The Role of BiP in Endoplasmic Reticulum-associated Degradation of Major Histocompatibility Complex Class I Heavy Chain Induced by Cytomegalovirus Proteins∗

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Human cytomegalovirus (HCMV) US11 and US2 proteins cause rapid degradation of major histocompatibility complex (MHC) molecules, apparently by ligating cellular endoplasmic reticulum (ER)-associated degradation machinery. Here, we show that US11 and US2 bind the ER chaperone BiP. Four related HCMV proteins, US3, US7, US9, and US10, which do not promote degradation of MHC proteins, did not bind BiP. Silencing BiP reduced US11- and US2-mediated degradation of MHC class I heavy chain (HC) without altering the synthesis or translocation of HC into the ER or the stability of HC in the absence of US11 or US2. Induction of the unfolded protein response (UPR) did not affect US11-mediated HC degradation and could not explain the stabilization of HC when BiP was silenced. Unlike in yeast, BiP did not act by maintaining substrates in a retrotranslocation-competent form. Our studies go beyond previous observations in mammalian cells correlating BiP release with degradation, demonstrating that BiP is functionally required for US2- and US11-mediated HC degradation. Further, US2 and US11 bound BiP even when HC was absent and degradation of US2 depended on HC. These data were consistent with a model in which US2 and US11 bridge HC onto BiP promoting interactions with other ER-associated degradation proteins.

Membrane and secreted proteins that fail to properly fold or assemble are degraded in a process known as ER-association degradation or ERAD (1–4), in which aberrant proteins are retrotranslocated across the ER membrane into the cytoplasm and degraded by proteasomes (4–8). Retrotranslocation in some instances appears to require Sec61 channels (2, 9–12) but may involve Derlins for other ERAD substrates (13–15). Polyubiquitination of ERAD substrates occurs in the cytoplasm in a process coupled to export and likely provides directionality to retrotranslocation (16–21). A complex of proteins, including p97 ATPase, ubiquitin fusion degradation-1 (Ufd1), and nuclear protein localization-4 (Npl4), forms an essential cytoplasmic component of the extraction and degradation machinery (18, 22–25). Some ERAD substrates may also be extracted from the ER membrane by the proteasome (26–29).

Less is known about how ERAD substrates are recognized and targeted to the retrotranslocon. ER chaperones, including BiP, calnexin (CNX), and calreticulin (CRT) promote folding of ER proteins. However, unsuccessful folding or assembly may force these chaperones to withdraw from folding cycles and target substrates for retrotranslocation (3, 8, 30). One process that determines the duration of retention of a glycoprotein in the ER involves the monitoring of N-linked oligosaccharides. CNX and CRT bind immature monoglucosylated proteins and promote ER retention, folding, and assembly (31). However, protracted cycles of CNX and CRT binding and release leads to binding of ERAD enhancing α-mannosidase-like (EDEM) protein that accelerates ERAD (32, 33). The yeast osteosarcoma-9 protein (Yos9p) and protein disulfide isomerase also select misfolded luminal glycoproteins for ERAD (34–36). However, interactions between EDEM, Yos9p, or protein disulfide isomerase and retrotranslocation channels or other ERAD components have not yet been described, so it is not clear how binding of these proteins promotes ERAD.

BiP recognizes hydrophobic regions of misfolded or partially assembled proteins, promoting ER retention and protein folding (3, 30, 37–39), in a process regulated by DnaJ-like proteins (39, 40). In yeast, BiP appears to play an important role in ERAD of ER luminal proteins. The role of BiP in ERAD can be genetically separated from its role in translocation, and degradation of ERAD substrates is slowed in yeast expressing mutant forms of BiP (9, 41, 42). BiP can function to reduce aggregation and maintain the solubility of ERAD substrates so that substrates are retrotranslocation-competent (42, 43). However, overexpression of wild-type BiP in the presence of mutant BiP did not overcome defects in ERAD, although substrates were no longer aggregated (42), suggesting other roles for BiP in ERAD. In mammalian cells, evidence for BiP function in ERAD involves

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3 The abbreviations used are: ER, endoplasmic reticulum; ERAD, ER-associated degradation; EDEM, ER degradation enhancing α-mannosidase-like; Ad, adenovirus; DTT, dithiothreitol; endoH, endoglycosidase H; HC, heavy chain; HCMV, human cytomegalovirus; ICP47, infected cell protein-47; MHC, major histocompatibility complex; PFU, plaque forming unit(s); siRNA, small interfering RNA; UPR, unfolded protein response; US, unique protein (Yos9p); mAb, monoclonal antibody; DOC, deoxycholate; CNX, calnexin; CRT, calreticulin.
correlations between the extent of BiP binding and rates of substrate release (26, 44–48). These results suggest that BiP stabilizes potential ERAD substrates and might transfer substrates onto other components of the ERAD machinery. BiP binds to the luminal side of the Sec61 translocon, sealing or gating the channel (49), and might therefore promote transfer of ERAD substrates to retrotranslocation channels, although there is no evidence for this at present. Arguing against this notion, the same BiP sequences are involved in binding both to ERAD substrates and the translocon (50). However, without mammalian cells lacking BiP or expressing mutant forms that overcome the native BiP, it is difficult to understand how BiP promotes ERAD.

HCMV glycoproteins US2 and US11 bind MHC class I and II proteins and trigger their degradation by proteasomes, a process mechanistically similar to ERAD, but with extremely rapid kinetics (12, 13, 15, 23, 51–55). MHC class I HC bound by US2 was proposed to be retrotranslocated across Sec61 channels (12), whereas Derlin-1 has been suggested to be the channel for US11-mediated degradation of HC (13, 15). Several cytoplasmic events, including polyubiquitination (20, 56) and the function of p97 ATPase (23, 57), are required for the degradation of MHC proteins. However, events in the ER lumen that promote US11- or US2-mediated degradation are poorly understood. Specifically, it is not clear how binding of US2 or US11 to normal MHC proteins triggers rapid ERAD. We and others have suggested that US2 and US11 might bridge MHC complexes onto cellular components of the ERAD machinery (58–60), but the identity of such luminal proteins has remained elusive.

In this study, we identified BiP as a protein bound by US11 and US2. Several homologous HCMV proteins, that bind MHC proteins but do not cause their degradation, did not bind BiP. Silencing of BiP did not alter the stability of HC in the absence of US11 or US2 but decreased US11- and US2-mediated degradation of HC. BiP promotes degradation of HC in US2- and US11-expressing cells by a mechanism distinct from maintaining HC solubility or “retrotranslocation competence.” US2 and US11 bound to BiP, even in the absence of HC, and bind directly to HC suggesting that the US proteins bridge BiP onto HC to promote binding onto other ERAD machinery.

**EXPERIMENTAL PROCEDURES**

Cells, Viruses, Antibodies, and Drugs—U373-MG human astroglial and Vero cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium containing 10% bovine growth supplement (HyClone) and antibiotics. 1858 melanoma cells were then identified using the ProFound software. Cells, Viruses, Antibodies, and Drugs—U373-MG human astroglial and Vero cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium containing 10% bovine growth supplement (HyClone) and antibiotics. 1858 melanoma cells were then identified using the ProFound software.

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Metabolic Labeling, Immunoprecipitation, and Western Blotting—Cells were radiolabeled 18 h after infection with Ad vectors or 4 h after infection with VSV. The cells were trypsinized, labeled in suspension with [35S]Met/Cys (300–500 μCi/ml, PerkinElmer Life Sciences), and the label was chased in medium containing a 10-fold excess of Met/Cys. For direct immunoprecipitations, cell extracts were made with Nonidet P-40-deoxycholate (Nonidet P-40-DOC) buffer. Proteins of interest were immunoprecipitated from clarified lysates with rabbit polyclonal antibodies to HCMV US2–11 proteins (61) and protein A-Sepharose, as described (53). For precipitation of HC with mAb HC10, lysates were heated to 60 °C for 1 h, cooled to room temperature, and clarified. Endoglycosidase H (endoH, New England Biolabs) analyses were performed as per the manufacturer’s instructions. Samples were subjected to electrophoresis using 8–12% polyacrylamide gels, the gels were fixed, dried, and exposed to PhosphorImager screens (Molecular Dynamics).

For assessing protein complexes, cells were lysed with 1% digitonin (Calbiochem) or 0.5% Nonidet P-40, and proteins were immunoprecipitated as above. Samples were subjected to SDS-PAGE and stained with silver reagent (Pierce) or were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore) and Western blotted using a chemiluminescence kit (New England Nuclear or Pierce). Blots were exposed to x-ray film, or bands were quantified using a Lumi-Imager (Roche Molecular Biochemicals). For Derlin-1, 5–6 × 10^6 cells were homogenized in 10 ml Hepes (pH 7.35), 1 ml EDTA, 250 mM sucrose, and postnuclear supernatants were centrifuged at 100,000 × g to obtain microsomes that were dissolved in digitonin buffer and diluted in 4× sample loading buffer before analysis by immunoblotting.

**Mass Spectroscopic Analyses**—Silver-stained protein bands were reduced in 10 mM dithiothreitol (DTT), alkylated in 10 mM iodoacetamide, and then subjected to in-gel trypsin hydrolysis (65). Peptides were purified and analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry using a cyano-4-hydroxycinnamic acid matrix (Sigma) on a Voyager DE-STR instrument (Applied Biosystems). Proteins were then identified using the ProFound software.

**RNA Silencing**—6 × 10^5 U373 cells in 100-mm dishes in 4 ml of serum-free Opti-MEM (Invitrogen) were incubated with 100 nM small interfering RNAs (siRNAs) and Oligofectamine (Invitrogen) as recommended by the manufacturer. The following oligonucleotides were used: (a) BiP siRNA #1 targeting nucleotides 684–704 (CCUUCGAUGUGUCUCUCUdTdT), (b) BiP siRNA #2 targeting nucleotides 1666–1686 (GAGGCCCAUUGAUACUGAdTdT), (c) Derlin-1 siRNA #1 targeting nucleotides 279–297 (GAGGCCCAGCAGACUAUUAdTdT), (d) Derlin-1 siRNA #2 targeting nucleotides 445–463.
(CGAUUUAAGCCUCGUAAUdTdT), (e) control siRNA #1 representing a scrambled sequence of BiP siRNA #1 (UGAC-UCCGUCUCGUUCAdTdT), and (f) control siRNA #2, targeting firefly luciferase (Dharmacon catalog # D-001210-02). After 4 h, 2 ml of Dulbecco’s modified Eagle’s medium containing 30% fetal bovine serum was added to each dish, and the cells were incubated an additional 14–16 h before the siRNA and Oligofectamine were removed, and Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum was added. 48 h after transfection, the cells were infected with Ad vectors for 18 h to express HCMV proteins. For silencing in smaller or bigger dishes, the proportion of reagents was scaled down or up accordingly.

Analysis of Induction of the Unfolded Protein Response—UPR induction was assessed by detecting the splicing of X-box-binding protein-1 (XBP-1) using reverse transcription-PCR. For positive controls, cells were either treated with 2 mM DTT or ing protein-1 (XBP-1) using reverse transcription-PCR. For accordingly.

Results

HCMV Glycoproteins US11 and, to a Lesser Extent, US2 Bind BiP—HCMV US2–US11 region encodes eight homologous ER-retracted type I membrane glycoproteins (66), and four of these (US2, US3, US6, and US11) inhibit the MHC class I or II antigen presentation pathways (51, 67). US2 and US11 cause degradation of MHC proteins (51, 61, 68). US3 binds MHC class I and II proteins promoting mislocalization but not degradation (51, 61, 68). US7, US8, US9, and US10 do not substantially affect MHC class I or II cell-surface expression or antigen presentation (61, 69), although US8 and US10 bind HC without causing degradation (70, 71). To identify cellular proteins associated with US11 and US2, the viral proteins were delivered using replication-defective Ad vectors, and radiolabeled US73 cells were extracted using 1% digitonin or 0.5% Nonidet P-40, and the US proteins were immunoprecipitated. Proteins were stained with silver reagent. D and E, infected cells were treated with 1 μM epoxomicin for 2 h, lysed with 1% digitonin, and US proteins were immunoprecipitated. Proteins were Western blotted for BiP (upper panels) or US proteins (lower panels).

FIGURE 1. US2 and US11 associate with BiP. Non-replicating Ad vectors were used to express various HCMV US2–US11 region proteins in US73 glial cells using 100 PFU/cell AdtetUS and 20 PFU/cell Adtet-trans viruses for 18 h. A and B, infected cells were radiolabeled with [35S]Met/Cys in the presence of 10 μM MG132 for 3 h, lysed in 0.5% Nonidet P-40, and US2, US3, US3/US2-TMCT, US7, or US11 immunoprecipitated with rabbit antibodies and precipitated proteins subjected to electrophoresis. C, infected cells were treated with 10 μM MG132 for 4 h, lysed in 0.5% Nonidet P-40, and US7 or US11 was immunoprecipitated. Proteins were stained with silver reagent. D and E, infected cells were treated with 1 μM epoxomicin for 2 h, lysed with 1% digitonin, and US proteins were immunoprecipitated. Proteins were Western blotted for BiP (upper panels) or US proteins (lower panels).

MHC class I or II
expressed at different levels consistently showed that US11 bound more BiP than US2, and US3, US9, and US10 bound little or no BiP. Moreover, US2 and US11 tend to be expressed at lower levels in HCMV-infected cells compared with AdUS2- or AdUS11-infected cells (66). However, BiP was detected even when US11 was expressed at very low levels (Fig. 1). US11 causes much more rapid and extensive degradation of MHC class I proteins, compared with US2 (12, 55). Thus, our observations provided a correlation between BiP binding and the capacity to promote MHC degradation. We note that an association of BiP with US11 was reported (13) when we were investigating the role of BiP in US11-mediated ERAD.

Silencing of BiP Reduces US11- and US2-mediated Degradation of MHC Class I—Although BiP is clearly important for ERAD in yeast, similar efforts to understand its function in mammalian ERAD have been slowed, because cells that do not express BiP or that express non-functional BiP are not available. We attempted to reduce BiP expression in U373 cells by using siRNAs. Cells were transfected with either of two different BiP siRNAs and then infected with Ad vectors to deliver US11 or other HCMV glycoproteins. In both US11-expressing and control cells, BiP siRNA #1 reduced steady-state levels of BiP by ~97% compared with a control siRNA (Fig. 2A). In other experiments, silencing with siRNA #1 was 90–96% compared with control siRNAs or transfection reagent alone, and there were no effects on actin levels (not shown). This extensive silencing was surprising, given the high levels of BiP present in cells. BiP siRNA #2 reduced BiP to 10–20% of that observed with a control siRNA (Fig. 2B). The presence of US11 did not have any effect on BiP silencing (Fig. 2, A and B). There was no additive or synergistic effect of both oligonucleotides, and two other BiP siRNAs were less effective (not shown). In cells treated with a control siRNA or with transfection reagent alone (no siRNA), US11 caused extensive degradation of MHC class I HC compared with cells expressing US7 or expressing no HCMV proteins, i.e. infected with Adtet-trans alone (Fig. 2C). As in previous experiments (59, 72), US11 caused loss of HC during the 10-min pulse period. By contrast, in cells expressing US11 and in which BiP was silenced with BiP siRNA #1, there was substantial stabilization of HC, compared with a control siRNA (Fig. 2C). There was ~5-fold more HC in the chase sample comparing cells expressing US11 that were transfected with BiP siRNA with US11-expressing cells transfected with a control siRNA or no siRNA. In eight independent experiments, BiP silencing with siRNA #1 decreased degradation of HC in the chase by 20–66% (1.5- to 5-fold) (not shown). There was no effect of BiP silencing on HC stability in US7-expressing cells. Silencing with BiP siRNA #2 reduced US11-mediated degradation of HC to 25–40% that observed with BiP siRNA #1, there was substantial stabilization of HC, compared with a control siRNA (Fig. 2C). There was ~5-fold more HC in the chase sample comparing cells expressing US11 that were transfected with BiP siRNA with US11-expressing cells transfected with a control siRNA or no siRNA. In eight independent experiments, BiP silencing with siRNA #1 decreased degradation of HC in the chase by 20–66% (1.5- to 5-fold) (not shown). There was no effect of BiP silencing on HC stability in US7-expressing cells. Silencing with BiP siRNA #2 reduced US11-mediated degradation of HC to 25–40% that observed with control siRNAs (Fig. 2D), confirming specificity for BiP. Note that US11 causes rapid degradation of HC, so that there is loss in pulse samples, and there is acquisition of antibody epitopes in chase samples when US11 is not expressed.

US2 also causes degradation of HC (12, 73), albeit less rapidly than US11. Moreover, there are mechanistic and qualitative differences between the US2- and US11-mediated processes (58, 60, 74–77). Despite these differences between US2 and US11, BiP siRNA #1 reduced HC degradation in US2-expressing cells by >6-fold compared with a control siRNA (Fig. 3). As with US11, a fraction of HC was degraded during the 10-min pulse period when BiP was not silenced. BiP silencing did not affect US2-mediated degradation of MHC class II α chain (not shown).

Silencing of CNX, CRT, and Derlin-1—Like BiP, CNX, and CRT bind HC and facilitate assembly of peptide-loaded class I complexes (8, 78). These chaperones may act in a redundant
fashion, e.g. CRT-negative cells can assemble class I heterodimers (79). To determine the roles of CNX and CRT in US11- and US2-mediated ERAD, we attempted to silence CNX and CRT. Unfortunately, cells transfected with several CNX and CRT siRNAs became vacuolated, rounded, exhibited reduced protein synthesis and, in some cases, lifted off the plastic dishes (not shown). This toxicity was never observed with BiP silencing until as much as 96 h following transfection (not shown).

Derlin-1 was implicated in US11-mediated degradation (13, 15), possibly acting as a retrotranslocation channel or as a transmembrane component of the ERAD pathway, signaling to p97 ATPase that participates in extraction (15, 23). Here, we tested whether silencing of Derlin-1 reduced degradation of HC in US11-expressing cells. Derlin-1 siRNA #2 reduced Derlin-1 levels measured in an immunoblot to 30% of that found in cells transfected with a control siRNA (Fig. 4A). Under the same conditions in US11-expressing cells, HC levels increased in chase samples by 3-fold compared with cells transfected with a control siRNA (Fig. 4B). Similarly, Derlin-1 siRNA #1 also reduced Derlin-1 expression and increased HC stability in US11-expressing cells (not shown).

Sequence analyses of our Derlin-1 siRNAs indicated that these oligonucleotides are unlikely to affect Derlin-2, although a role for Derlin-2 in US11-mediated degradation of HC has not been shown (13, 14). These results add to the evidence that Derlin-1 plays an essential role in US11-mediated ERAD and highlight further the utility of siRNA in characterizing ERAD in mammalian cells.

Effects of BiP Silencing on the Stability of HC in the Absence of US2 or US11 and on the Stability of US11 and US2 Proteins—To further understand the effects of BiP silencing, we characterized HC stability in cells not expressing US2 or US11. Silencing BiP had no effect on the stability of HC in the absence of US11 or US2 (Fig. 5A). To cause HC to accumulate in the ER, we delivered herpes simplex virus ICP47 into cells using an Ad vector. ICP47 inhibits the transporter associated with antigen presentation so that HC molecules do not bind peptides and are retained in the ER (63, 80). HC remained in an endoH-sensitive form in cells expressing ICP47, indicating retention in the ER (Fig. 5B, top panel). Without ICP47 (multiplicity of infection of 0), HC moved to the Golgi apparatus and acquired endoH resistance during a chase period. BiP silencing had no obvious effect on the stability of HC in
ICP47-expressing cells, although HC remained for relatively long periods in the ER (Fig. 5B). Therefore, the effects of BiP on HC stability were not observed when HC was retained in the ER but were specific to conditions in which US2 or US11 were present and caused degradation. US11, which is a relatively stable ER protein, and US2, which is less stable in cells, were not affected by BiP silencing (Fig. 5C).

Effects of BiP Silencing on Folding of Other ER Proteins and Induction of the UPRs—BiP is known to participate in the folding of numerous ER-resident proteins. VSV G protein has been routinely used to assess ER function. G protein depends on BiP for early folding events and export from the ER to the Golgi apparatus (81). The levels of glycosylated (endoH-sensitive) G protein produced in a short pulse of radiolabel in BiP-silenced cells was similar to control cells (Fig. 6), confirming that synthesis and translocation were not affected. The stability of G protein was also not altered, but approximately half of G protein remained endoH-sensitive after 60- and 120-min chases in BiP-silenced cells. Thus, as might be expected, a fraction of VSV G protein is not exported to the Golgi apparatus when BiP is reduced.

BiP acts to sense accumulation of misfolded proteins and participates in the UPR (82). UPR involves increased synthesis of CNX and CRT, chaperones that serve important roles in folding HC. Increased CNX and CRT might stabilize HC. However, BiP silencing did not significantly alter the steady-state levels of CNX (Fig. 7A) and increased CRT by only ~1.3-fold when normalized to β-actin (Fig. 7B). Experiments in which CNX and CRT were radiolabeled also showed no defects in protein expression (not shown). CNX and CRT protein levels are not the most sensitive measures of UPR, and splicing of XBP-1 occurs earlier (83). We found that DTT, a compound that provokes UPR, induced the majority (~60%) of XBP-1 to
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be spliced. By contrast, there was much less XBP-1 spliced (11.24%) in BiP-silenced cells 72 h after siRNA transfection (Fig. 7C). When we followed BiP silencing and XBP-1 splicing over the first 72 h after siRNA transfection, there was no substantial amount of XBP-1 splicing as BiP levels fell (not shown). It appeared that BiP silencing did not substantially induce UPR under our experimental conditions.

To determine whether UPR could affect US11-mediated HC degradation, cells were treated with DTT or heat to induce UPR. Very shortly after DTT treatment and under conditions in which UPR was first induced, HC synthesis was diminished, but US11-mediated degradation of HC occurred similar to that in untreated cells (Fig. 7D). Heat-treated cells expressed HC at levels more similar to untreated cells, and, again, there was no negative effect on US11-mediated HC degradation. At a time when UPR was more pronounced (3 h after DTT or heat treatment), HC expression was more similar to controls, and, again, US11-mediated degradation of HC was not affected. We concluded that UPR does not negatively impact US11-mediated HC degradation and cannot explain the stabilization of HC when BiP is silenced.

US2 and US11 Bind BiP in the Absence of MHC Proteins—To further characterize the role of BiP in ERAD, we investigated whether US2 and US11 could bind BiP in cells that did not express MHC proteins. We identified a melanoma cell line, 1858, that expressed no detectable MHC class I HC as assessed by Western blotting with mAb HC10 that recognizes most class I molecules (Fig. 8A). Moreover, no class II proteins were detected in these cells (not shown). BiP was co-precipitated with US2 and US11, but not with US3, from extracts of 1858 cells (Fig. 8B). These results demonstrate that US2 and US11 can bind to BiP without HC.

US2 is also degraded in cells, either coincident with HC degradation or in a distinct process (12, 55), but US11 is not (12, 55). One model, of how US2 and US11 function, suggests simultaneous binding of HC and BiP. Because US2-BiP complexes form in the absence HC, it was of interest to determine if US2 was degraded under these conditions. We compared the stability of US2 in U373 cells that express HC and 1858 cells that lack HC. Note that only the glycosylated, higher molecular weight species of US2 has been shown to be degraded in parallel with HC (12, 55). About 70% of the glycosylated form of US2 was degraded by 90 min in U373 cells, but much less (25%) was degraded in 1858 cells (Fig. 8C and D). We also observed that the proteasomal inhibitor epoxomicin stabilized the non-glycosylated form of US2, which has been hypothesized to be degraded in parallel with HC (12), in U373 cells but had no effect in 1858 cells (Fig. 8E). Therefore, although US2 can bind to BiP in the absence of HC, US2 degradation requires HC. Although factors other than HC could contribute to the difference in the rates of US2 decay, the most obvious conclusion is that US2 and HC are degraded simultaneously or that HC induces conformational changes in US2 to promote degradation of US2.

BiP Silencing Does Not Induce Aggregation of Class I HC—In yeast, ERAD substrates aggregate when BiP is mutated or when DnaJ-like co-chaperones are deleted, and it was proposed that BiP maintains substrates in a retrotranslocation-competent form (42, 43, 84). We tested whether BiP silencing promoted aggregation of HC in US11-expressing cells that were treated with the proteasome inhibitor epoxomicin to stabilize HC. Fig. 9A confirms that epoxomicin was functional, as evidenced by the appearance of the faster migrating, deglycosylated intermediate of HC, and Fig. 9B verifies that BiP was silenced and that this was not affected by epoxomicin. Radiolabeled cell extracts were fractionated on sucrose gradients, and HC was immunoprecipitated. Note that BiP silencing did not abolish all of the degradation of HC observed when epoxomicin was not present (not shown), and, thus, a fraction of the HC reached the cytoplasm and was present as deglycosylated form (Fig. 9C), as previously described (55). In cells transfected with a control siRNA, most HC was in the middle of the gradient (Fig. 9C). Silencing BiP increased the amount of HC slightly, even with epoxomicin present, but there was no increase in HC in the bottom fractions or in the pellet, suggesting no obvious aggregation of HC. Similar results were obtained in cells not treated with epoxomicin except that there was less HC (not shown).
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strates with downstream ERAD machinery or acts exclusively as a chaperone so that substrates are protected from ERAD machinery.

HCMV US2 and US11 have been useful molecular handles to study and characterize the ERAD machinery. This is based on observations that US2 and US11 promote similar interactions as those involved in the normal ERAD of cellular proteins. Unlike human immunodeficiency virus Vpu-triggered degradation of CD4 reconstituted in yeast (7), the existing evidence is that US2- and US11-mediated MHC protein degradation closely resembles “generic” ERAD. Consistent with this view, a number of the components of the mammalian ERAD pathway, including Sec61, Derlin-1, and p97 ATPase have been linked to ERAD by using US2 and US11 (12–15, 23, 57, 88). However, ER luminal components of the US2- and US11-mediated ERAD have not been identified. Such proteins might also participate in recognizing misfolded substrates during normal events in the ER.

Here, we demonstrated that two HCMV glycoproteins US2 and US11 bound BiP. US2 and US11 cause degradation of MHC proteins, whereas four other homologous proteins, US3, US7, US9, and US10, which do not cause MHC degradation, did not bind BiP. Of special interest was that US3, which shares most homology with US2, binds MHC proteins causing ER retention rather than degradation. A US3/US2 fusion protein that causes degradation also bound BiP. When BiP was silenced, US2- and US11-mediated degradation of HC was inhibited. BiP might bind US2 and US11 simply because these viral proteins are misfolded, whereas US3, US7, US9, and US10 are not. However, coupled with the much more extensive binding of US11 to BiP and the fact that US11 causes much more rapid degradation of HC, this seems far-fetched. Moreover, BiP silencing might be expected to reduce the stability of US2 and US11, and this was not the case. Importantly, the stabilities of HC, in the absence of US proteins, and of other ER resident proteins (G protein, CNX, and CTR) were also not affected by the loss of BiP. Thus, BiP was not obviously required to promote HC folding and maintain its stability in the absence of US11. Unlike the results from yeast, BiP did not appear to act by maintaining the “retrotranslocation-competence” (solubility) of HC in US11-expressing cells. Therefore, BiP is required for some step in the US2/US11-mediated degradation of HC, where BiP appears to act proactively in this process, rather than obviously to fold proteins.

Interpretation of these observations is made more complex because BiP is a multifunctional protein that plays numerous roles in maintaining the integrity of the ER. BiP acts to sense ER stress and triggers UPR (39). UPR could potentially up-regulate ER chaperones CNX and CRT that are required for folding of HC (8, 78). We found that CNX and CRT were not significantly increased, and there was no evidence of the earliest stages of UPR induction when BiP was silenced for up to 72 h. Evidence has been published that UPR is not required for US11-mediated HC degradation (89). More germane is that, when UPR was induced artificially, US11-induced degradation of HC was not altered. Therefore, BiP silencing does not reduce US11-mediated HC degradation by inducing UPR.

BiP also functions to gate or seal the translocon (49). With both HC and VSV G protein, we found no defects in the appear-

DISCUSSION

It is critical that proteins introduced into the ER be scrutinized and aberrant forms disposed of. Correct folding and strict quality control are a prerequisite not only for protein function but also to avoid activation of the immune system by misfolded, cell surface, or extracellular proteins. Recognizing ER proteins that require additional efforts to fold versus those that are terminally misfolded must occur, and a number of ER luminal proteins participating in this process have recently been described: EDEM, protein disulfide isomerase, and Yos9p (32–36, 85–87). However, we do not currently understand how binding of these proteins advances substrates along a pathway to degradation and, specifically, how ERAD substrates are targeted to retrotranslocation channels.

Numerous studies have implicated BiP in ERAD substrate recognition (4, 8, 39, 84). The best evidence that BiP acts proactively to promote ERAD comes from studies in yeast where certain BiP mutants exhibited reduced degradation (42, 43). These studies suggested that BiP can act to maintain substrates in a retrotranslocation-competent form and by other, uncharacterized mechanisms. In mammalian cells, beyond extensive correlation between BiP release and ERAD (44–48), it has been difficult to know whether BiP promotes association of sub-

Therefore, reducing BiP stabilizes HC without inducing aggregation.

FIGURE 9. HC does not aggregate when BiP is silenced. A, the function of epoxomicin was confirmed by infecting cells with Adtet-trans alone (Trans; 120 PFU/cell) or Adtet-US11 (100 PFU/cell) and Adtet-trans (20 PFU/cell) for 18 h and labeling the cells with [35S]Met/Cys for 10 min (P10), followed by a chase for 60 min (C60). HC was immunoprecipitated using mAb HC10. B, U373 cells were transfected with a control siRNA #1 (BiP scrambled) or BiP siRNA #1 for 48 h, then co-infected with AdtetUS11 (75 PFU/cell) and Adtet-trans (15 PFU/cell) for an additional 18 h. Infected cells were treated with 1 μM epoxomicin for 2 h, labeled with [35S]Met/Cys for 20 min, and lysed using 1% Triton X-100. A small aliquot of the extracts was used for immunoblotting BiP. C, extracts were centrifuged on 10–40% sucrose step gradients containing 0.1% Triton X-100. Fractions from gradients and pellets (P) were harvested and diluted in buffer containing DOC and SDS, and HC was immunoprecipitated using mAb HC10.
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ance of glycosylated proteins in BiP-silenced cells. This supports the notion that BiP silencing, to the extent we achieved, did not affect translation or translocation of HC into the ER. However, a fraction of VSVG protein was not exported from the ER to the Golgi apparatus in BiP-silenced cells. This likely relates to an important role for BiP in G protein folding (81), so that a fraction of G proteins is retained in the ER. Other proteins might also be misfolded when BiP is silenced, although this did not lead to instability of any of the proteins studied, including G protein, HC, US proteins, CNX, and CRT. Thus, if there is gross misfolding of ER proteins during transient reduction in BiP, this does not lead to increased protein turnover or marked pleiotropic effects in the ER. However, these results do leave open the possibility that the effects of BiP are indirect. For example, BiP might be required for folding of ER-resident proteins that are essential for ERAD. This problem is inherent in most (or all) studies of the role of ER luminal proteins in ERAD. Previous studies involving yeast BiP mutants, which concluded that BiP is essential for ERAD (9, 41, 42), and studies of EDEM involving overexpression (32, 33) similarly suffer from this caveat. Nonetheless, our studies provide the first solid evidence that BiP is functionally necessary for ERAD in mammalian cells advancing the status quo from correlations. Future studies based on silencing protocols should allow elucidation of the mechanism by which BiP promotes ERAD.

A number of observations support our working hypothesis that US2 and US11 bridge HC onto BiP and that this promotes interactions with other ERAD components. First, US2 and US11 bind BiP even in the absence of MHC proteins suggesting that these viral proteins can, themselves, interact directly with BiP. Second, US2 and US11 can bind directly to MHC proteins in vitro (54, 58). Third, it is well known that US2 and US11 link MHC proteins to components of the ERAD machinery, all cytoplasmic to date (12, 51, 57–59, 90). Fourth, US2 is normally degraded in cells expressing MHC proteins, and it has been proposed that a complex of US2-HC is recognized by ERAD machinery (12). However, we demonstrated that US2 was degraded poorly in cells lacking MHC proteins. Thus, when BiP or US2 is missing, HC is not degraded, and when HC is missing, US2 is not degraded. This implies that a trimolecular complex is formed, at least transiently. It is very difficult to demonstrate such a complex in cells by using immunoprecipitation, cross-linking, or gel filtration. Instead, the functional importance of such complexes awaits our efforts to construct mutant forms of US11 or US2 that do not bind BiP.

Based on the involvement of BiP in the US2/US11-mediated ERAD pathway, potential candidates for downstream components include the DnaJ-like proteins that collaborate with BiP in folding (39, 40, 91–93). However, arguing against their involvement are observations that DnaJ-like proteins normally recruit BiP, rather than vice versa (39, 40). Other ERAD proteins, including EDEM, Yos9p, or protein disulfide isomerase may be involved. Alternatively, BiP might be converted into a pro-ERAD factor, similar to the cytosolic Hsc70-interacting protein, which converts Hsc70 from a folding protein to a degradation factor in the ERAD of cystic fibrosis transmembrane conductance regulator (94). There is also the attractive model that BiP might bridge HC onto retrotranslocation channels. BiP binds and seals the luminal face of the Sec61 translocon (49, 50), and US2 and HC could be immunoprecipitated with Sec61 complexes (12). US2 and US11 could therefore promote interactions between HC and BiP that, in turn, associates with Sec61 retrotranslocation channels. It may be more difficult to extend this model to other ERAD substrates, because BiP may not bind simultaneously to Sec61 and ERAD substrates as the same BiP domains participate in binding Sec61 and ERAD substrates (50). However, there are multiple BiP molecules simultaneously bound onto ER proteins (95), and larger complexes containing BiP, ERAD substrates, other ERAD machinery and Sec61 may form. US11 and US2 may bind BiP in a manner that does not preclude BiP binding to Sec61. By this mechanism, the viral proteins make an “end run” around normal ERAD processes to promote ERAD in an illegitimate fashion.

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