

The HCMV Gene Products US11 and US2 Differ in Their Ability to Attack Allelic Forms of Murine Major Histocompatibility Complex (MHC) Class I Heavy Chains

By Robert P. Machold,* Emmanuel J.H.J. Wiertz,* Thomas R. Jones,‡ and H.L. Ploegh*

*From the *Center for Cancer Research, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; and ‡Department of Molecular Biology, Infectious Diseases Section, Wyeth-Ayerst Research, Pearl River, New York 10965*

Summary

Human cytomegalovirus downregulates the expression of human class I major histocompatibility complex (MHC) molecules by accelerating destruction of newly synthesized class I heavy chains. The HCMV genome contains at least two genes, US11 and US2, each of which encode a product sufficient for causing the dislocation of newly synthesized class I heavy chains from the lumen of the endoplasmic reticulum to the cytosol. Based on a comparison of their abilities to degrade the murine class I molecules H-2K^b, K^d, D^b, D^d, and L^d, the US11 and US2 gene products have non-identical specificities for class I molecules. Specifically, in human astrocytoma cells (U373-MG) transfected with the US11 gene, the K^b, D^b, D^d, and L^d molecules expressed via recombinant vaccinia virus are rapidly degraded, whereas in US2-transfected cells, only D^b and D^d are significantly destabilized. The diversity in HCMV-encoded functions that interfere with class I-restricted presentation likely evolved in response to the polymorphism of the MHC.

Class I MHC molecules present peptides derived from proteins degraded in the cytosol to cytolytic T cells, and as such are targets for viruses seeking to evade immune recognition (1). The ability to downregulate the expression of class I molecules in host cells has been documented for a number of viruses, including adenovirus (2), herpes simplex virus (3), and both mouse (4) and human cytomegalovirus (5). In HCMV-infected cells, newly synthesized class I heavy chains are rapidly degraded following their deposition in the lumen of the ER (6–8). The HCMV gene product US11 (“US11”), an ER-resident type I transmembrane glycoprotein, has been shown by transfection to be sufficient to cause the selective degradation of endogenous class I molecules (9). In the presence of US11, class I heavy chains are dislocated from the lumen of the ER into the cytosol, where they are deglycosylated by host *N*-glycanase, and then degraded by the proteasome (10). A second HCMV gene product, US2, has also been observed to cause the premature destruction of newly synthesized class I molecules, apparently by the same pathway as described above for US11 (9, 11). Since both US11 and US2 are expressed at the same stage of infection, we sought to determine why the virus might encode two separate proteins that appear to function in the same manner.

One possibility is that US11 and US2 recognize overlapping but nonidentical subsets of class I molecules. In the initial comparison of the US11 and US2 transfectants, only the degradation of endogenous human class I molecules was studied, and no differences in allelic selectivity between US11 and US2 were apparent (9–11). In human fibroblasts infected with HCMV, all endogenous class I molecules appeared to be destabilized (6–8). In mouse L cells (H-2k) transfected with HLA-B27, the human, but not murine class I molecules were degraded upon HCMV infection, but the reason for this difference was not established (6). In virus-infected cells, both US11 and US2 are presumably expressed, and these experiments therefore cannot resolve possible differences in substrate specificity between the two viral proteins. To probe the specificities of US11 and US2, we infected cell lines transfected with either US11 or US2 with a panel of recombinant vaccinia virus expressing different mouse class I heavy chains (H-2K^b, K^d, D^b, D^d, and L^d) to determine whether certain heavy chains would be resistant to US11- or US2-mediated degradation. We observe that US11 and US2 differ in their abilities to degrade murine class I heavy chains, and thus are not functionally redundant. Specifically, we find that US11 degrades K^b, D^b, D^d, and L^d efficiently, whereas US2 is most effective in

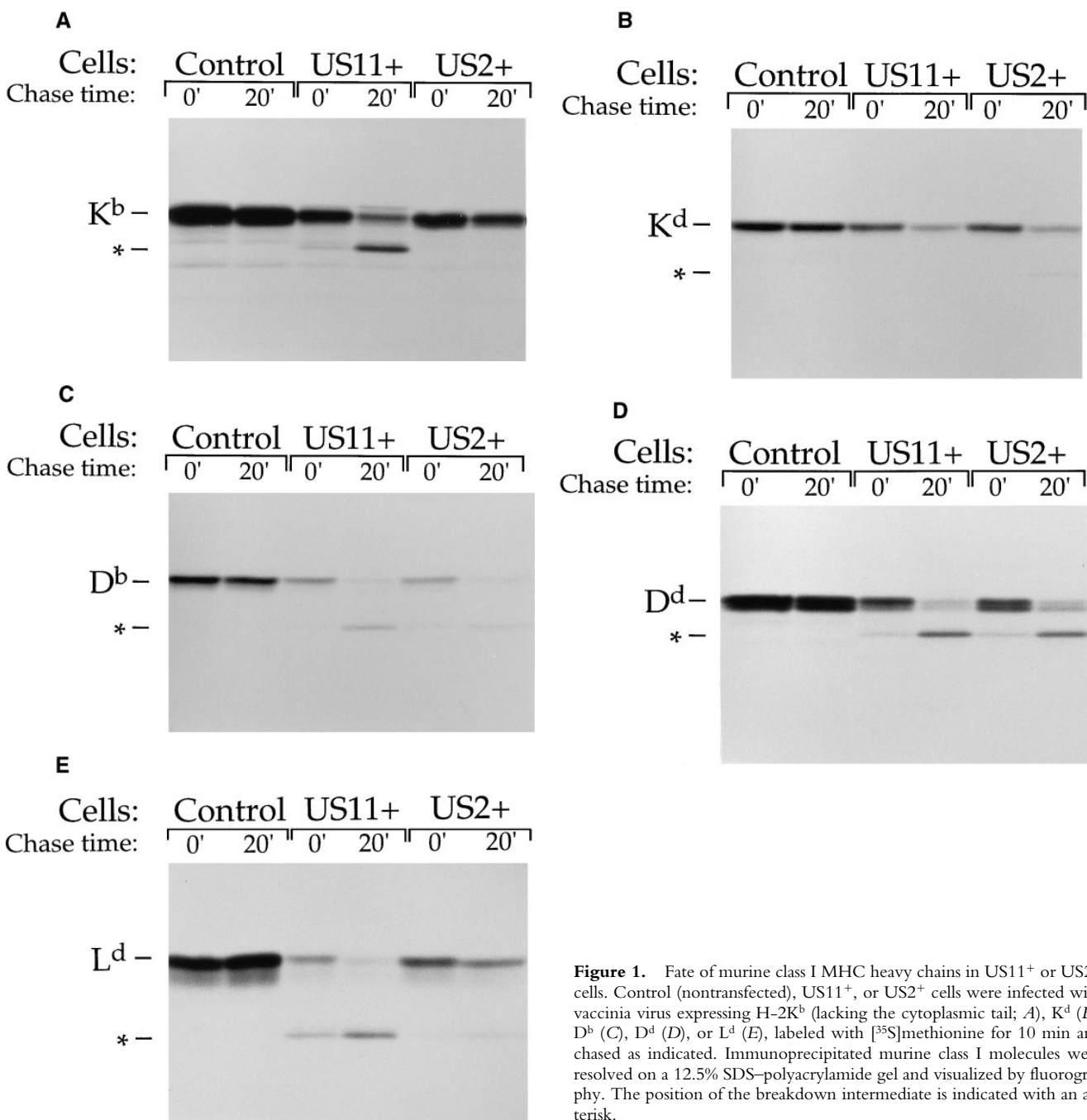


Figure 1. Fate of murine class I MHC heavy chains in US11⁺ or US2⁺ cells. Control (nontransfected), US11⁺, or US2⁺ cells were infected with vaccinia virus expressing H-2K^b (lacking the cytoplasmic tail; A), K^d (B), D^b (C), D^d (D), or L^d (E), labeled with [³⁵S]methionine for 10 min and chased as indicated. Immunoprecipitated murine class I molecules were resolved on a 12.5% SDS-polyacrylamide gel and visualized by fluorography. The position of the breakdown intermediate is indicated with an asterisk.

degrading D^b and D^d. We suggest that MHC polymorphism drives diversification of viral evasion strategies.

Materials and Methods

Cells and Vaccinia Virus Infections. U373-MG astrocytoma cells and the US11 and US2 transfectants prepared from this cell line have been described (9). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) FCS, penicillin (1:1,000 dilution U/ml), streptomycin (100 μg/ml), and puromycin (Sigma Chem. Co., St. Louis, MO) at a final concentration of 0.375 μg/ml. Recombinant vaccinia virus express-

ing H-2K^b (lacking the cytoplasmic tail), K^d, D^b, D^d, and L^d were obtained from Dr. J. Yewdell. Between 1 and 5 × 10⁶ cells per sample were detached by treatment with trypsin, resuspended in PBS supplemented with 1% FCS, penicillin, and streptomycin, and then infected for 45 min with recombinant vaccinia virus at a multiplicity of infection of 10, after which 10 ml of media was added. 5 h later, cells were starved in methionine/cysteine-free medium for 45 min with or without the proteasome inhibitor Cbz-LLL (10 μm final) (10) before labeling with 250 μCi/ml [³⁵S]methionine/cysteine (80:20). Labeling was terminated by addition of 1 mM cold methionine/cysteine to the labeling mix. Aliquots of cells were spun down at each chase point, and the cell pellets frozen before immunoprecipitation of class heavy chains.

Antibodies and Immunoprecipitations. For immunoprecipitation of mouse class I heavy chains, a rabbit polyclonal antiserum (RafHC) which recognizes non-assembled or unfolded heavy chains was used (12). Cell pellets were each lysed in 1 ml ice-cold lysis mix (0.5% NP-40, 50 mM Tris/HCl, pH 7.4, 5 mM MgCl₂, 1 mM PMSF, and 10 mM iodoacetamide), and the postnuclear supernatant precleared twice with 10% fixed *Staphylococcus aureus* before specific immunoprecipitation of murine class I heavy chains with RafHC. To enhance the visualization of degradation intermediates, the RafHC immunoprecipitates were boiled in denaturation buffer (2% SDS, 50 mM Tris/HCl, pH 7.8, 1 mM EDTA, 5 mM DTT), and the murine class I material re-immunoprecipitated with RafHC before gel analysis. The *N*-glycanase digestions in Fig. 2 were performed according to the manufacturer instructions (Boehringer Mannheim, Germany). SDS-PAGE and one dimensional isoelectric focusing were performed as described (13).

Results

The Murine Class I MHC Molecule H-2K^b Is Degraded by US11 but not by US2. To probe the specificity of the HCMV gene products US11 and US2 for class I MHC molecules, we infected parental U373-MG cells, and transfectants expressing either US11 or US2, with a panel of recombinant vaccinia virus encoding different mouse class I heavy chains, and then performed a pulse chase experiment (10-min labeling, 0-min and 20-min chase points) in the presence of the proteasome inhibitor Cbz-LLL (Fig. 1). At each chase point, aliquots of cells were pelleted and frozen at -80°C before lysis and immunoprecipitation with a rabbit anti-free heavy chain antiserum (RafHC). Immunoprecipitates were denatured by boiling in 2% SDS, and re-immunoprecipitated before analysis by SDS-PAGE. In the case of H-2K^b (lacking cytoplasmic tail) vaccinia virus-infected cells (Fig. 1 A), class I heavy chains synthesized in the 10-min pulse are stable throughout the 20-min chase in control cells and in US2⁺ cells. However, in US11⁺ cells, the fully intact K^b heavy chains observed immediately upon completion of labeling (Fig. 1 A, 0-min timepoint) are rapidly converted into a faster migrating species. If the pulse chase is performed in the absence of Cbz-LLL, then the K^b heavy chains in US11⁺ cells are fully degraded by 20 min, and no intermediates accumulate (data not shown).

US11 and US2 were equally capable of degrading the murine class I MHC heavy chains H-2D^b (Fig. 1 C), and D^d (Fig. 1 D). However, when challenged with the H-2L^d molecule (Fig. 1 E), US11 was more efficient than US2 at causing the breakdown of the heavy chains, as evidenced by the nearly complete conversion in US11⁺ cells of full-length L^d heavy chains into the breakdown intermediate over the 20-min chase. In the case of H-2K^d (Fig. 1 B), the heavy chains decay more rapidly in the presence of either US11 or US2 than in control cells, but little degradation intermediate accumulates in either transfectant, despite the presence of the inhibitor Cbz-LLL. This is most likely due to the comparatively lower rate of degradation for K^d, coupled with the earlier observation that Cbz-LLL retards, but does not block, the subsequent degradation of the heavy chain intermediate (10). Thus, while US2 can apparently

destabilize the K^d and L^d heavy chains to some extent, it does not cause their destruction at a rate sufficient for the intermediate to accumulate.

The K^b Degradation Intermediate Observed Is a Deglycosylated Heavy Chain. In the case of human class I MHC molecules, the US11-induced degradation intermediate that accumulates in the presence of Cbz-LLL is a deglycosylated heavy chain (10). To demonstrate that the K^b degradation intermediate observed in Fig. 1 is a deglycosylated heavy chain, we performed a pulse chase experiment (10-min label, 0-min and 20-min chase points) on K^b vaccinia virus infected US11 cells, and immunoprecipitated with RafHC the K^b heavy chains from lysates of cells removed at each chase point. Each immunoprecipitate was split in two, and either kept on ice (-) or treated with recombinant *N*-glycanase (+) before re-immunoprecipitation with RafHC. The samples were then split again and analyzed by SDS-PAGE (Fig. 2 A) or 1D-IEF (Fig. 2 B). As seen in Fig. 2 A, all of the K^b molecules at the beginning of the chase are fully susceptible to *N*-glycanase treatment (Fig. 2 A, 0-min timepoint), and no intermediate has yet accumulated. After 20 min, most of the heavy chains have been converted into the US11-induced fragment, which migrates at the same position as *N*-glycanase treated material (Fig. 2 A, 20-min timepoint). In hydrolyzing the *N*-glycosidic bond of the K^b

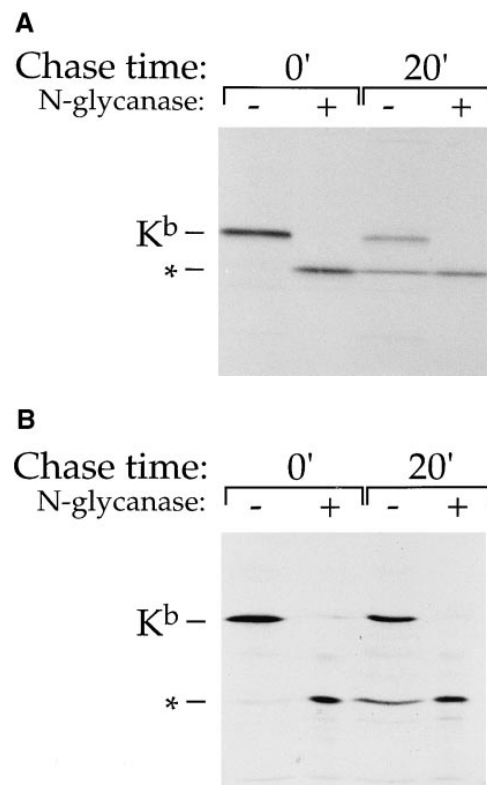


Figure 2. The K^b degradation intermediate is a deglycosylated heavy chain. US11⁺ cells were infected with vaccinia virus expressing H-2K^b (lacking cytoplasmic tail), labeled for 10 min with [³⁵S]methionine, and chased as indicated. Immunoprecipitated K^b heavy chains from each chase point were either kept on ice (-) or treated with recombinant *N*-glycanase (+) before resolution by SDS-PAGE (A) or 1D-IEF (B).

heavy chain's glycan, *N*-glycanase converts the Asn side-chain to an Asp, and thus imparts an additional negative charge upon the molecule. Isoelectric focusing of the samples in Fig. 2 A reveals that the fully intact K^b heavy chains present at the beginning of the chase have the same pI after *N*-glycanase treatment as the degradation intermediate observed after 20 min of chase (Fig. 2 B). We conclude that the mechanism of US11-mediated class I heavy chain degradation is similar for mouse and human molecules, and that in both cases, a deglycosylated heavy chain intermediate can be visualized when the proteasome inhibitor Cbz-LLI is present during the chase.

HCMV downregulates the expression of class I MHC molecules by dislocating newly synthesized class I heavy chains from the lumen of the ER back into the cytosol, where they are rapidly degraded (10). The virus encodes

two proteins, US11 and US2, that are each sufficient for causing the premature destruction of class I heavy chains (9, 11). We show here that US11 and US2 have nonidentical specificities for a panel of murine class I heavy chains. Over the 20-min chase period examined, US11 degraded H-2K^b, D^b, D^d, and L^d with similar kinetics, while US2 only degraded D^b and D^d efficiently. One possible explanation for the observed differences in class I heavy chain degradation for US11 and US2 positive cells is that each may interact with different regions of the class I heavy chain in the process of dislocating the latter into the cytosol. Due to the sequence variability between different class I alleles, this dual recognition strategy would allow the virus to down-regulate the expression of a broader range of class I molecules than might be possible with either US11 or US2 alone.

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Address correspondence to Hidde Ploegh, Center for Cancer Research, MIT, 40 Ames St. E17-322, Cambridge, MA 02139.

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