Immunity, Vol. 6, 613–621, May, 1997, Copyright ©1997 by Cell Press

The ER-Luminal Domain of the HCMV Glycoprotein US6 Inhibits Peptide Translocation by TAP

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Summary

Human cytomegalovirus (HCMV) inhibits MHC class I antigen presentation by a sequential multistep process involving a family of unique short (*US*) region–encoded glycoproteins. US3 retains class I molecules, whereas US2 and US11 mediate the cytosolic degradation of heavy chains by the proteosomes. In *US6*-transfected cells, however, intracellular transport of class I molecules is impaired because of defective peptide translocation by transporters associated with antigen processing (TAP). Peptide transport is restored in HCMV mutants lacking US6. In contrast to the cytosolic herpes simplex virus protein ICP47, US6 interacts with TAP inside the endoplasmic reticulum lumen, as shown by US6 derivatives lacking the transmembrane and cytoplasmic domains and by the observation that US6 does not prevent peptides from binding to TAP. Thus, HCMV targets TAP for immune escape by a molecular mechanism different from that of herpes simplex virus.

Introduction

A major immune defense against viral infections is mediated by cytotoxic T lymphocytes, which recognize and lyse infected cells upon engagement of the T cell receptor with major histocompatibility complex (MHC) class I molecules presenting viral peptides (Townsend and Bodmer, 1989). Such peptides are generated in the cytosol by proteasomes and translocated into the endoplasmic reticulum (ER) by a dedicated peptide transporter, or TAP, where they associate with empty MHC class I heterodimers consisting of heavy chains and β_2-microglobulin (Lehner and Cresswell, 1996). Only MHC class I molecules that have acquired peptides travel through the Golgi compartment to the cell surface to display their antigenic load.

Under selective pressure of this immune response, viral escape mechanisms that interfere at every step of the antigen presentation machinery have evolved (reviewed by Früh et al., 1997). In cells infected with adenovirus types 2 and 5, intracellular transport of MHC class I molecules is inhibited because of association with the E3-region E19 protein (Andersson et al., 1985; Burgert and Kvist, 1985), which contains an ER-retention motif in its cytoplasmic tail (Jackson et al., 1990). More recent work has unraveled an astonishing diversity in the ways in which members of the herpes virus family interfere with MHC class I antigen presentation. Epstein Barr virus prevents presentation of its latency-associated transcript *EBNA-1* via a stretch of repeat elements by an unknown mechanism (Levitskaya et al., 1995). Herpes simplex virus 1 and herpes simplex virus 2 express a small cytosolic protein, the immediate early gene *ICP47*, which inhibits TAP-dependent peptide translocation (Früh et al., 1995; Hill et al., 1995) by competing with peptides for binding to the cytosolic substrate-binding site of TAP (Ahn et al., 1996b; Tomazin et al., 1996).

Human cytomegalovirus (HCMV) uses an even more complex strategy to prevent MHC class I presentation. The immediate early gene *IE1* is not presented to T cells in the presence of the matrix protein pp65, indicating an antigen-specific mechanism (Gilbert et al., 1996). Moreover, a more general prevention of class I presentation is mediated by several gene products of the unique short (*US*) region (Jones et al., 1995). So far, three different gene products that independently modify the intracellular transport of MHC class I molecules have been identified. The glycoproteins US2 and US11 induce the rapid export of MHC class I heavy chains out of the ER into the cytosol, where they are destroyed by the proteasome (Wiertz et al., 1996a, 1996b). The related glycoprotein US3, however, retains fully assembled MHC class I heterodimers (Ahn et al., 1996a; Jones et al., 1996). As a result of the sequential expression of US3 and US11 during infection, MHC class I molecules are first retained and then degraded during the viral life cycle (Ahn et al., 1996a).

Here we identify a fourth glycoprotein of the HCMV *US* region, US6, which affects antigen presentation independent of US2, US3, or US11 and by a different molecular mechanism, despite its structural relatedness and similar subcellular localization. We show that US6 prevents peptide loading of MHC class I molecules by inhibiting TAP-mediated peptide translocation into the ER. Unlike ICP47, however, US6 inhibits TAP by acting in the ER lumen, without interference with the cytoplasmic substrate-binding site of TAP. Thus, TAP inhibition by US6 represents a novel way in which viruses escape immune detection by cytotoxic T lymphocytes.

Results

Four Glycoproteins of the HCMV US Region Independently Down-Regulate MHC Class I Surface Expression

To identify proteins encoded in the *US* region that would interfere with MHC class I antigen presentation, we expressed each open reading frame, tetracycline controlled, in HeLa cells by transient transfection. Two days

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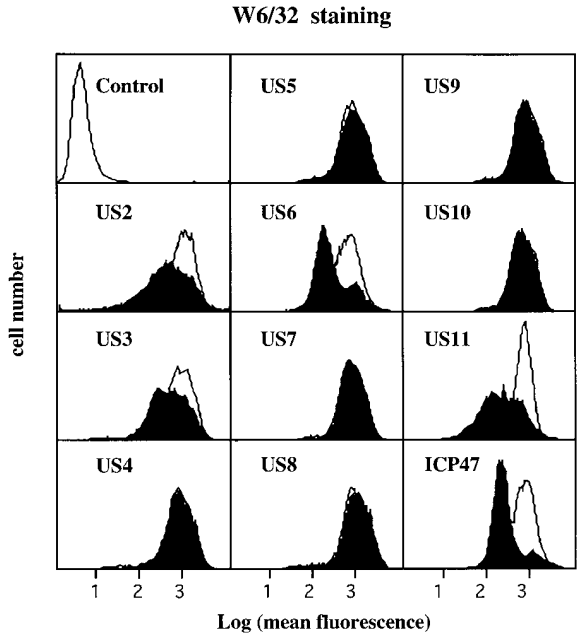

Figure 1. MHC Class I Surface Expression in HCMV US Protein–Expressing HeLa Cells

HeLa cells that stably express tetracycline transactivator (HtTa cells) (Gossen and Bujard, 1992) were transiently transfected with the indicated open reading frames of the HCMV *US* region (Chee et al., 1990), which had been inserted into pUHG10.3. Control cells were transfected with ICP47.pUHG10.3 (Früh et al., 1995). For half of the transfectants, tetracycline was removed (filled graphs) to induce US protein expression. After 48 hr, cell surface expression of MHC class I molecules was monitored by cytofluorometry using MAb W6/32 (Parham, 1983).

after removal of tetracycline, the surface expression of MHC class I molecules was analyzed by cytofluorometry using monoclonal antibody (MAb) W6/32, which specifically recognizes assembled heterodimers (Parham, 1983). A specific reduction of class I surface levels was observed only in cells transfected with *US2*, *US3*, *US6*, and *US11* (Figure 1), whereas surface expression of transferrin receptor as well as cotransfected CD4 was unaffected (data not shown). Expression of the other open reading frames did not affect surface expression (Figure 1), even when *US7–US10* were cotransfected together (not shown). Inhibition of class I antigen presentation by US2, US3, and US11 is in agreement with previous observations (Ahn et al., 1996b; Jones et al., 1996; Wiertz et al., 1996a, 1996b). Since such a function has not been noted for US6 we were interested in comparing the molecular mechanism of US6-mediated MHC class I down-regulation with that of US2, US3, or US11.

MHC Class I Molecules Are Empty in *US6*-Transfected Cells

Therefore, we established a stable HeLa cell line in which US6 expression can be up-regulated by tetracycline removal and studied the biosynthesis of MHC class I molecules by pulse-chase analysis (Figure 2). Similar amounts of W6/32-precipitable class I molecules were synthesized during the 15 min labeling period both in nontransfected and in *US6*-transfected HeLa cells (left lane),

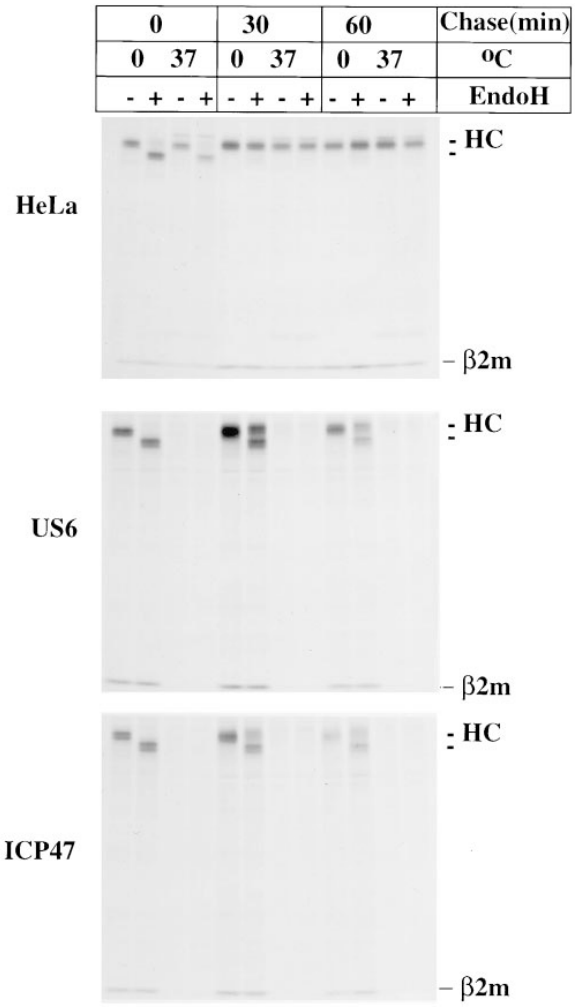

Figure 2. Intracellular Transport and Thermostability of MHC Class I Molecules in *US6*-Transfected HeLa Cells

Untransfected HeLa cells (top) or HtTa cells stably transfected with either *ICP47* (Früh et al., 1995) (bottom) or *US6* (middle) were metabolically labeled for 15 min and chased for the indicated time periods. After lysis in 1% NP40, lysates were divided into two aliquots and either kept on ice or incubated at 37°C for 1 hr. Heterodimeric MHC class I molecules were immunoprecipitated with MAb W6/32 (Parham, 1983) and treated with endoglycosidase H where indicated. Unlike human fibroblast cell lines transfected with *ICP47* (York et al., 1994), class I molecules do not completely remain EndoH sensitive in HeLa cells despite complete inhibition of TAP (Früh et al., 1995). It is possible that some of the haplotypes expressed in HeLa cells are transported in the absence of peptides or with bound signal sequence peptides, as described for HLA-A2.1 (Henderson et al., 1992; Wei and Cresswell, 1992). HC, heavy chain.

indicating that US6 operated at a posttranslational level. However, the intracellular transport of MHC class I molecules, as analyzed by EndoH digestion, was delayed in *US6*-transfected cells (Figure 2), whereas neither the transport of transferrin receptor nor that of EGF receptor was affected (data not shown). Some of the W6/32-reactive material remained in the ER even after chase periods of 60 min (Figure 2) or longer (data not shown), whereas all class I molecules in nontransfected cells had traveled through the Golgi by this time, as indicated

by their EndoH resistance. Thus it seemed that US6 acted by a mechanism different from that of US2 or US11, which reduce the half-life of heavy chains (Wiertz et al., 1996a, 1996b).

We examined the possibility that US6 retains MHC class I molecules similar to US3, but less efficiently. Solubilized US3-retained MHC class I molecules are resistant to increased temperatures (Ahn et al., 1996a) and thus contain peptides (Townsend et al., 1990). However, no class I heterodimers could be immunoprecipitated from *US6*-transfected cells after the lysates were incubated at 37°C for 1 hr at any time of the experiment (Figure 2). In contrast, class I molecules of nontransfected HeLa cells were partially thermostable after 15 min pulse and acquired full thermostability thereafter concomitant with their acquisition of EndoH resistance. This result suggested that class I molecules did not contain peptides in *US6* transfectants regardless of whether they remained in the ER or not. Thus, US6 seems to affect the maturation of MHC class I molecules by a mechanism different from that of US3.

Since "empty" class I molecules can also be observed in *ICP47*-transfected HeLa cells, we compared the intracellular transport and thermostability of *ICP47* transfectants and *US6* transfectants. As shown in Figure 2, the two cell lines were indistinguishable for both assays, suggesting that US6 and ICP47 prevent peptide loading of MHC class I molecules by a similar mechanism.

US6 Prevents Peptide Translocation by TAP

Since ICP47 inhibits TAP, we examined whether US6 directly affects peptide translocation. Using an established assay that monitors peptide translocation independent of MHC class I binding by ConA precipitation of peptides glycosylated upon entry into the ER (Neefjes et al., 1993) we compared TAP activity in nontransfected HtTa cells or in cells stably transfected with *US3*, *US11*, *ICP47*, or *US6* (Figure 3). No significant difference in the amount of glycosylated peptides was observed between noninduced or induced *US3* and *US11* cells (Figure 3) or U373 cells transfected with *US2* (data not shown). By contrast, peptide transport activity was completely inhibited in cells expressing US6, similar to *ICP47* transfectants or after ATP removal. Since peptide translocation was inhibited independently of MHC class I loading, we conclude that peptide loading and impaired MHC class I transport are downstream events of TAP inhibition by US6.

US6 Inhibits TAP in HCMV-Infected Cells

Recently, it was demonstrated that TAP transport is inhibited during HCMV infection (Hengel et al., 1996). Moreover, peptide translocation was restored upon deletion of a 15 kb fragment spanning *US1–US15*. To investigate whether *US6* alone or in combination with other genes encoded in this region is responsible for TAP inhibition during HCMV infection, we analyzed peptide transport activity in cells infected with a series of HCMV mutants (Jones et al., 1995) containing the deletions *IRS1–US6*, *US2–US11*, and *US6–US11* (Figure 4A). Human foreskin fibroblast (HFF) cells were infected either with wild-type HCMV or with the deletion mutants for

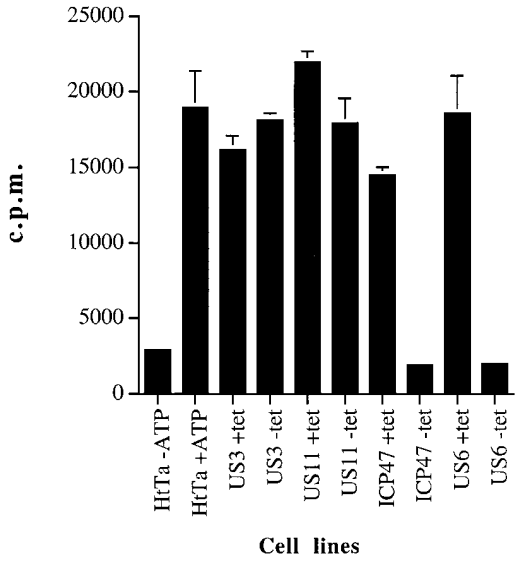

Figure 3. Peptide Translocation in *US*-Transfected HeLa Cells

Translocation of iodinated peptide RYNATGRL into the ER of nontransfected HtTa cells or cells stably transfected with *US3* (Ahn et al., 1996a), *US11* (Ahn et al., 1996a), *ICP47* (Früh et al., 1995), or *US6* was measured after tetracycline had been removed for 24 hr (−tet) to induce expression. First, 0.15 U streptolysin O (Murex) was activated for 10 min at 37°C in 100 μl of transport assay buffer (Neefjes et al., 1993) containing 4 mM dithiothreitol and 10 mM fresh ATP. Transfected or nontransfected HeLa cells (2×10^6) were resuspended in this solution, and after the addition of 2×10^6 cpm labeled RYNATGRL, cells were incubated at 37°C for 25 min. ConA precipitation was perfomed as described (Neefjes et al., 1993). As a control, no ATP was added and residual ATP was removed by apyrase in nontransfected HtTa cells prior to measurement of peptide transport. Results are presented as the mean of duplicate experiments (± standard deviation).

72 hr at a multiplicity of infection (moi) of 5, and peptide translocation was measured by the recovery of glycosylated ^{125}I-labeled reporter peptide library. In contrast to wild-type HCMV, none of these deletion mutants inhibited TAP-mediated peptide translocation in infected HFF cells, whereas peptide translocation was blocked when a synthetic version of ICP47 (sICP47) was added (Figure 4A).

Since the gene absent from all these mutant viruses is *US6*, a deletion mutant lacking this gene was constructed. HFF cells were infected for 72 hr at an moi of 5 with the mutant lacking the *US6* gene. As shown in Figure 4B, the absence of the US6 gene product restored TAP-dependent peptide translocation in HFF cells. These results clearly demonstrate that US6 is solely responsible for the inhibition of peptide translocation in HCMV-infected cells.

Subcellular Localization of US6

The observed functional similarity between ICP47 and US6 contrasts with their different predicted protein structure. ICP47 is a soluble protein that contains 89 amino acids and that localizes to the cytosol (York et al., 1994; Früh et al., 1995), whereas the open reading frame US6 encodes a predicted type I transmembrane glycoprotein of 184 amino acids with some degree of

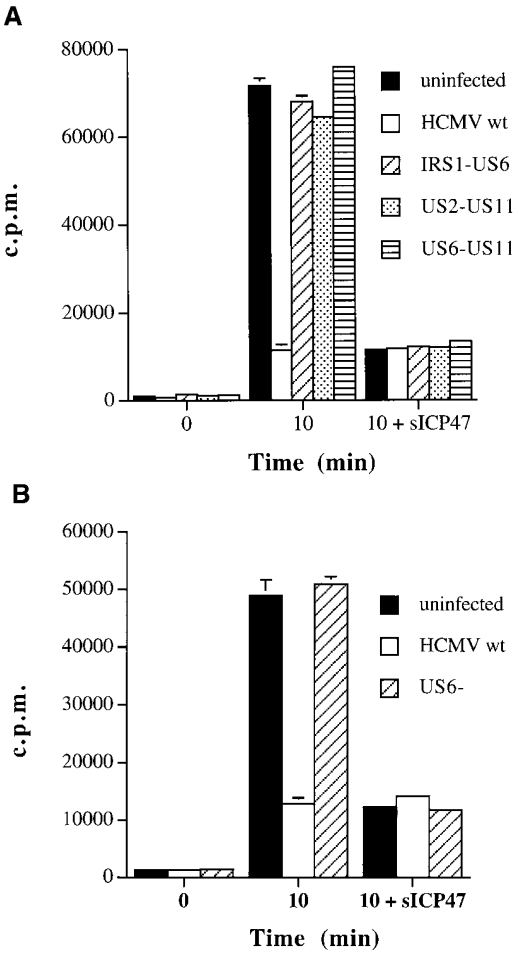

Figure 4. US6 Is Responsible for TAP Inhibition during HCMV Infection

Translocation of a reporter peptide library was measured in HFF cells infected with HCMV mutants containing deletions of multiple genes within the *IRS1–US11* region (A) or with only *US6* deleted (B). Peptide transport assays were performed as described in Experimental Procedures. As a control, peptide recovery after 0 min incubation or after the addition of sICP47 was measured. HCMV mutants *IRS1-US6*, *US2-US11*, *US6-US11*, as well as *US6* have been described previously (Jones et al., 1995).

sequence homology to the open reading frames *US2–US11* (Chee et al., 1990; Ahn et al., 1996a). To study the subcellular localization of US6, we raised antipeptide antibodies against the predicted luminal domain of US6 and compared by immunofluorescence analysis its intracellular localization with that of calnexin, an ER-resident chaperone (Degen and Williams, 1991). When HeLa cells transiently transfected with *US6* were probed with antiserum US6-N, a perinuclear staining was observed for transfected cells but not for nontransfected cells (Figure 5, arrows). A similar pattern was observed both for transfected and nontransfected cells with the calnexin-specific MAb AF8 (Figure 5, bottom). Moreover, costaining indicated that both proteins localized to the same compartment (Figure 5, middle). Thus, we conclude that US6 is an ER-resident glycoprotein as observed for US3 (Ahn et al., 1996a), US11 (Wiertz et al., 1996a) and a subpopulation of US2 (Wiertz et al., 1996b).

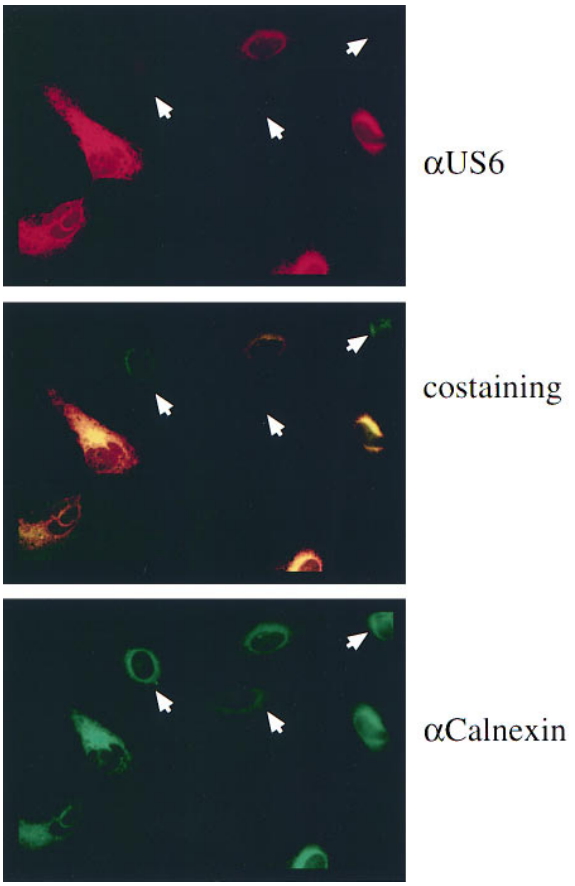

Figure 5. Subcellular Localization of US6

HtTa cells were transiently transfected with *US6* and grown in the absence of tetracycline for 48 hr. US6 was visualized using anti-US6N and goat-anti-rabbit rhodamine conjugate (Cappel). Calnexin was stained with AF8 (Sugita and Brenner, 1994) and visualized with goat-anti-mouse fluorescein isothiocyanate (Cappel). Nontransfected cells that express calnexin but not US6 are indicated by arrows. The same microscopic field was photographed at the excitation wavelengths of rhodamine (top), fluorescein (bottom), or both (middle).

The ER-Luminal Domain of US6 Is Responsible for TAP Inhibition

Since TAP inhibition by an ER-resident protein has not been observed previously, we wanted to know whether US6 inhibits TAP and/or is retained in the ER via its cytoplasmic, transmembrane, or luminal domain. Therefore, we constructed a series of fusion proteins and examined their intracellular localization and transport as well as their ability to reduce MHC class I surface expression and peptide translocation (Figure 6). When the cytoplasmic domain of CD8 was replaced with that of US6, surface levels of MHC class I remained unchanged (Figure 6, CD8US6). Moreover, the cytoplasmic tail of US6 was unable to prevent the intracellular transport of CD8, in contrast to the cytoplasmic tails of a number of ER-resident proteins (Jackson et al., 1990). Thus it seems that the cytoplasmic tail of US6 is not sufficient for either ER retention or TAP inhibition.

To examine whether the luminal domain of US6 alone would inhibit peptide transport, we replaced both the

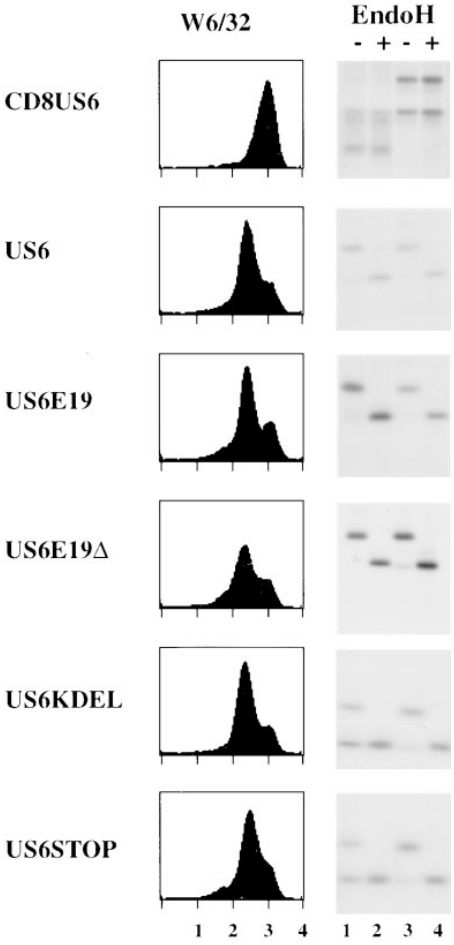

Figure 6. The Luminal Domain of US6 Is Sufficient for ER Retention and MHC Class I Down-Regulation

HtTa cells were transiently transfected with the indicated constructs in the absence of tetracycline. Thirty-six hours after transfection, MHC class I surface expression was monitored by cytofluorometry using MAb W6/32.

(Left) Results are graphically depicted as cell number versus mean fluorescence (log scale).

(Right) The US6 derivatives immunoprecipitated with anti-US6N from NP40 lysates of transiently transfected HtTa cells labeled for 30 min (lanes 1 and 2) and chased for 4 hr (lanes 3 and 4). Lanes 2 and 4 were treated with EndoH prior to SDS polyacrylamide gel electrophoresis. Immunoprecipitation shown in the top panel was performed with anti-CD8; all other immunoprecipitations were carried out with anti-US6N. Intracellular transport of CD8 is indicated by the molecular weight shift due to addition of sialic acid. Since CD8 does not contain N-linked carbohydrates, it is not affected by EndoH. Compared to nontransfected HtTa cells, peptide transport activity was reduced 50%–63% in all transfectants except for CD8-US6, in which no reduction was observed (data not shown).

predicted transmembrane domain and the cytoplasmic tail of US6 with that of CD8 and adenovirus E19, respectively. The resulting fusion protein US6E19 remained EndoH sensitive and down-regulated MHC class I surface expression as a result of TAP inhibition (Figure 6). Interestingly, ER retention as well as TAP inhibition was also observed for the construct US6E19Δ, which contains a deletion in the ER retention signal of the E19 tail (Jackson et al., 1990). These results suggested that the luminal domain of US6 is responsible for both ER retention and TAP inhibition.

To examine whether the luminal domain needs to be anchored in the membrane in order to inhibit TAP, we fused the luminal domain of *US6* to the sequence KDEL, which retains soluble proteins in the ER. In addition, we truncated the *US6* luminal domain by introducing a stop codon at amino acid position 139, before the predicted transmembrane domain. As shown in Figure 6, both US6KDEL and US6STOP remained EndoH sensitive, and both were able to prevent peptide translocation and thus class I surface expression. We conclude that the luminal domain of US6 is responsible for both inhibition of peptide translocation and ER retention of US6.

Association of US6 with the TAP/Class I Complex

It seemed likely that TAP inhibition and/or ER retention involved a direct interaction between US6 and TAP. However, neither of the two anti-US6 antisera coimmunoprecipitated TAP from lysates, even when mild detergents were used for solubilization (data not shown). This might be due to the possibility that these antisera are not able to recognize a US6/TAP complex because their epitopes are involved in TAP binding. Therefore, we used an antiserum against the E19 cytoplasmic tail to immunoprecipitate US6E19 from digitonin lysates of transiently transfected HeLa cells. We modulated the intracellular concentrations of both US6E19 and TAP by using tetracycline or interferon-γ, respectively. As a control, we immunoprecipitated the TAP/class I complex from HeLa cells using anti-TAP1 antiserum, which coprecipitates both MHC class I heavy chain and β_2-microglobulin (Figure 7, lane 1) and at least two additional proteins (arrows) with molecular weights corresponding to calreticulin (top band) and tapasin (bottom band), both of which are thought to be involved in class I binding to TAP (Sadasivan et al., 1996). Neither of these proteins was coprecipitated by anti-E19 from digitonin lysates of CD4E19-transfected cells (lane 2). In contrast, both TAP subunits as well as class I heavy chain and β_2-microglobulin coprecipitated with US6E19, as shown by reprecipitation with the respective antibodies (Figure 7, lanes 8–11). Moreover, the two additional proteins were coprecipitated by anti-E19, indicating that US6 does not disrupt the TAP/class I complex. Interestingly, increasing amounts of TAP/class I complex were coprecipitated upon decreasing US6E19 expression levels (lane 3–7).

We interpret this result as an indication of a specific saturable binding of US6, because upon saturation the ratio of free versus TAP-bound US6 increases with the intracellular concentration of US6. At high expression levels, relatively more unbound US6 will be precipitated if limiting amounts of the E19-specific antiserum are used, as is the case in the experiment shown in Figure 7. A very similar observation was made with ICP47: the majority of ICP47 remains free in the cytosol upon overexpression whereas most ICP47 is bound to TAP at low concentrations (Früh et al., 1995). Since binding to TAP is saturable it is unlikely that US6 is retained in the ER by binding to TAP, because US6E19 does not acquire EndoH resistance regardless of its expression levels

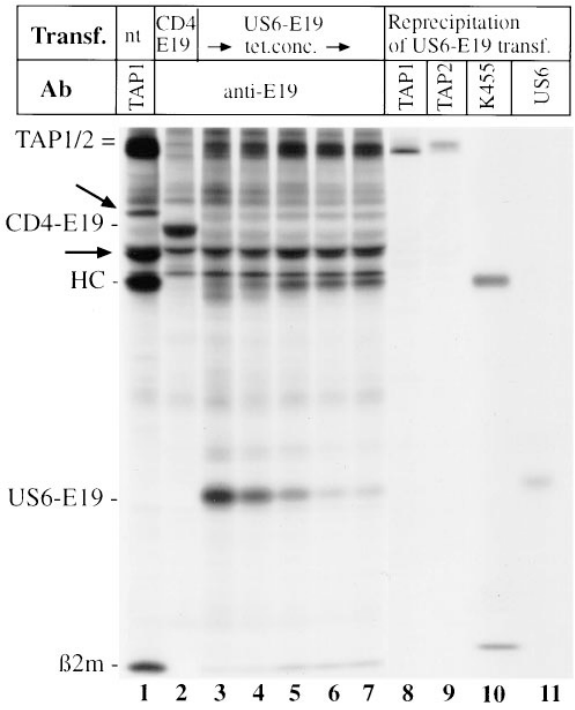

Figure 7. The Luminal Domain of US6 Interacts with TAP

HtTa cell were nontransfected (lane 1) or transfected with CD4E19.p-CMUII (Jackson et al., 1990) (lane 2) or pUHG10.3- US6E19 (lane 3–7). Two days after transfection, cells were metabolically labeled for 16 hr and proteins were solubilized in 1% digitonin and immunoprecipitated with anti-TAP1 (lane 1) or anti-E19 antiserum (Nilsson et al., 1989) (lane 2–7) and separated by SDS polyacrylamide gel electrophoresis (lanes 1–7). A fraction of the anti-E19 precipitate from US6E19-transfected cells was dissolved in 1% SDS and reprecipitated with anti-TAP1 (lane 8), anti-TAP2 (lane 9), K455 (13) (anti-class I [Andersson et al., 1985]) (lane 10), or anti-US6N (lane 11). (Lanes 1–7 and 8–11 are from different gels; hence the slightly different mobility of β_2-microglobulin). Prior to immunoprecipitation, cells were grown for 24 hr without tetracycline (lanes 1–3) or with 0.0001, 0.001, 0.01, or 0.1 μg/ml tetracycline (lanes 4–7) to achieve decreasing expression levels of US6E19. Proteins that are part of the TAP/class I complex as well as CD4E19 are indicated on the left. All other protein bands are nonspecifically immunoprecipitated since they are present in all precipitations. Cells were treated for 24 hr with interferon-γ (1000 U/ml) prior to labeling to increase TAP expression.

(Figures 6 and 7). However, the interaction of US6 with the TAP/class I complex might be required for the blocking of peptide translocation by TAP.

US6 Does Not Inhibit Peptide Binding to TAP

The association of US6 with the luminal portion of TAP is in contrast to ICP47, which approaches TAP from the cytosol. These different modes of interaction might be reflected in different molecular mechanisms of TAP inhibition. We demonstrated previously that ICP47 does not inhibit ATP binding to TAP but competes with peptides for binding to the substrate-binding site of TAP (Ahn et al., 1996b). Likewise, we did not observe an inhibition of ATP binding to TAP from US6-expressing cells (data not shown). To test whether US6 would interfere with peptide binding, we added labeled peptides carrying a photo-cross-linker to streptolysin O–permeabilized cells

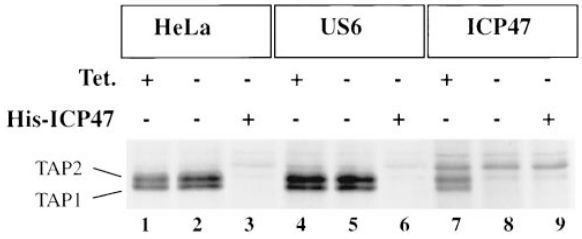

Figure 8. Peptide Binding to TAP in *US6*-Transfected HeLa Cells

Untransfected HtTa cells or HtTa cells stably transfected with *US6* or *ICP47* were grown in the presence or absence of tetracycline prior to streptolysin O permeabilization and incubation with photoreactive peptide KYWANATRSGL together with 1 μM purified recombinant His-ICP47 as described (Ahn et al., 1996b). After ultraviolet cross-linking, heterodimeric TAP molecules were precipitated with anti-TAP1 antiserum and separated by SDS polyacrylamide gel electrophoresis. The relative positions of TAP1 and TAP2 are indicated.

expressing US6 (Figure 8). As a control we used *ICP47*-transfected and nontransfected HeLa cells. In agreement with our previous observations (Ahn et al., 1996b), peptides could not be cross-linked to TAP isolated from ICP47-expressing cells (Figure 8, lane 8) or from HeLa cells treated with purified recombinant His-ICP47 (Figure 8, lane 3). By contrast, US6 expression did not prevent peptide binding (lane 5). Moreover, ICP47 binding to TAP was not affected by US6, since His-ICP47 inhibited peptide binding in US6-expressing cells (lane 6). We conclude that US6 interacts with a different domain of TAP compared to ICP47 and that US6 inhibits TAP by a different mechanism.

Discussion

The HCMV *US* region encodes a family of eight glycoproteins (US2, US3, US6, and US7–US11), all of which share some degree of sequence homology (Chee et al., 1990; Ahn et al., 1996a). Our data show that four members of this family (US2, US3, US6, and US11) are independently capable of preventing MHC class I surface expression. Although US7–US10 had no effect in our assay, we cannot rule out the possibility that a potential interference of US7–US10 with the class I pathway remained undetected by our analysis. For instance, they could affect class I haplotypes not present in HeLa cells or their influence may be too subtle to be detectable in transient experiments. However, since none of the three previously known evasion molecules (US2, US3, and US11) escaped detection by this approach, it seems likely that US7–US10 have other functions unrelated to antigen presentation. Thus, our data suggest that HCMV contains a total of four gene products interfering with the MHC class I antigen presentation pathway. The different mechanisms by which US2, US3, and US11 prevent antigen presentation have been described previously (Ahn et al., 1996a; Jones et al., 1996; Wiertz et al., 1996a, 1996b). Our finding that US6 inhibits peptide translocation by TAP, in contrast to all other US proteins, clearly separates the molecular action of US6 from the other US protein family members.

TAP inhibition has also been observed for the herpes simplex virus protein ICP47 (Früh et al., 1995; Hill et al.,

1995). However, several lines of evidence indicate that the molecular mechanism of TAP inhibition by US6 differs from that of ICP47. During peptide translocation, two steps clearly can be separated experimentally. The initial binding of peptides to TAP does not require ATP (Androlewicz and Cresswell, 1994; Androlewicz et al., 1994; Van Endert et al., 1994), and ATP binds to isolated ATP-binding domains of both TAP1 and TAP2 (Müller et al., 1994; Wang et al., 1994). However, peptide translocation seems to be coupled to ATP-binding and hydrolysis, since peptide transport is inhibited by nonhydrolyzable ATP analogs (Neefjes et al., 1993; Shepherd et al., 1993). Thus, following peptide binding, TAP might undergo ATP-driven conformational changes, releasing peptides from the substrate-binding site to enter the translocation channel. Whereas ICP47 interferes with the first step by competing with peptides for binding (Ahn et al., 1996b; Tomazin et al., 1996), US6 seems to interfere with the subsequent step(s). It might be that binding of US6 to the TAP/class I complex affects TAP conformation, thereby prohibiting peptides from entering the ER. Thus, US6 might be a useful tool to further dissect the molecular steps of peptide translocation.

The different subcellular localization of ICP47 compared to that of US6 together with their different modes of action also supports the hypothesis that the initial peptide binding occurs on the cytoplasmic side of TAP. This hypothesis was based on the findings that bound peptides are cross-linked to TAP residues corresponding to regions of the TAP sequence predicted, by theoretical considerations, to be on the cytoplasmic side (Nijenhuis and Hämmerling, 1996a; Nijenhuis et al., 1996b). Thus, our finding that the ER-luminal portion of US6 is responsible for TAP inhibition is consistent with the inability of US6 to prevent peptide binding to TAP.

Although all of the US proteins studied so far localize to the ER (Ahn et al., 1996a; Wiertz et al., 1996a) (US2 is present both in the ER and cytosol [Wiertz et al., 1996b]), it is not known what causes their ER retention. In analogy to the adenovirus immune evasion protein E19, it seemed possible that the ER-retention motif is separated from the business end of the molecules by the transmembrane domain. However, the cytoplasmic tails of US6 (Figure 6) or any of the other US proteins (our unpublished data) are insufficient for ER retention. Moreover, in the case of US6, both TAP binding and ER retention are mediated by the luminal domain. As discussed above, TAP binding cannot account for ER retention of US6. Therefore, we must assume that US6 is retained by another mechanism, for instance similar to that for misfolded cellular or viral proteins. Such unfolded proteins are usually found in association with ER-resident chaperones (Hammond and Helenius, 1994). However, the relevance of this association for ER retention of unfolded proteins is not known. Further work will be needed to clarify this point.

During the infectious cycle of HCMV, the mRNA for *US6* is most abundant at late times postinfection (Jones and Muzithras, 1991). Accordingly, TAP inhibition has been observed to increase steadily during infection and is complete only during the late phase of infection (Hengel et al., 1996). Thus, the inhibition of peptide loading onto MHC class I molecules seems to follow their retention by US3 during immediate early times and their destruction by US11 and US2 during early and late times of infection. The reason why HCMV has adopted such a sequential strategy to escape immune detection is presently not known. A possible explanation for the operation of several genes in different ways might be that each mechanism is leaky with respect to certain MHC class I haplotypes or certain peptides. For instance, US6 will be unable to prevent TAP-independent presentation of signal sequences generated inside the ER by signal sequence peptidases (Henderson et al., 1992; Wei and Cresswell, 1992). Thus, the different modes of action might be a backup system to ensure complete prevention of MHC class I antigen presentation and thus T cell recognition of HCMV infected cells.

Experimental Procedures

Constructs

The cloning and expression of *US3*, *US11*, and *ICP47* have been described (Früh et al., 1995; Ahn et al., 1996a). The open reading frames *US2*, *US4*, *US5*, *US6*, *US7*, *US8*, *US9*, and *US10* (Chee et al., 1990) were amplified by polymerase chain reaction using HCMV genomic cosmid pCM 1052 as template (Fleckenstein et al., 1982). All 5′-end primers contained the SacII restriction site CCGCGG followed by the sequence CCACCATG, corresponding to a consensus initiation signal (Kozak, 1984). The underlined start codon corresponded to the first three nucleotides of each open reading frame except for *US4*, for which the start codon was supplied by the primer since the *US4* open reading frame does not contain a start codon by itself. The 5′-end primers extended 20–30 nucleotides into the respective coding region, matching exactly the published sequence (Chee et al., 1990), with the exception of *US7*, for which a silent mutation was introduced by replacing the G at position 6 of the coding region with a T in order to remove a BamHI recognition site. All 3′-end primers matched the last 20–30 nucleotides of the coding sequences followed by a stop codon and a BamHI restriction site. Amplified DNA fragments were inserted as SacII/ Bam HI into the tetracycline-inducible expression vector pUHG10.3 (Gossen and Bujard, 1992).

Modified *US6* constructs were obtained by primer-directed PCR mutagenesis. CD8US6 contains the luminal and transmembrane domains of CD8 (to amino acid 212) followed by the predicted cytoplasmic tail of US6 (amino acid sequence ESTGRGIRRCGS). US6E19 and US6E19Δ contain amino acids 1–144 of wild-type US6 followed by the transmembrane domain of CD8 (amino acids 182–212 of wild-type CD8) and the cytoplasmic tail of E19 (amino acid sequence RSFIDEKKMP [E19] or KMP [E19Δ] [Jackson et al., 1990]). To generate US6KDEL, the amino acids KDEL were fused with amino acids 1–144 of US6. US6STOP was constructed by introducing a stop codon at amino acid position 139.

Antibodies

Antisera specific for US6 were raised against the synthetic peptides SSRDPKTLLSLSPRQQA (US6-N) and TYESTGRGIRRCGS (US6-C). Anti-TAP1 and anti-TAP2 antibodies were generated against the carboxy-terminal 21 amino acid residues of human TAP1 (Trowsdale et al., 1990) and human TAP2 (Kelly et al., 1992), respectively. In addition, the peptides contained a cysteine at their amino terminus for cross-linking to bovine serum albumin using N-succinimidyl-3-(2-pyridyldithio)propionate (Pierce). Rabbits were injected with 500 μg of the peptide–bovine serum albumin in complete Freund's adjuvants, followed by several injections of 100 μg in incomplete adjuvants. Antibodies to the carboxy-terminal region of E19 been described previously (Nilsson et al., 1989). MAb AF8 recognizing human calnexin was a kind gift from M. Brenner (Sugita and Brenner, 1994).

Viral Infections

HCMV wild-type strain AD169 was obtained from the American Type Culture Collection, and HCMV mutants were constructed as previously described (Jones et al., 1995). HFF cells were cultured as

adherent monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin, streptomycin, and 2 mM glutamine. HFF cell monolayers (10^7 cells) were infected with HCMV wild type or HCMV mutants at an moi of 5 in 10 ml of DMEM supplemented with 10% fetal calf serum, penicillin, streptomycin, and 2 mM glutamine. Two hours postinfection, medium was removed and 50 ml of the same medium was added. Cells were cultured for 3 days. Infection was assessed by the cytopathic effect observed in most of the cells 72 hours postinfection.

Transfections and Stable Cell Lines

HeLa cells containing the tetracycline regulatable transactivator (HtTa cells) (Gossen and Bujard, 1992) were transfected with 24 μg plasmid DNA by Ca^{2+}/PO_4^{-} precipitation as described by Jackson et al. (1990). For transient experiments, 10^6 HtTa cells were incubated with the transfection solution for 16 hr prior to washing three times with phosphate-buffered saline (PBS) to remove tetracycline. Transfectants were analyzed 48 hr after induction. To establish a stable cell line expressing US6, pUHG10.3-US6 was cotransfected with a plasmid conferring ouabain resistance (Yang et al., 1995). Stable clones were selected by adding ouabain (1 μM) for 24 hr each week. Single cell colonies were screened by cytofluorometry for tetracycline inducible reduction of MHC class I surface expression. Stable transfectants expressing US3, US11, or ICP47 have been described previously (Früh et al., 1995; Ahn et al., 1996a).

Metabolic Labeling and Immunoprecipitation

For pulse-chase experiments, 10^6 cells were methionine starved for 30 min prior to pulse labeling for the indicated times using 0.5 mCi of ^{35}S-labeled methionine (TranS-label, Amersham). The label was chased for various times with DMEM containing 10% fetal calf serum. For optimal labeling of TAP, cells were labeled for 16 hr with 0.4 mCi in DMEM containing 1% fetal calf serum dialyzed using a 12 kDa cutoff membrane. After labeling, cells were washed once with cold PBS and lysed using 1% NP40 (Sigma) in PBS or 1% digitonin (Calbiochem) in PBS for 30 min at 4°C. Immunoprecipitations and SDS gel assays were carried out as described (Yang et al., 1992). For EndoH treatment, immunoprecipitates were digested with 3 mU EndoH (Boehringer Mannheim) for 16 hr at 37°C in 50 mM NaOAc (pH 5.6), 0.3% SDS, 150 mM 2-mercaptoethanol.

Immunofluorescence and Cytofluorometry

For immunofluorescence transiently transfected HeLa cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 followed by incubation with antisera US6-N and MAb AF8 for 1 hr. Bound rabbit or mouse antibody was visualized with goat-anti-rabbit IgG fluorescein or goat-anti-mouse rhodamine conjugate, respectively (Cappel). Cytofluorometric analysis was performed using goat-anti-rabbit IgG fluorescein isothiocyanate to detect W6/32 specific for human MHC class I heterodimers (Parham, 1983).

Peptide Transport Assays

HCMV-infected HFF cell monolayers were detached with trypsin/EDTA, washed twice with transport buffer (130 mM KCl, 10 mM NaCl, 1 mM $CaCl_2$, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM HEPES [pH 7.3 with KOH]) at 4°C and then permeabilized (10^7 cells/ml) in transport buffer containing 4 U/ml of streptolysin O for 20 min at 37°C. Permeabilization was assessed by trypan blue exclusion. Permeabilized cells (10^6 cells/sample in Eppendorf tubes) were incubated for 10 min at 37°C with 10 μl of a radioiodinated peptide library (Heemels et al., 1993) and 10 μl of an ATP-generating system (50 μM ATP, 250 μM UTP, 2.5 mM creatine phosphate, and 8 U creatine phosphokinase) in a total volume of 100 μl at 37°C. When indicated, synthetic ICP47 87-mer was added to the translocation mixture in 10 μl at a final concentration of 10 μM. Peptide translocation was terminated by adding 1 ml of ice-cold stop buffer (transport buffer plus 10 mM EDTA, 0.02% sodium azide). Samples were centrifugated at 14,000 rpm; supernatant was removed; and 1 ml of ice-cold lysis buffer (0.5% NP40, 5 mM $MgCl_2$, 50 mM Tris-HCl [pH 7.5]) added. After 20 min, nuclei were removed by centrifugation at 14,000 rpm and the supernatant incubated with gentle agitation for 1 hr with 100 μl of ConA Sepharose beads at 4°C. Beads were washed three times with lysis buffer and radioactivity quantitated by γ-spectrometry.

Acknowledgments

Correspondence should be addressed to Y. Y. or K. F. We thank our colleagues Rohan Teasdale, Ann Fourie, and M. Jackson for constructs and helpful discussion and the Pharmaceutical Research Institute DNA and proteins core facilities for their excellent technical assistance. We also thank M. Brenner for antibody AF8 and D. Leturq for W6/32.

Received January 21, 1997.

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