Human Cytomegalovirus US2 Destabilizes Major Histocompatibility Complex Class I Heavy Chains

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Human cytomegalovirus (HCMV) infection causes down-regulation of major histocompatibility complex class I heavy chains. We determined previously that there are two HCMV loci which encode functions responsible for that phenotype and that US11 is one of these loci (T. R. Jones, L. A. Hanson, L. Sun, J. S. Slater, R. M. Stenberg, and A. E. Campbell, *J. Virol.* 69:4830–4841, 1995). Through the construction and analysis of defined viral mutants and stably transfected cell lines, we identify US2 as the other locus. US2 is expressed from very early through late times postinfection, with its predominant product being a relatively unstable 24-kDa endoglycosidase H-resistant glycoprotein. In cell lines constitutively expressing US2, free class I heavy chains are proximal targets for US2-induced degradation, shortly after their synthesis. Both US2 and US11 can function in concert with US3 to down-regulate class I. Beta-2-microglobulin-associated heavy chains which are retained in the endoplasmic reticulum as a result of binding to the US3 glycoprotein are susceptible to destabilization caused by both US2 and US11 gene products. Thus, three HCMV genes which affect either the stability or the transport of class I heavy chains have been identified. The observation that each of these proteins is most abundant early in the replicative cycle suggests that they may play an important immunomodulatory role in vivo prior to productive infection, either during the latent or persistent phase or during reactivation.

Evasion of the host's immune system is of paramount importance for the persistence of infectious agents. The human major histocompatibility complex (MHC) class I gene products have a central role in cytotoxic-T-lymphocyte (CTL)-mediated cellular immunity (50). The MHC class I complex is comprised of a heavy chain noncovalently associated with beta-2-microglobulin (β_2m) and an antigenic peptide. This complex is assembled in the endoplasmic reticulum (ER) and then transported to the cell surface, where it is recognized by specific CTLs (19). Thus, it is not surprising that many viruses encode proteins whose function(s) is geared toward interference with antigen presentation by modulation of MHC proteins (22, 37). Two such functions have been identified in adenoviruses. One function of the E1A gene of group A adenoviruses is to repress MHC class I heavy chain expression at the transcriptional level (37). The E3-19K gene products of group B to E adenoviruses bind and retain MHC class I heavy chains in the ER, preventing transport of the heavy chains to the cell surface (57).

Herpesviruses have been shown to affect antigen presentation by MHC class I proteins at the cell surface by acting at various points in the assembly and transport process. Herpes simplex virus (HSV) inhibits an early step: the delivery of cytosolic peptides to the ER (59). This is accomplished by HSV type 1 (HSV-1) ICP47, a cytosolic 9-kDa protein which binds to, and thereby inhibits, the cellular transporter associated with the antigen-processing function (TAP) (15, 21). Further studies have indicated that ICP47 blocks binding of peptides to the substrate binding site of TAP (2). Murine cytomegalovirus (CMV) alters an intermediate step in the pathway by retaining class I molecules intracellularly (13, 49), although down-regulation of class I synthesis also occurs in some cases (11). By yet

another mechanism, Epstein-Barr virus encodes a function which specifically inhibits presentation of its EBNA-1 protein, via *cis*-acting signals within this protein (35). Human CMV (HCMV) encodes multiple functions which down-regulate antigen presentation. For several years it has been known that MHC class I heavy chains are rapidly degraded, prior to their maturation, in HCMV-infected cells (3, 5, 52, 58). Recently, two additional functions were elucidated: retention of class I heavy chains in the ER and inhibition of cytosolic peptide transport (1, 20, 30).

We reported previously that HCMV contains two loci within the short component of its genome which function to down-regulate class I heavy chains by increased turnover (25). US11 was identified as the relevant gene of one locus. The glycoprotein product of this gene was recently shown to cause rapid dislocation of class I heavy chains from the ER to the cytosol, where the heavy chains are degraded by the proteasome (55). The second locus was determined to be within the HCMV US2 to US5 gene region (25). In this study, we used additional recombinant HCMV, with defined deletions, to determine that the US2 gene is the gene within the US2 to US5 locus which is sufficient for destabilization of MHC class I heavy chains. Like US11, US2 encodes a small endoglycosidase H-resistant glycoprotein with early-late expression kinetics and a short intracellular half-life. Furthermore, we demonstrate that US2 and US11 gene products can act in concert with HCMV US3 gene products, which retain class I heavy chains in the ER, to down-regulate MHC class I heavy chain expression.

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MATERIALS AND METHODS

DNA sequence. The numbering system of Chee et al. (12) was used for the HCMV strain AD169 DNA sequence (GenBank accession number X17403).

Virus and cells. HCMV strain AD169 was obtained from the American Type Culture Collection. The origins and growth of human foreskin fibroblast (HFF) and U373-MG astrocytoma (U373) cells were previously described (25). To obtain cell lines which constitutively express HCMV US2 under the control of the HCMV immediate-early enhancer-promoter, the US2 gene region was cloned as a 0.776-kb *Ban*II-*Xho*I DNA fragment (bases 193779 to 193003) into plasmid vectors pIE-puro, to yield pIEUS2-puro, and pIEsp-puro, to yield pIEspUS2-puro (25). 55-310 (US2⁺) cells and 55-212 (US2⁻) control cells are clonal lines derived from U373 cells after transfection of pIEspUS2-puro and pIEsp-puro, respectively, and selection in puromycin-containing medium. 166-213 (US2⁺) cells and 166-103 (US2⁻) control cells are clonal lines derived from HFF cells after transfection of pIEUS2-puro and pIE-puro, respectively, and selection in puromycin-containing media. 5-212 (US3⁺) cells were derived from U373 cells as previously described (30).

Recombinant mutant HCMV. The construction and isolation of β -glucuronidase-expressing HCMV deletion mutants were done by the general method described previously (28). Plasmids used to make the mutants contained the β -glucuronidase reporter gene flanked by about 1.5 kb of viral targeting sequences to direct homologous recombination with the HCMV genome. To replace HCMV open reading frames (ORFs) US11 through US4 with β -glucuronidase (i.e., to make RV8146) (Fig. 1B), pBgUS11/US4 was constructed. Sequentially, this plasmid contains the 1.8-kb *Pst*I-*Xba*I fragment (bases 202207 to 200391), β -glucuronidase, a 180-base fragment containing the HSV-1 *tk* polyadenylation signal, and the 1.7-kb *Eco*RV-*Sma*I fragment (bases 195083 to 193421). To replace HCMV ORFs US11 through US3 with β -glucuronidase (i.e., to make RV8173) (Fig. 1C), pBgUS11/US3 was constructed. Sequentially, this plasmid contains the 1.8-kb *Pst*I-*Xba*I fragment (bases 202207 to 200391), β -glucuronidase, the HSV-1 *tk* polyadenylation signal, and the 1.5-kb *Kpn*I-*Spe*I fragment (bases 194112 to 192648). Following cotransfection with HCMV wild-type strain AD169 genomic DNA, plaques containing β -glucuronidase-expressing virus were picked and plaque purified. The proper genomic organization of each mutant was verified by DNA blot hybridization analysis (data not shown) as previously described (28).

GST-US2 fusion protein. A glutathione S-transferase (GST)-US2 fusion protein was made with a modified vector, pGST-Nco (kindly provided by I. Mohr, Wyeth-Ayerst Research, Pearl River, N.Y.). This vector was derived by insertion of an in-frame *Nco*I site in place of the *Sma*I site within the polylinker of pGEX-2T (Pharmacia). By PCR, the portion of the US2 gene representing the region encoding amino acids 22 to 110 was amplified in a manner which resulted in the insertion of a 5' *Nco*I and a 3' *Eco*RI site to facilitate directional cloning between those same sites in pGST-Nco. Following transfection into *Escherichia coli* DH5, expression of the GST-US2 fusion protein was induced with IPTG (isopropyl- β -D-thiogalactopyranoside). After sonication of the induced bacteria, the fusion protein was located in the soluble fraction and purified by glutathione-Sepharose chromatography, according to the protocol of the manufacturer (Pharmacia).

Antibodies. Rabbit polyclonal antibody reactive with HCMV US11 protein was as previously described (28). Antibody to HCMV UL97 protein, obtained from rabbits immunized with a bacterially produced UL97 protein, was generously provided by M. van Zeijl (Wyeth-Ayerst Research). US2-reactive antibody was obtained from New Zealand White rabbits (Cocalico Biologicals, Reamstown, Pa.) with the GST-US2 fusion protein as the immunogen. Rabbit polyclonal antibody designated anti-HC, which reacts with free MHC class I heavy chains (5), was provided by H. Ploegh. Murine monoclonal antibodies W6/32 (4), specific for a conformation-dependent epitope on the heavy chain of human MHC class I proteins, and Ber-T9, specific for the human transferrin receptor, were purchased from Dako. Murine monoclonal antibody TP25.99 (14), specific for a conformation-independent epitope on human MHC class I heavy chains, was obtained from S. Ferrone (Department of Microbiology, New York Medical College, Valhalla) and R. Levy (SangStat Medical Corp., Menlo Park, Calif.). Murine monoclonal antibody 2H2.4 (47), specific for the HCMV 72-kDa IE1 protein, was purchased from Dupont, NEN.

Protein analysis. Metabolic labeling, immunoprecipitation, and immunoblotting were done as previously described (25, 29). For radiolabeling experiments involving nonradioactive chases, radioactive medium was removed and replaced with nonradioactive complete medium after two washes with phosphate-buffered saline; incubation was continued until the proper harvest time. Where indicated in Fig. 4, after immunoprecipitation, neuraminidase digestion was done for 1 h at 37°C; endoglycosidase H, N-glycosidase F, and O-glycosidase digestions were done for 18 h at 37°C under the recommended conditions (Boehringer Mannheim).

RNA analysis. Total cytoplasmic RNAs were analyzed by blot hybridization as described previously (28). US2-specific transcripts were identified through the use of a riboprobe synthesized *in vitro* from linearized pUS2-NX template. Essentially, pUS2-NX contains most of the US2 coding region (bases 193003 to 193760) downstream of a T7 RNA polymerase promoter.

RESULTS

Genetic analysis. As we reported recently (25), there are two loci within the S component of HCMV which independently

down-regulate expression of MHC class I heavy chains. While the relevant gene in one locus was identified as US11, the other locus was identified only as being in the US2 to US5 gene region, from comparison of data obtained with HCMV deletion mutants RV35 and RV798 (summarized in Fig. 1). Our previous experiments suggested that the relevant gene within the US2 to US5 gene region should be expressed throughout much of the replicative cycle, since recombinant deletion mutants of HCMV which lacked US11, but retained the US2 to US5 gene region, down-regulated MHC class I heavy chain expression at both early and late times postinfection (25). The expression kinetics of most of these genes were unknown, except for US3, which is expressed at immediate-early and very early times postinfection (24, 48, 53). To identify the gene in the US2 to US5 region responsible for the heavy chain down-regulation phenotype, several additional HCMV deletion mutants were constructed and analyzed. These new viral mutants included RV8146 and RV8173, which have the gene regions US4 to US11 and US3 to US11, respectively, deleted (Fig. 1). The new mutants were constructed by replacement mutagenesis by the homologous recombination technique described previously (28) and used to identify the role of US11 on class I heavy chain expression (25).

Class I heavy chain expression in cells infected by these new mutant viruses was assayed in metabolic radiolabeling-immunoprecipitation experiments (Fig. 2). Infected cells were radiolabeled at both early and late times postinfection; class I heavy chains were immunoprecipitated with the conformation-independent monoclonal antibody TP25.99. Consistent with previous results, substantial quantities of class I heavy chains were immunoprecipitated from lysates of uninfected and RV798-infected HFF cells (Fig. 2A). In contrast, greatly reduced amounts of MHC class I heavy chains were immunoprecipitated from lysates of cells infected with RV8146 and RV8173 at early times postinfection, amounts similar to those observed from infected cell lysates of other US11-negative mutants, RV699 and RV35, as well as from HCMV wild-type strain AD169-infected cell lysates. Similar results were observed at late times postinfection with infected HFF cells (data not shown) and infected U373 cells (Fig. 2E), an astrocytoma cell line which is permissive for HCMV. In control immunoprecipitations, we found similar levels of the HCMV IE1 protein or assembly protein precursor (Fig. 2B and F) and transferrin receptor (another cellular glycoprotein) (Fig. 2C and G). The data indicate that genes encompassing US3 to US5 are not required for class I heavy chain down-regulation. Furthermore, since the genotypic difference between mutants RV798 and RV8173 is the presence of US2 in the latter, US2 is therefore implicated in these genetic experiments (Fig. 1D).

Characterization of expression from US2. RNA and protein expression from HCMV US2 was analyzed (Fig. 3) and revealed early-late expression kinetics. In RNA blot analyses with a US2-specific riboprobe, a single 0.9-kb transcript was most abundant at early times (i.e., 3 to 16 h postinfection) but was also detected throughout the remainder of the replicative cycle (Fig. 3A and B). The size of this transcript is in good agreement with the predicted size of the US2 transcription unit plus a poly(A) tail (12, 54). Hydrophilicity analysis of the US2 ORF suggested that it may be a transmembrane glycoprotein, since it had amino- and carboxy-terminal hydrophobic domains which may correspond to the signal sequence and transmembrane domains, respectively, and one consensus N-linked glycosylation site within a hydrophilic domain (12, 54). To assess this prediction and other characteristics of the US2 gene product(s), protein expression from US2 was analyzed with polyclonal antibody obtained from rabbits immunized

FIG. 1. Organization of recombinant virus genomes and phenotype summary. (A) The first line is a schematic of the overall organization of the HCMV wild-type genome. Unique region sequences are indicated by lines; repeated region sequences are indicated by shaded boxes. Relevant *HindIII* fragments, within the L and S components, are indicated by letter designations (Q, X, and V) (42). The second line is an expansion of the wild-type *HindIII*-Q and -X regions of the S component. Significant ORFs are named in open boxes (4 is US4 and 5 is US5), and their orientations are noted with carets within the boxes (12). The position of the inverted short repeat (*IRS*) is indicated by the shaded rectangle. (B and C) Genomic organization of the indicated HCMV mutant. In each case, the first line is the organization of the parental AD169 wild-type genome and the second line represents the organization of relevant sequences of the linearized plasmid used to make the recombinant virus. The slanted lines indicate the boundaries of the viral flanking sequences which may be involved in homologous recombination to create the desired mutation. The region deleted in each mutant is indicated by a filled rectangle below the first line. Restriction sites are as follows: *EcoRV* (RV), *HindIII* (H), *KpnI* (K), *PstI* (P), *SacI* (Sa), *SmaI* (Sm), *XbaI* (Xb), and *XhoI* (X). Sites in parentheses no longer exist in the recombinant virus. pA, poly(A); Bgluc, β -glucuronidase. (D) Summary of MHC class I expression phenotypes of wild-type (WT) and mutant HCMV. For each virus, the gene region deleted and the virus' ability to down-regulate MHC class I heavy chains are indicated.

FIG. 2. Expression of MHC class I heavy chains in cells infected with HCMV mutants. (A to D) HFF cells were uninfected (U) or infected with the indicated virus (multiplicity of infection, 5 PFU/cell) and radiolabeled for 4 h at early times (6 to 10 h) postinfection (p.i.). (E to H) U373 cells were uninfected (U) or infected with the indicated virus (multiplicity of infection, 3 PFU/cell) and radiolabeled for 4 h at late times (74 to 78 h) postinfection. Proteins were harvested immediately after radiolabeling. (A and E) Class I heavy chains (HC) were immunoprecipitated with TP25.99 monoclonal antibody (MoAb). (B and F) To verify approximately equal rates of infection, the 72-kDa HCMV major immediate-early protein (IE1) was immunoprecipitated with the monoclonal antibody 2H2.4 and the HCMV UL80-encoded assembly protein precursor (pAP) was immunoprecipitated with the BX49 rabbit polyclonal antibody (29). (C and G) Immunoprecipitation of the cellular transferrin receptor (TfR) with monoclonal antibody Ber-T9 to verify approximately equal levels of expression of this cellular glycoprotein. (D and H) Immunoprecipitation of US2-encoded glycoprotein (gpUS2) with US2 polyclonal antibody (Ab). Molecular mass markers (in kilodaltons) are noted at the left of the gels.

with a GST-US2 fusion protein. Immunoblot analysis of proteins from wild-type HCMV-infected HFF cells indicated that US2 was expressed as an ~24-kDa protein throughout the replicative cycle, in agreement with the size deduced from the translated DNA sequence (12, 54). US2 protein expression was detected by 4 h postinfection, was most abundant 6 to 10 h postinfection, and persisted at lower but constant levels through late times postinfection (Fig. 3C and D). As expected, this protein was absent from RV798-infected cells but was present in cells infected with RV8173 and the other mutants used in the experiment which implicated US2 as the gene

responsible for down-regulation of class I heavy chains (Fig. 2D). The glycosidase sensitivity of the US2-encoded protein was determined with a metabolically radiolabeled and immunoprecipitated protein from HCMV-infected cells (Fig. 4). Three forms of the US2 gene product were detected. The most abundant was the 24-kDa protein detected in the immunoblot experiments (Fig. 3C and D). The two other forms, of approximately 21 and 14 kDa, were reproducibly detected and present at amounts about 10-fold less than that of the 24-kDa protein, which is the likely reason that they were not detected by immunoblot analysis. The 24-kDa protein is sensitive to both

FIG. 3. Expression of HCMV US2. (A and B) Total cytoplasmic RNA was isolated from uninfected (U) or HCMV-infected HFF cells (multiplicity of infection, 5 PFU/cell) at the indicated number of hours postinfection (p.i.). After agarose gel electrophoresis and blotting, the blots were hybridized with the US2-specific riboprobe. The position of cellular 18S rRNA is indicated. (C and D) Immunoblot analysis of US2-encoded protein. Blotted total cellular proteins from uninfected (U) or HCMV wild-type strain AD169- or RV798-infected HFF cells were probed with US2 polyclonal antibody (Ab). Molecular mass markers (in kilodaltons) are noted at the left of the gels.

endoglycosidase H and *N*-glycosidase F, indicating that it is indeed a glycoprotein (gpUS2) with N-linked sugars which are in the high-mannose form. gpUS2 was not sensitive to digestion with neuraminidase-*O*-glycosidase, indicating the apparent absence of O-linked glycans. In control experiments to verify enzyme activity, the HCMV UL55-encoded glycoprotein

(gB) was shown to be susceptible to *N*-glycosidase F, endoglycosidase H, and neuraminidase-*O*-glycosidase digestions, as expected (data not shown). The US2 21-kDa protein comigrates with the product of endoglycosidase H- or *N*-glycosidase F-digested gpUS2 and likely represents the unglycosylated form of the US2 gene product. A similar unglycosylated form of the primary US11 gene product (gpUS11) is detected in pulse-labeled proteins from HCMV-infected cells (data not shown). The 14-kDa protein is sensitive to *N*-glycosidase F, but it is insensitive to endoglycosidase H. Although this protein is a glycoprotein, the molecular composition of this putative minor US2 product has not been investigated further.

US2 is sufficient for MHC class I heavy chain down-regulation. The genetic data from experiments with the recombinant viruses indicated that in the context of HCMV infection, US2 is required for MHC class I down-regulation in the absence of US11. To determine if US2 expression is sufficient for this phenotype, transfected HFF and U373 cells which stably express US2 were constructed. This strategy was used previously to verify the function of gpUS11 (25). US2 was cloned in the pIE-puro expression vector such that its expression was under the control of the HCMV major immediate-early enhancer-promoter. Expression of US2 in clones obtained following transfection was assessed by immunoblot analysis. Numerous US2-expressing U373-derived cell lines were obtained, and each had a reduced steady-state level of MHC class I heavy chains compared to those of US2-negative control cell lines

FIG. 4. Glycosidase sensitivity of US2-encoded proteins. Uninfected (UNINF) or HCMV strain AD169-infected (multiplicity of infection, 5 PFU/cell) HFF cells were radiolabeled from 6 to 7 h postinfection (p.i.) (PULSE) and chased with unlabeled complete medium for 2 h (CHASE). After immunoprecipitation with US2 polyclonal antibody (Ab), precipitated proteins were digested with or without glycosidase as follows: without glycosidase (—) or with endoglycosidase H (EH), *N*-glycosidase F (NG), neuraminidase (N), or neuraminidase and *O*-glycosidase (N+O). Molecular mass markers (in kilodaltons) are noted at the left.

FIG. 5. Stability of class I heavy chains (HC) in US2⁺ U373 cells. Parental U373 cells (U373-MG), US2⁻ U373-derived cells (55-212), or US2⁺ U373-derived cells (55-310) were pulse-labeled (P) for 0.5 h and then chased for the number of hours postinfection indicated above the gels in unlabeled complete medium and immunoprecipitated with TP25.99 monoclonal antibody (MoAb) (A), Ber-T9 monoclonal antibody (B), or US2 polyclonal antibody (Ab) (C and D). The gel in panel D was exposed seven times longer than the gel in panel C to show the less abundant, 21- (♂) and 14-kDa (♀♀) US2-derived proteins. Molecular mass markers (in kilodaltons) are noted at the left of the gels. Tfr, transferrin receptor.

(data not shown). Each cell line was further examined by pulse-chase radiolabeling-immunoprecipitation experiments; representative results are shown in Fig. 5. In U373 parental cells and in stably transfected US2⁻ (55-212) control cells, class I heavy chains were stable throughout the 3.5-h chase period (Fig. 5A). In contrast, heavy chains were detected only in the pulse lane of US2⁺ (55-310) cells. Subsequent experiments indicated the half-lives of the heavy chains in 55-310 cells were less than 0.5 h (data not shown). As a control, the levels of the cellular transferrin receptor glycoprotein were similar in these cell lines (Fig. 5B). Immunoprecipitations with the US2 antibody confirmed the presence of the three US2 protein forms in the stably transfected cell lines, which were also detected in HCMV-infected cells (Fig. 4). Thus, these data confirm that US2 expression is sufficient and specific for the down-regulation of MHC class I heavy chains. Furthermore, US2-induced down-regulation, similar to the down-regulation caused by gpUS11, is due to instability of class I heavy chains (25, 55).

Class I heavy chain target population of gpUS2. During their synthesis, class I heavy chains are cotranslationally inserted into the ER via the translocation complex. In the ER, they initially associate with a chaperone protein(s) (i.e., calnexin) and β 2m, followed by association with antigenic peptide and TAP proteins (19). The noncovalent association of class I heavy chains with β 2m occurs at different rates, ranging from 5 min to 4 h, depending on the particular class I allele (23, 34). Thus, both β 2m-associated and free heavy chains exist in the ER. It is known that free heavy chains and some associated heavy chains are susceptible to US11-caused instability (data not shown and reference 55). The experiment shown in Fig. 5A utilized a monomorphic antibody which recognizes both the

β 2m-associated and free forms of class I heavy chains. Antibodies which differentiate between these forms were used in pulse-chase radiolabeling-immunoprecipitation experiments to assess the target population of class I heavy chain molecules by US2 gene products in stably transfected US2-expressing HFF cells (Fig. 6). All three forms of US2 gene products detected in HCMV-infected cells were detected in the US2⁺ HFF cells, and they retained the approximate 1-h half-life observed in the infected cells (Fig. 6C). When anti-HC antibody which recognizes only free heavy chains was used, heavy chains were unstable in the US2⁺ HFF cells (half-life, $\sim$ 0.5 h) but not in either control cell line (Fig. 6A). However, in immunoprecipitation experiments using antibody W6/32, which recognizes β 2m-associated heavy chains, the heavy chains were stable, although their abundance was drastically reduced compared to that in control cells (Fig. 6B). These results indicate that the free class I heavy chains are proximal targets of gpUS2 and that association with β 2m is not required. The reduction in the amount of β 2m-associated heavy chains in US2⁺ HFF cells is due to the small pool of stable free heavy chains (a result of gpUS2 activity) which can become associated with β 2m. In control cell lines, the amount of W6/32-precipitable heavy chains increases due to the association of β 2m with (previously) free heavy chains that occurs during the chase period. As in US2-expressing U373-derived cells, gpUS2 has no effect on the maturation of cellular transferrin receptor synthesis (Fig. 6C) synthesis or stability of β 2m (data not shown).

Effects of gpUS2 and gpUS11 on gpUS3-retained heavy chains. The HCMV US2 gene product is 50% similar to the putative HCMV US3 (i.e., the ORF which is adjacent to US2) gene product at the amino acid level (data not shown and reference 12). We and others have recently reported that US3 expresses

FIG. 6. Stability of free and β 2m-associated class I heavy chains (hc) in US2⁺ HFF cells. Parental HFF cells, US2⁻ HFF-derived cells (166-103), or US2⁺ HFF-derived cells (166-213) were pulse-radiolabeled and chased as described in the legend for Fig. 5. HCMV strain AD169-infected HFF cells (multiplicity of infection, 5 PFU/cell) were pulse-radiolabeled and chased beginning at 6 h postinfection in a similar fashion. Proteins were immunoprecipitated with either anti-HC antibody (HC Ab) (A), W6/32 monoclonal antibody (MoAb) (B), Ber-T9 monoclonal antibody (C), or US2 polyclonal antibody (D). Molecular mass markers (in kilodaltons) are noted at the left. Tfr, transferrin receptor.

a 21-kDa glycoprotein (called gpUS3) which binds and retains MHC class I heavy chains intracellularly, predominantly in the endoplasmic reticulum (1, 30). Unlike expression of US2 and US11, which occurs beginning at early times postinfection (i.e., after 3 to 4 h), expression of US3 is abundant at immediate-early and very early times postinfection (i.e., within the first 6 h) (24, 48, 53). In view of the progression of expression of *fosB* US3, followed by the expression of US11 and US2, we sought to determine whether heavy chains re-

FIG. 7. Susceptibility of class I heavy chains (hc) retained by gpUS3 to degradation by gpUS2 and gpUS11. Parental U373 cells or US3⁺ U373-derived cells were pulse-radiolabeled for 1 h prior to infection with either HCMV wild-type strain AD169 (US2⁺/US11⁺) or mutant RV798 (US2⁻/US11⁻), RV7119 (US2⁺/US11⁻), or RV8173 (US2⁻/US11⁻) at a multiplicity of infection of 5 PFU/cell in unlabeled complete medium. At 6 h postinfection, proteins were harvested and immunoprecipitated with either W6/32 monoclonal antibody (MoAb) (A and C) or Ber-T9 monoclonal antibody (B). Where indicated (+), immunoprecipitated proteins were digested with endoglycosidase H (Endo H). Molecular mass markers (in kilodaltons) are noted at the left. Tfr, transferrin receptor.

tained by gpUS3 were susceptible to degradation initiated by either gpUS2 or gpUS11 (Fig. 7). In this experiment, uninfected U373 parental cells and US3⁺ U373-derived cells were pulse-radiolabeled for 1 h and then infected with HCMV wild-type strain AD169 (US11⁺/US2⁺) or the mutant RV798 (US2⁻/US11⁻), RV7119 (US2⁺/US11⁻), or RV8173 (US2⁻/US11⁻). Each of these mutants has US3 deleted. Immunoprecipitations with W6/32 monoclonal antibody at 6 h postinfection revealed that the radiolabeled class I heavy chains were readily detectable in parental U373 cells infected with each of these viruses. Likewise, abundant levels of class I heavy chains were observed in US3⁺ cells infected with the US2⁻/US11⁻ mutant RV798. In contrast, radiolabeled class I heavy chains

were barely detectable, and thus unstable, in US3⁺ cells infected with mutant viruses which expressed US2 and/or US11. To confirm gpUS3 function in these experiments, the W6/32-derived immunoprecipitates were subjected to digestion by endoglycosidase H. As expected, radiolabeled heavy chains detected in US3⁺ cells, but not those from parental U373 cells, were sensitive to endoglycosidase H (Fig. 7C). These results verify the proper function gpUS3 (i.e., inhibition of transport of the class I heavy chains to the medial Golgi apparatus) and indicate that US3 can act in concert with either US2 or US11 to prevent cell surface expression of MHC class I complexes.

DISCUSSION

It is hypothesized that viruses manipulate the host's immune surveillance system in order to successfully establish and maintain a latent or persistent state (39, 40). Perhaps significant in this regard is the drastic reduction in cell surface expression of MHC class I proteins that has been observed in HCMV-infected cells (3, 25, 52, 58), which can be explained in part by the rapid turnover of MHC class I heavy chains in these cells. Portions of the US region of the HCMV genome, encoding the IRS1 to US11 genes, are dispensable for virus growth in culture, suggesting that they may be involved in virus-host interactions (27, 28, 32). We reported recently that HCMV contains two loci whose expression independently down-regulates class I heavy chains; US11 is the relevant gene in one locus (25, 55). This latter observation was confirmed recently by other laboratories (1, 20). Here, we report that the relevant gene in the second locus is US2.

Our identification of US2 was based on data obtained with defined HCMV deletion mutants and corroborated by the analysis of cell lines which constitutively expressed US2. Like US11, US2 expression results in the rapid degradation of MHC class I heavy chains shortly after their synthesis (Fig. 5 and 6). Although the homology between their primary translation products is extremely limited, both genes encode small N-linked glycoproteins which have a short intracellular half-life (1 h). gpUS11 has been localized to the ER (55) and is absent from gradient-purified virions (24). The presumed location of gpUS2 is also the ER, since it (i) induces instability of free class I heavy chains after their cotranslational translocation into the ER (Fig. 6) and (ii) has a single N-linked glycan which is in the immature (high-mannose) form (Fig. 4). Expression of US11 was shown to cause the rapid dislocation of class I heavy chains from the ER to the cytosol, resulting in their degradation by the proteasome (55). Analogous studies using US2⁺ cell lines revealed that heavy chain turnover initiated by gpUS2 occurs in an identical fashion (56). Specifically, newly synthesized MHC class I heavy chains were delivered to the cytoplasm through a process directly involving the mammalian Sec61 translocation complex (56), which functions in the cotranslational insertion of proteins across the ER membrane (18, 41).

Although the rapid degradation of free class I heavy chains by gpUS2 and gpUS11 is readily detected (Fig. 2) (24, 55), similar turnover of β 2m-associated heavy chains is observed only very shortly after β 2m association (55, 56). Thus, free heavy chains are the proximal targets of gpUS2 and gpUS11, while gpUS3 impairs the transport and maturation of β 2m-associated class I heavy chains via retention in the ER (1, 30). In the course of HCMV infection, US3 is expressed abundantly at immediate-early and very early times postinfection (24, 48, 53) while US2 and US11 are expressed with early-late kinetics, with high steady-state levels present during the first few hours of the early phase (Fig. 3) (24, 26). Although each of these viral proteins is relatively unstable (half life, 1 h), both gpUS2 and

gpUS11 are detected simultaneously in HCMV-infected cells from 3 to 6 h postinfection (Fig. 5 and 6) (24, 30). In U373-derived cell lines which constitutively express US3, gpUS3 does not retain all class I heavy chains with equal efficiencies. While all class I heavy chains were initially retained, some eventually acquired endoglycosidase H resistance and reached the cell surface, albeit with a substantial delay (30). The gpUS3 retention function can act in concert with the destabilization functions imparted by either gpUS2 or gpUS11 (Fig. 7). In HCMV-infected cells, the overall efficiency of MHC class I heavy chain down-regulation will be enhanced under these early conditions due to the cooperation of the multiple functions which are exerted by these three HCMV glycoproteins. Furthermore, the observed cooperation between gpUS3 and gpUS2 or gpUS11, in conjunction with the known mechanism of destabilization of MHC class I heavy chains by the latter HCMV glycoproteins, implies that gpUS3-retained heavy chains are located in proximity to the ER Sec61 translocation complex (55, 56).

Why should HCMV encode two proteins with apparently identical functions? While the answer to this question is unclear, it is possible that each glycoprotein does not recognize the full complement of human class I heavy chain alleles. While this hypothesis has not been rigorously examined, this appears to be the case for, at least, US2. When HCMV mutant-infected U373 and HFF cells are compared, degradation of class I heavy chains is more complete in the former than in the latter, although heavy chain synthesis is greater in U373 cells (Fig. 2). Similar results were also observed in cell lines which constitutively express US2. In pulse-chase experiments, small amounts of stable β 2m-associated heavy chains were observed in US2⁺ HFF cells (Fig. 6B). In contrast, no stable β 2m-associated heavy chains were detected in US2⁺ U373 cells (Fig. 3 of reference 30). Alternatively, subtle differences in the levels of either gpUS2 or interacting cellular proteins (e.g., Sec61) in the different cell types may cause this result. However, allelic preference may be common for viral glycoproteins which affect class I heavy chain expression, given the heterogeneity among these proteins, especially in the α 1 and α 2 domains (7). For example, adenovirus E3-19K glycoprotein, which binds and retains some class I heavy chains in the ER via recognition of the α 1 and α 2 peptide binding regions of these proteins, has preferences for the A2.1 and B7 alleles but not the B27 and Bw58 alleles (6, 33). Although the domain(s) of class I recognized by gpUS3 is unknown, HCMV gpUS3 functions similarly to E3-19K and appears to exhibit allelic preference in that certain class I heavy chains are retained intracellularly longer than others (30). Although definitive evidence for allelic preferences of human class I heavy chains by gpUS2 and gpUS11 has not been demonstrated, preferential recognition of mouse class I heavy chain alleles by these glycoproteins was recently observed (36a).

HCMV encodes multiple proteins which function to down-regulate the surface expression of MHC class I molecules in infected cells, including US11 (25), US3 (1, 30), and a putative protein(s) which interferes with peptide transport to the ER (20). Despite these functions, HCMV-specific CTLs which lyse infected fibroblasts in culture are generated *in vivo* (8, 9, 16). While it is believed that a specific CTL response is a major factor in limiting HCMV in immunocompetent humans (10), it is clear that the presence of HCMV-specific CTLs correlates with protection from HCMV disease after transplantation (36, 44, 46, 51). In HCMV-seropositive individuals, virus-specific CTLs recognize epitopes derived predominantly from pp65, a virion structural (matrix) protein (36, 38, 45). Thus, we propose that the combined effect of the multiple HCMV proteins which act to down-regulate MHC class I expression is to mod-

ulate, but not abrogate, the CD8⁺ CTL portion of the cellular immune response. These functions likely play a role in limiting the frequency or efficiency of CTLs directed against other HCMV epitopes, resulting in persistence, rather than elimination, of HCMV from the host.

The dominant target of CTLs after infection of mice by murine CMV is the immediate-early protein pp89 (31, 43). A similar CTL response in humans directed against the analogous protein of HCMV, IE1, is not observed (16). Since US2, US3, and US11 are expressed at immediate-early or early times postinfection, it is possible that they contribute to the absence of IE1 peptide presentation on the cell surface. However, Gilbert and colleagues reported recently that the presence of pp65 is sufficient to prevent cell surface expression of IE1-derived peptides, possibly due to posttranslational modification of IE1 (17). There is no obvious reason why US2, US3, and US11 might not also modulate the surface expression of IE1-derived peptides, especially under circumstances in which pp65 may not be present, perhaps during the viral latent or persistent phase or during reactivation. Further experimentation to determine the breadth of US2, US3, and US11 expression in infected human tissues, as well as studies utilizing the various HCMV mutants, with one or more of these viral genes deleted, is needed in order to properly define the impact of these genes on antigen presentation and CTL recognition.

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