Molecular Mechanism and Structural Aspects of Transporter Associated with Antigen Processing Inhibition by the Cytomegalovirus Protein US6*

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The human cytomegalovirus (HCMV) has evolved a set of elegant strategies to evade host immunity. The HCMV-encoded type I glycoprotein US6 inhibits peptide trafficking from the cytosol into the endoplasmic reticulum and subsequent peptide loading of major histocompatibility complex I molecules by blocking the transporter associated with antigen processing (TAP). We studied the molecular mechanism of TAP inhibition by US6 in vitro. By using purified US6 and human TAP co-reconstituted in proteoliposomes, we demonstrate that the isolated endoplasmic reticulum (ER)-luminal domain of US6 is essential and sufficient to block TAP-dependent peptide transport. Neither the overall amount of bound peptides nor the peptide affinity of TAP is affected by US6. Interestingly, US6 causes a specific arrest of the peptide-stimulated ATPase activity of TAP by preventing binding of ATP but not ADP. The affinity of the US6-TAP interaction was determined to 1 μM. The ER-luminal domain of US6 is monomeric in solution and consists of 19% α-helices, 25% β-sheets, and 27% β-turns. All eight cysteine residues are involved in forming a stabilizing network of four intramolecular disulfide bridges. Glycosylation of US6 is not required for function. These findings point to fascinating mechanistic and structural properties, by which specific binding of US6 at the ER-luminal loops of TAP signals across the membrane to the nucleotide-binding domains to prevent ATP hydrolysis of TAP.

During evolution vertebrates developed an adaptive immune system to protect themselves from a vast number of pathogens by using the host as a breeding ground. To eliminate intracellular pathogens, an efficient machinery for antigen processing has been evolved, and within this machinery the transporter associated with antigen processing (TAP)1 plays a key role (for review see Refs. 1 and 2). Cytosolic proteins including viral factors are mostly degraded via the ubiquitin-proteasome pathway (reviewed in Ref. 3). These cleavage products are transported by TAP into the lumen of the endoplasmic reticulum (ER) for chaperone-assisted loading onto MHC class I molecules (for review see Ref. 4). MHC-peptide complexes, optimally loaded with epitopes, overcome the ER “quality control” and continue their journey to the cell surface, where they are monitored by cytotoxic CD8⁺ T-lymphocytes. Recognition of viral epitopes presented on MHC molecules may lead to lysis of the virus-infected cell.

The TAP complex is a heterodimer consisting of TAP1 and TAP2, which both belong to the subfamily B of the ATP-binding cassette transporters (for review see Ref. 5). Each TAP subunit is composed of a hydrophilic transmembrane domain (TMD) followed by a nucleotide-binding domain (NBD). TMDs are thought to be responsible for peptide binding and translocation, whereas ATP hydrolysis at the NBDs provides the energy for substrate transport. In previous studies, the peptide-binding mechanism and substrate specificity of TAP have been analyzed in detail (for review see Ref. 5). From mutational analysis it has been concluded that both NBDs have distinct functions within the transport cycle (6–9). The allosteric coupling between peptide binding, ATP hydrolysis, and peptide translocation has been demonstrated recently (10).

Encoding a large DNA genome, herpes viridae have developed different strategies for evading immune recognition (11, 12). Two viral proteins have been identified to block TAP function as follows: ICP47 from herpes simplex virus and US6 from human cytomegalovirus. The human cytomegalovirus (HCMV) belonging to the β subgroup of herpesviruses is ubiquitous in the human population worldwide. After highly species-specific primary infection, the virus persists throughout the life of its host. In immunocompromised individuals like recipients of organ transplants, AIDS patients, and infants in utero, HCMV infection may cause fatal disease (for review see Ref. 13). The ER-resident type I transmembrane glycoprotein US6 has been identified to block TAP function and therefore helps infected cells to evade host immunity (14–16). US6 interacts with TAP via its ER-luminal domain (14) and inhibits ATP binding of the TAP complex (17). Although entirely unrelated, ICP47, an immediate early gene product of herpes simplex virus, focuses on the same target to shut down antigen processing (18, 19). However, in contrast to US6, ICP47 blocks a very early step in the translocation cycle, which is peptide binding to TAP (20, 21). As a consequence, ICP47 inhibits the cross-talk between peptide binding and ATP hydrolysis within TAP (10).

Viral proteins are important tools for the elucidation of molecular mechanisms of intracellular trafficking, which is ex-

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1 The abbreviations used are: TAP, transporter associated with antigen processing; DM, n-decyl-β-D-maltopyranoside; ER, endoplasmic reticulum; HCMV, human cytomegalovirus; MHC, major histocompatibility complex; NBD, nucleotide-binding domain; TMD, transmembrane domain; MALDI, matrix-assisted laser desorption ionization; HPLC, high pressure liquid chromatography; Ni-NTA, nickel-nitrioltriacetic acid; US, unique short.
exploited by viruses. The structure and function of ICP47 as well as the inhibition mechanism of TAP were analyzed in detail using isolated viral protein (22–24). In comparison to ICP47, the molecular mechanism of the US6 remained largely unknown mostly due to the lack of isolated viral protein. Therefore, quantitative aspects of the US6-TAP interaction as well as the structure and function of this viral TAP inhibitor were poorly defined. We studied the molecular mechanism and structural aspects of HCMV-US6 in vitro, using purified US6 and TAP co-reconstituted in proteoliposomes.

**EXPERIMENTAL PROCEDURES**

Expression of US6 in Insect Cells—To generate recombinant baculovirus for expression of US6(A147–183) in Sf9 insect cells, the US6 gene was amplified from phiDNA3.1-US6 by PCR. The cDNA was kindly provided by Dr. Hartmut Hengel (Robert-Koch-Institute, Berlin, Germany). The following primers were used for PCR amplification: primer US6forBanHI, 5'-AGAGGCGTTGACGATCGGCCATATGGAGCGGTCTTCGCG-3'; primer US6revNdeI, 5'-TGAGTGCCCTCGTACGATCATGTGAGGTGGAAGAATCCGTGCGTTCC-3'. The PCR product was cloned into pFastBac (Invitrogen) via 5' BanHI and 3' XbaI restriction sites. Recombinant baculovirus US6-BUS was generated as described in the Bac-to-Bac baculovirus instruction manual (Life Technologies, Inc.). The baculovirus harboring human TAP1 and TAP2 genes was described previously (25).

US6 were co-infected with baculovirus encoding US6(A147–183) term US6-BUS and human TAP1/2 term TAPI/2. 20 h after infection, cells were harvested, and microsomes were prepared as described (25). The expression of US6(A147–183), human TAP1, and TAP2 was analyzed by SDS-PAGE and immunoblotting using monoclonal antibody 148.3 against human TAP1 (25) and monoclonal antibody 148.3 against human TAP2 (26). US6-specific polyclonal antibodies were raised against the N-terminal residues 20–39 of US6. Peptide binding and transport assays were performed with insect cell microsomes as described previously (25, 27).

Expression and Purification of US6 from Escherichia coli—for E. coli expression of US6(A147–183) the following PCR primers were used: primer US6forNdeI, 5'-GGAGGCGTTGACGATCGGCCATATGGAGCGGTCTTCGCG-3'; primer US6revEcoRI, 5'-TGAGTGCCCTCGTACGATCATGTGAGGTGGAAGAATCCGTGCGTTCC-3'. The amplification product was cloned into pRSET-B (Invitrogen) via 5' NdeI and 3' EcoRI restriction sites yielding pRSET-US6. For expression of histidine-tagged US6(A147–183), E. coli BL21(DE3)pLysS (Invitrogen) was transformed with pRSET-US6. Expression was induced with 0.2 mM isopropyl-β-D-thiogalactoside at mid-logarithmic phase and 37 °C. Three hours after induction, cells were harvested by centrifugation at 5,000 × g for 15 min and lysed by sonication. The lysate was centrifuged at 10,000 × g for 15 min, and the soluble fraction was used for affinity chromatography. US6 was applied to C8-RP-HPLC (Amersham Biosciences) pre-equilibrated in PBS buffer (10 mM Na2HPO4/H3PO4, 120 mM KCl, 1 mM EDTA, pH 7.5) on ice. Inclusion bodies were pelleted at 5,000 × g for 1 h. The protein solution was subjected to reverse phase HPLC to be higher than 95%, and the identity was confirmed by MALDI mass spectrometry. Peptides were iodinated as reversed phase HPLC to be higher than 95%, and the identity was confirmed by MALDI mass spectrometry. Peptides were iodinated as described in the Bac-to-Bac baculovirus instruction manual (Life Technologies, Inc.). The baculovirus harboring human TAP1 and TAP2 genes was described previously (25).

Proteinase K Protection Assay—Peptides were synthesized by solid-phase technique by applying conventional Fmoc (N-(9-fluorenlymethoxycarbonyl) chemistry. The purity of the peptides was determined by reversed phase HPLC to be higher than 95%, and the identity was confirmed by MALDI mass spectrometry. Peptides were iodinated as described (25). In brief, radiolabeling was performed with 15 nmol of peptide and 1 μCi of Na[125I] (1 Ci = 37 GBq) using the chloramine-T method. Free iodine was removed by gel filtration through a Sephadex G-10 (Amersham Biosciences) column. The peptide binding affinity (Kd) and the amount of TAP in proteoliposomes were determined in buffer C by saturation binding assays as described (27, 29). The data were fitted by Equation 2, where B is the amount of bound peptide and |P| is the total peptide concentration.

To analyze the peptide transport, 50 μl of proteoliposomes were incubated with 1 μM [125I]-labeled RRYQKSTEL in 100 μl of buffer A for 2 min at 32 °C in the presence and absence of 3 mM ATP. The reaction was quenched by adding 500 μl of ice-cold assay buffer containing 9 μM non-labeled peptide (RRYQKSTEL). After 15 min of incubation on ice, the samples were centrifuged for 8 min (5,000 × g, 4 °C). The pellet was resuspended with 600 μl of buffer A and pelleted again. The supernatant was removed completely, and the transported substrate was determined by γ-counting.

**ATPase Assay**—The ATPase assays were performed essentially as described previously (10). In brief, 30 μl of buffer A supplemented with 1.8 mM magnesium, 9 mM Na2ATP, a mixture of ATP (3 mM) and [γ-32P]ATP (73 βc; 6.7 μCi) (Amersham Biosciences) were pre-equilibrated in PBS buffer (20 mM Na2HPO4/H3PO4, 120 mM KCl, 1 mM EDTA, pH 7.5). The protein concentration was measured by Micro BCA Protein Assay (Pierce) and by UV absorption at 280 nm.

Reconstitution of US6 and TAP into Proteoliposomes—To screen for activity of recombinant US6, we established an in vitro transport and inhibition assay with TAP reconstituted in proteoliposomes. Human Burkitt lymphoma cells (Raji cells) were propagated in roller bottles at 37 °C and 5% CO2 in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, and 40 units/ml each of penicillin and streptomycin. Microsomes were prepared as described (25). Protein concentration of the microsomes was determined by micro-bicinchoninic acid assay (Pierce). Aliquots were frozen in liquid nitrogen and stored at −80 °C. Microsomes were resuspended in buffer A (20 mM Tris/HCl, 145 mM NaCl, 2 mM KCl, 2 mM MgCl2, 0.5 mM NaN3, 20% glycerol, pH 7.4). For solubilization, 1 μl decyl-β-naltopyranoise (DM) (Calbiochem) was added to reach a ρ value of 2 (28), where ρ is determined in Equation 1,

\[
\rho = \frac{[\text{detergent}]}{[\text{lipid}]} - \text{CMC} \quad \text{(Eq. 1)}
\]

where CMC is the critical micellar concentration of the detergent (1.6 mM for DM). In microsomes, the lipid-to-protein ratio ([w/w]) was assumed to be 1. The mixture was incubated for 20 min at 4 °C, and insoluble material was removed by centrifugation at 100,000 × g for 1 h. Solubilized TAP complex was enriched by Superose 6 PC3.2/30 (Amersham Biosciences) equilibrated with buffer A containing 2.4 mM DM. TAP-containing fractions were pooled and subsequently used for reconstitution. For liposome preparation, a lipid mixture containing 23% (w/w) cholesterol, 10% (w/w) phosphatidic acid, and 67% (w/w) phosphatidylcholine (Avanti Polar Lipids) was dissolved in chloroform. The organic solvent was removed under a stream of nitrogen, and lipids were dried in vacuum. The lipid film was hydrated in buffer A yielding a lipid concentration of 8 mg/ml. Liposomes were extruded through a 1 mM polycarbonate filter using a LipoFast-Extruder (Avanti extrusion) followed by four cycles of freeze and thaw. Solubilized TAP and recombinant US6 were mixed with unilamellar liposomes yielding a final concentration of 20 μM US6, 1 μM TAP, and a ρ value of 0.14. For reconstitution, Bio-Beads SM 2 (Bio-Rad) were washed with buffer A containing 0.1% bovine serum albumin and applied to the mixture to remove the detergent. The Bio-Beads were pelleted by centrifugation at 2,000 × g for 2 min at 4 °C. The procedure was repeated four times. Finally proteoliposomes were collected by centrifugation at 20,000 × g for 15 min at 4 °C.

**Proteinase K Protection Assay**—Aliquots of proteoliposomes containing US6 and TAP were diluted 3-fold with digestion buffer (20 mM Tris/HCl, 145 mM NaCl, 2 mM KCl, 1 mM EDTA, 100 μM EGTA, and 140 mM NaCl, 2 mM KCl, 1 mM EDTA) for 30 min at 4 °C and incubated for 1 h on ice. Addition of phenylmethylsulfonl fluoride (3 mM) stopped the digestion. After centrifugation through a sucrose cushion (25,000 × g, 4 °C), the pellet was analyzed by SDS-PAGE and immunoblotting.

**Peptide Binding and Transport**—Peptides were synthesized by solid-phase technique by applying conventional Fmoc (N-(9-fluorenlymethoxycarbonyl) chemistry. The purity of the peptides was determined by reversed phase HPLC to be higher than 95%, and the identity was confirmed by MALDI mass spectrometry. Peptides were iodinated as described (25). In brief, radiolabeling was performed with 15 nmol of peptide and 1 μCi of Na[125I] (1 Ci = 37 GBq) using the chloramine-T method. Free iodine was removed by gel filtration through a Sephadex G-10 (Amersham Biosciences) column. The peptide binding affinity (Kd) and the amount of TAP in proteoliposomes were determined in buffer C by saturation binding assays as described (27, 29). The data were fitted by Equation 2, where B is the amount of bound peptide and |P| is the total peptide concentration.
fluid (Microscint, Packard Instrument Co.) to determine the release of inorganic phosphate by \(^{32}P\)-counting.

**Nucleotide Binding Assay**—Microsomes derived from Sf9 insect cells expressing TAP were solubilized in buffer A containing 1% (w/v) digitonin (Calbiochem) and incubated for 20 min on ice. Insoluble material was removed by ultracentrifugation at 100,000 \(\times g\) for 1 h at 4 °C. The supernatant was split in aliquots and incubated on ice with various concentrations of recombinant US6 (0–20 \(\mu\)M) and 1–3 nM TAP. Additional aliquots containing 5 \(\mu\)M isolated ICP47 or 0.8 \(\mu\)M peptide (RRYQKSTEL) were prepared. The final volume of each sample was adjusted to 400 \(\mu\)l. Each aliquot was incubated with 100 \(\mu\)l of \(\gamma\)-coupled ATP- or ADP-agarose (Sigma) for 1 h at 4 °C. After washing with 500 \(\mu\)l of buffer A containing 5 mM MgCl\(_2\) and 0.1% (w/v) digitonin, specifically bound protein was eluted by 10 mM MgATP in 50 \(\mu\)l of buffer A containing 0.1% (w/v) digitonin. The eluate was analyzed by SDS-PAGE and immunoblotting.

**Analysis of Disulfide Bonds and Free Cysteines**—The molecular mass of US6(Δ147–183) was analyzed by MALDI-mass spectrometry (Voyager, Perspective Biosystems, Framingham, MA) under non-reducing and reducing conditions (200 mM dithiothreitol, 5 min, 50 °C). Free cysteine residues of US6(Δ147–183) were determined with the Ellman's reagent (5,5'-dithio-bis(2-nitrobenzoic acid)) using glutathione as standard (30).

**Circular Dichroism Spectroscopy**—The concentration of US6(Δ147–183) was adjusted to 0.2 mg/ml in CD buffer (5 mM Na\(_2\)HPO\(_4\)/H\(_3\)PO\(_4\), 150 mM NaF, pH 7.4). CD spectra were recorded on a Jasco J-810 spectropolarimeter at 15 °C, using a 1-mm path length quartz cuvette. A scanning speed of 60 nm/min, a time constant of 0.5 s, and a bandwidth of 2 nm were used. The spectral resolution was 0.5 nm and 5 scans were averaged. Base-line spectra of CD buffer were background subtracted. The content of secondary structures was analyzed by CD Spectra Deconvolution software version 2.1 (Gerald Bohm, Bioinformatics, University of Halle, Germany).

**RESULTS**

**The ER-luminal Domain of US6 Blocks Peptide Transport in Insect Cells**—To study TAP inhibition by US6 in the absence of factors of the adaptive immune system, we co-expressed US6 and human TAP1/2 in Sf9 insect cells using recombinant baculoviruses. The ER-luminal domain of the type I membrane protein, US6(Δ147–183), was expressed with its original ER leader sequence (Fig. 1A). In microsomes prepared from infected insect cells, TAP1, TAP2, and US6 were detected by SDS-PAGE and immunoblotting. The ER-luminal domain of US6 blocks peptide transport but not peptide binding in insect cells. A, schematic illustration of the type I transmembrane glycoprotein US6 (upper panel) and US6(Δ147–183) expressed in insect cells (lower panel). The original signal sequence was used for ER targeting in insect cells. US6 contains 13 cysteines and one N-core glycosylation site at position 52. For expression in Sf9 insect cells, the TMD and the C-terminal cytosolic tail was deleted. B, US6(Δ147–183) and human TAP1/2 were co-expressed in Sf9 insect cells using recombinant baculoviruses. Microsomes prepared from insect cells 60 h after infection were analyzed by SDS-PAGE and immunoblotting using TAP and US6-specific antibodies. C, microsomes were incubated with radiolabeled peptide RRYQNSTEL (1 \(\mu\)M) for 10 min at 27 °C in the presence or absence of ATP (3 mM) and competitor peptide RRYQKSTEL (0.3 \(\mu\)M). Different microsomal preparations were normalized by their TAP concentration. After solubilization of the microsomes, glycosylated and therefore transported peptides were extracted by concanavalin A (ConA)-Sepharose and quantified by \(^{32}P\)-counting. D, microsomes were incubated with radiolabeled peptide RRYQKSTEL (0.3 \(\mu\)M) for 5 min at 4 °C in the absence of ATP. The amount of bound peptide was determined in presence and absence of US6. Unspecific binding was measured in the presence of a 400-fold excess of non-labeled peptide. Data are taken from duplicate measurements.
immunoblotting at an apparent molecular mass of 71, 72, and 16 kDa, respectively (Fig. 1B). In contrast to the expression in mammalian cells, US6(H9004147–183) was mostly non-glycosylated in insect cells. To analyze TAP inhibition by US6, peptide transport and binding assays were performed on isolated microsomes. TAP-dependent translocation of radiolabeled peptide was measured using a glycosylation assay as described previously (25, 31). We observed inhibition of ATP-dependent peptide transport across microsomes containing US6 (Fig. 1C). Peptide translocation was blocked to background level by an excess of non-labeled peptide. Transport inhibition by US6 was not complete, indicating either some cells were infected by BV-TAP1/2 but not by BV-US6 or that glycosylation, at least partly, stabilized US6. In contrast to the transport activity, peptide binding of TAP analyzed at 4°C was not affected by US6 (Fig. 1D). Background levels were observed in the presence of a 400-fold excess of non-labeled peptide.

Expression and Purification of the ER-luminal Domain of US6—Because US6 can inhibit TAP function even in cells lacking factors of the adaptive immune system, we were interested whether US6 is sufficient for blocking TAP function in vitro. We therefore expressed and purified the ER-luminal domain of US6 from E. coli. The construct lacks the ER leader sequence but contains a C-terminal His tag for one-step purification (Fig. 2A). Expression under control of the T7 promoter resulted in the accumulation of US6 in inclusion bodies. After solubilization in 8 M urea, US6 was purified by immobilized metal affinity chromatography using Ni-NTA-agarose in 6 M urea. After purification, we screened optimal refolding condi-
tions by varying the buffer, pH, ion strength, chaotropic agents, divalent cations, detergent, and redox-shuffling systems. The highest amount of refolded, soluble protein was obtained on removal of urea by dialysis under non-reducing conditions. Aggregates were removed by ultracentrifugation at 120,000 × g for 1 h at 4 °C. Expression, purification, and refolding of US6 were analyzed by SDS-PAGE under reducing and non-reducing conditions and immunoblotting using US6 specific antibodies. The recombinant protein has an apparent molecular mass of 16 kDa, which is in good agreement with 15.6 kDa calculated for His6-tagged US6(147–183). In non-reducing SDS-PAGE, US6 appears as monomer, and only a minor fraction was detected as dimer. Interestingly, we observe a weaker signal of the monomer after immunostaining under oxidative than under reducing conditions, indicating that the N-terminal epitope (residues 20–39) is less accessible possibly because of a compact structure stabilized by intramolecular disulfides. The overall purification and refolding efficiency was 30%, yielding 10 mg of US6 from 1 liter of E. coli culture (purity >95%). To analyze the formation of different disulfide isoforms, we performed 7 C8-reversed phase HPLC. Recombinant US6 eluted as a single peak at 35.4% acetonitrile, reflecting a single disulfide isomer (Fig. 2C). Next, the oligomerization state of recombinant US6 was studied by gel filtration (Superdex G-75). US6(147–183) eluted as a single peak at 16 kDa. Lysozyme (14.3 kDa) as reference eluted shortly after (Fig. 2D).

Recombinant US6 Inhibits Peptide Transport by TAP in Vitro—By functional reconstitution of TAP in proteoliposomes, it was possible to study peptide transport as well as the ATPase activity of the TAP complex in vitro (10). TAP was asymmetrically reconstituted in proteoliposomes with most of the nucleotide-binding domains facing outside. We utilized this system to screen the activity of recombinant US6. To analyze inhibition of peptide transport via TAP, US6 must be entrapped inside the proteoliposomes, as US6 binds to the ER-luminal side of TAP. For this purpose, recombinant US6 and solubilized
TAP were mixed with detergents and lipids and co-reconstituted by detergent removal (Fig. 3A). To demonstrate that US6 was entrapped inside of the liposomes, we performed proteinase K protection assays. As shown in Fig. 3B, the recombinant US6 was reconstituted in proteoliposomes and protected from digestion by proteinase K. After permeabilization of the liposomes by detergent (1% Nonidet P-40), US6 became accessible by proteinase K and was degraded.

Next we performed an in vitro peptide transport assay to study TAP inhibition by recombinant US6. We observed a drastic inhibition of ATP-dependent peptide transport activity in the presence of co-reconstituted US6 (Fig. 3C). Normal transport activity of TAP was detected in the absence of US6 or if bovine serum albumin instead of US6 was co-reconstituted. US6 added outside the proteoliposomes did not affect peptide translocation (data not shown), indicating that US6 could block TAP function only from a side opposite where peptide binding and ATP hydrolysis occur. In contrast, ICP47 added to the outside blocked TAP-dependent peptide transport into proteoliposomes (10).

Finally, we measured peptide binding to TAP at 4 °C applying half-saturating and full saturating concentrations of the radiolabeled peptide, RRYQKSTEL. At several peptide concentrations no effect of US6 on peptide binding was observed (Fig. 3D). Unspecific binding was determined in the presence of a 400-fold excess of non-labeled peptide. In summary, these data demonstrate the following: first, recombinant US6 is required and is sufficient for blocking TAP-dependent peptide transport into proteoliposomes (10).

Arrest of the ATPase Cycle of TAP by US6—Recently, we identified and analyzed the ATPase activity of the TAP complex (10). ATP hydrolysis is tightly coupled to peptide binding and blocked by the herpes simplex virus protein ICP47. Because US6 in contrast to ICP47 did not interfere with peptide binding, we were interested whether US6 could affect ATP hydrolysis of TAP. After reconstitution of TAP into proteoliposomes, in the presence and absence of recombinant US6, the ATPase activity was analyzed. We observed that the ATPase activity of TAP was stimulated by peptides as reported recently (10). Most interestingly, the ATPase activity was inhibited in the presence of co-reconstituted US6 (Fig. 4). This finding points to an exciting signaling mechanism how US6, which binds at the inside of the proteoliposomes, blocks ATP hydrolysis at the NBDs of TAP at the opposite side of the membrane.

US6 Prevents ATP Binding but Not ADP Binding to the TAP Complex—By having discovered the specific inhibition of the ATPase activity by US6, we were interested whether US6 could disrupt the coordinated dialogue between peptide binding and ATP hydrolysis or interfere with nucleotide binding of TAP directly. To investigate nucleotide binding of TAP, digitonin-solubilized TAP was mixed with purified US6 and incubated with ATP- or ADP-agarose at 4 °C. After washing, specifically bound proteins were eluted with MgATP and analyzed by SDS-PAGE and immunoblotting. As reported previously (32), we observed specific binding of the TAP complex to ATP- and ADP-agarose (Fig. 5A). However, binding of ATP to TAP was inhibited in the presence of US6. By varying the concentration
of US6, we quantitatively analyzed the US6-TAP interaction and determined the IC₅₀ value for TAP inhibition to be 1 μM (Fig. 5A). Next we were interested whether the herpes simplex virus protein ICP47 could revert the effect of US6 (Fig. 5B). ICP47 (5 μM) by itself has no effect on ATP binding to TAP as shown previously (20). In addition, specific binding of ICP47 to TAP does not revert the inhibition of ATP binding by US6. Similar results were obtained in the presence of peptides (0.8 μM RRYQKSTEL). In contrast to ATP binding, no effect of US6 on the ADP binding of TAP was observed (Fig. 5C). These findings are confirmed by alternative experiments. If TAP was bound to ATP-agarose, TAP was specifically eluted by US6. However, US6 did not elute TAP bound to ADP-agarose (data not shown). These data demonstrate that US6 binding to ER-luminal part of TAP affects ATP binding and subsequent ATP hydrolysis at the NBDs of TAP.

**Structural Analysis of the ER-luminal Domain of US6**—The cytomegaloviral protein US6 has a unique primary structure; no sequence homologues have been found in the data base. Heterologous expression and purification of active US6 from *E. coli* opens the field not only for mechanistic but also for structural studies. The ER-luminal domain of US6 contains eight cysteine residues. Thus we were interested which cysteines are free or involved in disulfide bond formation. By means of MALDI-mass spectrometry, the averaged mass of oxidized and reduced US6 (H₉₀₀₄₁₄₇–₁₈₃) was determined to 15,617 and 15,625 Da, respectively (Fig. 6, A and B). The masses differ by 8 Da, indicating addition of eight hydrogen atoms on reduction. Within the error limit (±0.05%), the average mass was in good agreement with 15,622 Da calculated for reduced His₆-tagged US6 (H₉₀₀₄₁₄₇–₁₈₃). C, US6(Δ147–183) was analyzed by circular dichroism spectroscopy. The spectra of active (solid line) and misfolded, inactive US6 (dotted line) are recorded under the same conditions (see under "Experimental Procedures"). Active US6 contains 19 ± 5% α-helices, 20 ± 6% antiparallel, and 5 ± 1% parallel β-strains, 27 ± 2% β-turn, and 33 ± 1% random coil. In comparison US6 refolded under reducing conditions (inactive) has below 4% α-helical and 44% random coil content.

**FIG. 6. Structural analysis of recombinant US6.** US6(Δ147–183) was analyzed by MALDI-mass spectrometry. Under non-reducing (A) and reducing conditions (200 mM dithiothreitol, 5 min at 50 °C) (B), the average mass (M + H)⁺ amounts to 15,617 and 15,625 Da, respectively. Within the error limit (±0.05%), the average mass was in good agreement with 15,622 Da calculated for reduced His₆-tagged US6(Δ147–183). C, US6(Δ147–183) was analyzed by circular dichroism spectroscopy. The spectra of active (solid line) and misfolded, inactive US6 (dotted line) are recorded under the same conditions (see under "Experimental Procedures"). Active US6 contains 19 ± 5% α-helices, 20 ± 6% antiparallel, and 5 ± 1% parallel β-strains, 27 ± 2% β-turn, and 33 ± 1% random coil. In comparison US6 refolded under reducing conditions (inactive) has below 4% α-helical and 44% random coil content.
Apartment from mechanistic aspects, we were interested in the structural properties of US6. It has been shown that the protein is glycosylated in vivo (14–16). US6 was found in association with the ER-resident chaperones calnexin and weakly with calreticulin (15). We provide clear evidence that US6 is sufficient for TAP inhibition, and N-core glycosylation at Asn-52 is not essential. We therefore suggest that glycosylation may assist efficient folding of US6.

At least four proteins encoded in the HCMV unique short (US) region are presently known to interfere with antigen processing. As described herein, US6 blocks the TAP-mediated peptide transport. The dislocation and mistargeting of MHC class I to proteasomal degradation is induced by US2 and US11 (35, 36). US3 retains MHC class I molecules in the ER (14, 37). These US gene products consist of an ER-luminal domain, a transmembrane domain, and a short cytosolic tail. The structure of the ER-luminal domain of US2 was recently determined. US2 possesses a typical Ig-like structure composed of seven connected β-sheets, packed face-to-face in a β-sandwich, and is tied up by one disulfide bond (38). Sequence alignments based on this structure were used to propose a similar fold for the other members of the US protein family including US6.

We provide several lines of evidence that US6 has a unique structure distinct from US2. First, secondary structure analyses demonstrate that the ER-luminal domain of US6 has about 20% α-helices and therefore differs from an Ig-like fold. More importantly, refolded US6 lacking the α-helical content was found to be inactive (Fig. 6C). These findings indicate that α-helices are essential for US6 function. Second, the site of N-linked glycosylation located at position Asn-52 of US6 was predicted to be a buried core residue in the proposed Ig fold. This is not confirmed by the fact that US6 is glycosylated in vivo (14–16). Third, Ig-like folds are held together by an essential disulfide bond. Based on the structure of US2, it was speculated that Cys-39 and Cys-127 form such disulfide bridges (38). The active luminal domain of US6 consists of eight cysteines. Our data show that all of them are involved in the formation of four intramolecular disulfide bonds. Endopeptidase cleavage and subsequent MALDI-mass spectrometry analysis show that Cys-127 forms a disulfide bridge with the neighboring Cys-129 and not with Cys-39. The N-terminal region of US6 seems to be stabilized by intramolecular disulfide bridges, because an N-terminal epitope is less accessible for antibodies if disulfide bonds are formed (Fig. 2).

In comparison to herpes simplex viral protein ICP47, US6 has a more complex structure. Residues 3–34 have been identified to be the active domain of ICP47 (22). The protein is unstructured in aqueous solution and forms a helix-linker-helix motif upon association with membranes (23, 24). In summary, we show that US6 possesses structural and mechanistic properties distinct from US2 and ICP47.

Recent investigations indicate that the C-terminal region of the ER-luminal domain of US6 might be critical for function, whereas the N-terminal region maintains the stability of US6 in vivo (17). Systematic truncation studies will elucidate the mechanistic and structural basis of the US6-TAP interaction. In this respect it will be interesting to determine the intramolecular cysteine network. With the in vitro system described, important questions about the interaction sites and the structure of US6 can be addressed.

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2 S. Hutschenreiter, C. Kyritsis, and R. Tampé, unpublished data.
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