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The Human Cytomegalovirus US11 Gene Product Dislocates MHC Class I Heavy Chains from the Endoplasmic Reticulum to the Cytosol

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Summary

Human cytomegalovirus (HCMV) down-regulates expression of MHC class I products by selective proteolysis. A single *HCMV* gene, *US11*, which encodes an endoplasmic reticulum (ER) resident type-I transmembrane glycoprotein, is sufficient to cause this effect. In US11$^+$ cells, MHC class I molecules are core-glycosylated and therefore inserted into the ER. They are degraded with a half-time of less than 1 min. A full-length breakdown intermediate that has lost the single N-linked glycan in an N-glycanase-catalyzed reaction transiently accumulates in cells exposed to the protease inhibitors LLnL, Cbz-LLL, and lactacystin, identifying the proteasome as a key protease. Subcellular fractionation experiments show this intermediate to be cytosolic. Thus, US11 dislocates newly synthesized class I molecules from the ER to the cytosol, where they are acted upon by an N-glycanase and the proteasome.

Introduction

Cytolytic T cells play a role in the elimination of virally infected host cells, and these cells require major histocompatibility complex (MHC) class I molecules to guide the attack (Townsend and Bodmer, 1989). A number of viruses are capable of manipulating the expression of the glycoproteins, the MHC class I products, that signal the presence of a virally infected cell to the immune system (Maudsley and Pound, 1991; Hill and Ploegh, 1995). A priori, every single step of the biosynthesis of MHC class I products is fair game for the virus.

Class I molecules consist of two protein subunits, the MHC-encoded glycoprotein heavy chain, and a small soluble polypeptide, β_2-microglobulin (β_2m). The short peptides that are presented by class I molecules fulfill an essential structural role and contribute to the formation of stable class I complexes (reviewed by Heemels and Ploegh, 1995). These peptides, 8–10 residues in length, are derived largely from cytosolic proteins, broken down by cytosolic proteases, amongst which the proteasome is the major proteolytic activity (Rivett, 1993). This multicatalytic complex (~700 kDa) exists as 20S proteasome, which also forms the core of the 26S (1500 kDa) proteasomal complex that degrades ubiquitin-conjugated proteins in an ATP-dependent manner. Nonubiquitinated proteins can also be degraded by the proteasomal complex. The resultant peptides enter the ER through a dedicated peptide transporter, the TAP (transporter associated with antigen processing) complex (Heemels and Ploegh, 1995).

Down-regulation of class I molecules in virally infected or transformed cells is a well-established phenomenon and often involves attenuation of transcription of the genes encoding the class I subunits (Maudsley and Pound, 1991). In addition, posttranscriptional mechanisms of down-regulation have been described and include the production of proteins that mediate retention of newly synthesized class I molecules in the ER. Such is the case for those strains of adenovirus that express the E3 19K protein (Burgert and Kvist, 1985), and this may also apply to mouse cytomegalovirus-infected cells, where ER retention of class I molecules has been observed (del Val et al., 1992). In both cases, the ER-retained class I molecules are relatively stable and persist for hours. More recently, it was shown that the Herpes simplex viruses 1 and 2 produce a small cytosolic protein, called ICP47, that blocks class I cell surface expression (York et al., 1994) by inactivating the TAP peptide transporter (Früh et al., 1995; Hill et al., 1995). By denying access of cytosolic peptides to the lumen of the ER, ICP47 prohibits stable assembly of class I molecules and prevents their expression at the cell surface.

A different member of the herpes virus family, human cytomegalovirus (HCMV), is also known to down-regulate expression of class I molecules, but apparently does so through rapid destruction of newly synthesized class I molecules (Beersma et al., 1993; Jones et al., 1995). We here describe experiments to show that the US11 gene product encoded by HCMV has the highly unusual property of being able to deliver class I heavy chains from the ER to a cytosolic environment, where they are exposed to N-glycanase and proteasomes to be destroyed.

Results

Class I Molecules Are Unstable in US11$^+$ Cells

The human astrocytoma cell line U373-MG transfected with the *US11* gene shows reduced levels of class I expression at the cell surface (Jones et al., 1995). In HCMV-infected fibroblasts, we observed rapid destruction of newly synthesized class I molecules (Beersma et al., 1993), an observation that was confirmed for the US11 transfectants. The rapidity of this destruction is apparent from an experiment in which US11$^+$ cells were pulse labeled for 60 s, followed by a chase up to 40 min (Figure 1A). The amount of labeled class I heavy chains increases between the 0 and 1 min chase points (Figure 1A, lanes 1 and 2), which we attribute to the time required

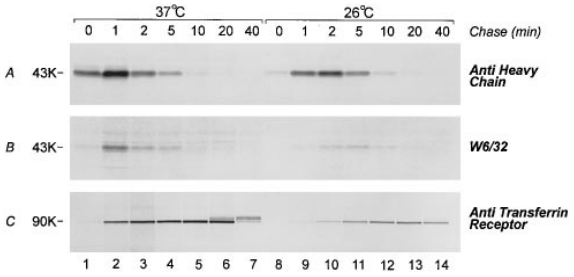

Figure 1. The Half-Life of Class I Heavy Chains in US11$^+$ Cells Is Less than 1 Minute

(A) Free class I heavy chains were immunoprecipitated with rabbit serum raised against the luminal domain of class I molecules (anti–heavy chain serum). US11$^+$ cells were pulse labeled with [^{35}S]methionine for 60 s, and chased as indicated, at 37°C (lanes 1–7) or 26°C (lanes 8–14). The immunoprecipitates were separated on a 12.5% SDS–polyacrylamide gel and visualized by fluorography.

(B) Class I heavy chain–β_2m complexes were immunoprecipitated with the monoclonal antibody W6/32; otherwise as A.

(C) Transferrin receptor was precipitated with the monoclonal antibody 66Ig10; otherwise as A.

for equilibration of intracellular methionine and methionyl tRNA pools at these short pulses, and the time required for completion of the polypeptide chain. From 1 to 2 min, over half of the radioactivity in the class I heavy chains is lost (lanes 2, 3), and beyond 20 min of chase, no heavy chains remain. This rapid disappearance cannot be counteracted by a reduction of temperature: at 26°C (Figure 1A, lanes 8–14) or 16°C (data not shown), we observed neither cessation of breakdown, nor the accumulation of breakdown intermediates. Kinetics of labeling and destruction of heavy chains, not surprisingly, were slower at the reduced temperatures. We conclude that breakdown of class I heavy chains takes place prior to their transport from the ER to the Golgi. Given the kinetics with which destruction occurs, the process is likely to be initiated immediately upon completion of the polypeptide chain. In cells that do not express US11, the class I heavy chains are stable over the time course observed here (Beersma et al., 1993; Jones et al., 1995; see also Figure 3B).

Class I heavy chain-β_2m complexes can be recovered by immunoprecipitation with the monoclonal antibody W6/32, whereas the rabbit anti–heavy chain serum used here is specific for free class I heavy chains (Stam et al., 1986). Both W6/32-reactive and anti–heavy chain-reactive class I molecules are stable in control U373-MG cells (Jones et al., 1995; see below), whereas both populations of class I molecule rapidly disappear from US11$^+$ cells (Figures 1A and 1B). The transferrin receptor (Figure 1C) (Beersma et al., 1993; Jones et al., 1995) does not show more rapid decay over the period of observation in US11$^+$ cells than is seen in control cells.

US11 Is an ER-Resident Protein

We performed immunoelectron microscopy on ultrathin cryosections of US11$^+$ cells and their untransfected counterparts. The rabbit anti-US11 antibody gave essentially no labeling on the untransfected cells (Figure 2A). In US11$^+$ cells, US11 immunoreactivity was detected exclusively in the ER (Figure 2B). Labeling over the Golgi area, cell surface, and nucleus did not exceed background levels (n = 20 cells).

Leucyl-leucyl-norleucinal Inhibits a Protease Involved in Class I Breakdown in US11$^+$ Cells and Leads to Accumulation of a 40 kDa Breakdown Intermediate

When cells were exposed to brefeldin A (BFA) to impede trafficking from the ER to the Golgi, no effect on the half life of class I heavy chains was seen in US11$^+$ cells (Figure 3). We observe a slight increase (Figure 3A, compare lanes 2 and 4 with lanes 6 and 8) in assembled class I complexes, as detected by the W6/32 antibody in US11$^+$ cells exposed to BFA. Because BFA impedes the flow of any free β_2m from ER to Golgi, the local increase in the concentration of β_2m is expected to favor complex formation. We conclude that transfer from the site of synthesis to a compartment distal to the block in trafficking imposed by BFA is not likely to be required for destruction of class I molecules in US11$^+$ cells.

The calpain I inhibitor leucyl-leucyl-norleucinal (LLnL) but not the calpain II inhibitor leucyl-leucyl-methional (data not shown) slowed down the disappearance of class I heavy chains in US11$^+$ cells (Figure 3). Its application resulted in the transient accumulation of an intermediate of lower molecular mass (40 kDa). The breakdown intermediate, to be further characterized below, was never observed in a complex with β_2m, and was unreactive with the W6/32 antibody specific for the assembled complex (Figure 3A, compare lanes 4 and 8 with 12 and 16). Application of LLnL to control cells (Figure 3B) failed to produce this breakdown intermediate. When untransfected cells were treated with LLnL or BFA, class I molecules, both assembled (W6/32 reactive) and free heavy chains, were essentially stable over the 20 min chase period (Figure 3B), during which class I heavy chains are destroyed completely in US11$^+$ cells. There appeared to be a modest effect of LLnL on the generation of W6/32 reactive assembled complexes (Figure 3B, compare lanes 2 and 4), possibly owing to inhibitory effects of LLnL on proteasomal protein breakdown (Rock et al., 1994). We also observed a slight reduction of free heavy chains in BFA-treated cells (Figure 3B, compare lanes 8 and 12), which may be attributable to increased complex formation as a result of a higher concentration of β_2m in the ER.

The Light Chain, β_2m Is Stable and Secreted in Increased Amounts in US11$^+$ Cells

Owing to the lack of heavy chains with which β_2m can pair (Figures 1 and 3; see the later time points), the excess of free β_2m in US11$^+$ cells behaves as a secretory protein and can be recovered from the supernate in increased amounts compared with control cells (Figure 4, compare lanes 2 and 6). Inclusion of the inhibitor LLnL has no effect on the quantity of β_2m released (Figure 4, lanes 2 and 4). Increased survival of class I heavy chains in the form of the 40 kDa breakdown intermediate induced by LLnL does not promote association with β_2m and an attendant reduction in the amount of β_2m secreted. Association of class I heavy chains with β_2m in the course of biosynthesis is a very rapid process (Heemels and Ploegh, 1995). Therefore the US11 gene

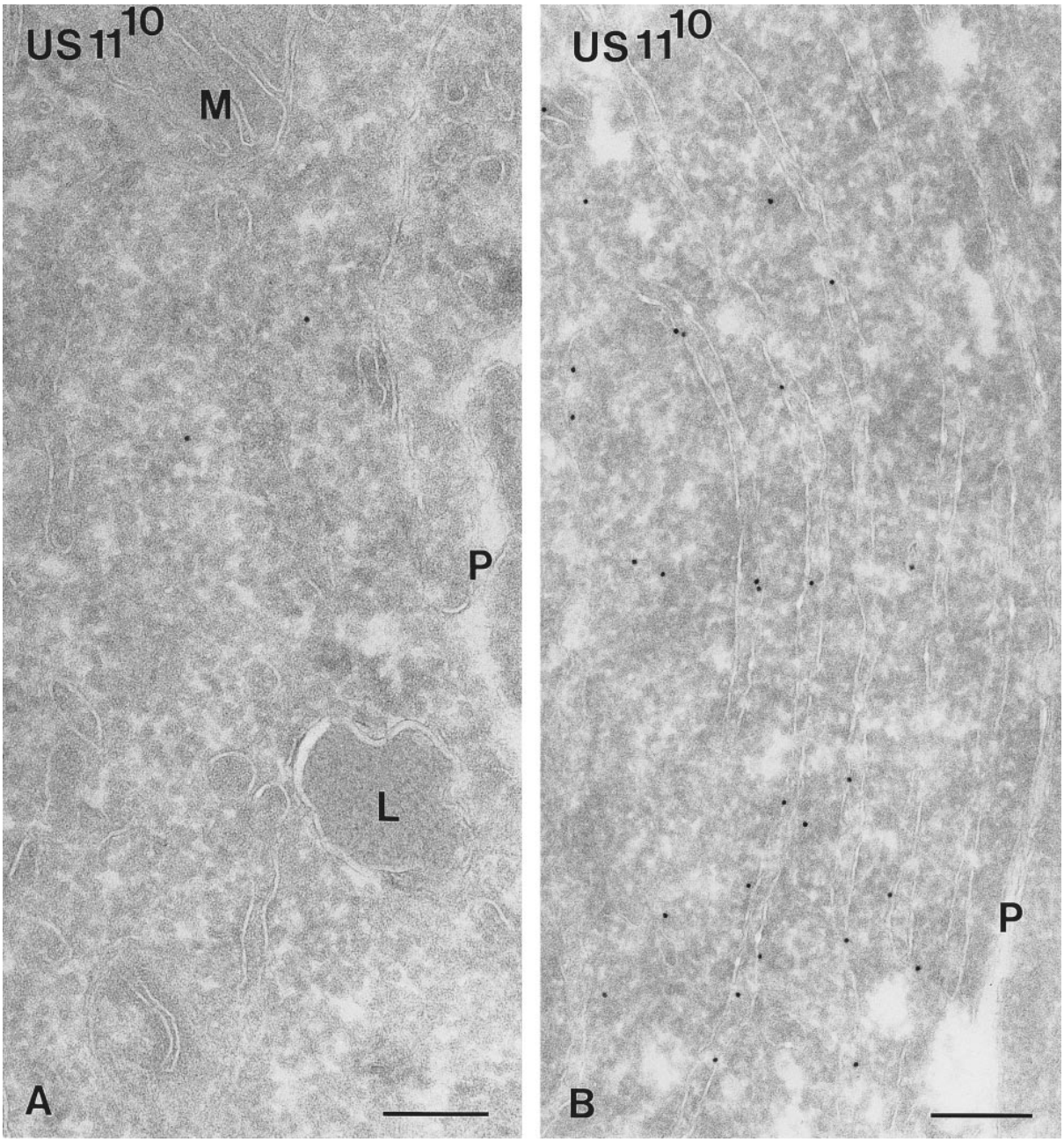

Figure 2. US11 is an ER-Resident Protein

Electron micrographs of ultrathin cryosections from a control cell (A) and a US11 transfected cell (B). The sections were immunogold labeled for US11 with 10 nm gold particles as indicated. In the control cell, two background gold particles can be seen; while in the US11 transfected cell, US11 is present in the ER (26/28 gold particles over the ER). L, lysosome; M, mitochondrion; P, plasma membrane. Bars, 0.2 μm.

product renders class I heavy chains less capable of associating with β_2m, either by altering the conformation of these heavy chains, or by transferring the heavy chain breakdown intermediate to a compartment where association with β_2m can no longer take place.

The Class I Breakdown Intermediate in US11$^+$ Cells Is Resistant to Endoglycosidase H

Upon observation of the 40 kDa breakdown intermediate in LLnL-treated US11$^+$ cells, we first considered the possibility of a proteolytic cleavage removing the cytoplasmic tail. Removal of this fragment would produce an intermediate of the observed mobility, and proteolysis of the COOH-terminal cytoplasmic tail is a rather frequent occurrence for class I molecules. Surprisingly, antibodies raised against a synthetic peptide (SDSAQGSDVSLTA) derived from the cytoplasmic COOH terminus of human class I molecules still recognized the breakdown intermediate, indicating its COOH terminus was largely, if not entirely, intact (data not shown). We next considered other modifications of the class I heavy chain and performed pulse-chase experiments in conjunction with digestion using the enzyme endoglycosidase H (endo H). Endo H cleaves high mannose–type oligosaccharides as

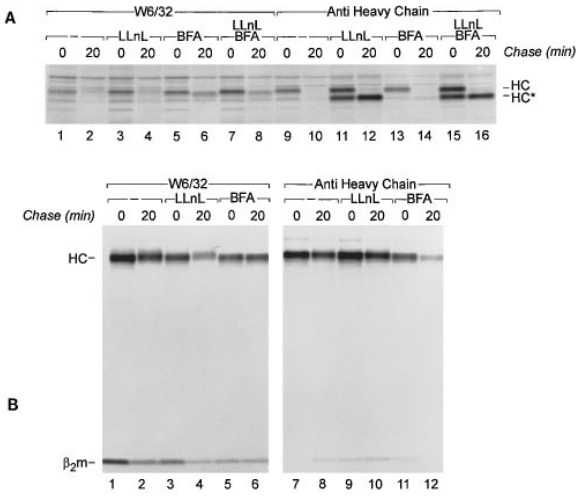

Figure 3. LLnL, but not BFA, Induces a Class I Breakdown Intermediate in US11$^+$ Cells

(A) Class I molecules were immunoprecipitated from US11$^+$ cells using the monoclonal antibody W6/32 (lanes 1–8), and rabbit heavy chain serum (lanes 9–16). The cells were pretreated with the appropriate inhibitors for 60 min, pulse labeled with [^{35}S]methionine for 15 min, and chased for 20 min. The proteins were separated on a 12.5% acrylamide gel. The positions of migration of core-glycosylated class I heavy chain (43–44 kDa; HC) and the breakdown intermediate (~40 kDa; HC with asterisk) are indicated.

(B) Class I molecules were precipitated from control cells using the monoclonal antibody W6/32 (lanes 1–6), and rabbit anti–heavy chain serum (lanes 7–12). The cells were pulse labeled with [^{35}S]methionine for 15 min and chased for 20 min. One hour prior to the pulse-chase experiment, the cells were incubated in methionine-free medium without (minus) or with LLnL or BFA.

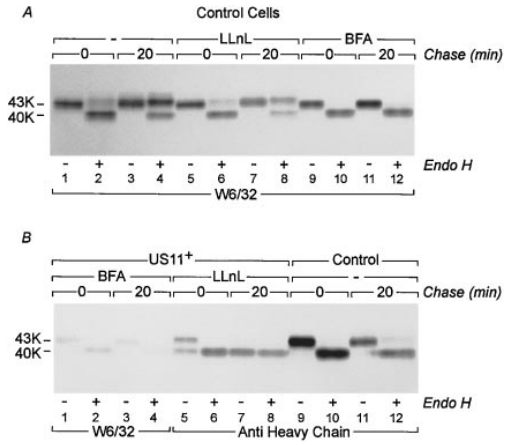

Figure 5. The Class I Breakdown Intermediate in US11$^+$ Cells Is Resistant to Endoglycosidase H

Control cells and US11$^+$ cells were preincubated in methionine-free medium without (minus) or with LLnL or BFA for 1 hr. Cells were labeled for 15 min with [^{35}S]methionine and chased for 20 min. Class I molecules were immunoprecipitated with W6/32 and rabbit anti–heavy chain serum. Samples were analyzed by SDS–PAGE before and after digestion with endoglycosidase H (indicated as minus and plus Endo H). W6/32 precipitates from control cells are shown in panel A. W6/32 and anti–heavy chain precipitates from US11$^+$ cells are shown in (B), lanes 1–8. Lanes 9–12: anti–heavy chain serum precipitates from the control cells. Note the endo H resistance of the breakdown intermediate (compare lanes 5 and 6 with 7 and 8).

commonly found on ER-resident N-linked glycoproteins. Figure 5A shows recovery of class I complexes using the W6/32 antibody from control cells exposed to LLnL or BFA. The expected acquisition of partial endo H resistance is observed for untreated cells at 20 min of chase (Figure 5A, compare lanes 2 and 4) and for LLnL-treated cells (Figure 5A, compare lanes 6 and 8); the reduction in total amount of W6/32 reactive complexes may again be attributable to reduction in peptide pools available for assembly (Rock et al., 1994). As expected, treatment with BFA at these relatively early chase points results in retention of full endo H sensitivity (Figure 5A, compare lanes 10 and 12). For US11$^+$ cells, only minor amounts of assembled W6/32 reactive complexes are detected (Figure 5B, lanes 1 and 2), almost all of which have disappeared by 20 min of chase (Figure 5B, lanes 3 and 4; see also Figure 1). In US11$^+$ cells exposed to LLnL, we observe the appearance of the 40 kDa intermediate, which persists for 20 min and is completely resistant to endo H (Figure 5B, lanes 5–8). The mobility of the intermediate is indistinguishable from that seen for endo H–digested free heavy chains from control cells (Figure 5B, lanes 9–12). We conclude that the breakdown intermediate that transiently accumulates in LLnL-treated US11$^+$ cells contains little if any N-linked glycans.

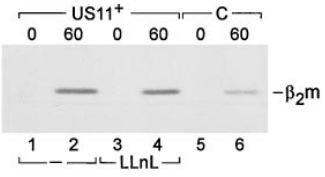

Figure 4. β_2m Is Secreted by US11$^+$ Cells in the Absence and Presence of LLnL

Cells were labeled with [^{35}S]methionine for 30 min. Supernatants were collected immediately after the labeling and after 60 min chase. The US11$^+$ cells were preincubated in methionine-free medium without (minus) or with LLnL for 1 hr. The control cells were preincubated in methionine-free medium. The β_2m protein was immunoprecipitated from the supernatants using the monoclonal antibody BBM.1 and, after separation on a 12.5% SDS polyacrylamide gel, visualized by fluorography.

N-Linked Glycans Are Removed from Class I Molecules in US11$^+$ Cells by an N-Glycanase Type Activity

A pulse-chase experiment performed on US11$^+$ cells in the absence or presence of LLnL establishes the precursor product relationship of the 43 and 40 kDa forms (Figure 6, lanes 1–14). Even though LLnL slows down proteolysis, it cannot prevent it: by 40 min of chase, most of the heavy chains synthesized in the 45 s pulse have disappeared (lane 14).

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) fails to distinguish between class I heavy chains deglycosylated using endo H and class I heavy chains isolated from tunicamycin (TM)-treated cells (Figure 6A, lanes 16 and 17); these differ by the presence of a single GlcNAc residue. We considered the possibility of an N-glycanase-type reaction, in which the N-glycosidic bond is hydrolyzed, and the Asn sidechain is converted to an

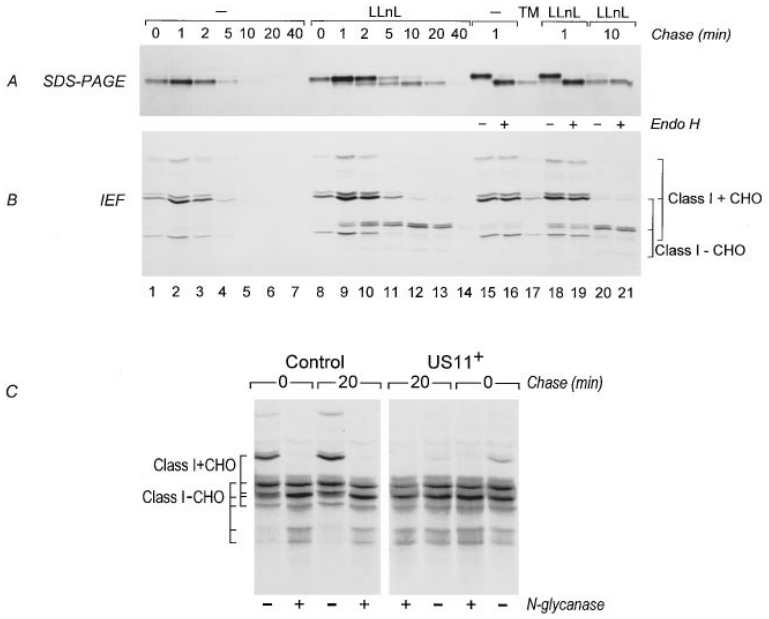

Figure 6. N-Linked Glycans Are Removed from Class I Molecules in US11$^+$ Cells by an N-Glycanase Type Activity

(A) The precursor-product relationship of the 43 kDa heavy chain and the 40 kDa breakdown intermediate was established in a 1 min pulse-chase experiment performed with US11$^+$ cells in the absence (minus) or presence of LLnL. Class I heavy chains were precipitated with rabbit anti–heavy chain serum and separated by SDS–PAGE. The cells were pulse labeled for 60 s and chased for the times indicated. Endo H digestion was performed for three samples (untreated cells, 1 min chase; LLnL-treated cells, 1 and 10 min chase). Samples before and after endo H digestion are shown in lanes 15–21. The class I heavy chains obtained in the presence of tunicamycin (TM; lane 17) are devoid of any carbohydrate.

(B) Isoelectric focusing (IEF) gel loaded in exactly the same order as the SDS–PAGE shown in (A). Note the shift in isoelectric point from the breakdown intermediate.

(C) US11$^+$ and control cells were labeled for 20 min and chased for 0 or 20 min. For US11$^+$ cells, LLnL was included in the labeling and chase medium. Class I heavy chains were digested with N-glycanase where indicated. Following complete denaturation in SDS, heavy chains were reimmunoprecipitated with rabbit anti–heavy chain serum, and analyzed by 1D IEF. In control cells, digestion of class I heavy chains with N-glycanase shifts the banding pattern to a more acidic position for both the 0 and 20 min time points (brackets; Class I–CHO). In US11$^+$ cells, some of the core-glycosylated precursor is observed at the 0 min time point and is still susceptible to N-glycanase digestion. Note that at the 20 min. timepoint, when only the breakdown intermediate is detectable (see Figure 3), digestion with N-glycanase is without effect on the IEF banding pattern. Moreover, the pattern observed in US11$^+$ cells is indistinguishable from that seen for N-glycanase-digested Class I heavy chains from control cells. The two panels shown are derived from the same electrophoretic separation, but different exposures were required to compensate for levels of incorporation of label. Cathode is at the top.

Asp residue (reviewed by Tarentino and Plummer, 1994). Such conversion should result in a change in isoelectric point of the class I heavy chains.

When the samples shown in Figure 6A are analyzed by one dimensional isoelectric focusing (IEF), we observe multiple bands in the anti–heavy chain precipitates. This multiplicity of bands is due to the simultaneous presence of the products of HLA-A, -B, and -C loci, each with a unique isoelectric point (Neefjes et al., 1986). Most of the class I heavy chains have isoelectric points well above the pKa of sialic acids or protein-bound phosphate moieties (Eichholtz et al., 1992). For each of the isoelectric species present at the beginning of the chase, the breakdown intermediate seen in LLnL-treated cells at later time points migrates at a more acidic isoelectric point (compare Figures 6A and 6B, lanes 1–7 and 8–14). Based on previous observations (Eichholtz et al., 1992) this change in isoelectric point is of a magnitude comparable to that seen for addition of a single sialic acid residue and thus most likely corresponds to the conversion of the amide (Asn) to the acid (Asp). For comparison, we have included class I molecules isolated from TM-treated cells (Figure 6, lane 17). In TM-treated cells glycosylation is suppressed, and the acceptor Asn residues persist as such without affecting isoelectric point. The presence of the single remaining GlcNAc residue as produced by digestion with endo H should not affect the isoelectric point of the class I heavy chains either, and indeed yields digestion products indistinguishable in focusing pattern from class I heavy chains isolated from TM-treated cells (compare lanes 15–17). In vitro treatment of core-glycosylated class I heavy chains with peptide N-glycanase yields class I heavy chains with isoelectric points identical to the breakdown intermediates seen in LLnL-treated US11$^+$ cells, and the breakdown intermediates in US11$^+$ cells are refractory to digestion with N-glycanase (Figure 6C). Combined, these data argue strongly in favor of the breakdown intermediate having lost all N-linked oligosaccharides in an N-glycanase catalyzed reaction, the polypeptide backbone remaining intact.

The Absence of N-Linked Glycans Does Not Affect Breakdown of Class I Heavy Chains in US11$^+$ Cells

TM treatment of control cells produces the expected nonglycosylated heavy chains that are stable during the 20 min chase (Figure 7A, compare lanes 1 and 2 with 3 and 4). In US11$^+$ cells exposed to TM, breakdown of class I molecules proceeds as it does in untreated US11$^+$ cells (Figure 7A, compare lanes 5 and 6 with 7 and 8). Inclusion of LLnL allows the visualization of the 40 kDa intermediate (Figure 7B, lanes 9–12), and there is no difference in mobility between precursor and breakdown intermediate in LLnL-exposed, TM-treated cells (Figure 7B, lanes 11 and 12). The increased survival of the breakdown intermediate in TM-treated cells exposed to LLnL may be deduced from a comparison of lanes 9 and 10 with 11 and 12 (Figure 7B) and is no less than that observed when comparing lanes 1 and 2 with 3 and 4 (US11$^+$ cells labeled in the absence of TM). The shift in mobility seen for US11 in TM-treated cells is consistent with the predicted presence of a single N-linked glycan. Note that US11, in both glycosylated and nonglycosylated form, is stable over the chase period during which class I heavy chains are degraded.

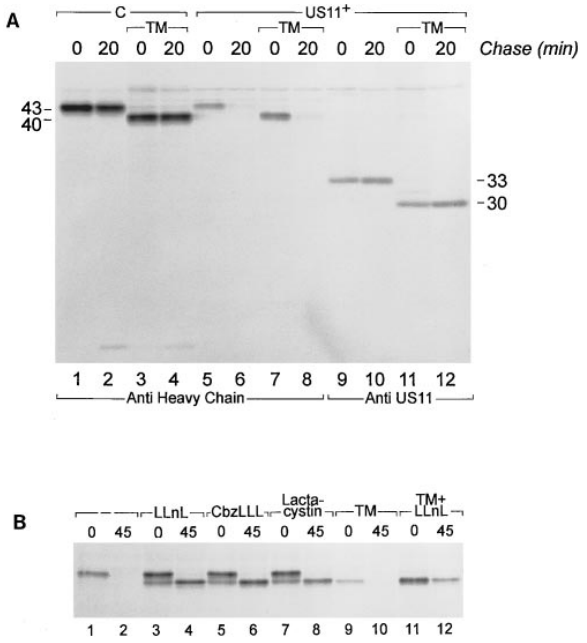

Figure 7. US11-Mediated Breakdown of Class I Molecules Does Not Require N-Linked Glycans, and Is Delayed by Inhibitors of the Proteosome

(A) Control cells (C) and US11$^+$ cells were incubated in methionine-free medium without or with tunicamycin (TM) for 1 hr. Cells were labeled for 15 min and chased for 20 min. Immunoprecipitations were performed with rabbit anti–heavy chain serum and with a rabbit serum against the US11 glycoprotein as indicated. The proteins were separated on a 12.5% polyacrylamide gel and visualized by fluorography. For control cells β_2m is visible near the dye front.

(B) US11$^+$ cells were pretreated with LLnL, Cbz-LLL, lactacystin, tunicamycin, or both LLnL and tunicamycin for 1 hr. Cells were labeled for 15 min and chased for 45 min in the continued presence of the inhibitors. The class I molecules were immunoprecipitated using the rabbit anti–heavy chain serum. The relevant section of the autoradiogram is shown.

Because in all likelihood there exist considerable pools of preexisting fully glycosylated US11 molecules when TM treatment and labeling are initiated, we cannot conclude that nonglycosylated US11 is still biologically active; but clearly, nonglycosylated class I molecules are fully susceptible to degradation imposed by the presence of the US11 product.

The Proteasome Inhibitors Carboxybenzyl-Leucyl-Leucyl-Leucinal and Lactacystin Produce a Class I Breakdown Intermediate Indistinguishable from that Seen in LLnL-Treated Cells

Peptide aldehydes such as LLnL have been widely used as inhibitors of proteases (Sherwood et al., 1993; Rock et al., 1994). Their precise target of inhibition is often difficult to assess, and the rank order of inhibitory potency on purified proteases derived from different sources, such as lysosomal or proteasomal, has been used as a criterion for identification of their intracellular targets (Rock et al., 1994). For example, whereas LLnL has been described as a calpain I inhibitor, it is known to inhibit other proteases, including lysosomal proteases and the proteasome (Rock et al., 1994). The compound carboxybenzyl-leucyl-leucyl-leucinal (Cbz-LLL) was described as a very potent inhibitor of proteasomes (Rock et al., 1994). We synthesized this compound using solution chemistry and found it to produce a class I breakdown intermediate indistinguishable from that produced in the presence of LLnL (Figure 7B, compare lanes 3 and 4 with 5 and 6). The antibiotic lactacystin (Fenteany et al., 1995) is perhaps the most specific inhibitor of proteasomes discovered yet: radioactive lactacystin labels only the β-type subunit of proteasomes, and the compound is strongly inhibitory on proteasomal proteolysis (Fenteany et al., 1995). Lactacystin, like LLnL and Cbz-LLL, produces the 40 kDa breakdown intermediate (Figure 7B, lanes 7 and 8). We conclude that the specificity profile of inhibition indicates the involvement of proteasomes in the destruction of class I heavy chains in US11$^+$ cells.

Subcellular Fractionation Shows Dislocation of the Class I Heavy Chain from the Microsomal Fraction to the Cytosol

Subcellular fractionation experiments were performed on control cells and US11$^+$ cells labeled in the presence of LLnL, as described in the Experimental Procedures. For both control and US11$^+$ cells, the 1000 g pellet (Figure 8, lanes 1 and 2) contains substantial amounts of class I heavy chains, β_2m, calnexin, and transferrin receptor (TfR). This pellet may contain unbroken cells, and larger cellular debris that may also trap soluble proteins. We therefore consider the distribution of the proteins of interest over the 10,000 and 100,000 g pellets and the 100,000 g supernatant to be the more significant parameters. In US11$^+$ cells, both intact heavy chains and some of the breakdown intermediates are present (Figure 8A, US11$^+$, lanes 1 and 2). Whereas in control cells almost all class I heavy chains were recovered in the 1000 and 10,000 g pellets (Figure 8A, Control, lanes 1–4) and no class I heavy chains were detectable in the 100,000 g supernate (Figure 8A, Control, lanes 7 and 8), the breakdown intermediate seen in LLnL-treated US11$^+$ cells was largely recovered from the 100,000 g supernate (Figure 8A, US11$^+$, lanes 7 and 8). We therefore conclude that US11 mediates the transfer of the class I heavy chain from the ER environment to the cytosolic compartment. In the same subcellular fractions, the light chain β_2m that is not associated with class I heavy chains (no coprecipitating heavy chains were observed; data not shown) sediments predominantly with the microsomal fractions, and its distribution is not noticeably different for control cells and US11$^+$ cells (Figure 8B). The intracellular compartments that contain β_2m must therefore remain largely intact in the course of homogenization. This finding also argues against breakage of intracellular organelles as the source of the soluble class I breakdown intermediate in US11$^+$ cells, and supports its cytosolic localization. Note that recovery of the class I breakdown intermediate using anti–heavy chain serum did not require the inclusion of detergent.

The distribution of US11 itself is indicative of a microsomal localization. Of note, the class I breakdown intermediate (Figure 8A, US11$^+$) is found in a compartment, the 100,000 g supernate, that is entirely devoid of US11 (Figure 8C, US11$^+$, lanes 7 and 8). Therefore, at the time that the breakdown intermediate has been generated,

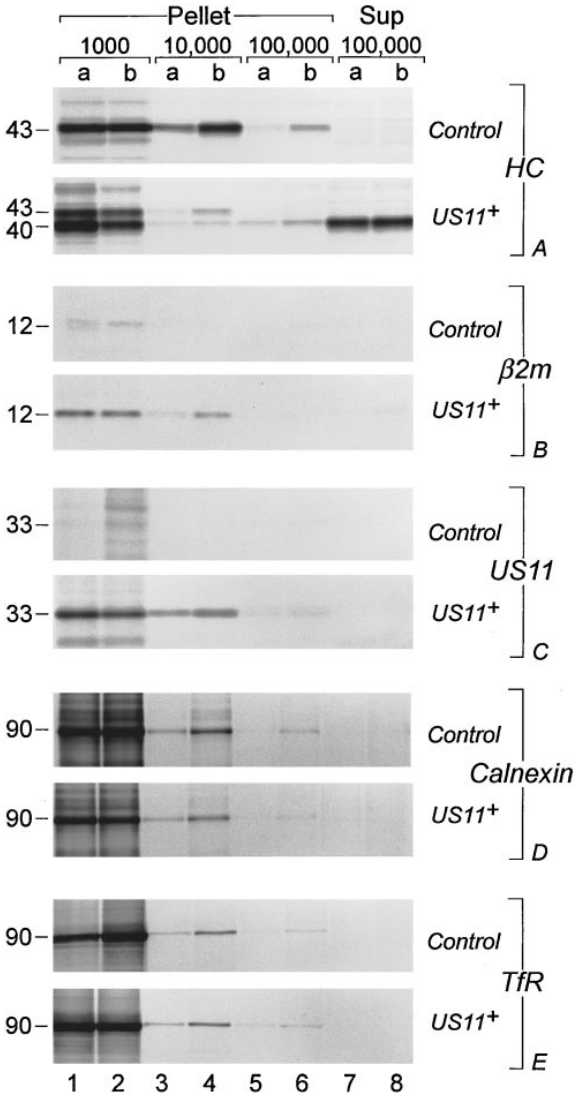

Figure 8. In US11$^+$ Cells, Treated with LLnL, Most Class I Heavy Chains Are Present in the Cytosol

Subcellular fractionation on US11$^+$ and control cells was performed as described in the Experimental Procedures. Class I heavy chains were precipitated from the subcellular fractions using the rabbit anti–heavy chain serum (A). Note recovery of the breakdown intermediate from the 100,000 g supernatant. The β_2m molecules were immunoprecipitated using the monoclonal antibody BBM.1 (B). The US11 glycoprotein could be recovered from US11$^+$ but not from control cells (C). Note that US11 sediments largely with the microsomal fractions and is absent from the 100,000 g supernatant, the compartment that contains the heavy chain breakdown intermediate. Calnexin (D) and transferrin receptor (TfR; [E]) were immunoprecipitated using the monoclonal antibodies AF8 and 66Ig10, respectively.

there can be no physical interaction between US11 and this intermediate. As further controls, we show that neither calnexin (Figure 8D), nor TfR (Figure 8E) are recovered from the 100,000 g supernatant.

Discussion

The present study reveals yet another astonishing example of how virus-infected cells may evade detection by the immune system through down-regulation of MHC class I molecules. In this regard, human cytomegalovirus has been an enigma: in HCMV-infected cells, there is a strong reduction in the cell surface display of host class I molecules, yet the gene(s) responsible, let alone the mechanism(s) by which this is accomplished, have remained obscure until recently.

A systematic search of the HCMV genome revealed the US11 gene as a major determinant of class I instability (Jones et al., 1995). With respect to class I heavy chain turnover, cell lines transfected with US11 behave in the same manner as HCMV-infected cells. Pulse-chase analysis revealed that all of the class I heavy chains synthesized in a 45 to 60 s pulse were broken down with an initial $t_{1/2}$ of <1 min. The occurrence of a core-glycosylated precursor to the breakdown intermediate in US11$^+$ cells shows that initial insertion of Class I heavy chains into the ER takes place normally. Polypeptide chain elongation proceeds at a rate of some 4 amino acids per second (Braakman et al., 1991), and thus the time required for completion of a 338-residue class I heavy chain (excluding signal peptide) would take ~80 s. Destruction of class I molecules in US11$^+$ cells is a process that most likely starts as soon as the polypeptide chain is completed, and must target molecules located in the endoplasmic reticulum, to which US11 itself is confined. No breakdown intermediates could be visualized unless protease inhibitors were included in the labeling and chase medium. An unexpected observation was the identity of a deglycosylated intermediate, the product of an N-glycanase-catalyzed reaction, that transiently accumulated in LLnL-treated US11$^+$ cells. The N-linked glycan itself plays no role in targeting class I heavy chains for destruction.

The specificity of the breakdown process deserves comment. US11$^+$ cells show no apparent growth disadvantage in tissue culture, suggesting that even if multiple substrates were being attacked by this US11-dependent process, no functions essential for growth are compromised. As examples, neither transferrin receptors nor calnexin are affected in their biosynthesis or intracellular distribution by the presence of US11. We favor the notion that specific breakdown of class I heavy chains is the consequence of their selective transfer to the cytosol.

There are at least three arguments in favor of the cytosolic disposition of the class I heavy chain breakdown intermediate. First is the occurrence of the nonglycosylated intermediate: in mammalian cells, N-glycanase has been reported as a cytosolic activity (Suzuki et al., 1994). While the published data are by no means conclusive, localization of N-glycanase to the ER would seem less plausible. It would entail the risk of inappropriately removing glycans from nascent or newly synthesized glycoproteins, which may depend on these glycans for entering the proper folding or sorting pathway (Hammond et al., 1994; Scheiffele et al., 1995). Is the presence of an N-linked glycan on the class I heavy chain incompatible with its re-entry into the cytosol via the translocation complex by which it entered the ER? The physical dimensions of the pore involved in translocation are not known (Simon and Blobel, 1991), but at least in bacteria, which possess a homologous translocation complex, nascent chains are accompanied into

the pore by a second polypeptide, the SecA protein, which transiently acquires a transmembrane orientation (Economou and Wickner, 1994; Kim et al., 1994). If two polypeptide chains can be accomodated simultaneously by the translocation complex, we see no obvious impediment to transport of a glycosylated polypeptide backbone out of the ER.

Second is the ability of a number of protease inhibitors to interfere with the breakdown of class I molecules. These inhibitors are the peptide aldehydes LLnL and CbzLLL, the latter of which was reported to show strong preference for inhibition of proteasomal proteolysis, at least in vitro. More convincingly, lactacystin, the only active site-directed inhibitor selective for the proteasomal β-type subunits (Fenteany et al., 1995), also inhibited class I breakdown and produced the nonglycosylated intermediate. Unless we assume the presence of a hitherto undiscovered proteolytic activity in the lumen of the ER that is also a target of inhibition for lactacystin, we consider this strong evidence in support of proteasome-mediated, and hence a cytosolic location for, US11-stimulated breakdown of class I molecules.

Third, the sedimentation behavior of class I molecules is altered dramatically in cells that express US11. Whereas in normal cells all class I heavy chains as well as other membrane proteins (TfR, calnexin, and US11 itself) sediment after 1 hr at 100,000 g, most of the 40 kDa nonglycosylated breakdown intermediates fail to do so under these conditions and are therefore cytosolic by definition (Lardy, 1965).

Current models suggest that, upon arrival of a so-called stop-transfer signal, type I membrane proteins anchor themselves firmly in the lipid bilayer by lateral diffusion out of the protein complex that mediates their cotranslational insertion (Simon and Blobel, 1991). The US11 product may allow newly synthesized class I molecules to exit the ER for the cytosol in a reaction that represents a reversal of the insertion process, by interference with the reception of this stop-transfer signal. Upon termination of the polypeptide chain the ribosome dissociates from the translocation complex. A failure to properly exit the translocation complex might allow the polypeptide chain to move in a backward motion towards the cytosol. Access of the newly synthesized class I heavy chain to cytosolic proteases or other proteins will likewise require the dissociation of the ribosome. In this model, a specific but transient interaction of the lumenal domains of US11 and class I ensures that only class I molecules, and not other membrane or secretory proteins, would suffer this fate. Any class I heavy chain that finds itself fully exposed to the cytosolic environment would be broken down rapidly, as are other type I membrane proteins inappropriately expressed in the cytosol (Townsend and Bodmer, 1989).

An alternative interpretation invokes the involvement of Kar2 or Bip-equivalents. The Kar2 protein in yeast is needed for efficient posttranslational translocation into the ER of secretory and membrane proteins in vivo and in vitro (Vogel et al., 1990; Sanders et al., 1992; Panzner et al., 1995). Its mechanism of action may involve repeated cycles of interaction and disengagement to produce a "pulling" action via a molecular ratchet, allowing entry and blocking slippage back into the cytosol of proteins in the course of translocation. This would be analogous to the function of the mitochondrial matrix Kar2 homolog, HSP70 (Schneider et al., 1994). It is at this stage that US11 could interfere with the interactions between class I heavy chains and the molecular ratchet. Having failed to properly engage this ratchet, for example because US11 and the Kar2 equivalent bind to the same key site, the class I molecule may slide back into the cytosol after completion of the polypeptide chain. However, Kar2/BiP did not appear to be required for the cotranslational mode of translocation (Görlich et al., 1992; Görlich and Rapoport, 1993; Panzner et al., 1995), and further experiments will be required to distinguish between the possibilities suggested here.

Little is known about the pathways in normal cells that lead to destruction of MHC class I molecules, especially of their intra membrane and cytoplasmic portions. Presumably, the lumenal segments are readily degraded when they enter the final part of the endocytic pathway, where the environment is reducing and where the low pH and abundant proteases finish them off. Indeed, for class I molecules that have exceeded their useful life span at the cell surface, destruction is preceded by loss of bound peptide and β_2m (not necessarily in that order) (Neefjes et al., 1993), and can be blocked to a significant extent through inactivation of the vacuolar proton-pumping ATPase by the macrolide antibiotic concanamycin B (R. Machold and H.L.P., unpublished data). Other type I membrane proteins may follow a similar general scheme for their catabolism. The involvement of proteasomes in the breakdown of the cystic fibrosis transmembrane conductance regulator (CFTR) protein was recently suggested, based on the inhibitory effects of Cbz-LLL and lactacystin, as well as the occurrence of ubiquitinated breakdown intermediates (Jensen et al., 1995; Ward et al., 1995). It should be noted that the mature form of the CFTR has some 80% of its total mass exposed on the cytosolic face, and that aberrantly folded CFTR decays with a $t_{1/2}$ of some 30 min. In the case of CFTR, there is no evidence that its membrane topology is altered prior to attack by cytosolic proteases.

Clearly, US11$^+$ transfectants reveal a novel pathway that alters the membrane dispostion and fate of a type I membrane protein. These findings have implications not only for our understanding of the biology of HCMV, but also for the biosynthesis and turnover of membrane proteins in general. Viruses have withstood the selective pressures exerted by the host environment through clever exploitation and evasion of cellular mechanisms, and have had tens of millions of years to hone these strategies. As far as class I expression is concerned, we refer to the recent finding (Früh et al., 1995; Hill et al., 1995) that HSV comes equipped with a potent inhibitor of the MHC-encoded peptide transporter, the TAP complex, thus thwarting presentation of viral antigen at a crucial step in the virus' lifecycle. Every single step in the biogenesis and intracellular transport of class I molecules is a potential target for these stealth strategies. The present example of US11 shows that this small type I membrane glycoprotein may accomplish the elimination of MHC class I products by effectively redirecting the class I heavy chain to the proteolytic machinery of

the cytosol. But these findings also raise the question whether degradation of membrane proteins in the ER may not more generally avail itself of this possibility. The carbohydrate moieties of membrane glycoproteins degraded prior to their leaving the ER fail to enter the secretory pathway, and instead may be broken down in the cytosol (Moore and Spiro, 1994). We suggest as a more general possibility that misfolded membrane proteins may be dislocated from the ER and be degraded by proteases in the cytosol, and not necessarily by proteases in the lumen of the ER. To sequester proteases from the environment into which proteins are inserted biosynthetically would prevent exposure of incompletely folded proteins to the risk of proteolytic damage. While we do not wish to suggest that the breakdown of ER-resident misfolded membrane proteins must obligatorily take place in the cytosol, the results presented here show that this route is in principle open in the mammalian cell, has been exploited by human cytomegalovirus, and is unlikely to be restricted to that single example. If one considers antigen presentation by MHC class I proteins as a process in essence focused on cytosolic proteins, our results would immediately suggest mechanisms by which peptides, derived from lumenally disposed proteins, may be generated by the cytosolic protease machinery and delivered to class I molecules by the MHC-encoded peptide transporter.

Experimental Procedures

Cells and Cell Culture

U373-MG astrocytoma cells and US11 transfectants prepared from this cell line have been described (Jones et al. 1994, 1995; Kim et al., 1995). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) fetal calf serum and puromycin (Sigma, St. Louis, MO) at a final concentration of 0.375 μg/ml.

Antibodies

For studying assembly of class I molecules, the mouse monoclonal antibody W6/32, specific for assembled class I molecules, the monoclonal antibody BBM.1 recognizing both free β_2m and class I heavy chain–associated β_2m, and the rabbit anti–class I heavy chain serum were used (see Beersma et al., 1993). The monoclonal antibody 66Ig10 recognizing the human transferrin receptor was used for isolation of TfR, as described (Beersma et al., 1993). Polyclonal rabbit serum reactive with US11 was described previously (Jones et al., 1991).

Biochemical Methods and Materials

Leucyl-leucyl-norleucinal (LLnL; Boehringer Mannheim, Germany) was dissolved in ethanol and used at a final concentration of 50 μM. TM and brefeldin A were from Sigma (Sigma Chemical Co., St. Louis). Treatment with endoglycosidase H (Endo H; New England Biolabs, Beverly) or N-glycanase (Boehringer Mannheim, Germany) were performed as suggested by the manufacturers. Lactacystin (Fenteany et al., 1995) was used at a final concentration of 20 μM. [^{35}S]methionine (1200 Ci/mmol) was from NEN–Du Pont.

Synthesis of Carboxybenzyl-Leucyl-Leucyl-Leucinal (Cbz-LLL)

Cbz-LLL was synthesized on 5 mmol scale using solution chemistry. First Cbz-Leucine-N-hydroxysuccinamide ester (Cbz-leu-Osu, Advanced Chemtech) was coupled to leucine. In brief, leucine and one equivalent of sodium bicarbonate were dissolved in water. Equimolar Cbz-leu-Osu was added as a solution in dioxane and the reaction stirred overnight. Dioxane was removed by rotary evaporation and the dipeptide was precipitated from the remaining water phase by addition of a citric acid buffer. The resulting precipitate was extracted into ethyl acetate and concentrated. Crude dipeptide was recrystallized from ethanol/water. Next, Leucinol (Advanced Chemtech) was coupled to Cbz-Leu-Leu using standard HoBt, DIC coupling in dioxane. The crude Cbz-Leu-Leu-Leucinol was purified by flash chromatography in methylene chloride/methanol 12:1 (v:v). Finally, Cbz-LLL was synthesized by oxidation of the corresponding alcohol using the Swern method. In brief, the tripeptide alcohol was dried and dissolved in methylene chloride. To an argon-flushed flask cooled to −78°C was added oxalyl chloride and dimethyl sulfoxide in methylene chloride. After 10 min, the peptide alcohol was added. Finally, the reaction was quenched with triethylamine and allowed to warm to room temperature. Water was added and the resulting crude aldehyde was extracted with three portions of methylene chloride. Pure Cbz-LLL was obtained by flash chromatography of the organic phase in methylene chloride/methanol 30:1. The purity and identity of the peptide aldehyde as well as intermediates in the synthesis were characterized by thin-layer chromatography and nuclear magnetic resonance spectroscopy. Cbz-LLL was dissolved in ethanol and used at a final concentration of 20 μM.

Pulse-Chase Analysis

Cells were detached by trypsin treatment and incubated in methionine-free Dulbecco's modified Eagle's medium with or without added inhibitors for 1 hr. Two million cells (15 min pulse; 250 μCi of label) or 5×10^6 cells (45–60 s; 500 μCi of label) were used per sample. Incorporation was terminated by the addition of nonradioactive methionine to a final concentration of 1 mM. Immediately after the chase, samples were placed on ice and lysed in 1 ml of ice-cold NP40 lysis mix. Preparation of lysates and immunoprecipitations were performed as described (Beersma et al., 1993).

Gel Electrophoresis

SDS–PAGE, one-dimensional IEF, and fluorography were performed as described (Ploegh, 1995).

Subcellular Fractionation

Cells ($n = 10^8$) were incubated in methionine-free medium with LLnL for 1 hr. Cells were then labeled with [^{35}S]methionine (250 μCi/ml) in a volume of 2 ml for 15 min. The cells were washed in homogenization buffer (0.25 M sucrose, 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA [pH 7.4] and the protease inhibitors leupeptin [1 μM], iodoacetamide [50 μM], phenylmethylsulfonyl fluoride [1 mM], and LLnL [20μM]) and resuspended in 2 ml of homogenization buffer. Cells were homogenized on ice using a Dounce homogenizer (30 or 50 strokes) with a tight fitting pestle (Thomas, pestle type A). The homogenate was spun at 1,000 g for 10 min, which yielded the 1,000 g pellet. The supernatant was subjected to 10,000 g centrifugation for 30 min, which yielded the 10,000 g pellet. Finally, the 10,000 g supernantant was spun at 100,000 g for 1 hr, which resulted in the 100,000 g pellet and the 100,000 g supernatant (cytosol). Lysis mix was added to all pellets (but not to the 100,000 g supernatant) and immunoprecipitations, SDS–PAGE, and fluorography were performed as described.

Immunoelectron Microscopy

U373 cells expressing US11 and control transfected cells were treated with LLnL as above, or taken directly and processed for immunoelectron microscopy as described (Slot et al., 1991; Liou and Slot, 1994) using rabbit anti-US11 antiserum and labeled with 10 nm protein A–gold particles. Sections were contrasted with uranyl and embedded in a mixture of methyl cellulose and uranyl as described (Liou and Slot, 1994).

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