TRAM1 Participates in Human Cytomegalovirus US2- and US11-mediated Dislocation of an Endoplasmic Reticulum Membrane Glycoprotein*\(^{\text{1,3}}\)

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The human cytomegalovirus proteins US2 and US11 have co-opted endoplasmic reticulum (ER) quality control to facilitate the destruction of major histocompatibility complex class I heavy chains. The class I heavy chains are dislocated from the ER to the cytosol, where they are deglycosylated and subsequently degraded by the proteasome. We examined the role of TRAM1 (translocating chain-associated membrane protein-1) in the dislocation of class I molecules using US2- and US11-expressing cells. TRAM1 is an ER protein initially characterized for its role in processing nascent polypeptides. Co-immunoprecipitation studies demonstrated that TRAM1 can complex with the wild type US2 and US11 proteins as well as deglycosylated and polyubiquitinated class I degradation intermediates. In studies using US2- and US11-TRAM1 knockdown cells, we observed an increase in levels of class I heavy chains. Strikingly, increased levels of glycosylated heavy chains were observed in TRAM1 knockdown cells when compared with control cells in a pulse-chase experiment. In fact, US11-mediated class I dislocation was more sensitive to the lack of TRAM1 than US2. These results provide further evidence that these viral proteins may utilize distinct complexes to facilitate class I dislocation. For example, US11-mediated class I heavy chain degradation requires Derlin-1 and SEL1L, whereas signal peptide peptidase is critical for US2-induced class I destabilization. In addition, TRAM1 can complex with the dislocation factors Derlin-1 and signal peptide peptidase. Collectively, the data support a model in which TRAM1 functions as a cofactor to promote efficient US2- and US11-dependent dislocation of major histocompatibility complex class I heavy chains.

HCMV\(^{2}\) can down-regulate cell surface expression of the immunologically important molecule major histocompatibility complex I to avoid immune detection by cytotoxic T cells (1, 2). More specifically, the HCMV US2 and US11 gene products alone can target the ER-localized major histocompatibility complex class I heavy chains for extraction across the ER membrane by a process referred to as dislocation or retrograde translocation. The N-linked glycan is then removed upon exposure to the cytosol by N-glycanase (3), followed by proteasomal destruction (4, 5). The HCMV US2 and US11 proteins utilize the ER quality control process to eliminate class I heavy cells in a similar manner as misfolded or damaged ER proteins (e.g. genetic mutants of α\(_1\)-antitrypsin (6) and the cystic fibrosis transmembrane conductance regulator protein (7)) are targeted for degradation (8). Hence, analysis of US2- and US11-mediated destruction of class I heavy chains provides an excellent system to delineate viral protein function as well as the ER quality control process.

ER and cytosolic proteins are required for US2- and US11-mediated dislocation/degradation of class I heavy chains. Some of these proteins have also been identified in the processing of aberrant ER polypeptides. The ER chaperones calnexin, calreticulin, and BiP have been implicated in US2-mediated class I destruction (9) as well as in the removal of some misfolded ER proteins (10). The ubiquitination machinery also participates in the extraction of class I heavy chains as ubiquitinated heavy chains are observed prior to dislocation (11, 12). For misfolded ER degradation substrates, ubiquitin conjugation enzymes (e.g. Ubc6p and Ubc7p/Cue1p) and ubiquitin ligases Hrd1p/Der3p, Doa10p, and Ubc1p have been implicated in the dislocation reaction (8). Interestingly, the ER membrane protein Derlin-1 along with SEL1L are involved in US11-mediated class I heavy chain degradation (13–15), whereas SPP is critical for US2-induced class I destabilization (16). The ubiquitinated substrates are dissociated by the AAA-ATPase complex composed of p97-Ufd1p-Npl4p (17) while docked to the ER through its interaction with VIMp (14) followed by proteasome destruction. The inhibition of the proteasome causes the accumulation of deglycosylated class I heavy chain intermediate in US2 and US11 cells, allowing the dislocation and degradation reactions to be studied as separate processes (4, 5).

Despite the identification of some cellular proteins that assist US2- and US11-mediated class I dislocation, the dislocation pore and accessory factors that mediate the efficient extraction of class I through the bilayer have yet to be completely defined. The current study explores the role of TRAM1 (translocating chain-associated membrane protein-1) in US2- and US11-mediated class I dislocation. TRAM1 is an ER-resident multispanning membrane protein that can mediate the lateral movement of select signal peptides and transmembrane segments from the
translocon into the membrane bilayer (18), a property that makes it uniquely qualified to participate in the dislocation of a membrane protein. TRAM1 has been cross-linked to signal peptides as well as transmembrane domains of nascent polypeptides during the early stages of protein processing (19–25). Interestingly, unlike the Sec61 complex and the signal recognition particle receptor, TRAM1 is not essential for the translocation of all membrane proteins into the ER (20, 21). Hence, TRAM1 may utilize its ability to engage hydrophobic domains to assist in the efficient dislocation of membrane proteins. In fact, association and TRAM1 knockdown studies demonstrate that TRAM1 participates in US2- and US11-mediated dislocation of class I heavy chains. Collectively, our data suggest for the first time that TRAM1 plays a role in the dislocation of a membrane glycoprotein.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—Human U373-MG astrocytoma cells and U373 cells that stably express HCMV US2, HCMV US11, and TRAM1HA were maintained in DMEM as described (26). TRAM1HA-expressing cells were generated by a retrovirus transduction protocol (26). Rabbit polyclonal anti-US11 and anti-protein disulfide isomerase were gifts from H. Ploegh (MIT). Rabbit polyclonal anti-US2 and anti-class I heavy chain antibodies were raised against the luminal domain of US2 (amino acids 15–140) or class I heavy chain (HLA-A2 allele, amino acids 25–366), respectively. The monoclonal antibodies W6/32 (27), HC10 (28), anti-hemagglutinin (HA) epitope tag (12CA5 (29)), anti-calnexin antibody (AF8 (30), a gift from M. Brenner (Harvard Medical School)), and anti-GAPDH (Chemicon) were utilized. The rabbit polyclonal anti-TRAM1 serum was raised against peptides corresponding to the TRAM1 amino acid sequence (amino acids 278–291 (RAENQKLDFST-

P-40 lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 20 mM MgCl2, 0.5% (v/v) Nonidet P-40), followed by incubation with the respective antibody and protein A-agarose beads (Repligen, Waltham, MA). Polypeptides were resolved using SDS-PAGE and subjected to immunoblot analysis.

Metabolic Labeling of Cells and Pulse-chase Analysis—Cells were subjected to pulse-chase analysis as previously described (31). The precipitates were resolved using SDS-PAGE (12.5%). The radioactive signal in the polyacrylamide gel was enhanced using Autofluor (National Diagnostics). The polyacrylamide gel was dried and exposed to autoradiography film for 2 days at −80 °C.

Subcellular Fractionation—Cells were resuspended in homogenization buffer (100 mM Tris, 150 mM NaCl, 250 mM sucrose, 1.5 μg/ml aprotinin, 1 μM leupeptin, and 2 μM phenylmethylsulfonyl fluoride) and mechanically homogenized using a 12-μm ball bearing homogenizer (Isobiotec, Heidelberg, Germany) with 15 passes. Unbroken cells and other debris were pelleted at 15,000 × g for 10 min at 4 °C, and the supernatants were centrifuged at 100,000 × g for 1 h at 4 °C. For immunoprecipitation studies, the 100,000 × g pellets were resuspended in Nonidet P-40 lysis buffer, and the respective polypeptides were immunoprecipitated as described above.

Generation of TRAM1 Small Hairpin RNAs (shRNA) Vectors—Oligonucleotides to TRAM1 cDNA (Table 1) were cloned into the RNai-Ready pSIREN-RetroQ vector (Clontech). These vectors were used to generate pseudotyped retrovirus used to transduce U373, US2, and US11 cells.

Quantitative Real Time PCR Analysis—Total RNA was isolated from cells using the Stratagene Absolutely RNA reverse transcription-PCR miniprep kit. Quantitative real-time PCR was performed as previously described at the Mount Sinai School of Medicine Quantitative PCR Shared Research Facility (32). In short, each sample was assayed in triplicate, and the fold-change values and control changes for each gene were calculated using the median threshold cycle. Primers for housekeeping genes were used for normalization (rps11, tubulin, and β-actin), and the copy number was determined using 2500 as an empirical estimate of the number of β-actin mRNA molecules/cell (33, 34).

RESULTS

TRAM1HA Is Stably Expressed in U373 Cells—The ability of TRAM1 to mediate the lateral movement of transmembrane domains between aqueous and hydrophobic environments suggests that it may participate in the US2- and US11-induced extraction of class I heavy chains from the membrane bilayer to the cytosol. To address this hypothesis, we generated a TRAM1 construct that contained the hemagglutinin epitope tag (AYPDYDVPDYA) at its amino terminus (TRAM1HA) that allows for the efficient recovery and detection of the TRAM1 protein. U373 (control), US2, and US11 cell lines that stably express TRAM1HA (U373TRAM1HA, US2TRAM1HA, and US11TRAM1HA) were generated by retrovirus transduction. TRAM1HA was recovered exclusively from U373TRAM1HA, US2TRAM1HA, and US11TRAM1HA cells (Fig. 1A, lanes 1, 2, 4, and 6) and not from U373, US2, and US11 cells (Fig. 1A, lanes 1, 3, and 5). These results confirm the expression of TRAM1 and the specific recovery of the polypeptide.

The localization of TRAM1HA to the ER was examined by its acquisition of an N-linked glycan. TRAM1HA molecules recovered from U373TRAM1HA, US2TRAM1HA, and US11TRAM1HA cells were subjected to endoglycosidase H treatment followed by immunoblot analysis (Fig. 1B). Endoglycosidase H is a bacterial enzyme that cleaves high mannose containing N-linked glycans from ER-resident polypeptides generating a faster
tides were detected by an anti-HA immunoblot. The glycosylated tides were observed from U373TRAM1-HA, US2TRAM1-HA, and US11TRAM1-HA cells lysed either in Nonidet P-40 or CHAPS. Unlike Nonidet P-40, CHAPS is a mild detergent that can preserve transient interactions among membrane proteins. The anti-HA precipitates and total cell lysates were subjected to immunoblotting analysis. TRAM1HA molecules were recovered under all lysis conditions (data not shown). Both US2 (Fig. 2A, lanes 2 and 5) and US11 (Fig. 2A, lanes 3 and 6) co-precipitated with TRAM1HA independent of lysis conditions. Together, these findings suggest a complex exists between viral proteins and TRAM1HA.

To confirm the specificity of the wild type US2 and US11 interaction with TRAM1, we examined the association of TRAM1HA with US2 (US2/CD4) (26) and US11 (US11Q192L) (37) mutants that do not target class I for destruction. TRAM1HA was recovered from US2/CD4TRAM1-HA and US11Q192L-TRAM1-HA cells lysed in either CHAPS or Nonidet P-40 buffer mix and subjected to immunoblotting analysis. As expected, TRAM1HA polypeptides were recovered from US2/CD4TRAM1-HA (Fig. 2B, lanes 1–4) and US11Q192L-TRAM1-HA cells (Fig. 2C, lanes 1–4) independent of lysis conditions. The anti-US2 or anti-US11 immunoblots revealed that the viral molecules co-precipitated with TRAM1HA only from cells lysed in CHAPS lysis buffer (Fig. 2, B and C, lane 10). These results demonstrate that only a transient interaction exists between the mutant US2 and US11 molecules with TRAM1HA. In contrast, wild type US2 and US11 continue to complex with TRAM1HA in both Nonidet P-40 and CHAPS lysis buffer. The specific interaction of US2 and US11 with TRAM1 implies that the viral gene products recruit TRAM1 to participate in class I dislocation.

Deglycosylated Class I Heavy Chain Intermediates Associate with TRAM1—An interaction between TRAM1 and class I polypeptides would strongly support the involvement of TRAM1 in class I heavy chain dislocation. Class I molecules can exist as glycosylated ER-resident species or deglycosylated intermediates in proteasome inhibitor-treated US2 and US11 cells (38). The observation of a deglycosylated class I molecule is indicative of the dislocation of the class I luminal domain into the cytosol. Therefore, experimental conditions were established to stabilize significant amounts of both glycosylated and deglycosylated class I heavy chains. US2 cells treated with different concentrations of proteasome inhibitor ZL3VS for up to 16 h were subjected to immunoblot analysis (Fig. 3A). In cells treated with ZL3VS, both glycosylated (HC(+CHO)) and deglycosylated heavy chain intermediates (HC(−CHO)) were observed (Fig. 3A, lanes 2–6), a result consistent with published data (11, 39). We observed decreased levels of class I molecules in cells treated with inhibitor for 16 h (Fig. 3A, lane 6), possibly due to the potential toxic effects of proteasome inhibition. Similar results were observed from US11 cells.3

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migrating species (35). Faster migrating TRAM1HA polypeptides were observed from U373TRAM1-HA, US2TRAM1-HA, and US11TRAM1-HA cells upon treatment with endoglycosidase H (Fig. 1B, lanes 2, 4, and 6) compared with nontreated samples (Fig. 1B, lanes 1, 3, and 5). The difference in the relative molecular weight of the two species (∼3–4 kDa) confirms that TRAM1HA acquires a single N-linked glycan, a result consistent with published data (36). In addition, TRAM1HA continues to interact with the Sec61β subunit of the translocon under mild lysis conditions (Fig. S1). These results demonstrate that the chimeric TRAM1HA molecule acts similarly to its endogenous counterpart and can be utilized to identify possible protein complexes between TRAM1 and viral gene products, as well as class I heavy chains.

TRAM1HA Complexes with Wild Type HCMV US2 and US11—The association of HCMV US2 and US11 with cellular factors SPP and Derlin-1 was an important finding implicating these cellular proteins in the dislocation/degradation of class I heavy chains (13, 16). Following this line of experiments, our initial studies examined whether TRAM1HA can complex with the viral proteins US2 and US11 (Fig. 2A) to propose a role for TRAM1 in the destruction of class I molecules. TRAM1HA was recovered from U373TRAM1-HA, US2TRAM1-HA, and US11TRAM1-HA cells lyzed in either Nonidet P-40 or CHAPS. Unlike Nonidet P-40, CHAPS is a mild detergent that can preserve transient interactions among membrane proteins. The anti-HA precipitates and total cell lysates were subjected to immunoblotting analysis. TRAM1HA molecules were recovered under all lysis conditions (data not shown). Both US2 (Fig. 2A, lanes 2 and 5) and US11 (Fig. 2A, lanes 3 and 6) co-precipitated with TRAM1HA independent of lysis conditions. Together, these findings suggest a complex exists between viral proteins and TRAM1HA.

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ditions of proteasome inhibition (ZL3VS, 2.5 μM for 14 h; Figs. 3 and 4 and Fig. S3) that fulfilled our criteria for levels of glycosylated and deglycosylated heavy chains and were not detrimental to cellular metabolism (Fig. S2). Hence, subsequent proteasome inhibitor experiments utilized these conditions.

To directly examine whether class I molecules complex with TRAM1HA, we performed an immunoprecipitation/immunoblot experiment using US2TRAM1-HA and US11TRAM1-HA cells treated with or without proteasome inhibitor (Fig. 3, B and C) lysed in Nonidet P-40 lysis buffer to exclude transient interactions. Total cell lysates were used as a reference for the migration pattern of TRAM1HA and glycosylated and deglycosylated class I heavy chains (Fig. 3, B lanes 5–7 and 12–14) and C (lanes 5–8 and 13–16). Similar amounts of TRAM1HA polypeptides were recovered from US2TRAM1-HA and US11TRAM1-HA cells (Fig. 3, B and C, lanes 1–4), independent of ZL3VS treatment. As expected, glycosylated and deglycosylated class I heavy chains accumulated in proteasome inhibitor treated US2TRAM1-HA (Fig. 3B, lane 14) and US11TRAM1-HA (Fig. 3C, lane 16) cells. Strikingly, deglycosylated class I intermediate exclusively co-purified with TRAM1HA from ZL3VS-treated US2TRAM1-HA (Fig. 3B, lane 11) and US11TRAM1-HA (Fig. 3C, lane 12) cells and not the glycosylated heavy chain from US3TRAM1-HA and US2TRAM1-HA (Fig. 3B, lanes 8–11) or US11TRAM1-HA (Fig. 3C, lanes 9–11) cells. The specific interaction of TRAM1 with the class I deglycosylated intermediate suggests that TRAM1 probably participates in both US2- and US11-mediated class I dislocation.

To further verify the complex between TRAM1 and the heavy chain intermediate, we showed that endogenous TRAM1 recovered from proteasome inhibitor-treated US2 and US11 cells associated with...
with the ER membrane, we performed an association experiment using ER-containing microsomes. Subcellular fractionation conditions were initially established using U373 and U373\textsuperscript{TRAM1-HA} cells treated with or without proteasome inhibitor. The homogenized cells were subjected to differential centrifugation (see “Experimental Procedures”), in which the nuclear material and cell debris were removed at 15,000 \( \times g \), whereas the ER-containing microsomes were collected in the 100,000 \( \times g \) pellet. The total cell lysates, 100,000 \( \times g \) pellet, 100,000 \( \times g \) supernatant, and anti-HA precipitates from the 100,000 \( \times g \) pellet were subjected to immunoblot analysis. The membrane proteins calnexin and class I heavy chains were localized to the 100,000 \( \times g \) pellet (Fig. 4A, lanes 4–6 and 16–18), whereas the cytosolic GFP molecule used to select for transductants fractionated exclusively with the 100,000 \( \times g \) supernatant (Fig. 4A, lanes 35 and 36). The specificity of the anti-HA immunoprecipitates was verified due to the lack of membrane or cytosolic proteins co-precipitating with TRAM\textsubscript{1-HA} (Fig. 4A, lanes 7–9, 19–21, and 31–33). These data demonstrate that the subcellular fractionation protocol allows for the exclusion of cytosolic material from the ER membrane fraction as well as the specificity of the anti-HA precipitations.

To directly address whether TRAM1 complexes with membrane-associated deglycosylated class I heavy chains, proteasome inhibitor-treated US2\textsubscript{TRAM1-HA} and US11\textsubscript{TRAM1-HA} cells were subjected to subcellular fractionation, and TRAM\textsubscript{1-HA} was recovered from the 100,000 \( \times g \) pellet. The precipitates, total cell lysates, and 100,000 \( \times g \) pellet were subjected to immunoblot analysis. Despite the presence of both the glycosylated and deglycosylated heavy chains in the total cell lysates and 100,000 \( \times g \) pellet of US2 and US11 cells (Fig. 4B, lanes 13–16 and 19–22), only deglycosylated intermediate co-precipitated with TRAM\textsubscript{1-HA} (Fig. 4B, lanes 18 and 24). These findings demonstrate that TRAM1 interacts with membrane-associated heavy chain intermediate, suggesting that it may play a direct role in the dislocation process.

**TRAM\textsubscript{1-HA} Complexes with Membrane-associated Ubiquitinated Species**—To examine whether TRAM1 associates with ubiquitinated dislocation intermediates, we performed an association experiment from membrane fractions. U373, U373\textsuperscript{TRAM1-HA} US2\textsuperscript{TRAM1-HA} and US11\textsuperscript{TRAM1-HA} cells treated with or without proteasome inhibitor were subjected to subcellular fractionation. The 100,000 \( \times g \) pellet and the anti-HA immunoprecipitates from the 100,000 \( \times g \) pellet were subjected to immunoblot analysis (Fig. 5). Polyubiquitinated species co-precipitated with TRAM\textsubscript{1-HA} from proteasome inhibitor-treated US2\textsuperscript{TRAM1-HA} and US11\textsuperscript{TRAM1-HA} cells. These polyubiquitinated species are probably derived from class I heavy chains or viral gene products (Fig. 5, lanes 5 and 7). These results suggest that TRAM1 associates with a subset of ER degradation substrates. As expected, the membrane proteins calnexin (Fig. 5, lanes 22–28) and TRAM\textsubscript{1-HA} (Fig. 5, lanes 36–42) were found in the 100,000 \( \times g \) pellet, whereas the cytosolic GFP molecule, used to select for transductants, fractionated exclusively with the 100,000 \( \times g \) supernatant\textsuperscript{2}. The specificity of the anti-HA immunoprecipitates was verified due to the lack of calnexin co-precipitating with TRAM\textsubscript{1-HA} (Fig. 5, lanes 15–21). These findings suggest that TRAM1 is a cellular
factor of the dislocation apparatus that interacts with dislocation intermediates prior to their complete extraction from the membrane bilayer.

Knockdown of TRAM1 Attenuates US2- and US11-mediated Class I Degradation—To substantiate the role of TRAM1 in class I dislocation, we examined class I protein levels in US2 and US11 cells knocked down for TRAM1. To that end, TRAM1 expression was attenuated using shRNAs to three distinct regions of TRAM1 (shTRAM1-R1, shTRAM1-R2, and shTRAM1-R3; Table 1). U373, US2, and US11 cells that stably express shTRAM1 constructs were analyzed using quantitative real time PCR (Fig. 6A) and immunoblot analysis (Fig. 6B). Cells transduced with shRNA to GFP (shGFP) were used as a control. The shTRAM1-R2-expressing (U373shTRAM-R2) cells were the most efficient at knocking down TRAM1 mRNA (~80%) (Fig. 6A and B, lanes 1–4). Equivalent levels of class I heavy chains (Fig. 5B, lanes 5–8) and GAPDH (Fig. 6B, lanes 9–12) polypeptides confirmed equal loading of cell lysates. Similar immunoblot results in US2 and US11 TRAM1 knockdown cells were observed. The results demonstrate that TRAM1 expression can be attenuated without being detrimental to cell viability.

To corroborate that TRAM1 is involved in the dislocation of class I heavy chains in US2 and US11 cells, a pulse-chase experiment was performed to examine levels of deglycosylated intermediate from US2shTRAM1-R2 and US11shTRAM1-R2 cells. Cells that stably express shRNA against GFP were used as a control. U373shGFP, U373shTRAM-R2, US2shGFP, US2shTRAM1-R2, US11shGFP, and US11shTRAM1-R2 cells treated with proteasome inhibitor were metabolically labeled with [35S]methionine for 15 min and chased up to 40 min (Fig. 7). As expected, the class I heavy chains from U373 cells

**FIGURE 4. TRAM1 complexes with a membrane bound class I intermediate.** U373 and U373TRAM-HA cells (A) and US2TRAM-HA and US11TRAM-HA cells (B) treated with or without proteasome inhibitor ZL3VS (2.5 μM, 14 h) were subjected to subcellular fractionation (see “Experimental Procedures”). The total cell lysates, 100,000 × g pellet, 100,000 × g supernatant, and anti-HA immunoprecipitates (IP) from the 100,000 × g pellet were subjected to anti-calnexin (A and B, lanes 1–12), anti-heavy chain (A and B, lanes 13–24), anti-GFP (A, lanes 25–36), and anti-HA (A, lanes 37–48; B, lanes 25–36) immunoblots. Calnexin, class I heavy chains (HC), HC(+CHO), and HC(−CHO), TRAM1-HA, immunoglobulin light chain (IgG LC), and molecular weight standards are indicated.
were stable throughout the chase independent of TRAM1 expression (Fig. 7, A (lanes 1–6) and B). In US2shTRAM1-R2 and US11shTRAM1-R2 cells, the accumulation of class I deglycosylated intermediate was delayed during the chase when compared with US2shGFP and US11shGFP cells (Fig. 7, A (lanes 7–18) and C and D (gray bars)). In addition, increased amounts of glycosylated heavy chains (Fig. 7, A and C and D (black bars)) were recovered during the chase from shTRAM1-R2-expressing cells. Equivalent levels of total class I were observed throughout the chase of TRAM1 knockdown cells. These findings demonstrate that the dislocation reaction was impaired in TRAM1 knockdown cells.

Strikingly, class I dislocation was more severely delayed in US11 TRAM1 knockdown cells than in US2 cells. Considering that US2 utilizes SPP (16), whereas US11 requires Derlin-1 and SEL1L (13, 15) to induce class I destruction, it is possible that US11-mediated class I dislocation is more dependent on TRAM1 than US2. These findings further suggest that the viral gene products rely on unique cellular proteins to effectively extract class I from the ER membrane. The ER degradation substrates may be extracted by different dislocins composed of distinct accessory factors that promote the efficient removal of degradation substrates. Alternatively, TRAM1 may alter the processing of a dislocation factor that is more specific for US11 than US2. Nevertheless, both of these paradigms support the notion that different cellular complexes mediated US2- and US11-induced class I dislocation.

Analysis of steady levels of class I from US2shTRAM1-R2 and US11shTRAM1-R2 cells treated with increasing concentrations of proteasome inhibitor revealed the significant accumulation of glycosylated class I molecules when compared with US2shGFP and US11shGFP cells (Fig. S5). The delayed accumulation of deglycosylated intermediate in US2 and US11 TRAM1 knockdown cells further suggests that TRAM1 may function as an efficiency factor for the dislocation of class I proteins. Collectively, the data imply that the lack of TRAM1 impedes US2- and US11-mediated class I dislocation/degradation.

**Tram1HA Associates with the Components of the Extraction Apparatus**—The dislocation of class I in US2 and US11 cells is mediated through the interaction with both ER and cytosolic proteins (40). Derlin-1 is essential for the dislocation of US11-mediated class I degradation (13, 14), whereas SPP is involved in US2-mediated class I destruction (16). To examine if Derlin-1 or signal peptide peptidase complexes with TRAM1, Derlin-1 (Fig. 8A) or SPP (Fig. 8B) was recovered from U373, U373TRAM1-HA, US2TRAM1-HA, and US11TRAM1-HA cells and subjected to immunoblot analysis. TRAM1HA molecules co-precipitated with Derlin-1 and SPP from all cells that expressed the chimeric molecules (Fig. 8A and B, lanes 2–4). Collectively, the interaction of TRAM1HA with components of the extraction apparatus further implies that TRAM1 probably plays

![Table 1](image1)

**Table 1. Oligonucleotides to generate shRNAs against TRAM1 and GFP**

| Protein | Nucleotides | cDNA target sequence | Hairpin sequence |
|---------|-------------|----------------------|-----------------|
| TRAM1-R1 | 196–215 | GCTACTGAAATACGAGTG | GATCCGCTAGCTGAAATACGAGTGCTTCTCCATAGAGAAAGGACACTGTAGTT |
| TRAM1-R2 | 571–590 | GAAGATATTCCTCGTCAGC | GATCCGAAGATATTCCTCGTCAGCTTCAAGAGAGCTGACGAGGA |
| TRAM1-R3 | 782–801 | GACTTCTGACTTTAATTCT | GATCCGACTTCTGACTTTAATTCTTTCAAGAGAAGAATTAAAGTCA |
| GFP (13) | 122–141 | GCAAGCTGACCCTGAAAGT | GATCCGCAAGCTGACCCTGAAAGTTTCAAGAGAAACTGCACTGATC |

**Figure 5. TRAM1 complexes with ubiquitinated species.** U373, U373TRAM1-HA, US2TRAM1-HA, and US11TRAM1-HA cells treated with or without proteasome inhibitor ZL3VS (2.5 μM, 14 h) were subjected to subcellular fractionation. The 100,000 × g pellet (membrane lysates) and anti-HA immunoprecipitates from the 100,000 × g pellet were subjected to anti-ubiquitin (lanes 1–14), anti-calnexin (lanes 15–28), and anti-HA (lanes 29–42) immunoblots. Ubiquitinated species, calnexin, TRAM1HA, and molecular weight standards are indicated.
a role in the dislocation of class I across the hydrophobic bilayer.

**DISCUSSION**

HCMV US2 and US11 efficiently promote the proteasome destruction of major histocompatibility complex class I heavy chains. The extraction of class I heavy chains across the ER presents a challenging task for the ER quality control machinery. It requires the transport of a Type I membrane protein across the hydrophobic bilayer of the ER. The cellular proteins that can possibly facilitate transfer of hydrophobic domains between the membrane bilayer and an aqueous pore are usually coupled with the translocation of nascent ER polypeptides (41). Consistent with this premise, TRAM1, a component of the translocon described as a “molecular chaperone” (18), can interact with both the translocation (Fig. S1) and dislocation machinery (Fig. 8), allowing it to play a dual role in processing of nascent polypeptides as well as to participate in the dislocation of ER proteins.

The knockdown of TRAM1 in US2 and US11 cells attenuated dislocation of glycosylated heavy chains, suggesting that TRAM1 may function as an efficiency cofactor for the rapid extraction of class I proteins from the ER (Fig. 7 and Fig. S5). Consistent with these data, TRAM1 complexes strongly with the wild type US2 and US11 proteins (Fig. 2), demonstrating that TRAM1 is probably recruited by the viral gene products to promote rapid dislocation of class I. Our findings are consistent with the hypothesis that US2 and US11 class I dislocation is dependent on a different set of cellular proteins (40). An alternative hypothesis is that the lack of TRAM1 alters the processing of an unidentified dislocation factor and thereby attenuates class I dislocation. Although this possibility could not be excluded, the association of TRAM1 with degradation intermediates (Figs. 3–5 and Fig. S3) strongly implies that it has a direct role in the dislocation reaction. Another possible explanation is that multiple dislocation apparatuses may mediate dislocation of class I. Hence, the lack of TRAM1 may overburden the other dislocon(s) during accumulation of degradation substrates and impede class I dislocation. Multiple dislocation apparatuses would ensure the efficient disposal of misfolded ER proteins. Both membrane and soluble misfolded ER proteins targeted for destruction make use of various degradation pathways and diverse ubiquitin-ligase complexes (42, 43). Therefore, it is possible that the cell utilizes multiple complexes to mediate efficient dislocation of proteins across the ER membrane.
TRAM1 most likely functions with the dislocon in a tightly linked process to enhance the dislocation of class I molecules. Consistent with this hypothesis is that TRAM1 complexes with the possible dislocon candidates Derlin-1 (Fig. 8) and the Sec61 complex (Fig. S1) (5, 13, 44). In addition, TRAM1 was up-regulated during an unfolded protein response and found to complex with p97 only under mild lysis conditions.4 We propose that TRAM1 may engage the dislocon to directly assist in the dislocation of class I or act as a scaffolding protein that stabilizes the extraction machinery for class I extraction. For disposal of misfolded ER proteins, yeast studies have postulated that Sec61p, Der1p, and Doa10 could be potential dislocon pores (45–48). Nevertheless, the yeast protein that would substitute for TRAM1 in the dislocation reaction has not yet been identified. The yeast TRAM1 homologue Lag1p participates in the ER-Golgi transport of glycosylphosphatidylinositol-proteins (49). Whether Lag1p is involved in dislocation remains to be seen. Indeed, it is most likely that the dislocon in both yeast and mammalian cells would be composed of proteins with multiple transmembrane domains and accessory factors that promote the robust dislocation of the degradation substrate.

Our study presents data that support a paradigm in which the multimembrane-spanning protein TRAM1 participates in the dislocation of ER class I molecules.

4 C. L. Ng and D. Tortorella, unpublished observations.
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US2- and US11-mediated class I dislocation as a likely component of the dislocon. The exact role of TRAM1 in dislocation of general misfolded ER proteins and the identity of protein complexes both upstream and downstream of TRAM1, such as ubiquitin ligases, requires further investigation. Even more intriguing is how TRAM1 may influence class I levels during an HCMV infection. Would the lack of TRAM1 or other cellular proteins involved in US2- and US11-mediated class I destruction allow rescue of class I molecules in HCMV-infected cells?

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