Human Cytomegalovirus Disrupts the Major Histocompatibility Complex Class I Peptide-Loading Complex and Inhibits Tapasin Gene Transcription

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Major histocompatibility complex class I (MHC I) molecules present antigenic peptides for CD8+ T-cell recognition. Prior to cell surface expression, proper MHC I loading is conducted by the peptide-loading complex (PLC), composed of the MHC I heavy chain (HC) and β2-microglobulin (β2m), the peptide transporter TAP, and several chaperones, including tapasin. Tapasin connects peptide-receptive MHC I molecules to the PLC, thereby facilitating loading of high-affinity peptides onto MHC I. To cope with CD8+ T-cell responses, human cytomegalovirus (HCMV) encodes several posttranslational strategies inhibiting peptide transport and MHC I biogenesis which have been studied extensively in transfected cells. Here we analyzed assembly of the PLC in naturally HCMV-infected fibroblasts throughout the protracted replication cycle. MHC I incorporation into the PLC was absent early in HCMV infection. Subsequently, tapasin neosynthesis became strongly reduced, while tapasin steady-state levels diminished only slowly in infected cells, revealing a blocked synthesis rather than degradation. Tapasin mRNA levels were continuously downregulated during infection, while tapasin transcripts remained stable and long-lived. Taking advantage of a novel method by which de novo transcribed RNA is selectively labeled and analyzed, an immediate decline of tapasin transcription was seen, followed by downregulation of TAP2 and TAP1 gene expression. However, upon forced expression of tapasin in HCMV-infected cells, repair of MHC I incorporation into the PLC was relatively inefficient, suggesting an additional level of HCMV interference. The data presented here document a two-pronged coordinated attack on tapasin function by HCMV.

Human cytomegalovirus (HCMV) belongs to the β-subgroup of herpesviruses, which are a family of viruses with a large double-stranded DNA genome. The HCMV genome carries approximately 200 genes, which are transcribed in a cascading fashion of immediate-early (IE), early (E), and late (L) genes. Completion of the protracted HCMV replication cycle takes 72 to 96 h. Like all herpesviruses, HCMV persists in the infected host for life, with alternating episodes of latent infection and recurrent replication. While being clinically symptomless in immunocompetent individuals, HCMV can cause severe disease in immunocompromised individuals, such as transplant and AIDS patients, reflecting the delicate balance between the immune system responding to the infection and viral evasion of immune control.

Antigen presentation to CD8+ T cells is a major defense mechanism against virally infected cells. The major histocompatibility complex class I (MHC I) antigen presentation pathway exposes peptide antigens on the cell surface to surveilling CD8+ T cells. Upon a fitting contact between the T cell receptor and an MHC I-peptide complex, the CD8+ T cell becomes activated to induce lytic destruction of the recognized target cell.

The antigenic peptides displayed by MHC I molecules on the cell surface are degradation products of proteins that have been targeted to the proteasome. To reach the luminal side of the endoplasmic reticulum (ER), the peptides are bound to the transporter associated with antigen processing (TAP) before being translocated across the ER membrane (43), where MHC I molecules encounter their peptide ligands. TAP is a heterodimeric ABC (ATP binding cassette) transporter consisting of the subunits TAP1 and TAP2. The efficient loading of peptides onto MHC I molecules requires participation of several additional chaperones. The peptide-receptive heterodimeric MHC I, comprising the MHC I heavy chain (HC) and β2-microglobulin (β2m), is recruited to the peptide-loading complex (PLC). In the PLC, MHC I is bridged to TAP via the chaperone tapasin (48). Tapasin connects PLC constituents but also exerts a critical quality control on the MHC I loading process that determines the release of MHC I molecules and cell surface expression (23, 57). In addition, calreticulin and the oxy-
doreductase ERp57 are found as further constituents of the PLC (34, 44). Within the PLC, tapasin and ERp57 form a stable interaction through a disulfide bond (41, 42). Recently, it was shown that this specific interaction promotes loading of high-affinity peptides even in the presence of an excess of low-affinity peptides (54).

Most of the constituents of the MHC I antigen presentation pathway are extensively controlled on the transcriptional level. Expression of the genes encoding MHC I, β2m, TAP1, TAP2, and tapasin is strongly induced by gamma interferon (IFN-γ) (1, 26, 35). In contrast, ERp57 is not elevated by IFN-γ stimulation, but under these conditions, the majority of the existing molecules are recruited to the PLC (42). Analysis of the mouse tapasin promoter showed that several gamma activated sequence (GAS) elements located shortly upstream of the transcription start site are functional. Enhancement of gene transcription by IFN-γ was found to be dependent on secondary transcription factors such as interferon regulatory factor 1 (IRF-1) (1, 22). Conversely, a transcriptional repressor belonging to the Krüppel family of zinc finger proteins, PRDM-1 (IRF-1) (1, 22), is also found that US3 is able to inhibit the function of tapasin (37) and that of protein disulfide isomerase (PDI) (38). US3 is a short-lived protein which is expressed only during the IE phase of HCMV gene expression (13, 49), implying that the inhibition of tapasin through US3 is restricted to a few hours of HCMV infection. US6 binds to TAP and blocks the peptide supply, preventing the formation of ternary MHC I (3, 19, 32). US6 interacts with both TAP subunits to inhibit peptide transport, thereby freezing the PLC in a complex with TAP and US6 (15). Reports on as yet undefined mechanisms suggest that US10, US8, and UL82/pp71 might also be involved in evasion of antigen presentation by MHC I molecules (11, 50, 51).

Here we analyzed the formation of the PLC throughout the HCMV replication cycle in detail. Deficient association of MHC class I heterodimers with the PLC was observed early during the course of infection. In addition, HCMV blocked nascent tapasin gene transcription and protein neosynthesis. Forced tapasin transcription in HCMV-infected cells only partly reconstituted the incorporation of MHC I into the PLC and the density of MHC I molecules on the cell surface, pointing at possible unidentified mechanisms by which HCMV prevents proper PLC assembly and further impedes peptide loading of MHC I.

**MATERIALS AND METHODS**

**Cell lines and antibodies.** MRC-5 (ATCC CCL171) and HEK 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), penicillin, streptomycin, and glutamine. The STC anti-tapasin rabbit antisera recognizing the C terminus of tapasin was used for immuno-
For immunoblotting, adjusted protein amounts were separated by SDS-PAGE. Proteins were blotted onto a Protran membrane and incubated with specific antibodies, followed by peroxidase-conjugated secondary antibodies and detection using the enhanced chemiluminescence substrate ECL Plus (Amer sham Biosciences).

Northern blotting and semiquantitative reverse transcription-PCR (RT-PCR). RNA was isolated using a QiAshredder and RNeasy kit (Qiagen). Equal amounts of total RNA (2 to 5 μg) were subjected to gel electrophoresis and cross-linked to a nylon membrane. Hybridization, washing, and detection were performed using standard techniques. Equal loading of RNA was confirmed by ethidium bromide staining of agarose gels and reblotting of nylon membranes with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. Detection of specific transcripts was performed with digoxigenin (DIG)-labeled probes amplification by the primers listed in Table 1.

For OneStep RT-PCR (Qiagen), 20 ng of total RNA was applied, and intron-overlapping primers were used when possible (Table 2). For semiquantitative analysis, the number of cycles was adjusted to 30 cycles. PCR products were run over­lapping primers were used when possible (Table 2). For semiquantitative analysis, the number of cycles was adjusted to 30 cycles. PCR products were run.

**RESULTS**

Altered composition of the PLC in HCMV-infected cells. HCMV inhibitors interfering with the MHC I pathway of antigen presentation have been studied extensively in transfected cells overexpressing the inhibitors, whereas no comprehensive approaches to analyze the PLC in the context of HCMV infection have been reported so far. In a previous study, we noted that MHC I and tapasin might be absent from TAP1/2 immunoprecipitates of HCMV-infected cells at 72 h postinfection (p.i.) (18). This hypothesis prompted us to monitor the composition of the PLC throughout HCMV infection. MRC-5 fibroblasts were infected with the HCMV AD169-derived mutant HB5 (6), lacking the genes US2 to US6, to exclude the effects of previously described inhibitors. The cells were radio­ labeled for 6 h. Lysis was performed using the mild detergent digitonin, which preserves the PLC for precipitation by antibodies directed against various components of the PLC. PLC assembly was analyzed at 8, 24, 48, and 72 h p.i. IFN-γ treat­ ment of mock-infected cells enhanced the bands corre­sponding to tapasin, MHC I HC and βm, ERp57, and TAP1/2 (Fig. 1A, lane 11), and the expected band shift resulting from endothelial cell attachment (endo-N-acyetylglucosaminidase) was evident (endo H) treatment confirmed the identities of tapasin and MHC I HC (indicated by arrows in Fig. 1A, lane 2). The experiment revealed two interesting phenotypes. First, as the replication cycle proceeded, the tapasin band weakened in comparison to that for mock-infected cells. The first indication was observed at 24 h p.i. (Fig. 1A, lane 5), but it was most pronounced at 72 h p.i. both in wild-type AD169-infected cells and in HB5-infected cells (Fig. 1A, lanes 9 and 10). Second, MHC I HC and βm had already disappeared from the PLC in HB5-infected cells at 24 h p.i. (Fig. 1A, lane 5), which was a surprising observation since the gene for US2 is deleted from HB5 and, consequently, fewer MHC I molecules are degraded. Interestingly, the two observed phenotypes followed different kinetics. Whereas the band for tapasin disappeared slowly, MHC I coprecipitation was reduced already at 8 h p.i. and was completely abolished at 24 h p.i. (Fig. 1B).

To further examine the changes taking place in the forma­tion of the PLC during HCMV infection, we made use of specific antibodies directed against distinct PLC components for analysis of MHC I incorporation into the PLC. Since HCMV produces highly glycosylated viral FcγRs, i.e., TRL11- and IRL11-encoded gp34 and UL19-118-encoded gp68, which have high affinities for rabbit IgG (4, 33), we constructed an HB5 (ΔUS2-US6)-derived mutant lacking gp34 and gp68 (ΔΔUS2-US6) to avoid precipitation of FcγRs (causing background bands as demonstrated in Fig. 1C). The cells were infected with HCMV ΔΔUS2-US6 for 48 h, a time point at which a reduced level of tapasin incorporation into the PLC was observed and no MHC I HC-βm complexes were coprecipitated with anti-tapasin antibodies (Fig. 1A). Using MAbs W6/32, recognizing only heterodimeric MHC I HC-βm complexes and not free HC, the levels of radiolabeled MHC I in mock-

**TABLE 1. Primers for Northern blot probes**

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**TABLE 2. Primers for RT-PCR**

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**TABLE 3. Composition of the PLC throughout HCMV infection.** MRC-5 fibroblasts were infected with the HCMV AD169-derived mutant HB5 (6), lacking the genes US2 to US6, to exclude the effects of previously described inhibitors. The cells were radio­ labeled for 6 h. Lysis was performed using the mild detergent digitonin, which preserves the PLC for precipitation by antibodies directed against various components of the PLC. PLC assembly was analyzed at 8, 24, 48, and 72 h p.i. IFN-γ treatment of mock-infected cells enhanced the bands corresponding to tapasin, MHC I HC and βm, ERp57, and TAP1/2 (Fig. 1A, lane 11), and the expected band shift resulting from endothelial cell attachment (endo-N-acyetylglucosaminidase) was evident (endo H) treatment confirmed the identities of tapasin and MHC I HC (indicated by arrows in Fig. 1A, lane 2). The experiment revealed two interesting phenotypes. First, as the replication cycle proceeded, the tapasin band weakened in comparison to that for mock-infected cells. The first indication was observed at 24 h p.i. (Fig. 1A, lane 5), but it was most pronounced at 72 h p.i. both in wild-type AD169-infected cells and in HB5-infected cells (Fig. 1A, lanes 9 and 10). Second, MHC I HC and βm had already disappeared from the PLC in HB5-infected cells at 24 h p.i. (Fig. 1A, lane 5), which was a surprising observation since the gene for US2 is deleted from HB5 and, consequently, fewer MHC I molecules are degraded. Interestingly, the two observed phenotypes followed different kinetics. Whereas the band for tapasin disappeared slowly, MHC I coprecipitation was reduced already at 8 h p.i. and was completely abolished at 24 h p.i. (Fig. 1B).

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HCMV virions. This was also the case for TAP1 and TAP2 but expression controls the induced tapasin expression triggered by (Fig. 2A, lanes 8 to 10 and 17 to 19). Hence, HCMV gene amounts in cells treated with UV-inactivated HCMV particles and 12 to 15), which contrasted with the clearly increased eventually recognizable (Fig. 2A, compare lane 1 with lanes 4 to 6 observed in infected cells, but a moderate reduction was even-

By immunoblotting. No drastic effect on tapasin amounts was tion of US2 and HCMV-infected cells were determined. Due to the dele-

3476 HALENIUS ET AL. J. VIROL.

Reduced biosynthesis of tapasin. Tapasin is known to be a long-lived protein (5). To assess the time point at which the reduction of newly synthesized tapasin results in a loss of overall protein abundance, the steady-state levels of tapasin were examined in comparison with other PLC components. From HCMV-infected cells, anti-tapasin recovered only 22% of MHC I in comparison to MHC I from mock-infected cells, anti-ERp57 recovered 21%, anti-TAP1 recovered 33%, and anti-calreticulin recovered 51% (Fig. 1E). The relative amount of MHC I recovered by anti-tapasin compared to W6/32-immunoprecipitated MHC I heterodimers was determined to be 25% in the case of mock-infected cells, whereas for infected cells it was 10% (Fig. 1F). This implies that whereas the calreticulin-MHC I interaction seems to be maintained in infected cells, the recruitment of MHC I heterodimers to the PLC is disturbed independent of the downregulation of tapasin molecules.

Reduced biosynthesis of tapasin. Tapasin is known to be a long-lived protein (5). To assess the time point at which the reduction of newly synthesized tapasin results in a loss of overall protein abundance, the steady-state levels of tapasin were examined in comparison with other PLC components during the progression of HCMV replication. Cell lysates from AD169- and US2-US11 mutant-infected cells were analyzed by immunoblotting. No drastic effect on tapasin amounts was observed in infected cells, but a moderate reduction was eventually recognizable (Fig. 2A, compare lane 1 with lanes 4 to 6 and 12 to 15), which contrasted with the clearly increased amounts in cells treated with UV-inactivated HCMV particles (Fig. 2A, lanes 8 to 10 and 17 to 19). Hence, HCMV gene expression controls the induced tapasin expression triggered by HCMV virions. This was also the case for TAP1 and TAP2 but not for ERp57. The reduction occurred independently of the US2 to US11 genes, as revealed by cells infected with the HCMV ΔUS2-US11 deletion mutant. We also detected MHC I HC by immunoblotting and found only moderate changes in the steady-state levels during HCMV AD169 wt infection, whereas in ΔUS2-US11 mutant-infected cells, MHC I HC was abundantly detected, reaching a level close to that seen after treatment of cells with IFN-γ or inactivated HCMV particles (Fig. 2A). Taken together, the data from the immunoblot analysis demonstrated only a slow diminishment of tapasin steady-state levels in infected cells, suggesting a blocked synthesis rather than enhanced protein degradation.

To illustrate this hypothesis, tapasin biosynthesis was investigated in more detail by shortened metabolic pulse labeling of cells for 60 and 180 min. Under these conditions, the viral effect on tapasin neosynthesis was much more pronounced (Fig. 2B). Reduction of tapasin biosynthesis could be observed at 24 h.p.i., and at 48 and 72 h.p.i., tapasin was barely detectable (equal translation rates of samples were verified [data not shown]). In pulse-chase experiments using metabolically labeled cells, we observed no change in the half-life of tapasin upon HCMV infection (data not shown). Taken together, the data set presented in Fig. 2 strongly argues that the loss of tapasin precipitation from radiolabeled HCMV-infected cells was not due to protein degradation but rather to blocked de novo synthesis.

Reduced levels of tapasin mRNA in HCMV-infected cells. Since existing tapasin molecules are stable in HCMV-infected cells and the rate of newly synthesized molecules is downregulated, we set out to analyze whether this regulation occurs at the transcriptional or translational level. Northern blot analysis was performed at different time points along the HCMV replication cycle. Similar to tapasin protein levels, tapasin transcript levels were found to be reduced continuously in comparison to the mock control level, eventually reaching very low levels (Fig. 3A). This effect was seen in cells infected with the HCMV wt strain AD169 and was reproduced with the ΔUS2-US11 mutant virus (Fig. 3B). In contrast to the results of

<table>
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<th>Primer Tm (°C)</th>
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TABLE 3. Primers for qRT-PCR
immunoblot analysis (Fig. 2A), there was no difference in MHC I transcripts between AD169- and US2-US11 mutant-infected cells, and the overall level of MHC I mRNA was induced throughout HCMV infection. Interestingly, TAP1 and TAP2 RNAs were also reduced in HCMV-infected cells, but the effect appeared later in infection and was clearly visible at 72 h p.i. At 24 h p.i., the TAP1 RNA level was even slightly upregulated. The β2m RNA level was only marginally affected by HCMV infection at 72 h p.i. By analyzing transcripts in cells treated with UV-inactivated HCMV particles, the strength of the transcriptional upregulation in the absence of viral gene expression was assessed (Fig. 3C). Transcription of MHC I was found to be induced in cells treated with inactivated HCMV, reaching levels seen in IFN-γ-stimulated cells. Similarly, the steady-state level of tapasin transcripts was upregulated in cells infected with UV-inactivated HCMV, strongly contrasting with the progressive decline of tapasin mRNA induced by replicating HCMV.

FIG. 1. HCMV impairs PLC assembly. MRC-5 cells were mock or IFN-γ (500 U/ml, 72 h) treated or infected with HB5, AD169, or ΔΔvFcγR virus at a multiplicity of infection (MOI) of 5. (A) Cells were labeled for 6 h and then lysed at the indicated time points of infection. Lysates were snap-frozen in liquid N2 and stored at −80°C. After thawing, proteins were recovered by immunoprecipitation using anti-tapasin antibodies. Arrows indicate endo H (EH)-deglycosylated proteins in lane 2. The smear produced by the HCMV-encoded vFcγR gp34 is indicated. (B) Quantification of tapasin and MHC I HC precipitation from HB5-infected cells. The band intensities are displayed relative to those for the mock control at 8 h (lane 1 in panel A; depicted as time point 0). (C) HCMV-encoded FcγRs gp34 and gp68 are precipitated by rabbit antiserum. Cells were labeled for 3 h and lysed at 48 h p.i. Using rabbit anti-tapasin antiserum, precipitation of viral FcγRs was demonstrated. The vFcγR deletion mutant (ΔΔvFcγR) lacks the bands caused by the highly glycosylated vFcγR proteins gp34 and gp68 seen in the AD169 lane. (D) Analysis of PLC composition. Cells were labeled for 3 h and lysed at 48 h p.i. Immunoprecipitations were performed with the indicated antibodies. Immune complexes were separated by 10 to 11.5% SDS-PAGE. Marked bands indicate primary targets of the antibodies. (E) Quantification of PLC components. Diagrams show intensities of bands corresponding to the PLC components indicated above, relative to the maximum intensity. The antibody used for immunoprecipitation is indicated on the x axis. Numbers beneath show the percentages of bands from ΔΔvFcγR-infected cells in comparison to bands from mock cells. n.d., not determined. (F) Quantification of the amount of MHC I HC coprecipitated by anti-tapasin antibodies in comparison to the amount of MHC I HC precipitated by W6/32.
HCMV inhibits tapasin gene expression at a pretranscriptional level. In order to test whether the tapasin mRNA is destabilized, e.g., by a degrading microRNA (miRNA), or if HCMV interferes with tapasin gene expression on a pretranscriptional level, the stability of tapasin RNA was measured. Cells were treated with solvent (dimethyl sulfoxide [DMSO]) only or with actD for 0, 5, 10, and 24 h, starting at 36 h p.i. Tapasin mRNA was analyzed by semiquantitative RT-PCR (Fig. 3D). Tapasin mRNA is relatively stable, and the half-life exceeded the time frame of the experiment. Successful actD blocking of the RNA polymerase was confirmed by the disappearance of SOCS3 mRNA, which has been shown to have a half-life of only 30 min (59). In line with the previous results obtained by Northern blotting, tapasin mRNA was already reduced at 36 h p.i. in infected cells without actD treatment (Fig. 3). Interestingly, actD treatment of infected cells did not reveal a decrease in tapasin mRNA stability. This finding strongly argues against a destabilization of tapasin transcripts. Rather, we considered it more likely that the reduced mRNA level was caused by events taking place at a pretranscriptional level. However, conclusions concerning transcriptional activity based on levels of total RNA may be deceptive in cases of long-lived transcripts, as for tapasin. Hence, we took advantage of a novel method in which nascent RNA is isolated from the total pool of cellular RNA subsequent to RNA labeling by 4sU (9). This method allows for comparison of transcriptional regulation of different genes. To test the power of the applied method, the kinetics of transcriptional regulation of individual target genes in IFN-γ- and tumor necrosis factor alpha (TNF-α)-treated cells were included in this experimental setting (Fig. 4). In parallel, transcriptional induction was measured for TAP1, TAP2, and MHC I at 8 h post infection, whereas the tapasin transcripts were downregulated at this time point, implying different regulatory mechanisms of their respective promoters. The same pattern of gene regulation was observed in cells infected with the ΔUS2-US11 mutant virus. Interestingly, a strong downregulation of TAP1 and TAP2 could be observed between 8 and 24 h p.i., while transcription of tapasin was only weakly affected at this time point. Tapasin transcription continued to decrease throughout the HCMV replication cycle. In contrast, MHC I transcription accelerated toward E/L times of HCMV infection preceding the increased abundance at the protein level (Fig. 2A). After an initial induction, TAP1 transcripts remained approximately at control levels. TAP2 transcription, on the other hand, was repressed, although the effect was not as pronounced as that for tapasin. Taken together, the data show that the initial peak of transcription was inhibited at later time points for both TAP1 and TAP2, whereas tapasin gene expression, followed by that of TAP2, became repressed irreversibly early during HCMV infection.

Tapasin association of MHC I HC-β2m heterodimers is impaired in HCMV-infected cells. The transcriptional block explains the absence of newly synthesized tapasin in HCMV-infected cells. As demonstrated above, the lack of MHC I HC-β2m incorporation into the PLC was observed earlier in infection (Fig. 1A, lane 5), when tapasin protein was still abundantly detectable in Western blots (Fig. 2A). We therefore wondered whether newly synthesized tapasin rather than aged molecules is necessary for MHC I recruitment and incorporation into the PLC. To approach this issue, we nucleofected siRNA directed against tapasin transcripts into MRC-5 cells and assessed the PLC formation by immunoprecipitation. Cells infected with the ΔΔLvFc-γR HCMV mutant were analyzed in parallel. Knockdown of tapasin was successful, as tapasin biosynthesis was strongly reduced with increasing concentrations of siRNA (Fig. 5A). At 48 h p.i., the HCMV-mediated reduction of tapasin biosynthesis was as efficient as inhibition in mock-infected cells by siRNA treatment (Fig. 5A and B). Comparing the level of MHC I coprecipitation by tapasin-specific antibodies and the levels of tapasin (Fig. 5A, IP [biosynthesis] and IB [total]), we noticed that in mock-infected cells the efficiency of MHC I coprecipitation was rather pro-
portional to the total level of tapasin, and not to the amount of newly synthesized molecules (compare white bars in Fig. 5C with black and white bars in Fig. 5B), excluding the possibility that newly synthesized tapasin molecules preferentially bind to MHC I HC-β2m complexes. To obtain a more precise estimation of the amounts of MHC I HC-β2m molecules available for PLC recruitment, an endo H treatment was performed subsequent to precipitation by MAb W6/32 (Fig. 5A). Whereas endo H-resistant forms of MHC I have been transported beyond the cis-Golgi compartment, the endo H-sensitive forms localize to earlier compartments, where formation of the PLC is taking place. Remarkably, although the overall MHC I level was reduced in US2-US11 mutant, the amount of endo H-sensitive MHC I molecules in infected cells was increased (Fig. 5A, lanes 5 and 6, and C, black bars). Despite the 1.5-fold increase of endo H-sensitive forms was found (Fig. 5A, lanes 5 and 6, and C, black bars), demonstrating that the lack of MHC I recruitment to tapasin cells is not a consequence of reduced numbers of MHC I molecules. In addition to the ΔΔΔvFcyR mutant, lacking the US2 to US6 genes, this phenotype was also observed in RV35 (ΔUS6-US11) (27)-infected cells (Fig. 6), suggesting that the US2 to US11 genes are not responsible for the disturbed tapasin interaction with MHC I HC-β2m heterodimers. Based upon the findings that (i) MHC I molecules are not limited for PLC incorporation into HCMV-infected cells and (ii) MHC I association with tapasin is not directly linked to tapasin neosynthesis, we concluded that the transcriptional block of tapasin cannot be the cause of the lack of MHC I recruitment to the PLC in HCMV-infected cells.

Restoration of MHC I cell surface expression after forced expression of tapasin in HCMV-infected cells. To examine the relative impact of inhibited tapasin transcription in the context of HCMV infection, we reconstituted newly synthesized tapasin molecules in HCMV-infected cells. MRC-5 cells were transduced with either a tapasin-expressing or control lentiviral vector before PLC assembly was assessed by tapasin-specific antibodies under mock infection conditions or HCMV infection at 48 h p.i. Biosynthesis of tapasin was restored to levels above those observed in mock-treated cells, although HCMV reduced the lentiviral tapasin overexpression compared to that in mock-infected cells (Fig. 7, upper panel). Remarkably, there was no further increase of MHC I-tapasin association in mock-infected fibroblasts as a result of tapasin overexpression. Likewise, tapasin synthesis in AD169-infected cells largely failed to restore MHC I incorporation into the PLC, while a marginal gain of MHC I coprecipitation was seen.
in ΔΔvFγR-infected cells (Fig. 7). Clearly, MHC I association in HCMV-infected cells did not reach the levels observed in mock-infected cells. As an independent tool, anti-TAP1 antibodies were applied to analyze PLC assembly (Fig. 7, second panel from top), basically confirming the PLC composition revealed by anti-tapasin antibodies. Again, MHC I association by forced tapasin synthesis was marginally induced in ΔΔvFγR- and AD169-infected cells and did not change the levels of MHC I as revealed by MAb W6/32 (Fig. 7, third panel from top). Taken together, the data showing the incomplete restoration of MHC I incorporation by overexpressed tapasin confirm the previous finding that an independent HCMV-induced factor prevents MHC I association with tapasin molecules. Since overexpression of tapasin had no or only a marginal effect on MHC I recruitment to the PLC, it is assumable that HCMV targets a molecule other than tapasin.

In the experiments described above, conditions of forced tapasin expression were used to study early events of PLC assembly restricted to a time interval of 3 h. To study the outcome of retained tapasin neosynthesis on steady-state levels on the cell surface, FACS analysis was performed. First, lentivirus-transduced control or tapasin-expressing MRC-5 cells were mock treated or infected with HB5. Cell surface expression of MHC I was determined by MAb W6/32 throughout the HB5 replication cycle. Interestingly, mock-treated tapasin-expressing MRC-5 cells had smaller amounts of MHC I than control MRC-5 cells (Fig. 8A). Tapasin transduction of MRC-5 cells was repeated three times with the same outcome, convincing us that selectively overexpressed tapasin indeed retards MHC I trafficking to the cell surface. This was confirmed in a pulse-chase experiment (data not shown), consistent with earlier reports (45, 46). In contrast, in HCMV-infected cells, the trend was reversed and MHC I was consistently induced in the tapasin-expressing MRC-5 cells.

FIG. 4. Newly synthesized tapasin RNA is downregulated throughout the HCMV replication cycle. MRC-5 cells were mock (m) or IFN-γ/TNF-α treated (500 U/ml and 25 ng/ml, respectively) or infected with AD169 or the ΔUS2-US11 HCMV deletion mutant at an MOI of 5. At the indicated time points, cells were incubated for 1 h with 4sU prior to RNA preparation and isolation of 4sU-incorporated RNA. qRT-PCR was performed, and relative quantification was carried out by normalization against 18S rRNA. Reactions were performed in duplicate. Data for one of two independent experiments with very similar outcomes are shown. Black bars indicate pulse-labeled newly synthesized RNA, and gray bars indicate total RNA.
Already at 24 h p.i., the reduced MHC I cell surface expression observed in mock-infected tapasin-expressing MRC-5 cells became reversed (Fig. 8B). At 48 h p.i., the impact of restored tapasin synthesis was even stronger (Fig. 8A and B), before it dropped at 72 h p.i. In addition, the effect was followed up with viruses allowing different degrees of MHC I expression: AD169, HB5 (US2-US11), and the mutant. The more MHC I expression the different viruses allowed for, the larger was the increase of MHC I due to forced tapasin expression (Fig. 8C). However, the relative gains of MHC I cell surface expression were similar for all viruses (Fig. 8D). In conclusion, the data provided proof of principle that restoration of tapasin expression is able to convey a higher level of MHC I cell surface expression in HCMV-infected cells, albeit to a rather limited extent, possibly due to the inhibitory effect of HCMV targeting MHC I HC-

DISCUSSION

In this study, we analyzed the fate of the PLC in the context of all phases of the protracted HCMV replication cycle. A defective incorporation of MHC I heterodimers into the PLC was observed, and specifically, the presence of tapasin was strikingly reduced. While the stability of tapasin remained un-

FIG. 5. Blocked interaction between MHC I and tapasin in HCMV-infected cells. (A) MRC-5 cells were subjected to nucleofection with siRNA targeting tapasin transcripts or with a random sequence (ctrl) and then infected as indicated. Cells were metabolically labeled for 3 h prior to lysis at 48 h postnucleofection/postinfection. Lysates were analyzed by anti-tapasin (STC) or anti-MHC I (W6/32) immunoprecipitation (IP) or by immunoblotting (IB) with the indicated antibodies. (B) Diagram showing relative intensities of bands corresponding to tapasin (Tpn) IB (black bars) and IP (white bars). (C) Relative intensities of endo H (EH)-sensitive MHC I molecules (black bars) and tapasin-coprecipitated MHC I (white bars).

FIG. 6. Blocked interaction between MHC I and tapasin is independent of the US2 to US11 genes. MRC-5 cells were infected with the ∆∆∆vFcγR mutant, lacking the US2 to US6 genes, and the RV35 mutant (∆US6-US11), as indicated, at an MOI of 5. Cells were metabolically labeled for 3 h prior to lysis at 48 h p.i. Lysates were analyzed by anti-tapasin (STC), anti-ERp57, or anti-MHC I (W6/32) immunoprecipitation. Subsequent to the W6/32 precipitation, the samples were treated by endo H. Marked bands indicate endo H-sensitive MHC I.
affected, an immediate effect on its biosynthesis was found. In accordance, tapasin mRNA was constantly diminishing in HCMV-infected cells, but only the selective analysis of newly synthesized RNAs revealed the instantly occurring transcriptional inhibition of the tapasin gene by HCMV. Unexpectedly, forced tapasin overexpression in HCMV-infected cells only marginally rescued PLC recruitment of MHC I, while at least augmenting surface MHC I. This relatively inefficient rescue suggests that additional unidentified factors may be involved in the distraction of MHC I heterodimers from the PLC. Taken together, our data establish for the first time that HCMV targets the transcription of selected genes of the MHC I pathway and prohibits MHC I association with the PLC.

Transcriptional control of tapasin gene expression. The lack of tapasin biosynthesis in HCMV-infected cells is a result of viral inhibition of tapasin transcription. The overall levels of tapasin transcripts gave the impression that the RNA was downregulated at a slower pace than the observed reduction of protein neosynthesis. Only the selective measurement of newly transcribed mRNAs revealed the immediate repression of tapasin followed by TAP2 transcription. Based upon these findings, it is tempting to speculate that newly synthesized tapasin transcripts preferentially become translated into protein, while another, long-lived pool of mRNAs still exists without being translated. Alternatively, an HCMV-encoded miRNA could interfere with the translation of tapasin, similar to the case described for the NKG2D ligand MICB (47). Indeed, in a collaboration with O. Mandelboim, who performed bioinformatic screens for targets of HCMV-encoded miRNAs, a high-score target of the HCMV-encoded miRNA miRUS25.2 was identified at the tapasin 3′-untranslated region.

FIG. 7. Forced expression of tapasin is not sufficient to rescue MHC I incorporation into the PLC in HCMV-infected cells. Tapasin-transfected or control MRC-5 cells were mock infected or infected with AD169 or with the HB5 (∆US2-US6)-derived ∆∆FcvR HCMV deletion mutant, as indicated, at an MOI of 5. Cells were labeled for 3 h prior to lysis at 48 h p.i. Immunoprecipitations were performed as indicated. Proteins were separated by 10 to 11.5% SDS-PAGE.

FIG. 8. Tapasin overexpression induces MHC I cell surface expression on HCMV-infected cells. Tapasin-expressing or control MRC-5 cells were mock treated or infected with AD169, HB5, or the US2-US11 deletion mutant at an MOI of 5. MHC I cell surface expression was determined by FACS analysis using W6/32. (A) MHC I detection at 48 h p.i. for mock and HB5 infections. (B) Mean changes in fluorescence intensity (ΔMFI) between tapasin-expressing and control MRC-5 cells at various time points of HB5 infection. (C) ΔMFI values between tapasin-expressing and control MRC-5 cells at 72 h p.i. for the indicated infections. (D) MFI values from the experiment shown in panel C, depicted as fold MFI values measured for tapasin-expressing MRC-5 cells in comparison to control MRC-5 cells.
the HCMV tegument protein pp71 (UL82) genes outside the US2region. We wondered whether the regulation of transcriptional corepressor Daxx is located next to the extended MHC class II locus, and interestingly, the gene tapasin is located in the extended MHC class II locus, and interestingly, the gene for the transcriptional corepressor Daxx is located next to tapasin. We wondered whether the regulation of tapasin transcription by HCMV might be part of a broader effect and analyzed the transcriptional activities of neighboring genes of tapasin, including Daxx, RGL2, and 18SRP) (21). However, the transcription of none of these was affected by HCMV infection (data not shown), indicating that the early repression of tapasin transcription is astonishingly gene selective.

This might suggest that tapasin is regulated in HCMV-infected cells either by inhibitory viral factors or by mobilization of cellular factors that have the propensity to repress the transcription machinery directly. Indeed, it was recently described that the transcriptional repressor PRDM-1 (positive regulatory domain I) targets the tapasin promoter (10). PRDM-1 was found to be able to inhibit IFN-γ-induced transcription of tapasin, qualifying this factor to exert a possible function in tapasin gene repression during HCMV infection.

Phenotypic disruption of the PLC. Most studies analyzing the molecular function of HCMV-encoded MHC inhibitors (i.e., US2, US3, US6, US10, and US11) have been performed with experimental systems of isolated gene expression, highlighting the sophisticated and redundant control mechanisms of this virus over the MHC I pathway of antigen presentation (3, 19, 28, 29, 32, 37–39, 55, 56). These established inhibitors interact with defined target molecules, i.e., MHC I, TAP1/2, and tapasin. Our finding that the disruption of PLC assembly was not only evident in cells infected with HCMV wt strains but also seen with ΔUS genes deletion mutants indicates that the effects must be caused by other factors, thus excluding the previously described MHC I inhibitors. The following two dominant phenotypic changes of the PLC could be defined by immunoprecipitation analysis of radiolabeled cells: (i) a lack of tapasin synthesis and (ii) disruption of molecular interactions between PLC components.

Multiple effects target tapasin in HCMV-infected cells. Previous work by Park et al. (37) revealed that the US3 glycoprotein acts as an inhibitor of tapasin function, resulting in impaired peptide loading onto MHC I. However, the immediate-early kinetics of US3 transcription (49) and the short half-life of the US3 glycoprotein (13) exclude a sustained effect of US3 on tapasin function during the long course of HCMV replication. The early onset and sustained nature of blocked tapasin gene expression by HCMV, as documented here, could complement the transient US3-mediated effect in an optimal way by depleting this factor critical for the selection of MHC I epitopes. We found it remarkable that HCMV limits its effect to the replenishment of tapasin and does not downregulate overall levels of this PLC component. However, the restoration of the HCMV-induced effect on the PLC by ectopic expression of tapasin was grossly incomplete. This implies that, in fibroblasts, the impact of retarded tapasin transcription is diminished due to a redundant and dominant interference of HCMV with MHC I maturation, as illustrated by the inhibited association of MHC I HC-β2m heterodimers with tapasin. Indeed, augmentation of tapasin levels did not correct the deficient recruitment of MHC I HC-β2m heterodimers to tapasin (Fig. 7). Moreover, the level of MHC I is also not the limiting factor, since high levels of endo H-sensitive MHC I heterodimers were found in ΔUS2-6- and ΔUS6-11-infected cells. The obvious conclusion is that HCMV is able to keep these molecules from interacting. Such a posttranslational mechanism would perfectly complement the time-limited effect of US3 (37) and the successive suppression of tapasin transcription.

Remarkably, whereas the disruption of MHC I interaction could be documented for tapasin, ERp57, and TAP, the MHC I-calreticulin interaction was not reduced in infected cells (Fig. 1D). This drew our attention to a recent publication by Elliott, Springer, et al. (24) in which an additional function outside the PLC was described for calreticulin. Not by affecting the rate of MHC I molecules leaving the ER but by redirecting suboptimaly loaded MHC I molecules from post-ER compartments back to the ER, calreticulin mediated a further point of quality control for MHC I. Accordingly, defects in peptide loading (e.g., TAP deficiency) induced the colocalization of MHC I and calreticulin in the ER-Golgi intermediate compartment (ERGIC) and the cis-Golgi compartment. It is tempting to hypothesize that the MHC I-calreticulin interaction we observed in HCMV-infected cells might reflect an accumulation of calreticulin and MHC I in the ERGIC and the cis-Golgi compartment. The increased portion of endo H-sensitive MHC I molecules in HCMV-infected cells supports the assumption that MHC I is trapped in the ER, ERGIC, and/or cis-Golgi compartment. Besides calreticulin, tapasin, TAP, and peptide-free MHC I have also been reported to cycle between the ER, ERGIC, and cis-Golgi compartment (12, 24, 40). In such a dynamic scenario, HCMV is apparently confronted with many opportunities to take control over checkpoints of PLC assembly and disassembly.

While the primary goal of established HCMV stealth features is the quantitative downregulation of the bulk of MHC class I molecules from the cell surface (16, 20), the targeting of tapasin is supposed to exert a qualitative influence. Tapasin promotes loading of immunodominant peptides (7), which allowed us to assume that deficiency of tapasin function in HCMV-infected cells could alter the quality of HCMV-specific CD8+ T-cell responses. Since HCMV infects many cell types and cycles through productive as well as latent phases of infection, the precise consequences of tapasin downregulation on HCMV infection biology remain to be established. Another conundrum of HCMV infection is the extreme convergence of CD8+ T-cell responses to a few epitopes, despite the very large number of HCMV antigens produced in infected cells (30, 58).
In view of this peculiarity of the cytotoxic T-lymphocyte response to HCMV, we must consider the possibility that it is the sustained tapasin deficiency in HCMV-infected cells that contributes to the apparently ineffective presentation of a large majority of HCMV epitopes.

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