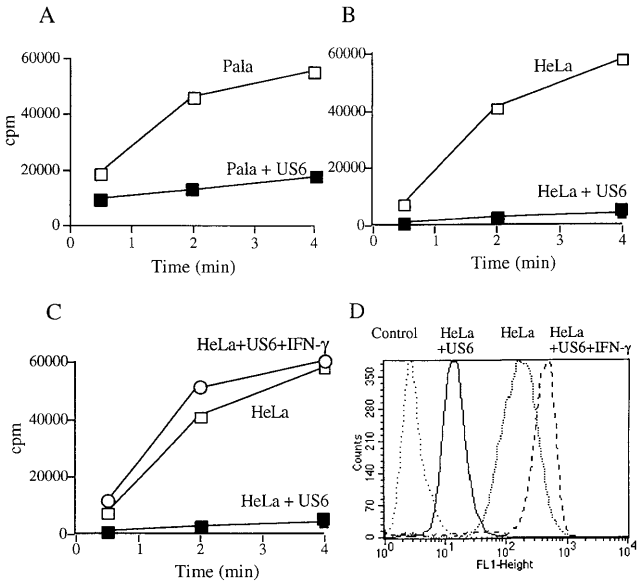

FIG. 4. TAP-mediated peptide translocation is inhibited in US6-transfected cells and can be overcome with IFN-γ. Streptolysin-O (Murex)-permeabilized US6 and control-transfected Pala cells (*A*), HeLaM (*B*), and HeLaM cells that had been pretreated with IFN-γ (200 unit/ml) for 48 hr (*C*) were incubated with an iodinated reporter peptide at 37°C for the indicated time period, and the reaction was stopped by lysis with 3% Triton X-100, as described in *Materials and Methods*. Translocation into the ER was assessed by binding of the glycosylated reporter peptide to concanavalin A-Sepharose beads and counting on a γ-counter. HeLaM, US6-transfected HeLaM, and US6-transfected HeLaM cells that had been pretreated with IFN-γ were analyzed for class I surface expression by flow cytometry, using the mAb w6/32 and fluorescein-conjugated rabbit anti-mouse IgG (*D*). The control was a nonspecific isotype-matched monoclonal antibody.

cells transfected with the herpes simplex-derived ICP47 gene that blocks translocation of peptide into the ER (33–35).

US6 Associates With the TAP Complex and Inhibits TAP-Dependent Peptide Translocation. We and others have assessed TAP mediated peptide translocation in Streptolysin-O (Murex) permeabilized cells *in vitro* (19, 36, 37). To determine whether the impaired maturation of class I molecules reflects a lack of available class I binding peptide in the lumen of the ER, we examined the effect of US6 expression on peptide translocation by TAP. The rate of peptide translocation was compared between US6 and control-transfected cells (Fig. 4). TAP-mediated peptide translocation, as assessed by the glycosylation of an iodinated reporter peptide, was inhibited in both HeLaM and Pala US6-transfected cells, the effect being more marked in US6-transfected HeLaM cells. This inhibition was independent of the concentration of ATP used. Treatment of US6-transfected HeLaM cells with interferon (IFN)-γ overcame the inhibition in peptide translocation (Fig. 4*C*) and resulted in an increase in surface expression of MHC class I (Fig. 4*D*). The increased cell surface expression of class I in US6-transfected cells was observed by 48 hr, and by 72 hr was comparable with that of IFN-γ-treated control HeLaM cells (not shown). These results suggest that the impaired class I maturation and reduced class I cell surface expression is a consequence of inhibition of peptide translocation by TAP, resulting in a decreased amount of peptide in the lumen of the ER.

Efficient binding of peptide by class I molecules requires the assembly of a complex intracellular structure whose components include TAP, tapasin, class I heavy chain, β_2-microglobulin, and calreticulin (24). While it is not known how US6 inhibits TAP function, it was important to determine whether US6 is associated with the TAP/class I complex and whether US6 expression affects assembly of this complex. Comparison of TAP and tapasin immunoprecipitations in [^{35}S]methionine-labeled US6 and vector-transfected cells indicated that assembly of the TAP/class I complex appeared normal in US6 expressing cells. Pulse–chase analysis revealed that TAP stability in US6-transfected cells was unaltered (data not shown). Furthermore, no protein corresponding to US6 was seen in TAP immunoprecipitations from metabolically labeled cells, even when the solubilization was performed in digitonin, a mild detergent that maintains the association of proteins in the TAP complex. Using a different approach, we immunoprecipitated TAP from US6-transfected and control cells and looked for associated proteins by Western blot analysis. Following TAP immunoprecipitation, the dissociated complex was separated by SDS/PAGE, transferred to Immobilon-P membrane, and probed with antisera specific for TAP1, tapasin, class I, and US6 (Fig. 5). In both US6 and control cells, class I, tapasin, and β_2-microglobulin (not shown) associate with TAP as was reported (24). In US6-transfected cells, the US6 protein also associates with the TAP/class I complex, although it was not possible to determine if the association with TAP was direct or required an intermediate protein, such as tapasin.

DISCUSSION

In this study we show that the HCMV US6 gene encodes a 22-kDa glycoprotein that binds the TAP complex and inhibits peptide translocation from the cytosol to the ER. By blocking the delivery of TAP-dependent peptides to the ER, US6 prevents the assembly of class I molecules, resulting in de-

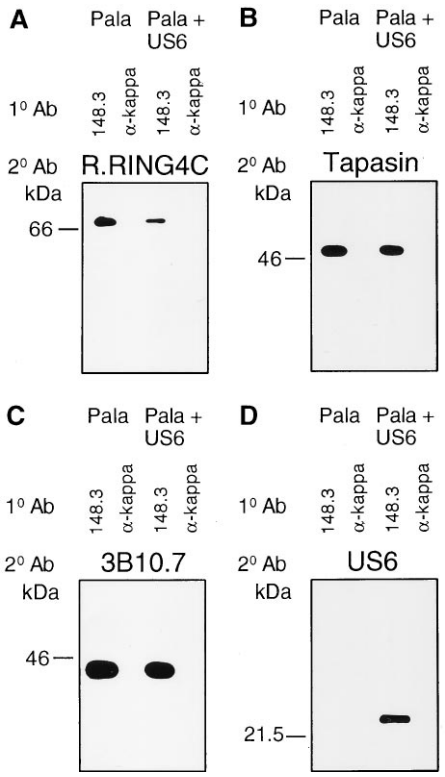

FIG. 5. US6 associates with the TAP/tapasin/class I complex. Digitonin lysates (1%) from US6 and control-transfected PaLa cells were immunoprecipitated with TAP1-specific (148.3) and control (κ immunoglobulin light chain-specific) mAb, coupled to A-15 m Sepharose beads. Precipitated proteins were eluted in 0.1% SDS/0.05% Triton X-100 buffer, separated by 12.5% SDS/PAGE under nonreducing conditions, transferred to Immobilon P membrane, and probed with TAP-specific, R.RING 4C (*A*), tapasin-specific, R.tapasinN (*B*), class I heavy chain-specific, 3B10.7 (*C*), and anti-US6 (*D*) antibodies.

creased MHC class I expression at the cell surface. We have shown that the functional consequence of this block is an inability to present endogenous antigen to CTL.

Since the initial observation that HCMV infection causes a decrease in cell surface class I, this virus has been found to encode four genes whose products are individually capable of blocking class I expression. Three of the four genes now described target different points of the class I assembly pathway. By inhibiting TAP-dependent peptide transport, US6 targets a vulnerable step of class I assembly. It was shown previously that HCMV infection of fibroblasts increases mRNA levels of TAP1 and TAP2 (7) and enhances expression of TAP1 and TAP2 (10). It would therefore seem appropriate for the virus to target TAP as a potential site of inhibition, and thus prevent peptide gaining access to the lumen of the ER. In this regard US6 most closely resembles the herpes simplex virus-encoded ICP47 protein, which also binds TAP and inhibits peptide transport (33–35). Indeed the ICP47 protein alone appears sufficient to cause the reduction in class I seen in herpes simplex virus-infected cells. The intracellular location of US6 has not been directly determined, but its association with TAP and sensitivity to endo H digestion are consistent with US6 being an ER-resident glycoprotein with a short cytoplasmic tail, while ICP47 is a cytosolic protein that inhibits TAP apparently by attaching to its peptide binding site (38, 39). Therefore, these two viral proteins probably use different mechanisms to inhibit TAP. How a lumenal protein inhibits TAP-dependent peptide translocation is not known. US6 associates with the TAP complex and could interfere with TAP function in number of ways. These include a direct association with TAP during its assembly, which may require association with calnexin (24), or interference with its association with other components of the complex. We found no evidence to suggest that US6 affects assembly of TAP or its association with the class I complex. Nor is it at present clear whether US6 associates directly with TAP or requires an intermediary such as tapasin, which mediates class I binding to TAP (24).

In US6-transfected HeLaM cells the inhibition of TAP-dependent peptide translocation could be overcome by treatment with IFN-γ, and was associated with restoration of surface class I expression. IFN-γ has been shown to induce expression of TAP (40), MHC class I, and tapasin (P.J.L. and P.C., unpublished observations) and, depending on the level of US6 expression, it appears that increased levels of these class I components can compensate and overcome the US6 inhibition of TAP. Furthermore, IFN-γ is reported to restore class I expression and antigen presentation in both HCMV and murine CMV-infected cells (41, 42). While the effect of IFN-γ on US2, -3, and -11 gene products has not been reported, overcoming the US6-induced TAP inhibition may contribute to the rescue of class I expression seen in IFN-γ treated, HCMV-infected cells.

There are several reasons why HCMV may have developed multiple independent mechanisms to prevent MHC class I presentation. The US3 gene product is the only immediate early gene product to interfere with MHC class I expression and inhibit maturation and transport of class I in the ER in an allele-specific manner (13). Both the US2 and US11 gene products cause the rapid degradation of MHC class I molecules. Based on a comparison of their ability to degrade murine class I molecules, it has been proposed that these two gene products have nonoverlapping specificities for class I molecules and may have evolved in response to the polymorphism of the MHC (43). Although the US6 gene should prevent class I loading of all TAP-dependent peptides, it would not be predicted to affect signal sequence derived peptides that, for example, can access HLA-A2 in a TAP-independent manner (44, 45). Furthermore, the effect of US6 may be overcome by IFN-γ. Therefore no single mechanism is likely to shut off antigen presentation by all class I alleles. By developing independent but synergistic mechanisms of interfering with class I, HCMV may completely shut down antigen presentation by the majority of class I molecules. In support of this, our CTL data shows that US6 prevents presentation of endogenous antigen, but there is sufficient class I at the cell surface to allow peptide sensitization for CTL lysis. In contrast, it was shown (7) that if HLA-A2 positive fibroblasts were infected with HCMV at a high multiplicity of infection, peptide sensitization did not result in CTL lysis.

Despite the elaborate mechanisms employed by HCMV to evade CTL detection, virus-specific CTL are readily detectable at high frequencies in HCMV-infected subjects and play an important role in the control of viral disease (1, 46). What is striking about the HCMV-specific CTL response is that the majority of CTL are focused on the pp65 structural protein (47, 48). In a quantitative analysis of human CTL responses to HCMV, Wills *et al.* (48) showed that in six healthy subjects, between 70 and 90% of all CTL recognizing HCMV-infected cells were pp65-specific. CTL specific for the HCMV immediate-early gene product (IE-1) were detected, although at a much lower frequency, and it has been reported that only pp65-specific CTL clones were able to lyse HCMV-infected targets (47). pp65 is a structural protein and is present in significant amounts immediately after infection, being derived from input virus and does not require *de novo* synthesis. Peptides from this protein will therefore be able to access class I molecules before the HCMV-encoded genes have had time to exert their effect on class I expression.

In summary, we have shown that the US6 gene encodes a 22-kDa glycoprotein that binds to the TAP/class I complex

and inhibits translocation of peptide into the lumen of the ER. In the absence of available peptides, class I molecules are unable to assemble and are retained in the ER, resulting in an inability to prevent endogenous antigen to CTL. This is the first example of an ER-resident viral glycoprotein to bind TAP and inhibit the transport of peptides.

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