Human Cytomegalovirus US3 Chimeras Containing US2 Cytosolic Residues Acquire Major Histocompatibility Class I and II Protein Degradation Properties

Mathieu S. Chevalier and David C. Johnson*

Department of Molecular Microbiology and Immunology, Oregon Health and Science University, Portland, Oregon 97239

Received 2 October 2002/Accepted 17 January 2003

Human cytomegalovirus (HCMV) glycoprotein US2 increases the proteasome-mediated degradation of major histocompatibility complex (MHC) class I heavy chain (HC), class II DR- α and DM- α proteins, and HFE, a nonclassical MHC protein. US2-initiated degradation of MHC proteins apparently involves the recruitment of cellular proteins that participate in a process known as endoplasmic reticulum (ER)-associated degradation. ER-associated degradation is a normal process by which misfolded proteins are recognized and translocated into the cytoplasm for degradation by proteasomes. It has been demonstrated that truncated forms of US2, especially those lacking the cytoplasmic domain (CT), can bind MHC proteins but do not cause their degradation. To further assess how the US2 CT domain interacts with the cellular components of the ER-associated degradation pathway, we constructed chimeric proteins in which the US2 CT domain or the CT and transmembrane (TM) domains replaced those of the HCMV glycoprotein US3. US3 also binds both class I and II proteins but does not cause their degradation. Remarkably, chimeras containing the US2 CT domain caused the degradation of both MHC class I and II proteins although this degradation was less than that by wild-type US2. Therefore, the US2 CT and TM domains can confer on US3 the capacity to degrade MHC proteins. We also analyzed complexes containing MHC proteins and US2, US3, US11, or US3/US2 chimeras for the presence of cdc48/p97 ATPase, a protein that binds polyubiquitinated proteins and likely functions in the extraction of substrates from the ER membrane before the substrates meet proteasomes. p97 ATPase was present in immunoprecipitates containing US2, US11, and two chimeras that included the US2 CT domain, but not in US3 complexes. Therefore, it appears that the CT domain of US2 participates in recruiting p97 ATPase into ER-associated degradation complexes.

The betaherpesvirus human cytomegalovirus (HCMV) genome includes a cassette of genes, US2-US11, encoding eight membrane glycoproteins that are of similar size and show some limited homology one to another (16). Four of these viral glycoproteins (US2, US3, US6, and US11) allow HCMV to escape recognition by CD4⁺ or CD8⁺ T lymphocytes (12). Of these four, US2 and US11 increase the degradation of class I proteins by the proteasome (17, 31). US2 also causes the degradation of two proteins of the class II pathway, DR- α and DM- α (4, 29), as well as of HFE, a nonclassical major histocompatibility complex (MHC) class I protein involved in the regulation of iron (2). US3 does not alter the stability of MHC proteins but rather causes the retention of class I complexes in the endoplasmic reticulum (ER) (1, 18) and inhibits the association of invariant chains with class II DR- $\alpha\beta$ dimers in the ER, causing the mislocalization of class II complexes and reduced peptide loading (12).

The molecular mechanisms involved in the US2- or US11-mediated degradation of MHC class I or II proteins are not well understood. Upon binding of US2 in the ER, there is retrotranslocation of the class I heavy chain (HC) through Sec61 protein translocation pores into the cytoplasm (32).

When cells are treated with proteasome inhibitors, class I HC accumulates in the cytosol as a soluble, deglycosylated intermediate (31, 32). Such deglycosylated intermediates of HC are also observed in cells that do not express its binding partner, β_2 microglobulin (14). It has been shown that the polyubiquitination of class I HC is required for extraction and proteolysis, although ubiquitin addition to the cytoplasmic (CT) domain of HC is not required (19, 25, 26, 28). By contrast, class II DR- α chains remain associated with ER membranes in cells expressing US2 and treated with proteasome inhibitors (29). In this aspect, it is interesting to note that degradation intermediates of class II DR- β chains are also found in the ER membrane (6). It appears that an active form of the proteasome is required in order to extract class II α and β chains from the membrane, as has been found for several other membrane proteins acted upon by the ER-associated degradation pathway (6, 13, 22).

It was recently shown that deletion of the CT domain or both the CT and transmembrane (TM) domains of US2 produces molecules that can bind well to both class I HC and class II DR- α proteins without causing their degradation (4, 7, 8, 30). Therefore, binding of US2 to both class I and II proteins is not in itself sufficient to promote retrotranslocation and degradation. We hypothesized that the US2 CT domain binds cellular components of the ER-degradation pathway that are responsible for initiating the degradation of MHC proteins.

In this study, we characterized the CT domain of US2 fur-

* Corresponding author. Mailing address: Department Of Molecular Microbiology and Immunology, Mail code L-220, Oregon Health and Science University, Portland, OR 97239. Phone: (503) 494-0834. Fax: (503) 494-6862. E-mail: johnsoda@ohsu.edu.

ther, determining whether it was capable of conferring on a homologous HCMV glycoprotein, US3, the capability to cause MHC protein degradation. Three chimeric US3 proteins were constructed so that the US3 CT, TM, or CT and TM domains were replaced with those of US2. Chimeras of US3 carrying the CT domain of US2 were able to significantly increase the degradation of both HC and DR- α . We also investigated whether US2 and chimeric proteins could bind cdc48/p97 ATPase, a member of the AAA ATPase family (20), involved in the extraction of polyubiquitinated substrates from the ER membrane during ER degradation (5, 24, 33). p97 ATPase was found in association with US2, US11, and two chimeras containing the CT domain of US2, but not with US3. These studies provide further evidence for a link between the US2 CT domain and cellular factors, including cdc48/p97 ATPase, that promote the degradation of MHC proteins by the ER degradation pathway.

MATERIALS AND METHODS

Cells. U373-MG, a human glioblastoma cell line (American Type Culture Collection, Rockville, Md.), was grown in Dulbecco's modified Eagle's medium (Mediatech, Herndon, Va.) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah) and penicillin-streptomycin (BioWhittaker, Walkersville, Md.). U373-CIITA-HIS16 cells, denoted here as His16, were derived from U373-MG cells by stable transfection with the class II transactivator (CIITA) gene (29) and were propagated in Dulbecco's modified Eagle's medium lacking histidine and supplemented with 10% fetal bovine serum and 0.5 mM histidinol (Sigma, St. Louis, Mo.).

Antibodies. Mouse monoclonal antibody (MAb) anti-human class I W6/32 (correctly assembled MHC class I) (23) was obtained from the American Type Culture Collection. MAb HC10 (free HC) (27) was a gift of H. Ploegh (Harvard University Medical School). MAb specific for HLA-DR- α (DA6.147) (11) was obtained from Peter Cresswell (Yale University). Rabbit anti-peptide sera (US2N and US3N) specific for US2 and US3 have been described previously (12, 29). A murine MAb against the mammalian form of cdc48/p97 ATPase was purchased from Research Diagnostics (Flanders, N.J.).

Mutagenesis of HCMV US3 glycoprotein. Three chimeric US3/US2 fusion proteins were engineered, swapping US2 sequences in place of the equivalent US3 sequences without any further modification of the original US3-coding sequences. US3 with the cytosolic tail of US2 (US3/US2-332) contains the US2 CT sequence (D¹⁸⁶-C¹⁹⁹) inserted in place of the US3 CT sequence (R¹⁸²-I¹⁸⁶). US3/US2-322 has US3 sequences with the TM and CT domains of US2 (H¹⁶¹-C¹⁹⁹) in place of the TM and CT domains of US3 (L¹⁶²-I¹⁸⁶). US3/US2-320 has the US2 TM domain (H¹⁶¹-V¹⁸⁵) in place of the US3 TM domain and lacks the CT domain of US3. All the mutants were constructed after first using PCR, producing a silent mutation at the 3' extremity of the coding sequence for the luminal domain of US3 (ends at F¹⁸¹) to create a unique *EcoRI* restriction site. The modified US3-coding sequences were then ligated with hybridized, phosphorylated (T4 kinase; New England Biolabs) oligonucleotides that were synthesized with an *EcoRI* site at the 5' end, followed by the correct coding sequences for TM and CT US2 for chimera 322, TM US3 and CT US2 for chimera 332, or TM US2 for chimera 320. Each of these constructs present in pUC19 was confirmed entirely by DNA sequence analyses, and then the US3/US2 chimeras were removed by using restriction enzymes and ligated into plasmid pAdtet7 downstream of an HCMV immediate-early promoter element regulated by a tetracycline transactivator element (9).

Construction of adenovirus (Ad) vectors expressing US3/US2 chimeras. The construction of replication-defective Ad viruses in which the HCMV glycoprotein genes were coupled to a promoter that is regulated by a tetracycline transactivator protein and inserted in place of Ad E1 sequences has been described in detail (4, 29). Briefly, US3/US2 chimeric genes were inserted into pAdtet7 plasmids and these were cotransfected along with *psi5* Ad DNA into 293-*cre4* cells which express the *cre* recombinase (3). Viruses produced by *cre*-mediated recombination were passaged three times on 293-*cre4* cells and then grown and titrated on 293 cells.

Metabolic labeling of cells and immunoprecipitation of HCMV glycoproteins. His16 cells (6×10^6 to 7×10^6 cells) were coinfecting with Ad vectors expressing HCMV glycoproteins (using 10 to 100 PFU/cell) and Adtet-trans (2 to 20 PFU/

cell), the latter to provide expression of the tetracycline transactivator protein. The cells were labeled with [³⁵S]methionine-cysteine (Promix; Amersham Pharmacia Biotech, Piscataway, N.J.), cell extracts were made by using 1% NP-40–0.5% deoxycholate in Tris buffer, and extracts were clarified by centrifugation at 60,000 to 100,000 $\times g$ for 30 to 60 min. For direct-immunoprecipitation reactions, extracts were incubated with antibodies specific for MHC class I or II proteins or with US2 or US3 for 2 h, followed by incubation with protein A-agarose (Amersham Pharmacia Biotech). The protein A-agarose beads were washed, and precipitated proteins were eluted by the addition of 2 $\times$ Laemmli sodium dodecyl sulfate (SDS) sample buffer containing 4% SDS, 2% β -mercaptoethanol, 100 mM Tris-HCl (pH 6.8), and 20% glycerol and boiled for 5 min (4). Sequential immunoprecipitations were performed as described previously (4, 29). Briefly, His16 cells in suspension were incubated for 60 to 90 min in media lacking methionine and cysteine and supplemented with a 30 μ M concentration of the proteasome inhibitor carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132; Calbiochem, San Diego, Calif.). Following this treatment, the cells were radiolabeled with 1 mCi of [³⁵S]methionine-cysteine/ml for 20 min in the presence of MG132. Cell extracts were prepared with mild detergent (1% digitonin; Calbiochem), and these preparations were preadsorbed by incubation with a preimmune serum and protein A-agarose. MHC class I proteins were immunoprecipitated with MAb W6/32, and class II complexes were immunoprecipitated with MAb DA6.147. Antigen-antibody complexes were captured by the addition of protein A-agarose beads and washed with buffer containing 0.1% digitonin and 1 mM phenylmethylsulfonyl fluoride (Sigma); then the proteins were eluted by the addition of Tris saline containing 1% SDS and boiled for 10 min. The eluted proteins were diluted 10-fold with Tris saline containing 0.5% NP-40, and the remaining primary antibody was captured by incubation with protein A-agarose for 2 h. A second immunoprecipitation was then performed by the addition of rabbit anti-US2 or anti-US3 polyclonal sera for 2 h, followed by protein A-agarose for 2 h. Proteins were loaded onto 12% polyacrylamide gels and subjected to electrophoresis, and the gels were incubated with Enlightening (New England Nuclear, Beverly, Mass.), dried, and exposed to X-ray film (Eastman Kodak Company, Rochester, N.Y.) or analyzed by PhosphorImager system BAS 2500 (Molecular Dynamics, Sunnyvale, Calif.).

Immunoblotting. Extracts of His16 cells infected with Ad vectors expressing US2, US3, or US3/US2 chimeras were made with 1% digitonin as described above. Extracts were mixed with anti-US2 or anti-US3 polyclonal sera, antigen-antibody complexes were captured by the addition of protein A-agarose beads, and beads were washed with buffer containing 0.1% digitonin and 1 mM phenylmethylsulfonyl fluoride (Sigma). Immunoprecipitated proteins were eluted by the addition of 2 $\times$ Laemmli SDS-sample buffer as described above. Proteins immunoprecipitated in this way or cell extracts not immunoprecipitated were then subjected to electrophoresis in SDS–10% polyacrylamide gels, and proteins were transferred onto Immobilon membranes (BioWhittaker), which were incubated in Tris-buffered saline (TBS) with 3 mg of bovine serum albumin/ml–1% fish gelatin–0.5% polyvinylpyrrolidone for 1 h. After three washes in TBS–0.1% Tween 20, the membranes were incubated for 1 h with a MAb specific to mammalian cdc48/p97 ATPase (Research Diagnostics) diluted 1/1,000 in TBS–0.1% Tween 20. The membranes were washed and then incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Amersham Pharmacia Biotech). Samples were visualized by using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

RESULTS

Expression of HCMV US3/US2 chimeric proteins. A series of chimeras containing the luminal domain of US3 and the TM and CT domains of US2 in place of homologous US3 sequences were constructed. These mutant US3/US2 molecules were expressed by using replication-defective (E1[−]) Ad vectors that do not express detectable quantities of Ad proteins. These vectors were made as described previously (4, 29) by inserting chimeric US3/US2 genes into a shuttle plasmid, pAdtet7. In this plasmid, the HCMV genes were expressed from a basal HCMV IE promoter regulated by a tetracycline transactivator sequence. Coinfection of cells with Adtet-trans, which delivers the transactivator protein into cells, allowed expression of the HCMV proteins.

Figure 1A depicts the panels of US3/US2 chimeric glyco-

FIG. 1. Expression of HCMV US3/US2 chimeric proteins. (A) Schematic representation of US3/US2 chimeras. Wild-type US3 contains an ER-luminal domain (hatching), a predicted TM domain (black), and a short CT domain (hatching). Wild-type US2 also contains a luminal domain (white), a TM domain (grey), and a CT domain (white). US3/US2 chimeric proteins were denoted by three numbers representing, sequentially, the luminal, TM, and CT domains. US3/US2-332 has US3 sequences except for the US2 CT domain substituting for the US3 CT domain, US3/US2 contains the US2 TM and CT domains, and US3/US2-320 lacks a CT domain. (B) Sequences of the predicted CT domains of US2 and US3. (C) Expression of wild-type US2, US3, and chimeras in His16 cells after the cells were coinfectd with AdtetUS2, AdtetUS3, or vectors expressing the chimeras (100 PFU/cell) and Adtet-trans (20 PFU/cell) for 16 h. Cells were radiolabeled with [³⁵S]methionine-cysteine for 5 min, and US2, US3, or chimeric proteins were immunoprecipitated with rabbit anti-US2 or US3 polyclonal sera. Molecular size marker proteins are indicated to the left.

proteins that were constructed. US3/US2-332 contained large-ly US3 sequences, with the US2 CT domain replacing that of US3. US3/US2-322 contained US3 luminal sequences, with the US2 TM and CT domains replacing those of US3. US3/

US2-320 contained the US2 TM domain but lacked a CT domain. Figure 1B compares the CT domains of US2 and US3. The US2 CT domain contained 14 residues, including numer-ous hydrophobic residues, e.g., methionine and phenylalanine.

The US3 CT was much shorter, with only 5 amino acids, including two arginines.

The expression of US3/US2 chimeric proteins was examined in His16 cells, U373 microglial cells stably transfected with CIITA and capable of expressing MHC class II proteins (29). The cells were infected with Ad vectors expressing wild-type US2 or US3 or each of the individual chimeric US3/US2 proteins. The cells were radiolabeled, and wild-type and mutant proteins were immunoprecipitated with polyclonal antibodies. Cells radiolabeled with [³⁵S]methionine in a short pulse, e.g., 5 min, exhibited two US2 species as previously described (4, 29). US2 is 199 amino acids in length and expected to be 23 kDa without glycosylation and migrates at ~27 kDa when glycosylated. The slower migrating protein was glycosylated, while the faster migrating species was not modified with N-linked oligosaccharides. By contrast, US3 appeared as a single glycosylated species, as described previously (12). Each US3/US2 chimeric protein appeared as a single band, or this was largely so under these labeling conditions. US3 is 186 amino acids long, expected to be 21.5 kDa without glycosylation and ~24 kDa when glycosylated. US3/US2-322 was ~25.5 kDa, as expected, and based on the addition of 11 residues, US3/US2-332 was ~26 to 26.5, as expected, and US3/US2-320 was 23 kDa, as predicted.

Binding of US3/US2 chimeras to class I and II proteins.

Binding of the US3/US2 chimeric proteins to either MHC class I or class II DR complexes was examined by sequential immunoprecipitation. The MHC complexes were immunoprecipitated first, the complexes were denatured, and then US2, US3, or chimeras were immunoprecipitated with rabbit polyclonal antibodies. Such assays measure whether or not class I or II proteins are associated with US2 or US3 but are not absolutely quantitative and instead measure the presence or absence of MHC proteins when US2 or US3 is precipitated (4, 12, 29). In previous experiments, the specificity of these coimmunoprecipitations was demonstrated by several means: no US2 was immunoprecipitated in conjunction with the transferrin receptor (29), and a control antibody did not precipitate US3 (12). We also analyzed directly the proteins that were immunoprecipitated with anti-US2 or anti-US3 antibodies to control for the amounts of US2 or US3 or chimeric proteins expressed in cells (Fig. 2, upper panel). After sequential immunoprecipitation, we detected the glycosylated US2 and US3 associated with MHC class I complexes (W6/32), and there was also binding of all of the US3/US2 chimeric proteins to class I (Fig. 2, middle panel). It appeared that the amount of US3 present in class I complexes was less than that observed for US2 and all the US3/US2 chimeric proteins and that this appeared to be partially due to the reduced expression of US3 in the cells (Fig. 2, upper panel). Similarly, US2, US3, and all the chimeric US3/US2 proteins were observed in the MHC class II complexes (DA6.147 [Fig. 2, lower panel]). As in a previous study (4), there was much less US2 and US3 associated with class II complexes than there was with class I complexes, and this may be related to the decreased rates of synthesis of class II proteins in the cells or the reduced stability of class II complexes containing US2 and US3 in digitonin. We concluded that all the US3/US2 chimeras bind to MHC class I and II proteins approximately as well as US2.

US3/US2 chimeric proteins containing the cytosolic domain

FIG. 2. Sequential immunoprecipitation of US3/US2 chimeric proteins with MHC class I and II proteins. His16 cells were coinfectd with Ad vectors expressing US3/US2 chimeric proteins or US2 or US3 (25 PFU/cell) and Adtet-trans (5 PFU/cell) for 13 h and then incubated with proteasome inhibitor MG132 (30 μ M) for 60 to 90 min. The cells were radiolabeled with [³⁵S]methionine-cysteine for 20 min in the presence of MG132. Cell extracts were made by using 1% digitonin buffer and US2 or US3 immunoprecipitated directly with polyclonal anti-US2 or -US3 antibodies (top panel). Alternatively, MHC proteins were immunoprecipitated with MAb W6/32 (middle panel) or class II complexes were precipitated with MAb DA6.147 (bottom panel) and then the precipitated proteins were denatured and reimmunoprecipitated with anti-US2 or -US3 polyclonal antibodies. It was previously reported that the amount of DR- α in immunoprecipitates was less than that of class I MHC due to the use of different antibodies as well as different rates of synthesis between class I and class II proteins (4).

of US2 cause degradation of MHC class I and II proteins. To measure the degradation of MHC proteins caused by US3/US2 chimeras, His16 cells expressing class I and II proteins were radiolabeled for 1 min, the label was chased for 15 or 30 min, and the MHC proteins were immunoprecipitated. The pulse samples were not shown in these experiments, as they do not serve as a reference point. In a previous study (29), US2 caused the rapid degradation of MHC proteins even when they were labeled for 1 min. Thus, comparisons were made to the amount of class I HC or class II DR- α present after a 15- or 30-min chase period in US3-expressing cells. Previously, it was shown that class I and II proteins were as stable in cells expressing US3 as in control cells not expressing US3 (12). In the experiments shown here, we did not compare class I or II proteins to uninfected cells because Ad vectors upregulate MHC proteins

FIG. 3. Degradation of class I and II proteins by US3/US2 chimeric mutants. His16 cells were coinfecting with Ad vectors expressing US3/US2 chimeras, US3, or US2 (100 PFU/cell) and Adtet-trans (20 PFU/cell) for 12 to 16 h. The cells were trypsinized, washed, and radiolabeled in suspension with [35 S]methionine-cysteine for 1 min; then the label was chased for 15 or 30 min. MHC class I HC was immunoprecipitated with MAb HC10 (A), or class II complexes were immunoprecipitated with MAb DA6.147 (B). The gels were subjected to phosphorimager analyses to determine the quantities of HC and class II DR- α . The graphs in each panel represent the mean of three different experiments, with standard deviations calculated. Each value for HC or DR- α observed after a 15-min chase was compared to the value obtained for US3-expressing cells after a 15-min chase period (arbitrarily set at 100). Similarly, experimental values obtained after a 30-min chase period were compared to those for US3-expressing cells chased for 30 min. The pulse samples are not shown because there was degradation during labeling (29), and these do not serve as a point of reference.

and thus it is more reasonable to compare the effects of US2 to those of US3, both expressed with Ad vectors. As expected, expression of wild-type US2 caused a marked loss of expression of class I HC, only ~10% of the class I HC remaining after either a 15- or 30-min chase period compared with that in cells expressing US3 (Fig. 3A). The data shown in the graphs are the means from three different experiments. Two of the chimeric US3/US2 glycoproteins, US3/US2-322 and US3/US2-332, both containing the US2 CT domain, caused significant degradation of class I HC, ~75% of the class I HC being lost during 15- or 30-min chases. US3/US2-320 displayed a limited level of degradation, ~20 to 50% of that observed in cells expressing wild-type US2, suggesting that the TM domain of US2 also confers some limited degradation capacity on US3.

US2 also causes the degradation of MHC class II DR- α , while US3 does not affect the stability of DR- α (4, 12, 29). Chimeras US3/US2-322 and US3/US2-332 caused the degradation of class II DR- α , and there was a loss of 70 to 80% of the DR- α in the 15- or 30-min chase periods compared with that in US3-expressing cells (Fig. 3B). There was also more limited degradation with US3/US2-320. We concluded that the CT domain of US2 can confer the DR- α and class I HC degradation properties of US2 to US3.

Analysis of interactions between US2, US3, and chimeric US3/US2 proteins and the cdc48/p97 ATPase. Recent studies have identified the cdc48/p97 ATPase as a component of a complex of proteins involved in the extraction of polyubiquitinated proteins from the ER membrane before these proteins

are degraded by the proteasome (15, 21, 24). cdc48/p97 ATPase was found in association with MHC class I proteins in cells expressing US11, and it was proposed that the p97 ATPase is involved in the US11-triggered extraction of class I from the ER membrane (33). To further characterize the mechanism of degradation of both the MHC class I and II proteins by US2, it was of interest to determine whether cdc48/p97 ATPase was found in complexes with US2 and with the US3/US2 chimeras. His16 cells expressing US2, US3, US11, or the US3/US2 chimeras were incubated with proteasome inhibitors, and the US glycoproteins were immunoprecipitated from digitonin cell extracts. The immunoprecipitated proteins were separated on SDS-polyacrylamide electrophoresis gels and then transferred to Immobilon membranes, which were incubated with cdc48/p97 ATPase-specific antibodies (Fig. 4A). cdc48/p97 ATPase was found in both US2 and US11 samples but was absent or not detected in US3 samples. We also detected smaller amounts of p97 ATPase in US3/US2-322 and US3/US2-332 samples. There was little or no p97 ATPase in US3/US2-320 immunoprecipitates. A contaminating protein was precipitated with anti-US2, -US3, and -US11 antibodies (Fig. 4A, asterisk). This protein was not observed in the Western blots of whole cell extracts but was observed in immunoprecipitates from cells not expressing US2 (Fig. 4B). Thus, this contaminant was apparently a cellular protein precipitated with any of the rabbit sera and different from p97 ATPase. We concluded that the US2 CT domain is sufficient in order to recruit p97 ATPase

FIG. 4. Interactions of US proteins with cdc48/p97 ATPase. (A) His16 cells were infected with Ad vectors expressing US2, US3, US3/US2 chimeric proteins, or US11 (25 PFU/cell) and Adtet-trans (5 PFU/cell) for 16 h and then incubated with MG132 (30 μ M) for 90 min. US2, US3, or US11 was immunoprecipitated from cell extracts made by using 1% digitonin, and proteins were separated on SDS-polyacrylamide gels. Proteins were transferred to membranes, and p97 ATPase was detected by incubating membranes with monoclonal anti-p97 ATPase antibodies, secondary antibodies, and a chemoluminescent substrate. Molecular size markers of 132 and 78 kDa are indicated to the left of the panel. The asterisk represents a contaminating or cross-reacting cellular protein of about 110 kDa that was precipitated with rabbit serum. IP, immunoprecipitate. (B) The contaminating 110-kDa protein was also detected in the immunoprecipitates involving anti-US2 antibodies and uninfected cells. In cells infected with AdtetUS2, both the contaminating protein and p97 ATPase were detected.

into complexes that ultimately lead to the degradation of MHC proteins.

DISCUSSION

In a previous study, the US2 sequences important for binding to MHC class I HC and class II DR- α and for the US2-initiated degradation of these proteins were defined (4). Mutant forms of US2 in which the 20-amino-acid signal sequence was replaced by a heterologous signal sequence were as active as wild-type US2 in causing the degradation of class I and II proteins. Moreover, a mutant lacking these residues and 7 additional residues caused the degradation of MHC proteins. We concluded that the N terminus of US2 is not required for the binding or degradation of both MHC proteins. By contrast, removal of the CT domain of US2 (US2 1-186) dramatically reduced the degradation of both class I and II proteins. A mutant lacking both the CT and TM domains (US2 1-160) lost all degradation activity. In other studies, US2 1-186 produced by *in vitro* translation did not degrade class I HC (7). Thus, US2 must retain cytosolic sequences and be anchored in the ER membrane in order to cause degradation. US2 1-186 and

1-160 mutants were especially interesting because both bind well to class I and II proteins yet do not cause their degradation.

To further examine the role of the US2 CT domain in MHC degradation, we engineered chimeric proteins where the US2 CT domain replaced that of US3 or where both the TM and CT domains replaced those of US3. The degree of homology between these HCMV glycoproteins is significant (55% amino acid similarity; 25% amino acid identity), especially in the luminal domains that bind both MHC proteins, and it appears likely that US2 and US3 evolved from a common ancestor by gene duplication. Nevertheless, the effects of US2 and US3 binding on MHC proteins differ dramatically. US3 retains class I in the ER (1, 10, 18) and reduces invariant chain assembly with class II complexes so that class II is mislocalized (12). With substitution of the US2 CT domain, US3 acquired the capacity to degrade both class I and II proteins although this was less than that obtained with wild-type US2. A US3 molecule with US2 TM and CT domains also caused the degradation of MHC proteins, but there appeared to be little or no increase in the levels of degradation compared with those of US3/US2-332. These results prove that US2 CT sequences are not only necessary for degradation but also sufficient to confer degradation function on a homologous but inactive glycoprotein. This further supports the hypothesis that cytosolic factors involved in extracting ER-resident proteins into the cytoplasm for degradation interact with the US2 CT domain.

In considering our results, one might suggest that the US3/US2 chimeras are misfolded in a manner that promotes the nonspecific degradation of MHC proteins. This possibility appears unlikely because the TM and/or CT domains of US2 were swapped in place of those of US3 and these sequences are unlikely to perturb the luminal domain of US3. Moreover, these chimeras bound MHC proteins approximately as well as did US2, arguing against the notion that they are grossly misfolded. Previously, a mutant US2 lacking a critical cysteine (involved in a luminal disulfide) and thought to be grossly misfolded did not bind MHC proteins (4). Since the US3/US2 chimeras were able to bind MHC proteins, we do not believe that they promote the degradation of MHC proteins by a mechanism different from that of US2.

There was also a reproducible but smaller degree of degradation in cells expressing US3/US2-320, a molecule containing the US2 TM but lacking any CT domain. The relatively low level of degradation observed with 320 was reminiscent of the small amount of degradation caused by truncation mutant US2 1-186, which lacks the US2 CT domain (4). Therefore, it appears that the CT domain is not absolutely required for degradation and that the low level of degradation occurs without this domain. It is nevertheless possible that the deletion of 13 residues of the CT domain of US2 does not necessarily remove all of the amino acids exposed in the cytoplasm. Exposure of US2 residues on the CT face of the ER has been predicted using software programs and not tested experimentally. These results are consistent with a role for the US2 TM domain in promoting the assembly of ER degradation complexes on MHC proteins. In previous studies, truncation mutant US2 1-160, which lacks both the TM and CT domains, was completely inactive in causing degradation of MHC proteins (4).

Although US3 acquired some ability to degrade MHC pro-

teins with the addition of US2 TM and CT sequences, the degree to which these chimeras functioned was, in every case, less than that with wild-type US2. Thus, the luminal domain of US2 improves the efficiency of degradation. Both US2 and US3 bind to MHC proteins (Fig. 2) (12). The interaction between US2 and MHC proteins is short-lived, as the MHC proteins and US2 are degraded within minutes. By contrast, US3 interacts with MHC complexes for hours (12). Thus, there are major differences in the binding of US2 and US3 to both class I and II proteins and this is accompanied by recruitment of different sets of ER-resident proteins onto MHC proteins and different fates of the MHC proteins. Apparently, US2 luminal domains are better able to interact with the ER-resident proteins involved in extraction and degradation. By contrast, US3 appears to primarily alter the assembly and forward transport of MHC proteins (12).

One cellular protein that is involved in the degradation of MHC class I by the related HCMV glycoprotein US11 is cdc48/p97 ATPase. Ye et al. inhibited US11-mediated class I HC retrotranslocation from the ER to the cytoplasm by expressing a dominant-negative form of p97 ATPase (33). Moreover, they demonstrated that the association of p97 ATPase with MHC class I HC was more pronounced in US11-expressing cells than that in control cells. Polyubiquitinated substrates are preferentially bound by p97 ATPase in complex with two adaptor proteins, Ufd1 and Npl4. This association triggers retrotranslocation of the substrates from the ER membrane, followed by proteasome-mediated degradation (24). Ye et al. proposed that p97 ATPase extracts class I HC from ER membranes in US11-expressing cells. Our results support the hypothesis that p97 ATPase is also an important component of the US2-mediated retrotranslocation and degradation of MHC proteins. We found p97 ATPase in complexes containing US2 or US11 and MHC proteins, but not in US3 complexes. Two of the chimeric US3/US2 proteins, US3/US2-322 and US3/US2-332, also bound detectable amounts of p97 ATPase. Therefore, the CT domain of US2 appears necessary for the recruitment of p97 ATPase into a complex that leads to the degradation of MHC proteins.

ACKNOWLEDGMENTS

This work was supported by NIH grants from the National Eye Institute (EY11245) and the National Cancer Institute (CA73996).

We are grateful to Claire Dunn and Nagendra Hegde for helpful discussions and for reviews of the manuscript. Tiffani Howard was invaluable in preparing the figures.

REFERENCES

- Ahn, K., A. Angulo, P. Ghazal, P. A. Peterson, Y. Yang, and K. Fruh. 1996. Human cytomegalovirus inhibits antigen presentation by a sequential multistep process. *Proc. Natl. Acad. Sci. USA* 93:10990–10995.
- Ben-Arieh, S. V., B. Zimmerman, N. I. Smorodinsky, M. Yaacubovitch, C. Schechter, I. Bacik, J. Gibbs, J. R. Bennink, J. W. Yewdell, J. E. Coligan, H. Firat, F. Lemonnier, and R. Ehrlich. 2001. Human cytomegalovirus protein US2 interferes with the expression of human HFE, a nonclassical class I major histocompatibility complex molecule that regulates iron homeostasis. *J. Virol.* 75:10557–10562.
- Chen, L., M. Anton, and F. L. Graham. 1996. Production and characterization of human 293 cell lines expressing the site-specific recombinase Cre. *Somat. Cell. Mol. Genet.* 22:477–488.
- Chevalier, M. S., G. M. Daniels, and D. C. Johnson. 2002. Binding of human cytomegalovirus US2 to major histocompatibility complex class I and II proteins is not sufficient for their degradation. *J. Virol.* 76:8265–8275.
- Dai, R. M., and C. C. Li. 2001. Valosin-containing protein is a multi-ubiquitin chain-targeting factor required in ubiquitin-proteasome degradation. *Nat. Cell Biol.* 3:740–743.
- Dusseljee, S., R. Wubolts, D. Verwoerd, A. Tulp, H. Janssen, J. Calafat, and J. Neefjes. 1998. Removal and degradation of the free MHC class II beta chain in the endoplasmic reticulum requires proteasomes and is accelerated by BFA. *J. Cell Sci.* 111:2217–2226.
- Furman, M. H., H. L. Ploegh, and D. Tortorella. 2002. Membrane-specific, host-derived factors are required for US2- and US11-mediated degradation of major histocompatibility complex class I molecules. *J. Biol. Chem.* 277:3258–3267.
- Gewurz, B. E., E. W. Wang, D. Tortorella, D. J. Schust, and H. L. Ploegh. 2001. Human cytomegalovirus US2 endoplasmic reticulum-luminal domain dictates association with major histocompatibility complex class I in a locus-specific manner. *J. Virol.* 75:5197–5204.
- Gossen, M., and H. Bujard. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA* 89:5547–5551.
- Gruhler, A., P. A. Peterson, and K. Fruh. 2000. Human cytomegalovirus immediate early glycoprotein US3 retains MHC class I molecules by transient association. *Traffic* 1:318–325.
- Guy, K., V. Van Heyningen, B. B. Cohen, D. L. Deane, and C. M. Steel. 1982. Differential expression and serologically distinct subpopulations of human Ia antigens detected with monoclonal antibodies to Ia α and β chains. *Eur. J. Immunol.* 12:942–948.
- Hegde, N., R. A. Tomazin, T. W. Wisner, C. Dunn, J. Boname, D. M. Lewinsohn, and D. C. Johnson. 2002. Inhibition of HLA-DR assembly, transport, and loading by human cytomegalovirus glycoprotein US3: a novel mechanism for evading major histocompatibility complex class II antigen presentation. *J. Virol.* 76:10929–10941.
- Ho, S., S. Chaudhuri, A. Bachhawat, K. McDonald, and S. Pillai. 2000. Accelerated proteasomal degradation of membrane Ig heavy chains. *J. Immunol.* 154:4713–4719.
- Hughes, E. A., C. Hammond, and P. Cresswell. 1997. Misfolded major histocompatibility complex class I heavy chains are translocated into the cytoplasm and degraded by the proteasome. *Proc. Natl. Acad. Sci. USA* 94:1896–1901.
- Jarosch, E., C. Taxis, C. Volkstein, J. Bordallo, D. Finley, D. H. Wolf, and T. Sommer. 2002. Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. *Nat. Cell Biol.* 4:134–139.
- Jones, T. R., L. K. Hanson, L. Sun, J. S. Slater, R. M. Stenberg, and A. E. Campbell. 1995. Multiple independent loci within the human cytomegalovirus unique short region down-regulate expression of major histocompatibility complex class I heavy chains. *J. Virol.* 69:4830–4841.
- Jones, T. R., and L. Sun. 1997. Human cytomegalovirus US2 destabilizes major histocompatibility complex class I heavy chains. *J. Virol.* 71:2970–2979.
- Jones, T. R., E. J. Wiertz, L. Sun, K. N. Fish, J. A. Nelson, and H. L. Ploegh. 1996. Human cytomegalovirus US3 impairs transport and maturation of major histocompatibility complex class I heavy chains. *Proc. Natl. Acad. Sci. USA* 93:11327–11333.
- Kikkert, M., G. Hassink, M. Barel, C. Hirsch, F. J. van der Wal, and E. Wiertz. 2001. Ubiquitination is essential for human cytomegalovirus US11-mediated dislocation of MHC class I molecules from the endoplasmic reticulum to the cytosol. *Biochem. J.* 358:369–377.
- Langer, T. 2000. AAA proteases: cellular machines for degrading membrane proteins. *Trends Biochem. Sci.* 25:251–257.
- Lord, J. M., A. Ceriotti, and L. M. Roberts. 2002. ER dislocation: Cdc48p/p97 gets into the AAAct. *Curr. Biol.* 12:R182–R184.
- Mancini, R., C. Fagioli, A. Fra, C. Maggioni, and R. Sitia. 2000. Degradation of unassembled soluble Ig subunits by cytosolic proteasomes: evidence that retrotranslocation and degradation are coupled events. *FASEB J.* 14:769–778.
- Parham, P., and H. L. Ploegh. 1980. Molecular characterization of HLA-A, B homologues in owl monkeys and other nonhuman primates. *Immunogenetics* 11:131–143.
- Rabinovich, E., A. Kerem, K.-U. Frohlich, N. Diamant, and S. Bar-Nun. 2002. AAA-ATPase p97/Cdc48p, a cytosolic chaperone required for endoplasmic reticulum-associated protein degradation. *Mol. Cell. Biol.* 22:626–634.
- Shamu, C. E., C. M. Story, T. A. Rapoport, and H. L. Ploegh. 1999. The pathway of US11-dependent degradation of MHC class I heavy chains involves a ubiquitin-conjugated intermediate. *J. Cell Biol.* 147:45–58.
- Shamu, C. E., D. Flierman, H. L. Ploegh, T. A. Rapoport, and V. Chau. 2001. Polyubiquitination is required for US11-dependent movement of MHC class I heavy chain from endoplasmic reticulum into cytosol. *Mol. Biol. Cell* 12:2546–2555.
- Stam, N. J., T. M. Vroom, P. J. Peters, E. B. Pastoors, and H. L. Ploegh. 1990. HLA-A- and HLA-B-specific monoclonal antibodies reactive with free heavy chains in Western blots, in formalin-fixed, paraffin-embedded tissue sections and in cryo-immuno-electron microscopy. *Int. Immunol.* 2:113–125.
- Story, C. M., M. H. Furman, and H. L. Ploegh. 1999. The cytosolic tail of class I MHC heavy chain is required for its dislocation by the human cyto-

- megalovirus US2 and US11 gene products. *Proc. Natl. Acad. Sci. USA* **96**:8516–8521.
29. **Tomazin, R., J. Boname, N. R. Hegde, D. M. Lewinsohn, Y. Altschuler, T. R. Jones, P. Cresswell, J. A. Nelson, S. R. Riddell, and D. C. Johnson.** 1999. Cytomegalovirus US2 destroys two components of the MHC class II pathway, preventing recognition by CD4⁺ T cells. *Nat. Med.* **5**:1039–1043.
30. **Tortorella, D., C. M. Story, J. B. Huppa, E. J. Wiertz, T. R. Jones, I. Bacik, J. R. Bennink, J. W. Yewdell, and H. L. Ploegh.** 1998. Dislocation of type I membrane proteins from the ER to the cytosol is sensitive to changes in redox potential. *J. Cell Biol.* **142**:365–376.
31. **Wiertz, E. J., T. R. Jones, L. Sun, M. Bogoy, H. J. Geuze, and H. L. Ploegh.** 1996. The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell* **84**:769–779.
32. **Wiertz, E. J., D. Tortorella, M. Bogoy, J. Yu, W. Mothes, T. R. Jones, T. A. Rapoport, and H. L. Ploegh.** 1996. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature* **384**:432–438.
33. **Ye, Y., H. H. Meyer, and T. A. Rapoport.** 2001. The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature* **414**:652–656.