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A Viral ER-Resident Glycoprotein Inactivates the MHC-Encoded Peptide Transporter

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Summary

Human cytomegalovirus inhibits peptide import into the endoplasmic reticulum (ER) by the MHC-encoded TAP peptide transporter. We identified the open reading frame *US6* to mediate this effect. Expression of the 21 kDa US6 glycoprotein in human cytomegalovirus–infected cells correlates with the inhibition of peptide transport during infection. The subcellular localization of US6 is ER restricted and is identical with TAP. US6 protein is found in complexes with TAP1/2, MHC class I heavy chain, β_2-microglobulin, calnexin, calreticulin, and tapasin. TAP inhibition, however, is independent of the presence of class I heavy chain and tapasin. The results establish a new mechanism for viral immune escape and a novel role for ER-resident proteins to regulate TAP via its luminal face.

Introduction

Cytomegaloviruses (CMVs) belong to the β subfamily of herpesviruses, which are large DNA-containing enveloped viruses. Human CMV (HCMV) is an important pathogen causing both acute and chronic infections in the immunologically immature and in the immunocompromised host (Ho, 1982). CMV genes are expressed in a cascade fashion characteristic of herpesviruses during the immediate-early (IE), early, and late phases of infection. CMVs have evolved specific functions to escape cellular immune responses (reviewed by York, 1996). Both HCMV and mouse CMV interfere with the surface expression of major histocompatibility (MHC) class I molecules and antigen presentation to $CD8^+$ T lymphocytes at multiple checkpoints (Barnes and Grundy, 1992; Del Val et al., 1992; Hengel et al., 1995; Jones et al., 1995). In HCMV-infected fibroblasts, the formation of ternary class I heavy chain–β_2-microglobulin (β_2m)–peptide complexes is drastically reduced during the early and late phase of infection (Beersma et al., 1993; Yamashita et al., 1993; Warren et al., 1994).

In the MHC class I pathway of antigen presentation, antigenic peptides generated by cytosolic proteases must be translocated by the ATP-dependent transporter associated with antigen processing (TAP) across the endoplasmic reticulum (ER) membrane for assembly into ternary MHC class I complexes (reviewed by Yewdell and Bennink, 1992; by Heemels and Ploegh, 1995; and by Koopmann et al., 1997). TAP is a heterodimer composed of two homologous proteins, TAP1 and TAP2, both encoded in the MHC. Both subunits are predicted to span the ER membrane 6–10 times with small loops penetrating the cytosol and ER lumen and to possess a large cytosolic domain containing an ATP-binding cassette. The transport of peptides by TAP requires two coupled but independent events. In the first step, the peptide is bound to the cytosolic face of TAP, before it is subsequently translocated in an ATP-dependent manner and released into the lumen of the ER (Androlewicz et al., 1993; Neefjes et al., 1993; Shepherd et al., 1993; van Endert et al., 1994). Recently, the herpes simplex virus 1 (HSV-1) ICP47 protein was demonstrated to inhibit the peptide transport by blocking the peptide-binding site of TAP (Ahn et al., 1996b; Tomazin et al., 1996).

The assembly of MHC class I heavy chain with β_2m and peptide is assisted by transient interactions with molecular chaperones in the ER. Calnexin has been shown to interact with free class I heavy chains (Degen and Williams, 1991; Rajagopalan and Brenner, 1994), and calreticulin binds human class I/β_2m dimers (Sadasivan et al., 1996). MHC class I heterodimers associate with TAP via the TAP1 subunit (Androlewicz et al., 1994; Ortmann et al., 1994; Suh et al., 1994) mediated by an 48 kDa ER glycoprotein, tapasin (Sadasivan et al., 1996). Binding of high-affinity peptides to class I molecules leads to the dissociation of TAP–class I complexes and the exit of ternary class I complexes from the ER (Ortmann et al., 1994; Suh et al., 1994).

The down-regulation of MHC class I expression during permissive HCMV infection was attributed to two gene regions of the HCMV genome, one of which is the gene *US11* (Jones et al., 1995). We have recently described that HCMV infection results in an inhibition of peptide translocation into the ER despite augmented TAP expression in HCMV-infected cells. This effect was not mediated by the gene *US11* and was found to be absent from cells infected with a HCMV deletion mutant, ts9, lacking the genes *US1* through *US15* (Hengel et al., 1996). Ploegh and coworkers have elegantly demonstrated that the *US11*- and *US2*-encoded glycoproteins target class I heavy chains from the ER to the cytosol for rapid proteolytic degradation (Wiertz et al., 1996a, 1996b).

Here we describe the identification of the HCMV gene *US6* encoding a 21 kDa glycoprotein preventing peptide translocation by TAP. In US6-expressing HeLa cells, MHC class I molecules do not acquire peptides and lack transport out of the ER. The subcellular distribution of gpUS6 shows a pattern identical with TAP1, and gpUS6 maintains complete sensitivity to endoglycosidase H

(endo H), indicative of ER-resident proteins. gpUS6 is demonstrated to associate with the TAP–tapasin–MHC–calreticulin complex as well as with calnexin gpUS6 prevented the peptide import into microsomes prepared from mutant cell lines deficient for either MHC class I or for tapasin, indicating that these molecules are not required to block TAP. Both the inhibition of TAP via its ER luminal face and the retained peptide binding to TAP in the presence of gpUS6 underscore a markedly different behavior from ICP47 of HSV-1 and establish a new molecular mechanism to regulate this transporter.

Results

HCMV *US6* Affects MHC Class I Surface Expression, Antigen Presentation to CD8$^+$ Cytotoxic T Lymphocytes, and Peptide Transport into the ER

The absence of peptide transport inhibition in human fibroblasts permissively infected with the HCMV AD169–derived deletion mutant ts9 suggested that the putative inhibitor may reside within the gene region lacking in ts9, that is, *US1* through *US15*. To search for the viral genes that mediate TAP inhibition, we cloned and stably expressed the open reading frames *US1*, *US2*, *US3*, *US4*, *US5*, *US6*, *US7*, *US8*, *US9*, *US10*, *US12*, and *US13* in HLA-A2$^+$ 293 kidney cells and HeLa cells. The transfectants were screened for antigen presentation to HLA-A2 allospecific CD8$^+$ cytotoxic T lymphocyte (CTL) clones (Goulmy et al., 1984), class I surface expression, and TAP-mediated peptide transport. The isolated genes *US2* (data not shown) and *US6* proved to reduce both surface expression of class I molecules and recognition by CD8$^+$ CTL (Figures 1A and 1B). In contrast, the surface expression of CD44 molecules on HeLa cells was not affected by *US6* expression (Figure 1B). In HeLa or 293 cells stably transfected with *US6* or infected with a recombinant vaccinia virus expressing *US6*, a drastic reduction of ATP-dependent peptide transport by TAP was found (Figure 1C). This inhibition was similar to the inhibition seen in transfectants stably expressing the TAP inhibitor ICP47 of HSV-1 (Figure 1C) (Fruh et al., 1995; Hill et al., 1995). Likewise the US6 sequence tagged with the hydrophilic FLAG sequence at the C-terminus inhibited peptide translocation by TAP (Figure 1C). We conclude that HCMV US6 is able and sufficient to interrupt the MHC class I pathway of antigen presentation by reducing the peptide translocation into the ER.

MHC Class I Molecules in HeLa-US6 Transfectants Do Not Aquire Peptides

Peptide-filled MHC class I complexes are characterized by stability at 37°C in 1% NP40 lysate and transport to the medial-Golgi where their carbohydrate moieties acquire resistance to cleavage by endo H (Townsend et al , 1990). To determine whether MHC class I molecules in HeLa-US6 transfectants are loaded with peptide or not, HeLa control cells and HeLa-US6 cells were metabolically labeled with [^{35}S]methionine for 15 min and lysed in 1% NP40 buffer. The lysates were split and aliquots chased for 60 min at 37°C or 4°C, respectively. MHC class I molecules were precipitated with either the conformation-dependent monoclonal antibody (MAb) W6/32 detecting β_2m-associated class I heavy chains (Parham et al., 1979) or MAb HC10 recognizing nonassembled class I molecules (Stam et al., 1986). Half of each precipitate was subjected to endo H digestion and separated by SDS polyacrylamide gradient gel electrophoresis (SDS-PAGE). As depicted in Figure 1D, the formation of MHC class I complexes that remained endo H sensitive was diminished in HeLa-US6 cells. Most strikingly, almost all MHC I complexes formed in HeLa-US6 transfectants were unstable at 37°C, while in HeLa control cells most β_2m-associated class I heavy chains remained stable at 37°C and aquired resistance to endo H cleavage. Conversely, the level of nonassembled MHC class I heavy chains recognized by MAb HC10 was increased in US6-expressing HeLa cells compared to controls (Figure 1D, bottom). Taken together, the results confirm defective peptide loading onto heavy chain/β_2m heterodimers in the presence of the US6 protein resulting in a reduced exit of stably formed MHC class I molecules from the ER.

Synthesis of US6 Protein Correlates with Inhibition of Peptide Transport during Permissive HCMV Infection

As in other herpesviruses, CMV replication is tightly regulated in a multistep process. During productive infection, cellular transcription factors initiate the transcription of IE genes that induce the expression of several sets of early genes, most abundantly expressed 6–60 hr postinfection. Early proteins are required for viral DNA replication followed by the synthesis of late proteins (approximately 48–96 hr postinfection), many of which are incorporated into the virion or aid the process of progeny assembly. The kinetics of US6 protein expression in HCMV wild-type strain AD169–infected fibroblasts during the course of permissive infection was assessed after metabolic labeling and immunoprecipitation with a polyclonal rabbit antiserum raised against synthetic peptide corresponding to amino acids 20–29 of the US6 sequence. From parallel cultures of the same experiment, ATP-dependent peptide translocation by TAP was assessed using the peptide RYWANATRSF. As shown in Figure 1E, the continuous decline in peptide transport correlated with US6 protein synthesis, which was maximal at 72 hr postinfection. Pulse-chase experiments indicated that the US6 polypeptide has a half time of approximately 3 hr (data not shown). We conclude that US6 protein synthesis starts during the early phase and reaches peak levels at 72 hr postinfection in the late phase of the viral replication cycle, while, inversely, TAP-dependent peptide translocation into the ER is progressively decreased.

Subcellular Distribution of the US6 Protein

The putative amino acid sequence of *US6* codes for a type Ia transmembrane protein with a protein core of 21 kDa and a single potential N-linked glycosylation site. To study the subcellular distribution of the US6 protein, confocal laser scanning microscopy of *US6*-transfected HeLa cells was performed using an affinity-purified rabbit antiserum recognizing the luminal domain of the protein. In paraformaldehyde-fixed detergent-solubilized

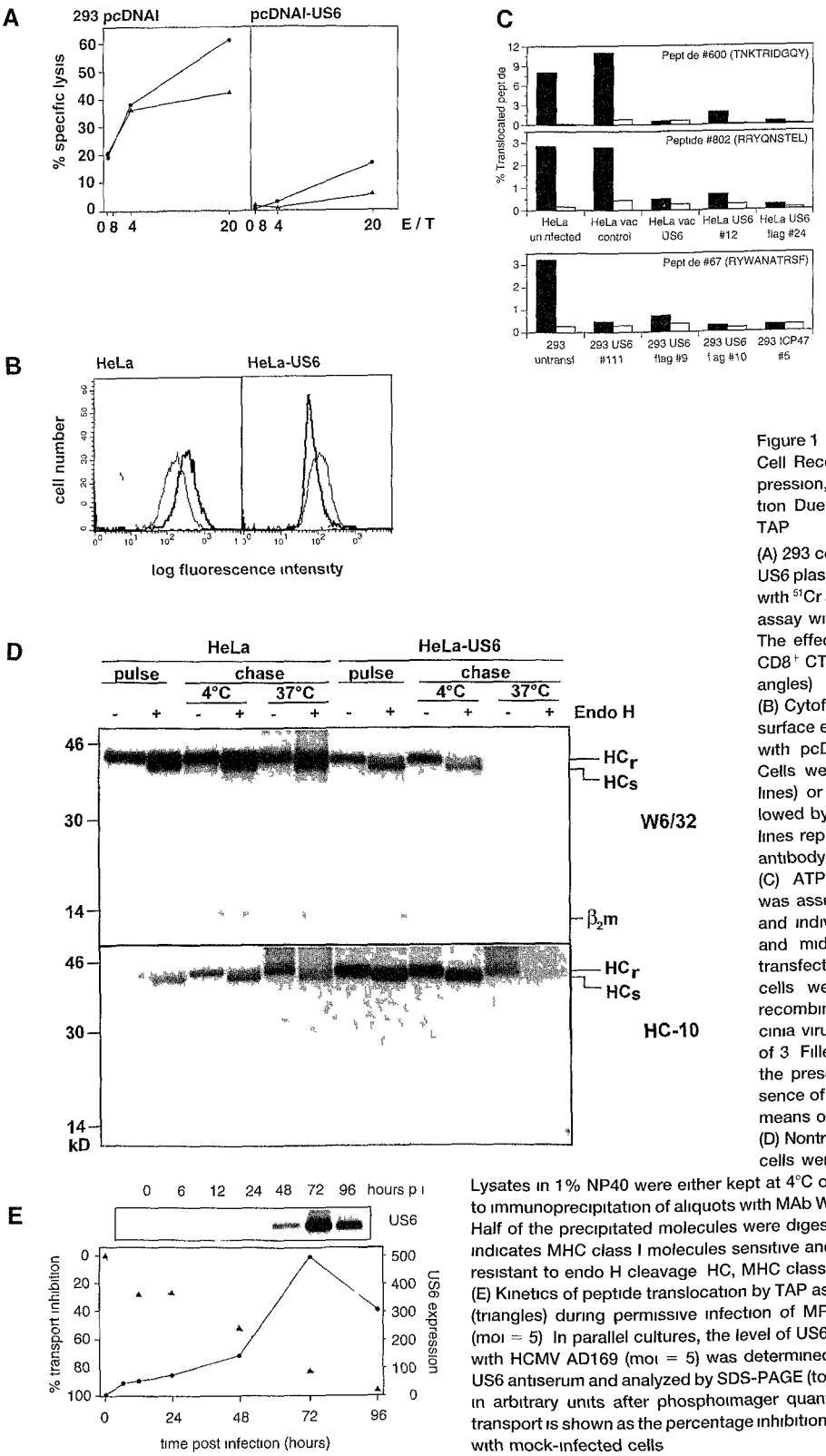

Figure 1 US6 Expression Prevents CD8+ T Cell Recognition, MHC Class I Surface Expression, and MHC Class I Complex Formation Due to Inhibited Peptide Transport by TAP

(A) 293 cells stably transfected with pcDNAI-US6 plasmid or the vector alone were labeled with ^{51}Cr and tested in a 4 hr standard release assay with graded number of effector cells The effectors were the HLA-A2 allospecific CD8+ CTL clones IE2 (circles) and JS132 (triangles)

(B) Cytofluorometric analysis of MHC class I surface expression of HeLa cells transfected with pcDNAI-US6 and HeLa control cells Cells were stained with MAb W6/32 (bold lines) or anti-CD44 MAb (narrow lines) followed by goat-anti mouse IgG-FITC Dotted lines represent control staining with second antibody only

(C) ATP-dependent peptide translocation was assessed for permeabilized HeLa cells and individual US6 transfected clones (top and middle) and 293 cells and 293-US6 transfectants, respectively (bottom) HeLa cells were infected overnight with US6-recombinant vaccinia virus or control vaccinia virus at a multiplicity of infection (moi) of 3 Filled bars represent transport rates in the presence of ATP, open bars in the absence of ATP for control The data represent means of duplicate values

(D) Nontransfected and US6-transfected HeLa cells were metabolically labeled for 15 min Lysates in 1% NP40 were either kept at 4°C or incubated at 37°C for 60 min prior to immunoprecipitation of aliquots with MAb W6/32 (top) and MAb HC-10 (bottom) Half of the precipitated molecules were digested with endo H or mock treated s indicates MHC class I molecules sensitive and r indicates MHC class I molecules resistant to endo H cleavage HC, MHC class I heavy chains

(E) Kinetics of peptide translocation by TAP assessed with peptide RYWANATRSF (triangles) during permissive infection of MRC-5 fibroblasts with HCMV AD169 (moi = 5) In parallel cultures, the level of US6 expression in MRC-5 cells infected with HCMV AD169 (moi = 5) was determined by immunoprecipitation with anti-US6 antiserum and analyzed by SDS-PAGE (top) US6 expression (circles) is shown in arbitrary units after phosphoimager quantitation of the US6 bands Peptide transport is shown as the percentage inhibition of the transport rate (9 2%) obtained with mock-infected cells

cells a typical ER-like staining pattern was observed (Figure 2A), while HeLa control cells were negative (data not shown). The localization of US6 in the ER was confirmed by a nearly perfect colocalization with the ER marker protein BiP (Vaux et al., 1990) (data not shown) and with TAP1, which is primarily located in the ER and can reach cisternae of the *cis*-Golgi (Kleijmeer et al., 1990; Russ et al., 1995) (Figure 2B). The distribution pattern of US6 clearly differed from that of the ER Golgi intermediate compartment (ERGIC) marker ERGIC-p53

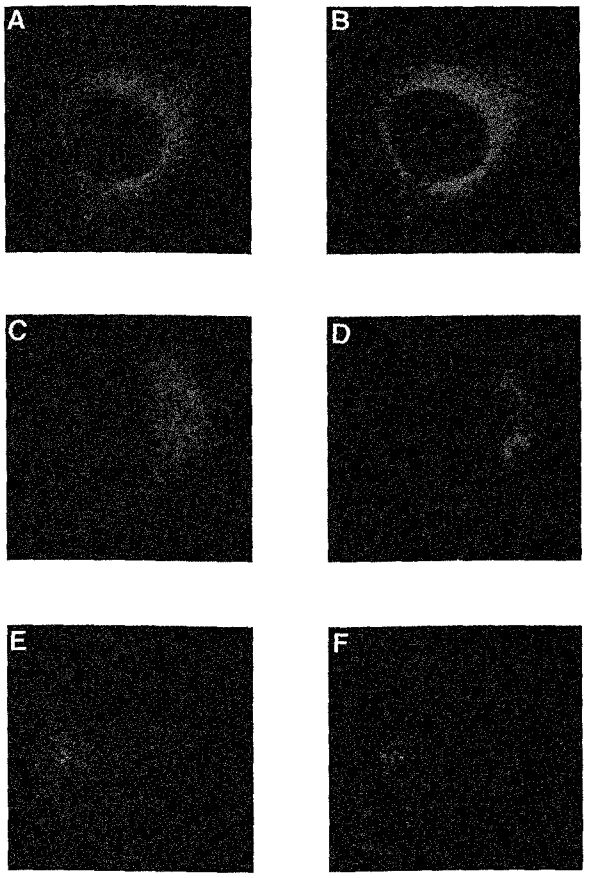

Figure 2 Subcellular Distribution of US6 Visualized by Confocal Laser Scanning Microscopy

HeLa-US6 transfectants were pretreated with 500 U/ml IFNγ for 48 hr before paraformaldehyde fixation and solubilization with 0 1% NP40 Cells were double-stained with (A) anti-US6 antiserum and (B) anti-TAP1 MAb 1 28 and goat anti-rabbit IgG-FITC and goat anti-mouse IgG-TRITC HeLa-US6 cells were double stained with (C) anti-US6 antiserum and (D) mouse MAb G1/93 reactive with p53, a marker protein of the ERGIC Second antibodies as in (A) and (B) HeLa-US6 cells stained (E) with anti-US6 antibodies and (F) mouse MAb CM1A10 recognizing coat proteins of the Golgi Second antibodies as above

(Schweizer et al., 1990) (Figure 2D) and the staining obtained with CM1A10, a coatomer-specific MAb binding to *cis*- and medial-Golgi cisternae (Palmer et al., 1993) (Figure 2F). The data documented a superimposed distribution of US6 and its target, TAP, within the cell and suggested that the US6 polypeptide is a transmembrane ER-resident protein.

gpUS6 Interacts with Multiple ER Proteins Including TAP1/2

To test whether US6 interacts directly with TAP, HeLa-US6 transfectants were incubated with interferon-γ (IFNγ) to stimulate TAP synthesis and labeled overnight with [^{35}S]methionine before lysis in digitonin buffer US6 protein was immunoprecipitated from lysates and recovered immune complexes were eluted and analyzed by PAGE. Bands of approximate molecular weights of 97, 70, 55, 48, and 44 kDa were coprecipitated with US6, a protein of 21 kDa (Figure 3A). Of these, only the 48, 44, and 21 kDa bands were found completely sensitive to endo H, indicating N-linked glycosylation and retention of these molecules in the ER. To characterize the gpUS6-associated proteins further, their pattern was analyzed from the HeLa-US6 transfectant pretreated with IFNγ for 48 hr or not. This proved the polypeptides of 70, 48, and 44 kDa to be inducible by IFNγ while the intensity of the other bands remained constant (data not shown).

To identify the components of the US6 complex, the immunoprecipitate recovered from a digitonin lysate of HeLa-US6 cells was heated in NP40 lysis buffer containing 1 5% SDS, resulting in release of the proteins (Figure 3B, lane 1). After dilution to a final SDS concentration of 0.15% and preclearing of anti-US6 antibodies, reimmunoprecipitation was performed from the supernatant. Reprecipitation with antibodies specific for TAP1 and TAP2 (Figure 3B, lane 2), free class I heavy chain (Figure 3B, lane 3) and calnexin (Figure 3B, lane 4) yielded prominent bands with the expected molecular weight of the proteins in addition to a weaker 21 kDa band corresponding to reassociated gpUS6. Reprecipitation with an anti-calreticulin antibody (Figure 3B, lane 5) recovered no band corresponding to calreticulin but minute amounts of gpUS6, whereas reprecipitation with anti-BiP was negative (Figure 3B, lane 6). In an independent reimmunoprecipitation experiment, antibodies recognizing tapasin (Ortmann et al., 1994; Sadasivan et al., 1996) yielded a band of the appopriate size (48 kDa) from US6 complexes present in a digitonin lysate (Figure 3C, lane 2). In addition, a protein of 12 kDa representing β_2m was precipitated from US6 complexes by MAb BBM1 (Figure 3C, lane 3). To decide whether calreticulin participates in the gpUS6 complex, an immunoprecipitate recovered by anti-calreticulin antibodies (Figure 3D, lane 1) was dissolved in 1.5% SDS and the supernatant precipitated with anti-US6 antibodies As demonstrated in Figure 3D, lane 3, this procedure yielded bands corresponding to TAP, tapasin, and MHC class I but also small amounts of gpUS6.

In conclusion, the data suggest that gpUS6 interacts with the recently described transient assembly complex containing TAP1/2, tapasin, class I heavy chain, β_2m, and calreticulin (Sadasivan et al., 1996). In addition, gpUS6 associates with the ER-resident chaperone calnexin. This interaction may be independent of the complex formation with TAP, since previous studies indicated that in human cells calnexin is not associated with the class I–TAP complex (Ortmann et al., 1994; Sadasivan et al., 1996).

gpUS6 Does Not Prevent Peptide Binding to TAP

The cytosolic TAP inhibitor ICP47 was shown to compete with the ATP-independent binding of peptides to the transporter (Ahn et al., 1996b, Tomazin et al , 1996). Using a photoactivable radioiodinated ^{125}I-TYDNK TRA(Tpa) peptide, we tested whether the binding of peptides to TAP can occur in the presence of gpUS6. Increasing amounts of the photopeptide were incubated with streptolysin O (SLO)–permeabilized HeLa-US6 or

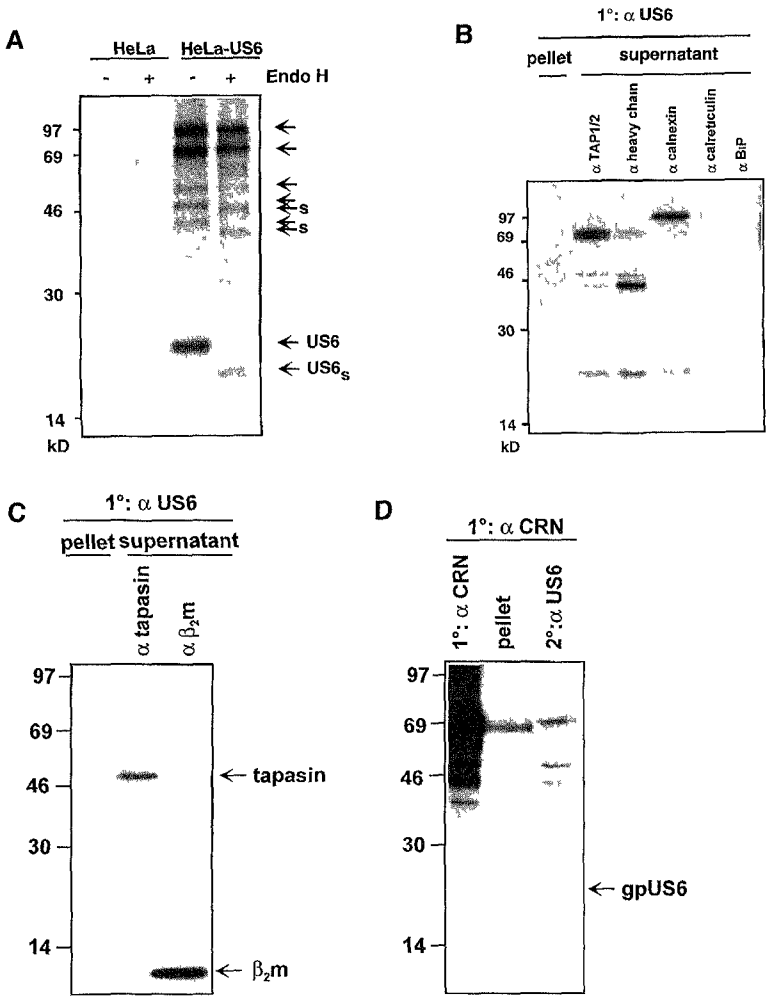

Figure 3. Identification and Characterization of US6-Associated ER Proteins

(A) HeLa and HeLa-US6 transfectants were metabolically labeled overnight and lysed in digitonine lysis buffer. gpUS6 was immunoprecipitated by rabbit anti-US6 antiserum, and proteins were separated by 10%–15% PAGE. US6-associated proteins are indicated by arrows. Immune complexes retrieved from digitonin lysates were mock-digested or digested with endo H. s indicates bands with a mobility shift after endo H digestion.

(B) HeLa-US6 cells were metabolically labeled as described in (A) and lysed in digitonin lysis buffer. Material precipitated with anti-US6 antiserum was heated and dissolved in 1% NP40/1.5% SDS buffer. Proteins not dissociated from the protein A Sepharose pellet were analyzed on lane 1. Aliquots of the supernatant were reprecipitated with anti-TAP1 MAb TAP1.28 and anti-TAP2 MAb TAP2.70 (lane 2), rabbit anti–heavy chain antiserum (lane 3), anti-calnexin MAb AF8 (lane 4), rabbit anti-calreticulin antiserum (lane 5), and rabbit anti-BiP antiserum (lane 6).

(C and D) HeLa-US6 cells were pretreated with 500 U/ml IFN-γ before metabolically labeled as described in (A) and lysed in digitonin lysis buffer. (C) One aliquot of the lysate was precipitated with anti-US6 antiserum. The precipitate was heated and dissolved in 1% SDS. Proteins not dissociated from the protein A Sepharose pellet were analyzed in lane 1. Aliquots of the supernatant were reprecipitated with rabbit anti-gp48 (tapasin) (lane 2) antiserum and MAb BBM1 specific for human β_2m (lane 3). (D) The lysate was precipitated with rabbit anti-calreticulin antibodies. Aliquots of these immune complexes were either directly analyzed (lane 1) or heated and dissolved in 1% SDS. Proteins not dissociated from the protein A Sepharose pellet were analyzed in lane 2. The supernatant was reprecipitated with anti-US6 antiserum (lane 3).

HeLa control cells in the absence of ATP at 4°C. Ultraviolet crosslinking and immunoprecipitation with TAP-specific antibodies from HeLa-US6 cells resulted in bands of about 70 kDa, the intensity of which was not reduced compared to the HeLa control (Figure 4) but was drastically reduced after addition of recombinant ICP47 protein, which blocks peptide binding to TAP (Figure 4, lanes 4 and 8). This result indicates that gpUS6 does not affect peptide binding to TAP. Furthermore, the ICP47-mediated competitive inhibition of peptide binding is independent of the presence of gpUS6. Thus the mechanism employed by US6 for the blockade of peptide transport is different from ICP47.

gpUS6 Does Not Require MHC Class I and Tapasin to Block TAP

To address the role of class I heavy chains or tapasin for the inactivation of TAP1/2 by gpUS6, US6 protein was translated in vitro in the presence of microsomes prepared from HLA-A$^-$, -B$^-$, -C$^+$ tapasin-negative LCL721.220 and HLA-A, -B, -C–negative but tapasin-positive LCL721.221 mutant cells (DeMars et al., 1985; Greenwood et al., 1994; Grandea et al., 1995; Sadasivan et al., 1996) and the microsomes were assayed for ATP-dependent peptide import (Figure 5). In the presence of US6, microsomes of both cell lines completely failed to accumulate glycosylated peptides, while translation of Saccharomyces cerevisiae α-factor mRNA as a control had no effect. Thus, class I heavy chains and tapasin are dispensable for the functional inactivation of TAP1/2. Furthermore, this finding illustrates that in vitro translated US6 protein is able to reach preformed TAP complexes to exert its blocking activity.

Discussion

Despite augmented levels of TAP expression, fibroblasts permissively infected with HCMV exhibit a down-modulation in the capacity to translocate peptides across the ER membrane. This effect is detectable not earlier than 12 hr postinfection and progressively increases during infection (Hengel et al., 1996). This kinetics is consistent with the appearance of a viral inhibitor

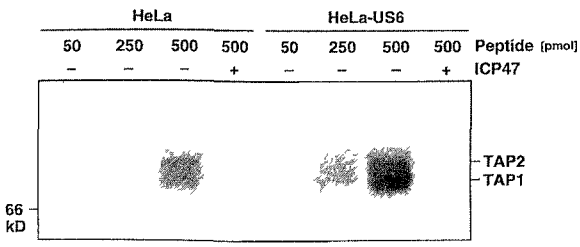

Figure 4. gpUS6 Does Not Prevent Peptide Binding to TAP

SLO-permeabilized HeLa or HeLa-US6 cells were incubated with titrated amounts of the photolabile peptide ^{125}I-TYDNKTRA(Tpa) without (lanes 1–3 and 5–7) or with recombinant ICP47 protein (lanes 4 and 8). After ultraviolet crosslinking, TAP1 and TAP2 molecules were immunoprecipitated by MAb TAP1.28 and TAP2.70 and analyzed by SDS-PAGE.

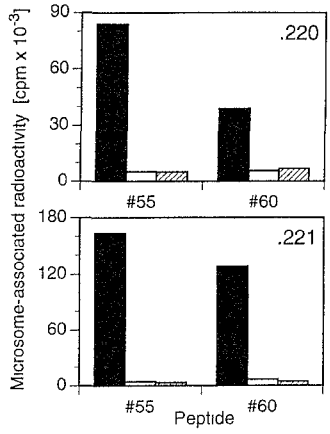

Figure 5. Inhibiton of Peptide Transport by US6 Is Independent of Tapasin and MHC Class I Heavy Chain

US6 protein was in vitro translated into microsomes of tapasin-deficient 721.220 cells and HLA-A, -B, -C–deficient 721.221 cells. The transport capacity of microsomes was determined with glycosylatable peptides 55 (RYWANATRSA) and 60 (RYWANATRSQ). Filled bars represent in vitro–translated control mRNA, transport in the presence of ATP; open bars represent in vitro translation of US6 mRNA, transport in the presence of ATP; hatched bars represent in vitro translation of control mRNA, no ATP added.

the expression of which is relatively low after the onset of the early phase and peaks in the late phase 72 hr postinfection. Here we describe the *US6* gene product to cause the inhibition of TAP-mediated peptide transport. As a consequence of TAP inhibition by gpUS6 and other independent gene functions expressed earlier during HCMV infection (vide infra), the formation of MHC class I complexes, their transport to the cell surface and antigen presentation to CD8$^+$ T cells is abolished in HCMV-infected cells (Beersma et al., 1993; Yamashita et al., 1993; Warren et al., 1994; Hengel et al., 1995). Transient expression of the isolated *US6* gene by recombinant vaccinia virus or stable expression of gpUS6 in transfected cells consistently resulted in a diminished peptide transport function of human cells. The maximum of gpUS6 expression was found to occur at approximately 72 hr postinfection, which perfectly matches the slow kinetics of TAP inhibition. A HCMV deletion mutant, ts9, lacking the genomic region encompassing US6, was previously shown not to impair peptide transport (Hengel et al., 1996). Finally, in cells expressing the HCMV *US11* (Hengel et al., 1996) or *US2* genes (data not shown), which are sufficient to down-regulate MHC class I expression, the peptide transport function is not affected. We conclude that in all likelihood, gpUS6 represents the only relevant HCMV gene product mediating TAP inhibition in the course of HCMV infection.

The reduced transport capacity of gpUS6-expressing cells is demonstrated in vitro using nonnatural peptide sequences that are retained in the ER through an N-linked carbohydrate. Our biochemical analysis of HeLa-US6 cells provided evidence that the vast majority of class I molecules expressed in HeLa cells fails to be loaded with peptides as indicated by lacking thermostability and ER retention. Thus, the impairment of peptide transport is not restricted to selected peptide sequences but appears to apply to the function of TAP in general. Only a small minority of class I ligands may be generated in the ER lumen itself or get access to the ER independent of TAP (reviewed by Momburg et al., 1994a).

The US6 open reading frame encodes a type I transmembrane protein of 21 kDa containing a single N-glycosylation site. We found the whole population of gpUS6 glycosylated. The carbohydrate moiety may contribute to the stability of gpUS6 since the half-life of US6 is reduced in tunicamycin-treated cells (H. H., unpublished data). The complete sensitivity of gpUS6 to endo H indicates efficient retention in the ER consistent with the superimposable immunofluorescence staining for gpUS6 and the ER marker protein BiP. The perfect colocalization with TAP1 appears to make the latter a dedicated target for gpUS6. Since gpUS6 does not contain the C-terminal KKXX consensus motif for ER retention of transmembrane proteins (Jackson et al., 1990), it is unclear by which mechanism gpUS6 is retained in the ER. We have found a prominent association of gpUS6 with the ER-resident chaperone calnexin that associates with incompletely folded glycoproteins through a lectin-like activity (Ou et al., 1993). In contrast to gpUS6 complexes with TAP that are severely reduced or undetectable in NP-40 lysates compared with digitonin lysates, association of gpUS6 and calnexin is less affected by the stronger detergent NP-40 (data not shown). It is conceivable that gpUS6 molecules containing as many as 11 cystein residues slowly attain a mature conformation and that calnexin retains immature gpUS6 molecules in the ER by high-affinity binding.

Although the functional phenotype of HSV-1 ICP47 and HCMV gpUS6 appears similar, these proteins utilize entirely different mechanism for the inhibition of TAP. While the soluble 9 kDa protein ICP47 binds to the cytosolic face of TAP1/2 dimers with high affinity and inhibits peptide association with the transporter in a competitive fashion (Ahn et al., 1996b; Tomazin et al., 1996), gpUS6 does not interfere with peptide binding, which is an ATP-independent and temperature-insensitive step thought to precede the energy-consuming translocation of bound substrate (van Endert et al., 1994). It is unknown how TAP facilitates vectorial transport of peptides varying greatly in their amino acid composition and length

against a concentration gradient (reviewed by Androlewicz and Cresswell, 1996, and by Koopmann et al., 1997). It can be envisaged that amphipathic membrane-spanning segments form a pore that allows the transit of hydrophilic substrates through the lipid bilayer. The energy dependence of peptide translocation and the presence of two nucleotide-binding cassettes within the TAP1/2 dimer suggests that conformational changes of the transporter itself are essentially involved. A stable intercalation of gpUS6 with ER-luminal loops or membrane-spanning segments of TAP1/2 might disturb conformational changes leading to abrogation of transport.

In a minimal model, the inactivation of peptide transport could be explained by the physical association of gpUS6 and TAP without further factors being involved. We have shown here that gpUS6 associates with the multimeric TAP-associated complex rather than disrupting it. This raises the possibility that the interaction with TAP might not be sufficient to prevent peptide transport but requires a cellular cofactor to mediate this effect. Our results obtained with the tapasin-deficient .220 mutants and with HLA-A, B, C–deficient 221 mutant cell indicate that at least these components are not essential for US6-mediated TAP inhibition. The role of calreticulin and calnexin remains to be addressed. The decreased presence of class I heavy chains in the TAP-associated complex at late time points during HCMV infection (Hengel et al., 1996) further strengthens the notion that class I heavy chains are dispensable for TAP inhibition by gpUS6. We favor the idea that gpUS6 directly contacts the TAP1/2 heterodimer, the latter being simultaneously associated with tapasin, MHC class I/β_2m, and calreticulin (Sadasivan et al., 1996). This physical interaction possibly involves a luminal region encompassing residues 78–96 of gpUS6 because an antiserum recognizing this region failed to coprecipitate TAP (H. H., unpublished data) whereas the N-terminal US6 epitope 20–29 allows coimmunoprecipitation as shown here.

Available biochemical evidence suggests that calnexin dissociates from human class I-β_2m heterodimers before the latter associate with calreticulin and with tapasin–TAP (Ortmann et al., 1994, Sadasivan et al., 1996; Solheim et al., 1997). Thus, calnexin does not participate in the human TAP-associated complex, which is in clear contrast to findings in murine cells (Suh et al., 1994, 1996). Therefore, it seems likely that gpUS6 binds to calnexin directly and independent of its association with TAP complexes.

Among the herpesviruses, CMVs have evolved the most extensive genetic repertoire to evade the MHC class I–restricted T lymphocyte response of the host. Mouse CMV expresses three early gene functions that interfere with the MHC class I pathway of antigen presentation (Thale et al., 1995; Kleijnen et al., 1997; Ziegler et al., 1997). HCMV is expressing a cascade of four consecutive US gene functions interrupting the class I pathway of antigen presentation in a general manner. The (IE) protein gpUS3, which is expressed in the very beginning of permissive infection, impairs the transport of MHC class I complexes (Ahn et al., 1996a; Jones et al., 1996). The *US2*- and *US11*-encoded glycoproteins misdirect nascent class I heavy chains into the cytosol where they are rapidly degraded by the proteasome (Wiertz et al., 1996a, 1996b). These genes are abundantly expressed up to 24 hr postinfection but poorly transcribed at later times (Jones and Muzithras, 1991; Tenney and Colberg-Poley, 1991). By contrast, the appearance of gpUS6 is maximal 48–96 hr postinfection when other genes interfering with the MHC class I pathway of antigen presentation become almost silent. The action of gpUS6 at late times of infection may limit the presentation of abundantly expressed structural antigens of the virion like glycoprotein B. Indeed, the CTL response against HCMV glycoprotein B was reported to be relatively weak and predominantly restricted by MHC class II rather than MHC class I (Borysiewicz et al., 1988; Hopkins et al., 1996).

The multitude of stealth genes in the genomes of CMV may be required for several reasons: first, to cover the protracted replication cycle, which takes at least 72 hr in the case of HCMV, and second, to compensate for the opposite effects on MHC class I by cytokines like IFNγ, type I IFNs, and TNFα (Hengel et al., 1994; Hengel et al., 1996) that are produced in infected tissues. Finally, the great demand to regulate the presentation function of a high number of MHC class I alleles and their peptides in different cell types may have favored the diversification of the HCMV US genes and their functions. Our screening procedure failed to identify *US3* as an inhibitor of antigen presentation (Ahn et al., 1996a, Jones et al., 1996). In fact, this could be due to a preference of the US3 glycoprotein for certain human MHC class I alleles (Jones et al., 1996)

Altogether, it appears a fascinating feature of HCMV to use proteins encoded within a single cluster of related genes, i.e. gpUS3 (Ahn et al., 1996a; Jones et al., 1996) controlling ER export of peptide loaded MHC class I molecules on the one hand and gpUS6 controlling ER import of peptides on the other hand, to escape recognition by class I–restricted CTL.

Experimental Procedures

Cell Lines and Antibodies

Human fetal lung fibroblasts, MRC-5 (Bio-Whittacker), in passages 6–16, 293 human kidney cells (ATCC CRL 1573), and human HeLa cells (ATCC CCL-2) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and 2 mM glutamine The human lymphoblastoid cell lines LCL721 220 and LCL 721 221 (DeMars et al., 1985, Greenwood et al., 1994) were grown in RPMI 1640

Antibodies used were the following MAb W6/32 recognizing HLA class I heavy chain/β_2m dimers (Parham et al., 1979) was obtained from the ATCC (HB 95), MAb HC-10 recognizing unassembled class I heavy chains with a preference for HLA-B and -C alleles but also HLA-A locus products (Stam et al., 1986), MAb BBM1 detecting human β_2m (ATCC HB-28), anti-CD44 MAb BA06 was from Oncogene Science (Uniondale, NY), polyclonal rabbit antibodies to calreticulin and BiP were from StressGen (Victoria, British Columbia, Canada), MHC I heavy chain rabbit antiserum was a kind gift from Dr H L Ploegh (Cambridge, MA), MAb AF8 to calnexin was a kind gift from Dr M Brenner (Boston, MA), anti-TAP1 MAb TAP1 28 and anti-TAP2 MAb TAP2 70 have been described (Nijenhuis et al., 1996), polyclonal rabbit antibodies to tapasin (Rgp48N) (Sadasivan et al., 1996) were kindly donated by Dr P Cresswell (New Haven, CT) Polyclonal rabbit antiserum was raised by immunization of rabbits with KLH-coupled synthetic peptides of amino acids 20–29 of the US6 sequence

Viruses and Infection Condition

Virus stocks of HCMV strain AD169 were prepared as described (Hengel et al., 1995) For infections, subconfluent monolayers of fibroblasts were incubated with HCMV at an multiplicity of infection of 5 and centrifuged at 800 × g for 30 min to increase the efficiency of infection. Infections of subconfluent HeLa cells with vaccinia virus was performed at an multiplicity of infection of 3 overnight.

Cytolytic Assay

The generation and maintenance of the CD8$^+$ CTL clone IE2 was described in detail earlier (Goulmy et al., 1984). Clone JS132 was kindly donated by Dr. Jannie Borst, Amsterdam. Target cells were labeled with ^{51}Cr and tested in a 4 hr standard release assay with graded numbers of effector cells. In all experiments, effector-to-target ratios ranged from 20 1 to 0.8:1 Spontaneous ^{51}Cr release in the experiments given did not exceed 30% of the maximal release values measured in the presence of 1% Triton X-100

Cloning and Expression of the HCMV *US6* Gene and Construction of a *US6* Vaccinia Virus Recombinant

The open reading frame of the *US6* gene was cloned after PCR amplification from HCMV AD169 DNA (forward primer 5′-CGCG GGGGATCCGCCGCCATGGATCTCTTGATTCGTCTC-3′, backward primer 5 -CGCGGGTCTAGAGAATTCGCATCAGGAGCCACAACG TCG-3 , resulting in an amplification product of 591 bp) into the pcDNAIneo expression vector (Invitrogen, San Diego, CA). The US6 construct containing the 3 24 bp FLAG sequence (Eastman Kodak, New Haven, CT) was obtained using the backward primer 5′-CGC CCCTCTAGATTACTACTTGTCATCGTCGTCCTTGTAGTCCT CGAG GATATCGGAGCCACAACGTCG AATGGGACG-3′ The PCR product was cloned into the 5′ BamHI and 3′ Xbal restriction sites of pcDNAIneo Intervened by a short spacer (DILE), the hydrophilic FLAG sequence (DYKDDDDK) was fused to the *US6* coding sequence Human 293 kidney cells and HeLa cells were transfected with plasmid DNA by calcium phosphate precipitation. Cell clones were selected in the presence of 0.5 mg/ml G418 and tested for gpUS6 protein expression by immunoprecipitation with US6-specific antibodies.

The open reading frame of the US6 gene sequence was cloned after PCR amplification from HCMV AD169 DNA (forward primer 5 -CGCGGGGGATCCGCCGCCATGGATCTCTTGATT CGTCTC-3′, backward primer 5 -CGCGGGTCTAGAGAATTCGCATCAGGAGCC ACAACGTCG-3′ into the 5′ BamHI and 3 EcoRI sites of plasmid p7 5K131 (Schlicht and Schaller, 1989) This plasmid was used for the construction of the vaccinia recombinant virus vacUS6 by homologous recombination with the vaccinia strain Copenhagen. The recombinant vaccinia virus vacUS6 expressing *US6* were selected by infecting tk-143 cells as described (Volkmer et al , 1987)

Peptide Translocation Assay

The transport assays were performed essentially as described (Neefjes et al , 1993; Momburg et al , 1994b) Peptides 67 (RYWA NATRSF), 600 (TNKTRIDGQY), and 802 (RRYQNSTEL) were radiolabeled with ^{125}I by chloramine-T–catalyzed iodination After trypsinization, HCMV-infected fibroblasts, vaccinia virus–infected HeLa cells, or transfectants were permeabilized with SLO (2.5 U/ml) Next, 1 25 × 10^6 cells per sample were incubated with peptide (1 μM) and 10 mM ATP in 0 1 ml incubation buffer (130 mM KCl, 5 mM HEPES [pH 7 3], 10 mM NaCl, 1 mM $CaCl_2$, 2 mM EGTA, 2 mM $MgCl_2$) for 20 min at 37°C Following lysis in 1% NP40, the glycosylated peptide fraction was isolated with 30 μl concanavalin A–Sepharose slurry and quantified by γ-counting Concanavalin A recovered counts per minute were expressed as percentage of input counts per minute.

Preparation of Microsomes, In Vitro Translations, and Peptide Transport Assay

Microsomes were prepared according to Scheele (1983). In vitro translations were performed using microsomes from the human lymphoblastoid cell lines LCL721 220 and LCL 721.221 *US6* mRNA was transcribed from pcDNAIneo-US6 using T7 RNA polymerase (Promega, Heidelberg, Germany) according to the instructions of the supplier. *US6* mRNA or, for control, S cerevisiae α-factor mRNA was translated in the presence of microsomes. 50 pmol radioiodinated peptide and 10 mM ATP were added in a final volume of 50 μl incubation buffer containing 0.1% BSA and incubated for 20 min at 37°C. Then microsomes were lysed in 1% NP40 and glycosylated peptides recovered with concanavalin A–Sepharose and quantitated by γ-counting

Metabolic Labeling and Immunoprecipitation

Immunoprecipitation was performed as described previously (Hengel et al., 1995, 1996). In brief, semiconfluent layers of HeLa cells were incubated with IFNγ (500 U/ml) and labeled overnight with [^{35}S]methionine and [^{35}S]cysteine (1200 Ci/mmol; Amersham, Braunschweig, Germany) at a concentration of 350 μCi/ml and lysed in lysis buffer (140 mM NaCl, 20 mM Tris [pH 7.6], 5 mM $MgCl_2$, 0.2 mM phenylmethylsulfonyl fluoride, leupeptin and leustatin) with 1% digitonin ^{35}S incorporation into proteins was quantitated in all experiments by liquid scintillation counting of a TCA precipitate or an aliquot of the lysate All lysates used for immunoprecipitation were adjusted to ensure comparability in quantitative terms. After removal of nuclei by centrifugation, lysates were precleared with preimmune rabbit serum and protein A Sepharose. Immune complexes were eluted with sample buffer and analyzed by 10%–15% PAGE. Gels were treated with Amplify (Amersham), dried, and exposed to Bio-MaxMR films (Kodak) at −70°C for 1–7 days In some experiments, bands were quantitated using a Storm 860 Molecular Imager (Molecular Dynamics, Sunnyville, CA). Digestion of immune complexes with 2 mU per sample endo H (Boehringer Mannheim, Germany) was performed at 37°C overnight

In reimmunoprecipitation experiments, HeLa-US6 and control cells were metabolically labeled overnight and lysed in 1% digitonin lysis buffer followed by immunoprecipitation with anti-US6 antibodies. After washing precipitated proteins were dissolved in 1% NP40 lysis buffer containing 1.5% SDS and heated to 65°C for 35 min. After dilution to a final SDS concentration of 0 15% anti-US6 antibodies were removed by two rounds of incubation with protein A Sepharose before reimmunoprecipitation with the appropriate antibodies and protein A Sepharose.

Photocrosslinking of Peptide

We used 10^7 SLO-permeabilized HeLa or HeLa-US6 cells for photocrosslinking with the radioiodinated peptide ^{125}I-TYDNKTRA(Tpa) (4-[trifuoromethyl-diazirinyl]phenylalanin) 4°C by irradiation of the suspension at 254 nm with an ultraviolet lamp as described (Nijenhuis et al., 1996). After 5 min exposure, cells were lysed with buffer containing 1% NP40 for 30 min at 4°C Nuclei were pelleted for 5 min at 2000 × g TAP was immunoprecipitated from the supernatant and immunoprecipitated using MAbs TAP1.28 and TAP2.70 and analyzed by 10% SDS-PAGE. As an inhibitor of peptide binding to TAP, recombinant ICP47 protein (Fruh et al., 1995) was used at a concentration of 30 μg/ml.

Flow Cytometry

HeLa cells were preincubated in 5% goat serum and then stained with MAbs Bound antibodies were visualized by addition of fluorescein-conjugated goat anti-mouse antibodies (Dianova, Hamburg, Germany). As a negative control cells were incubated with the second antibody alone A total of 10^4 cells was analyzed for each histogram on a FACScan IV (Becton Dickinson, San Jose, CA)

Confocal Laser Scanning Microscopy

Subconfluent layers of HeLa-US6 cells were grown on glass coverslips. Cells were rinsed with phosphate-buffered saline (PBS) and fixed with 3% (wt/vol) paraformaldehyde in PBS for 20 min. After blocking unreactable aldehyde groups with 50 mM NH_4Cl and 20 mM glycine in PBS, cells were permeabilized with 0 2% Triton X-100 in PBS. To block nonspecific binding of antibodies, coverslips were incubated in 0.2% (wt/vol) fish skin gelatin in PBS (Sigma, St. Louis, MO) Double immunofluorescence was performed by incubating primary antibodies together with 0.2% gelatin in PBS for 45 min. After extensive washing with PBS, the cells were incubated again with 0 2% gelatine and stained with second antibodies, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G

(IgG) (Dianova) and rhodamine-conjugated goat anti-mouse IgG (Dianova), in 0.2% gelatine for 45 min. After washing with PBS, the glass coverslips were mounted on glass slides with Histosafe (Camon, Wiesbaden, Germany). The mounted cells were analyzed with a laser scanning confocal microscope (Leitz DM IRB, Leica Scanner TCS 4D).

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