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Forced interaction of cell surface proteins with Derlin-1 in the endoplasmic reticulum is sufficient to induce their dislocation into the cytosol for degradation

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ABSTRACT

Aberrantly folded proteins in the endoplasmic reticulum (ER) are rapidly removed into the cytosol for degradation by the proteasome via an evolutionarily conserved process termed ER-associated protein degradation (ERAD). ERAD of a subset of proteins requires Derlin-1 for dislocation into the cytosol; however, the molecular function of Derlin-1 remains unclear. Human cytomegalovirus US11 exploits Derlin-1-dependent ERAD to degrade major histocompatibility complex class I (MHC-I) molecules for immune evasion. Because US11 binds to both MHC-I molecules and Derlin-1 via its luminal and transmembrane domains (TMDs), respectively, the major role of US11 has been proposed to simply be delivery of MHC-I molecules to Derlin-1. Here, we directly tested this proposal by generating a hybrid MHC-I molecule, which contains the US11 TMD, and thus can associate with Derlin-1 in the absence of US11. Intriguingly, this MHC-I hybrid was rapidly degraded in a Derlin-1- and proteasome-dependent manner. Similarly, the vesicular stomatitis virus G protein, otherwise expressed at the cell surface, was degraded via Derlin-1-dependent ERAD when its TMD was replaced with that of US11. Thus, forced interaction of cell surface proteins with Derlin-1 is sufficient to induce their degradation via ERAD. Taken together, these results suggest that the main role of US11 is to recruit MHC-I molecules to Derlin-1, which then mediates the dislocation of MHC-I molecules into the cytosol for degradation.

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1. Introduction

During protein synthesis, membrane and secretory proteins enter the endoplasmic reticulum (ER) in an unfolded state and undergo a folding process, with the aid of various ER-resident chaperones, to reach their native states [1–3]. A considerable fraction of proteins eventually fail to fold properly because of the intrinsic inefficiency of the folding process in the ER. These unfolded proteins are often toxic to the cell and are thus rapidly removed from the ER to the cytosol for degradation by the proteasome [4,5]. This series of events, collectively referred to as endoplasmic reticulum-associated protein degradation (ERAD), employs multiple pathways with well-conserved and specific components to degrade various ERAD substrates. ERAD involves translocation of ERAD substrates across the ER membrane and therefore requires a protein-conducting channel or dislocon. Although the identity of the channel remains unknown, a variety of different proteins have been suggested as the channels of distinct ERAD pathways. Among those are transmembrane E3 ligases [5,6], and Derlin-1 [7,8].

Human cytomegalovirus (HCMV) US11 triggers ERAD of MHC-I molecules to escape an attack by cytotoxic T cells, which recognize the viral antigens presented by MHC-I molecules and kill the infected cells [9]. The US11-induced ERAD pathway of MHC-I molecules requires Derlin-1 to dislocate MHC-I molecules from the ER into the cytosol (Fig. 1A) [7,8]. US11, a type I transmembrane protein, interacts with MHC-I molecules through its ER luminal domain. Moreover, US11 binds to Derlin-1 via its transmembrane domain (TMD) [10]. A point mutation in the TMD of US11, which disrupts interaction with Derlin-1, deprives US11 of its ability to induce ERAD of MHC-I molecules [8]. Thus, it has been proposed, although never tested directly, that the main function of US11 is simply to capture MHC-I molecules and convey them to Derlin-1. Once MHC-I molecules are trapped by Derlin-1, they are dislocated from the ER to the cytosol with the aid of cytosolic proteins, such as p97 and its cofactor Ufd1/Npl4p [11]. The AAA ATPase p97 binds to Derlin-1 directly, or indirectly, through interaction with VIMP-1 [7], and provides the energy required for dislocation of the ERAD substrate. Immediately after dislocation into the cytosol, the N-linked glycan moiety of dislocated MHC-I molecules is removed by a cytosolic *N*-glycanase [12]. The N-linked glycan-free MHC-I molecules are then rapidly degraded by the proteasome [12].

Derlin-1 was initially believed to be the protein-conducting channel that directly mediates dislocation of MHC-I molecules

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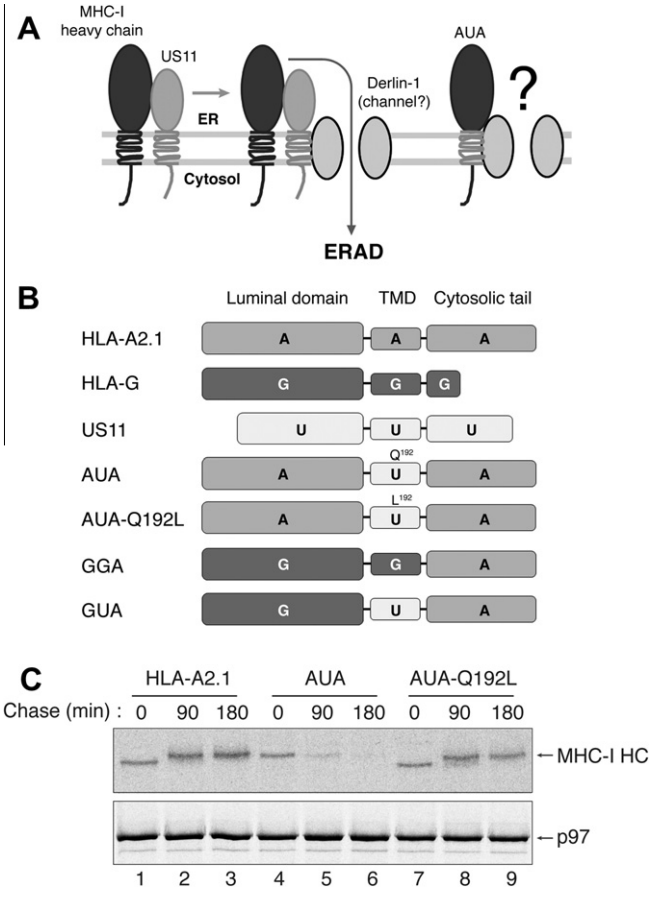

Fig. 1. Forced interaction of MHC-I molecules with Derlin-1 induces their degradation in the cytosol. (A) Working model for the US11 function in the endoplasmic reticulum-associated protein degradation (ERAD) of MHC-I molecules (B) Schematic diagram of protein constructs used in this study and (C) The HLA-A2.1 hybrid (AUA) containing the TMD of US11 is rapidly degraded through interaction with Derlin-1. HeLa cells transiently expressing HLA-A2.1, AUA, or AUA-Q192L were metabolically labeled with ^{35}S-methionine/cysteine for 10 min and chased for the indicated times. Cell lysates in 1% NP-40 were subjected to immunoprecipitation with mAb BB7.2. Immunoprecipitation for p97 (lower panel) indicates that the same amount of HeLa cell lysate was used for each sample in the experiment. MHC-I HC, MHC-I heavy chain.

through the ER membrane to the cytosol during US11-induced ERAD, but direct evidence regarding the function of this protein as a channel is lacking. Derlin-1 is a mammalian homologue of the yeast *DER1*, which is one of the first proteins identified as being involved in ERAD [13]. *DER1* deletion in yeast causes a profound defect in the degradation of secreted ERAD substrates, but not in ERAD of integral membrane proteins [14]. In contrast, Derlin-1 has been implicated in ERAD of both soluble and integral membrane proteins, including the Hong Kong (NHK) variant of α1-antitrypsin [15], ATP-sensitive potassium channels [16], and the ΔF508 mutant of cystic fibrosis transmembrane conductance regulator (CFTR) [17,18]. Derlin-1 has six TMDs with the two termini facing the cytosol, and multimerizes; thus, it may function as a homomeric channel. Alternatively, Derlin-1 may form a channel together with other components of ERAD complexes, such as the E3 ubiquitin ligase, HRD1, which has been shown to function as a protein-conducting channel for a subset of luminal and transmembrane proteins [6,19]. Although HRD1 seems to play no role in US11-induced ERAD of MHC-I molecules [20,21], it has been shown that HRD1 is required for ERAD of unfolded β_2m-free MHC-I molecules [20]. Despite the crucial role of US11 in this process, the function of US11 has not yet been clearly defined. In this study, therefore, we attempted to elucidate how US11 induces the Derlin-1-dependent ERAD of MHC-I molecules.

2. Materials and methods

2.1. Cell culture and transfection

HeLa and U373-MG cells were cultured in DMEM supplemented with 7% fetal bovine serum (Hyclone), 2 mM Glutamax-I, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were transfected with Lipofectamine-2000 (Invitrogen), and all assays were performed 24–48 h after transfection. U373-MG cells stably expressing HCMV US11 were described [22].

2.2. Plasmids

The plasmids pcDNA3.1-HLA-A2.1, pcDNA3.1-HLA-G/A-tail (GGA), pUHD10.1-US11, pUHD10.1-US11-Q192L were described [22,23] and used as template for generating chimeras. The AUA, GUA, and AUA-Q192L constructs were generated by overlapping extension PCR, and the resulting PCR products were inserted into pcDNA3.1 or pcDNA3.1-puro. The plasmid pCMV-VSV-G was a generous gift of Dr. Robert Weinberg (Whitehead Institute). The VUV and VUV-Q192L constructs were generated by overlapping extension PCR and sub-cloned into pCMV (Clontech), generating pCMV-VUV and pCMV-VUV-Q192L, respectively.

2.3. Antibodies

The monoclonal antibody (mAb) BB7.2 and the mAb HC10 were purified from the supernatant of mouse hybridoma cells. The mAbs 4H84 (Santa Cruz), G233 (Abcam), and anti-VSV-G (Santa Cruz) were purchased. Rabbit antisera against p97 and US11 were generated by immunization with synthetic peptides corresponding to the 792nd–806th amino acids of p97 and the 42nd–56th amino acids of HCMV US11, respectively. Anti-Derlin-1 rabbit serum was generated by immunization with the two synthetic peptides of Derlin-1, each covering the 239th–251st amino acids and the 221st–235th amino acids.

2.4. Pulse-chase and immunoprecipitation

Pulse-chase and immunoprecipitation were performed as previously described [22]. For sequential immunoprecipitation, transfected cells were starved in methionine/cysteine-free DMEM (Invitrogen) for 1 h and labeled with ^{35}S-methionine/cysteine (PerkinElmer) for 1 h. Then, the cells were lysed with 1% digitonin buffer (1% digitonin in 25 mM HEPES [pH 7.4], 150 mM NaCl, 1 mM PMSF, and 10 μM leupeptin). Post-centrifugation supernatants were pre-cleared with protein G sepharose (PGS, Amersham) at 4 °C for 1 h. The pre-cleared supernatants were incubated with indicated antibody at 4 °C overnight. After addition of PGS, samples were incubated for an additional 1 h. Material bound to PGS was precipitated, washed twice with 0.1% digitonin buffer, and eluted in 100 μl of Re-IP buffer (1.5% SDS and 2 mM DTT in PBS). The eluates were diluted in 900 μl of 1% NP-40 in PBS and treated with indicated antibody at 4 °C overnight. After addition of PGS, the incubation was continued for 1 h. Material bound to PGS was washed three times with 1% NP-40 in PBS and eluted by boiling for 10 min. Samples were separated by SDS–PAGE and analyzed by autoradiography. For Endo H analysis, material bound to PGS was eluted in Endo H buffer (50 mM sodium acetate, pH 5.6, 0.3% SDS, and 150 mM β-mercaptoethanol) by boiling for 10 min. Each eluate was divided into two and incubated overnight at 37 °C in the presence or absence of Endo H (5 mU, Roche).

3. Results

3.1. The MHC-I-US11 hybrid AUA is rapidly degraded via Derlin-1-dependent ERAD pathway in the absence of US11

Although the exact mechanism by which US11 triggers the ERAD of MHC-I molecules remains unclear, it has been proposed that US11 captures MHC-I molecules and simply delivers them to Derlin-1 [8], as depicted in Fig. 1A. To directly test this proposed mechanism of US11 function, we constructed a hybrid of human MHC allele HLA-A2.1, in which the TMD was replaced with the TMD of US11 (Fig. 1B), and compared its stability with that of wild-type HLA-A2.1 (Fig. 1C). HeLa cells transiently expressing wild-type HLA-A2.1 or one of the HLA-A2.1 hybrids (AUA or AUA-Q192L) were metabolically labeled with ^{35}S-methionine/cysteine for 10 min, chased for 90 or 180 min. HLA-A2.1, AUA, and AUA-Q192L were then immunoprecipitated using monoclonal antibody (mAb) BB7.2 that specifically reacts with the assembled ER luminal domain of HLA-A2.1. While the wild-type HLA-A2.1 remained stable even after a chase time of 180 min (Fig. 1C, lanes 1–3), AUA was rapidly degraded over this chase period (lanes 4–6). This rapid degradation requires physical interaction with Derlin-1, as the degradation of AUA-Q192, which contains a point mutation that would abrogate interaction with Derlin-1, was significantly delayed (Fig. 1C, lanes 7–9). The reduced half-life of AUA could be due to a failure of AUA to fold properly; thus, it would become a natural substrate of Derlin-1-dependent ERAD. This, however, is unlikely because AUA and AUA-Q192L were successfully recovered by mAb BB7.2 (Fig. 1C, lanes 4 and 7), which specifically reacts with the conformation-sensitive epitope of HLA-A2.1 [24]. US11 itself was shown to be a substrate of ERAD, but its degradation occurred independently of Derlin-1 [8], reflecting that the degradation of AUA occurs through a pathway distinct from US11 degradation.

To confirm that AUA interacts with Derlin-1 using sequential immunoprecipitation, which involves the denaturation of protein complexes, we required an antibody that specifically recognizes denatured forms of HLA-A2.1, but not other MHC-I alleles expressed in HeLa cells [25]. However, no such antibody was available. Therefore, we modified another MHC-I molecule, HLA-G, which is the non-classical MHC-I molecule specifically expressed in human placenta, and used mAb 4H84, which is specific for denatured HLA-G molecules. The MHC-I hybrid GUA, which consists of the luminal domain of HLA-G, the US11 TMD, and the cytosolic tail of HLA-A2.1, was also significantly less stable than the GGA hybrid, which contains the TMD of HLA-G instead of the US11 TMD (Fig. 2A, compare lanes 1–3 and 4–6). To confirm that GUA indeed interacts with Derlin-1 through the US11 TMD, we performed

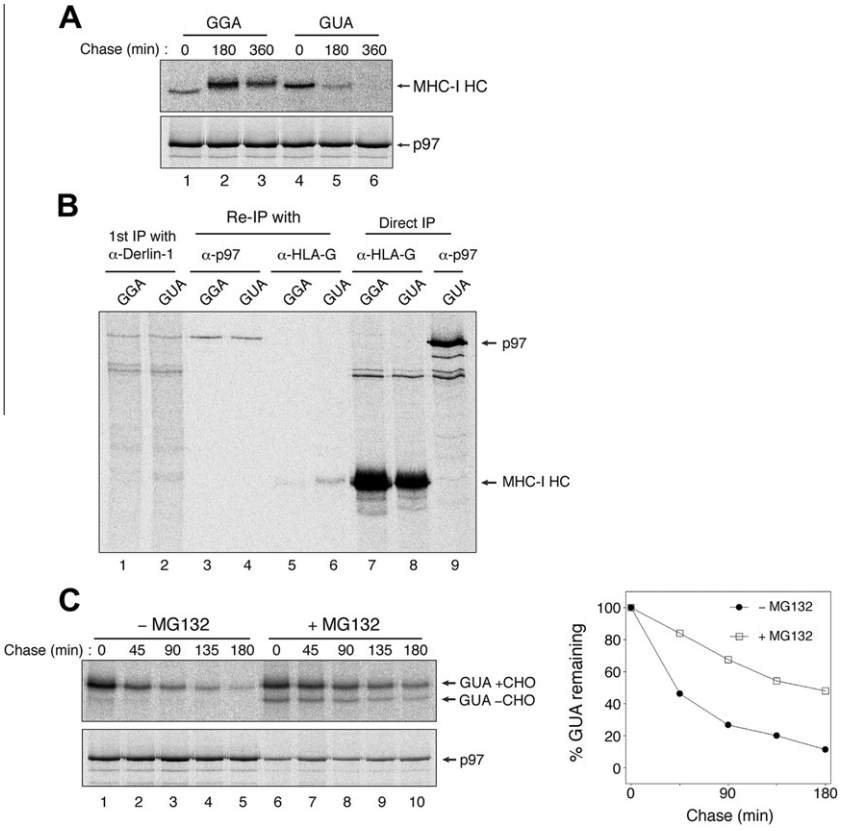

Fig. 2. Degradation of MHC-I molecules induced by forced interaction with Derlin-1 occurs in the cytosol. (A) The HLA-G hybrid (GUA), which contains the TMD of US11 and the HLA-A2.1 cytosolic tail, is degraded over the chase period while GGA remains largely unchanged. HeLa cells transiently expressing GGA or GUA were metabolically labeled for 10 min and chased for the indicated times. Cell lysates in 1% NP-40 were subjected to immunoprecipitation with mAb G233 for assembled HLA-G/β_2m or anti-p97 antibodies. (B) GUA, but not GGA, associates with Derlin-1. HeLa cells transiently expressing GGA or GUA were metabolically labeled with ^{35}S-methionine/cysteine for 1 h and subjected to lysis in 1% digitonin. Then, cell lysates were subjected to immunoprecipitation with anti-Derlin-1 antibodies, and co-precipitated material was boiled in DTT/SDS-containing buffer. After centrifugation, the resulting supernatant was diluted 10-fold in 1% NP-40 and subjected to a second round of immunoprecipitation with anti-p97 antibodies or mAb 4H84. Re-immunoprecipitation of p97 indicates that the precipitation efficiency of Derlin-1 is comparable between samples. (C) GUA is degraded by the proteasome in the cytosol. HeLa cells transiently expressing GUA were treated with either DMSO or 30 μM MG-132 for 2 h, metabolically labeled for 10 min, and chased for the indicated times in the presence of either DMSO or 30 μM MG-132. The graph shows quantification of the experiment. All experiments were performed multiple times with similar results, and the data shown are representative of all results.

sequential immunoprecipitation experiments: HeLa cells transiently expressing GGA or GUA were metabolically labeled and lysed in 1% digitonin buffer. The solubilized lysates were subjected to immunoprecipitation with anti-Derlin-1 antibody, and the precipitate was boiled in SDS-containing buffer to disrupt all protein–protein interactions. After 10-fold dilution in 1% NP-40, the sample was subjected to a second immunoprecipitation with anti-p97 antibody, or with mAb 4H84. Because it has been reported that endogenous Derlin-1 was not readily detectable by metabolic labeling and autoradiography presumably due to its slow turnover rate [8], p97, a Derlin-1-binding partner during ERAD, was used to show that precipitation efficiency was comparable between samples. While comparable amounts of p97 were co-precipitated with Derlin-1 (Fig. 2B, lanes 3 and 4), only GUA was co-precipitated with Derlin-1 (Fig. 2B, lane 6) even though the steady-state level of GUA was lower than that of GGA (Fig. 2C, compare lanes 7 and 8). Thus, these results clearly demonstrated that the degradation of GUA was induced by its physical interaction with Derlin-1 through the US11 TMD.

The US11-mediated/Derlin-1-dependent ERAD pathway involves dislocation of its target proteins into the cytosol, where they are degraded by the proteasome. To examine whether degradation of GUA also occurs through this route, we examined the degradation of GUA in the presence or absence of MG132, a specific inhibitor of proteasomes (Fig. 2C). Upon MG132 treatment, the deglycosylated form of GUA accumulated (Fig. 2C, lanes 6–10), indicating that the ER luminal domain of GUA was exposed to a cytosolic *N*-glycanase. Additionally, degradation of GUA was significantly delayed in the presence of MG-132 (Fig. 2C), indicating that GUA degradation, induced by forced interaction with Derlin-1, is also mediated by proteasomes in the cytosol. These results strongly suggest that degradation induced by forced interaction with Derlin-1 shares the pathways downstream of US11-induced ERAD.

3.2. Degradation by forced Derlin-1 interaction is not specific to MHC-I proteins

US11 specifically targets MHC-I molecules and directs them to destruction via the Derlin-1-dependent ERAD pathway; thus, it was possible that the ERAD induced by forced interaction with Derlin-1 shown here could be restricted to MHC-I molecules and their homologues. To examine whether non-MHC-I molecules could also be degraded by this manipulation, we investigated vesicular stomatitis virus glycoprotein (VSV-G), another type I transmembrane protein expressed at the cell surface, and constructed a hybrid VSV-G in which the TMD was replaced with that of US11 (VUV). To test whether VUV is degraded via the Derlin-1-dependent pathway, as shown for AUA or GUA, we compared the stability of VUV with that of the wild-type VSV-G or VUV-Q192L by pulse-chase experiments (Fig. 3A). While the level of wild-type VSV-G was largely unchanged after a chase of 180 min (Fig. 3A, lanes 1–3), the level of VUV was markedly reduced (Fig. 3A, lanes 4–6). The hybrid containing the mutant US11 TMD defective for Derlin-1 interaction (VUV-Q192L) remained stable during the chase, as did the wild-type VSV-G (Fig. 3A, lanes 7–9). Thus, ERAD induced by forced interaction with Derlin-1 was not specific to MHC-I molecules.

To confirm that the degradation of VUV was induced by physical interaction with Derlin-1, we examined whether the wild-type VSV-G, VUV, and VUV-Q192L interacted with Derlin-1, using sequential immunoprecipitation (Fig. 3B). As expected, VUV, but not VSV-G or VUV-Q192L, was co-precipitated with Derlin-1 (Fig. 3B, lane 8), demonstrating that the degradation of VUV was mediated by its physical interaction with Derlin-1.

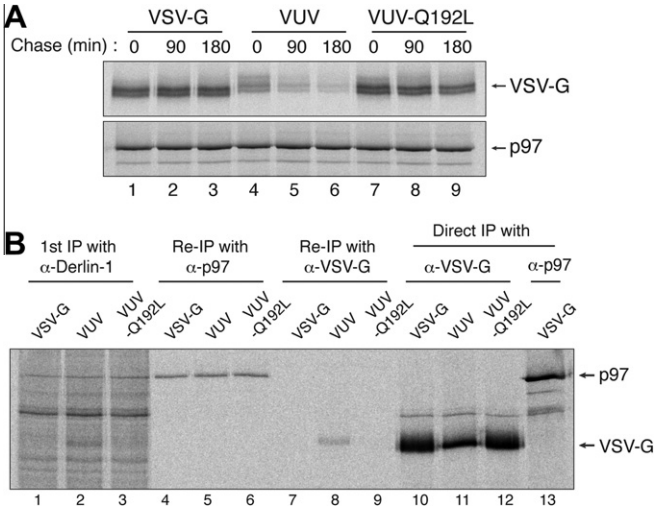

Fig. 3. Forced interaction of a viral protein with Derlin-1 is sufficient to induce the degradation of the viral protein. (A) VSV-G hybrid (VUV) that contains the TMD of US11 becomes a substrate of Derlin-1-dependent ERAD. HeLa cells transiently expressing VSV-G, VUV, or VUV-Q192L were metabolically labeled for 10 min, chased for the indicated times, and lysed in 1% NP-40. The cell lysates were then subjected to immunoprecipitation with anti-VSV-G antibodies or anti-p97 antibodies. (B) VUV, but not VSV-G or VUV-Q192L, binds to Derlin-1. HeLa cells transiently expressing VSV-G, VUV, or VUV-Q192L were metabolically for 1 h and subjected to lysis in 1% digitonin. Then the cell lysates were subjected to immunoprecipitation with anti-Derlin-1 antibodies, and co-precipitated material was boiled in DTT/SDS-containing buffer. After centrifugation, the resulting supernatant was diluted 10-fold in 1% NP-40 and subjected to a second round of immunoprecipitation with anti-p97 antibodies or anti-VSV-G antibodies. All experiments were performed multiple times with similar results, and the data shown are representative of all results.

3.3. Derlin-1 plays a key role in the dislocation of MHC-I molecules from the ER to the cytosol during US11-induced ERAD

US11-induced ERAD of MHC-I molecules involves dislocation of MHC-I molecules from the ER membrane into the cytosol, suggesting the existence of a protein-conducting channel, which may involve Derlin-1 [7,8]. If Derlin-1 itself is the protein-conducting channel, or at least a key component of this channel, it is reasonable to assume that Derlin-1 interacts its ERAD substrates throughout the dislocation process. To examine whether Derlin-1 interacts with ERAD substrates not only at the pre-dislocation stages, but also during their transit or exposure to the cytosol, we analyzed the status of N-linked glycans of MHC-I molecules that had been co-precipitated with Derlin-1 or p97 (Fig. 4). The majority of MHC-I molecules co-purified with p97 were deglycosylated, which are most likely MHC-I molecules that had been trimmed by a cytosolic *N*-glycanase (Fig. 4, lanes 5 and 6). In contrast, the majority of MHC-I molecules that had been co-precipitated with Derlin-1 retained N-linked glycans, which was indicative of the pre-dislocation status of the MHC-I luminal domain, which bears the N-linked glycan. Interestingly, however, a minor, but reproducibly detectable, fraction of Derlin-1-bound MHC-I molecules was deglycosylated, indicating that they had been exposed to the cytosol (lanes 3 and 4). Although unlikely, it is possible that ERAD substrates initially bind to Derlin-1 at the early stages of ERAD, then dissociate from Derlin-1 before dislocation, move to the cytosol through an unknown channel, and again bind to Derlin-1 after the luminal domain has been exposed to the cytosol. Another possible, but more feasible, scenario would be that Derlin-1 receives MHC-I molecules from US11, initiates and plays a key role in their dislocation across the ER membrane, and retains them until the N-linked glycan of the MHC-I luminal domain has been removed by a cytosolic *N*-glycanase.

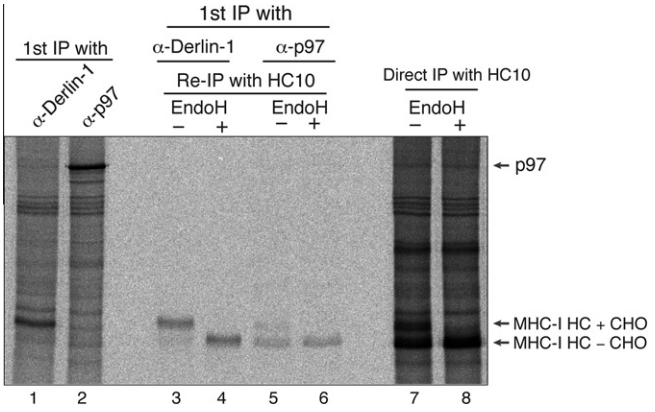

Fig. 4. Derlin-1 retains the MHC-I molecules that have been dislocated to the cytosolic face of the ER membrane. U373-MG cells stably expressing US11 were treated with 30 μM MG-132 for 2 h, metabolically labeled with ^{35}S-methionine/cysteine for 1 h, and subjected to lysis in 1% digitonin. Then, cell lysates were subjected to immunoprecipitation with anti-Derlin-1 antibodies or anti-p97 antibodies, and co-precipitated material was boiled in DTT/SDS-containing buffer. After centrifugation, the resulting supernatant was diluted 10-fold in 1% NP-40 and subjected to a second round of immunoprecipitation with mAb HC10 for denatured MHC-I heavy chains. Then, precipitates were treated with endoglycosidase H (Endo H) at 4 °C overnight. All experiments were performed multiple times with similar results, and the data shown are representative of all results.

4. Discussion

By making use of MHC-I hybrids, of which the TMDs were replaced with the US11 TMD, we demonstrated that the main function of US11 is indeed to commandeer MHC-I molecules, which would otherwise leave the ER for expression at the cell surface, to Derlin-1 for dislocation; however, we cannot completely exclude additional functions of US11. Indeed, it has been reported that US11 induces unfolded protein response (UPR), which may facilitate removal of MHC-I molecules from the ER into the cytosol [26]. This may explain why AUA is degraded with slower kinetics while MHC-I molecules are degraded with fast kinetics in US11-expressing cells [9,22]. In addition, US11 may possess some functions that are executed by HRD1, as the ERAD of β_2m-deficient misfolded MHC-I molecules, but not US11-induced ERAD, requires functional HRD1 [20].

Co-immunoprecipitation experiments (Fig. 4) with Derlin-1 revealed that, although Derlin-1 mainly interacted with the glycosylated MHC-I forms, a small fraction of the Derlin-1-bound MHC-I molecules lacked the N-linked glycan. This could imply that MHC-I molecules are bound to Derlin-1 from the initial stage of dislocation to the point when the N-linked glycan of the ER luminal domain is removed by the cytosolic *N*-glycanase. Thus, it is likely that Derlin-1 participates in the entire MHC-I dislocation process during US11-induced ERAD. Conversely, the majority of MHC-I molecules bound to p97 are deglycosylated, suggesting that p97 preferentially binds to MHC-I molecules that have been exposed to the cytosol, and extract them from Derlin-1 for degradation by the proteasome. This model is consistent with a recent finding that p97 extracts the deglycosylated forms of the NHK variant of α1-antitrypsin from Derlin-1 and release them into the cytosol for degradation [15].

In summary, forced interaction of non-ERAD substrates with Derlin-1 was sufficient to induce their dislocation into the cytosol, where they are degraded by the proteasome. Furthermore, Derlin-1 interacted with its ERAD substrates even when their ER luminal domain had been exposed to the cytosol, indicating that Derlin-1 interacts with the ERAD substrates throughout the dislocation process. To fully understand the molecular function of Derlin-1 during US11-induced ERAD of MHC-I molecules, reconstitution of this process with fully defined components will be required. Nonetheless, our current findings strongly suggest that Derlin-1 plays a key role in the dislocation process, rather than merely functioning as a bystander-regulator of dislocation or a receptor for ERAD substrates in the early stages of HCMV US11-induced ERAD of MHC-I molecules.

Acknowledgments

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