Adapter-mediated Substrate Selection for Endoplasmic Reticulum-associated Degradation*

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During endoplasmic reticulum (ER)-associated degradation (ERAD), a relatively small number of ubiquitin ligases (E3) must be capable of ubiquitinating an assortment of substrates diverse in both structure and location (ER lumen, membrane, and/or cytosol). Therefore, mechanisms that operate independently of primary sequence determinants must exist to ensure specificity during this process. Here we provide direct evidence for adapter-mediated substrate recruitment for a virus-encoded ERAD E3 ligase, mK3. Members of an ER membrane protein complex that normally functions during major histocompatibility complex class I biogenesis in the immune system are required for mK3 substrate selection. We demonstrate that heterologous substrates could be ubiquitinated by mK3 if they were recruited by these ER accessory molecules to the proper position relative to the ligase domain of mK3. This mechanism of substrate recruitment by adapter proteins may explain the ability of some E3 ligases, including cellular ERAD E3 ligases, to specifically target the ubiquitination of multiple substrates that are unrelated in sequence.

Ubiquitin-regulated pathways intersect with virtually all aspects of cell biology. This is certainly true of protein quality control pathways, including those that operate to degrade proteins from the ER lumen and membrane. This essential pathway, known as ER-associated degradation (ERAD), prevents the toxic accumulation of misfolded proteins through the regulated degradation of target substrates. Initiation of ERAD involves substrate recognition leading to ubiquitination mediated by ubiquitin ligases (E3). Multiple cellular E3 ligases have been identified that associate with the ER membrane, including Hrd1, Doa10 (known as TEB4 in mammals), and gp78 (1, 2). These ligases are known to ubiquitinate a multitude of diverse substrates. However, the mechanisms by which substrates are selected remain poorly understood. Although evidence exists for direct binding of some substrates to E3 ligases (3, 4), cofactor molecules in the ER lumen, membrane, and cytosol appear to provide an essential substrate recruitment function (1, 2, 5–9). Indeed, it is now appreciated that E3 ligases in the ER membrane associate with a complex set of accessory molecules that, collectively, facilitate ERAD. The complexity of these systems confounds the characterization of substrate selection, but the fact that ERAD has been implicated in numerous diseases (10) magnifies the importance of attaining a fuller understanding of substrate recruitment/selection.

Members of the RING finger domain-containing E3 ligase family are known to play a critical role in ERAD (1, 2). In general, RING E3 ligases have been divided into two broad classes, single- and multi-subunit (11). Single-subunit E3 ligases possess discrete domains that mediate substrate binding and ubiquitin-conjugating enzyme recruitment. In contrast, multi-subunit E3 ligases are dependent upon a complex of protein subunits that act together to mediate substrate binding and ubiquitin conjugation. The mK3 protein, encoded by the murine γ-herpesvirus 68, is a member of a family of E3 ligases found in several γ-herpesviruses and poxviruses, as well as in eukaryotes. These molecules are membrane-anchored and possess a cytosol-facing RING domain of the RING-CH subtype (12, 13). Like many of its viral homologs, mK3 is a presumed single-subunit E3 ligase. MK3 is employed by the virus to interfere with the host immune response by inhibiting the major histocompatibility complex (MHC) class I antigen presentation pathway (14). In the presence of mK3, which localizes to the ER membrane, nascent class I heavy chains (HC) are ubiquitinated, leading to their rapid degradation in a proteasome-dependent fashion (15). This ubiquitination is known to require a cytosolic tail on the class I HC (15, 16). Furthermore, class I HC that are incapable of associating with the class I peptide-loading complex in the ER, consisting of TAP-1/2, tapasin, and additional accessory proteins (17), are resistant to mK3-mediated ubiquitination (18). Interestingly, the stable expression and function of mK3 require TAP-1, TAP-2, and tapasin. In fact, mK3 associates with this complex even in the absence of the class I HC (18, 19). Furthermore, only the class I HC (and not TAP-1, TAP-2, or tapasin) is detectably ubiquitinated and rapidly degraded in the presence of mK3 (20). Thus, it was initially assumed that mK3 would bind directly to a unique determinant on the peptide-loading complex-associated class I HC; this would be consistent with a single-subunit E3 ligase.

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plex for stable expression, led to an alternative model to explain the specificity of mK3 for MHC class I HC; the association of mK3 with TAP-1/2 and tapasin positions its RING-CH domain such that only the tail of class I HC but not the cytosolic domains of other proteins within the peptide-loading complex can be ubiquitinated (21).

In this study, we show that a protein unrelated to class I HC in sequence can, upon association with the peptide-loading complex, be ubiquitinated by mK3. In addition, changing its relative position within the peptide-loading complex can alter the fate of this novel substrate. These data provide direct evidence to support an “adapter-mediated” model of substrate recruitment in which the peptide-loading complex functions as an “adapter complex” for mK3 and serves to recruit the substrate (class I HC) and orient the RING-CH domain of mK3. Additionally, our results highlight the distinction between substrate recruitment by adapters and specific requirements of the E3 ligase for ubiquitination of the substrates. Indeed, the features of class I HC ubiquitination by mK3 (a cytosolic tail with either lysine, serine, threonine, or cysteine residues (22)), held true for our unrelated chimeric substrate. The relationship of mK3 with viral and cellular E3 ligases, including Doa10/TEB4 (23), suggests that studies of substrate recruitment/selection by this E3 ligase can provide insights that can inform our understanding of this process by other E3 ligases.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Retroviral Vector Transduction—**Experiments were performed using the 3KO murine embryo fibroblast line (β2m−/−, class I 3K−/−, and class I D−/−) (18) and a tapasin-deficient murine embryo fibroblast line (Tp−/− and H-2b; 24) into which various constructs were introduced. 293T cells (25) were used for the production of ecotropic retrovirus vectors following transient transfection. All cells were maintained in complete RPMI 1640 medium (Mediatech, Manassas, VA) supplemented with 10% fetal calf serum (HyClone, Logan, UT), 1 mM HEPES (Invitrogen), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 100 units/ml penicillin/streptomycin (all from Mediatech). Cells were cultured for 24 h in the presence of 100 units of mouse γ-interferon/ml to increase transcription of the peptide-loading complex protein genes. Retrovirus-containing supernatants were generated using the Vpack vector system (Stratagene, La Jolla, CA) for transient production in 293T cells to generate ecotropic virus for infection. Packaging cells were transfected using FuGENE 6 (Roche Diagnostics) according to the supplier’s instructions. Virus-containing supernatants were collected 48–72 h post-transfection and added to target cells in the presence of hexadimethrine bromide (Polybrene) (Sigma) at a concentration of 8 μg/ml. Stable transductants were produced by selection with the appropriate antibiotics, at the following concentrations: puromycin, 2.5 μg/ml; Zeocin, 0.2 mg/ml; hygromycin, 0.2 mg/ml; geneticin, 0.5 mg/ml.

**DNA Constructs—**The vectors used for expression of each cDNA were all murine stem cell virus-derived nonreplicating retroviral vectors. Each vector is bicistronic with the gene of interest upstream of an internal ribosome entry site element that precedes the antibiotic resistance marker. pMIP and pMIB are derivatives of pMSCV-IRES-GFP (pMIG (26)) in which the green fluorescent protein cDNA was replaced with the puromycin and bleomycin (Zeocin) resistance genes, respectively. pMIN and pMIH have been described (16) and encode neomycin and hygromycin resistance, respectively. All cDNAs used in this study were expressed from one of these vectors. The cDNA clones for soluble human β2m, mouse B7.2 (CD86), MHC class I Ld heavy chain, and mouse tapasin were all obtained by reverse transcription-PCR using human- and mouse-derived cells as RNA sources, respectively. Generation of the Ld T134K mutant and the mK3 cDNA have been described (19, 28). Transmembrane versions of human β2m all contain the entire open reading frame of hβ2m, including the N-terminal signal peptide fused in frame with various C-terminal domains. Tβ2m.B7.B7 consists of hβ2m fused to residues 208–284 of murine B7.2 (TM and cytosolic domains). Tβ2m.Tp.Tp consists of hβ2m fused to residues 381–442 of murine tapasin (TM and cytosolic domains). Tβ2m.B7.Tp consists of hβ2m fused to residues 208–239 of B7.2 (TM domain) and residues 418–442 of tapasin (cytosolic tail). These chimeric constructs were generated by overlap-extension (fusion) PCR with the respective templates. Truncations of cytosolic domains were generated by PCR in which the reverse oligonucleotide incorporated a stop codon at the desired location. The class I Ld tail-deletion mutant (HC-ΔCyt) retained only six residues (KRRRN). The Tβ2m.B7.B7 tail-deletion mutant (Tβ2m.B7.ΔCyt) retained only five residues (CHRRP); the two arginine residues are lysines in the native B7.2 sequence). All site-directed mutagenesis was performed using the QuickChange mutagenesis kit (Stratagene) according to the manufacturer’s instructions. A sequence comparison of the cytosolic tail sequences present in the various constructs is given in Figs. 1 and 6. The correct sequence for all of the constructs was confirmed by DNA sequence analysis.

**Antibodies—**BBM1 recognizes native and denatured hβ2m (29). Anti-ubiquitin monoclonal antibody (mAb) P4D1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). mAb 64-3-7 recognizes open forms (unassembled) of the Ld class I HC or epitope-tagged class I heavy chains; mAb 30-5-7 is specific for fully conform (β2m-associated) class I Ld; 5D3 is a hamster anti-mouse tapasin mAb; all have been described previously (18). Rabbit antiserum against C-terminal sequences of mK3 (residues 167–187), and mouse TAP-1 have been described (18). mAb ACTN05 (C4) against actin was purchased from Abcam (Cambridge, MA). Mouse anti-CD86 (B7.2 clone GL1) was purchased from eBioscience (San Diego).

**Flow Cytometry—**All flow cytometric analyses were performed using a FACSCalibur (BD Biosciences), and data were analyzed using CellQuest software (BD Biosciences). Cells were stained on ice with the appropriate dilution of the indicated unconjugated primary antibodies. Phycoerythrin-conjugated goat anti-mouse IgG (Pharmingen) was used to visualize the staining levels of the primary antibodies. For low pH treatment of cells to denature class I molecules, cells were suspended in 0.5% glycine buffer, pH 2.8, for 3 min at 37 °C. Excess complete culture medium was added to neutralize the samples, which were then treated as above for flow cytometric analysis.
Immunoprecipitation—For co-immunoprecipitation, cells were lysed in phosphate-buffered saline (PBS) containing 1% digitonin (Wako, Richmond, VA), 20 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride (both from Sigma), and Protease Inhibitor Cocktail III (Calbiochem). Separately, protein A-Sepharose beads (GE Healthcare) were incubated for 4 h at 4 °C with saturating amounts of antibody. Excess antibody was removed from beads by washing three times with 0.1% digitonin/PBS buffer. Post-nuclear lysates were incubated with immobilized antibody overnight at 4 °C. Beads were washed four times with 0.1% digitonin/PBS buffer, and proteins were eluted by boiling in nonreducing lithium dodecyl sulfate sample buffer (Invitrogen). Sequential immunoprecipitations were performed as described above with the exception of the elution step following the primary immunoprecipitation, which was performed by boiling in 0.5% SDS in 10 mM Tris-Cl, pH 6.8.
ERAD Substrate Recruitment by Adapter Proteins

A

| Molecules present | Staining |
|-------------------|----------|
|                   | ![Graphs](beta_m.png) |

β2m

Folded Class I HC

Untreated | Low pH 3 min

B

| mK3 | Endo H |
|-----|--------|
| -   | -      |
| +   | +      |

IP: HC

Blot: Ub

-60

Blot: HC

-40
Samples were diluted with 1% IGEPAL CA-630 (Nonidet P-40) (from Sigma) in PBS for a final concentration of 0.1% SDS. Supernatants were incubated with the second protein A-immobilized antibody overnight at 4 °C. Beads were washed four times with 0.1% IGEPAL CA-630 in PBS, and proteins were eluted by boiling in nonreducing lithium dodecyl sulfate sample buffer. Direct denaturing immunoprecipitations were performed essentially as described above, except that IGEPAL CA-630 was substituted for digitonin. Cells were lysed in buffer containing 1% IGEPAL in PBS and protease inhibitors. Post-nuclear lysates were denatured by the addition of 0.5% SDS and then boiled for 5 min. Samples were diluted with 1% IGEPAL CA-630 buffer for a final concentration of 0.1% SDS prior to incubation with immobilized antibody. Beads were washed four times with 0.1% IGEPAL/PBS, and proteins were eluted by boiling in nonreducing lithium dodecyl sulfate sample buffer. For endoglycosidase-H (endo-H) treatment of precipitates, post-immunoprecipitation samples were eluted by boiling in 10 mM Tris-Cl, pH 6.8, 0.5% SDS. Eluates were mixed with an equal volume of 100 mM sodium acetate, pH 5.4, and either digested (or mock-digested) at 37 °C for >1 h with 1 milliunit of endoglycosidase H (New England Biolabs, Ipswich, MA) that was reconstituted in 50 mM sodium acetate, pH 5.4.

**SDS-PAGE and Immunoblotting**—For immunoblot of cell lysates, cells were lysed in 1% IGEPAL/PBS. Post-nuclear lysates were mixed with lithium dodecyl sulfate sample buffer and 2-mercaptoethanol (1% final concentration). Protein content was determined using the BCA protein assay from Thermo Scientific (Rockford, IL). Samples were electrophoresed on NuPAGE SDS-polyacrylamide gels (Invitrogen). Separated proteins were then transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked for 1 h with either 5% dried milk, 0.1% Tween 20 (Sigma), 0.1% SDS in PBS, or 0.1% Tween 20, 0.1% SDS. The latter formulation (no milk) was used for the ubiquitin blots. After washing three times with 0.1% Tween 20, 0.1% SDS in PBS, membranes were incubated with the appropriate dilution of primary antibody for >1 h, washed three times, incubated with appropriate biotin-conjugated secondary antibodies for 1 h, followed by incubation with streptavidin-conjugated horseradish peroxidase (Zymed Laboratories Inc.) for 1 h. Membranes were incubated with SuperSignal West Femto chemiluminescent substrate (Thermo Scientific) and visualized using x-ray film or a Chem-Doc imager (Bio-Rad).

**Pulse-Chase Metabolic Labeling**—Cells (at 10⁷ cells/ml) were preincubated for 20 min in Cys- and Met-free medium (Sigma) with 5% dialyzed fetal calf serum and labeled with Express [³⁵S]Cys/Met labeling mix (PerkinElmer Life Sciences) at 200 μCi/ml for 10 min. Chase was initiated by the addition of medium containing excess unlabeled Cys/Met (5 mM each), and samples were taken at the appropriate chase time points.

Where indicated (Fig. 5), the proteasome inhibitor MG132 (Calbiochem) was added during the chase period at a concentration of 50 μM. Immunoprecipitations were performed from lysates of labeled cells as described above. Samples were subjected to SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride membranes. Membranes were fixed with methanol for 10 min, dried, and then exposed to BioMax-MR film (Eastman Kodak Co.).

**Statistical Analysis**—Data are representative of three independent experiments and are presented as the means ± S.E. Statistical analysis was performed using a two-tailed Student’s *t* test at a significance of *p* < 0.05.

**RESULTS**

**Experimental Model**—MK3 represents a unique system with which to study substrate selection by E3 ligases during ERAD, due in part to its defined set of interacting proteins and its restricted pool of available substrates (Fig. 1A). Furthermore, the degradation pathway downstream from mK3 has clear similarities to ERAD induced by cellular E3 ligases because many of the same molecules are involved (30). The mK3 protein functions to inhibit MHC class I antigen presentation during virus infection (31). Class I molecules are comprised of a type I transmembrane protein, the HC, and a soluble light chain (β₂m), which assemble in the ER lumen along with a peptide antigen. Once assembled with peptides, class I molecules traffic to the cell surface where the peptides are displayed for sampling by CD8+ T cells, which can respond if they detect the presence of “foreign” peptides (virus-derived, for example) bound to the class I HC. Peptide acquisition by class I HC is aided by the peptide-loading complex, which includes the polytopic transmembrane peptide transporter TAP (a heterodimer of TAP-1 and TAP-2) and tapasin (among other molecules; see Fig. 1A). The type I transmembrane protein tapasin stabilizes the TAP-1/2 heterodimer and bridges TAP-1/2 with class I (17). Importantly, the functional interaction between class I HC and mK3 leading to ubiquitination occurs in the context of the peptide-loading complex in the ER membrane (18).

Available data indicate that the specificity of mK3 for the class I HC, and resistance of β₂m, tapasin, or TAP-1/2 to ubiquitination, is due to the relative position of each of these molecules within the peptide-loading complex (see Fig. 1A). A prediction of this model is that any protein that can associate with TAP/tapasin in a similar orientation as class I HC will be a target for mK3-mediated ubiquitination, so long as the new potential substrate possesses a cytosolic domain of at least 13 residues, including a ubiquitin-acceptor residue (16, 22). The fact that entry of the class I HC into the peptide-loading complex during biogenesis involves complex interactions with tapasin presented a difficulty for directly testing our model. Indeed, class I HC with a point mutation that prevents tapasin binding

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**FIGURE 2.** Tβ₂m, B7. B7 is membrane-anchored and supports class I expression and mK3 function. A, 3KO fibroblasts (β₂m−/−, class I Kβ−/−, and class I Dβ+−/−) were stably transduced with class I HC (Lα), hβ₂m, and Tβ₂,m.B7.B7, as indicated. Cells were then stained for surface expression of hβ₂m (top row) or fully confined complex of class I HC (bottom row), with or without low pH treatment to denature surface class I molecules. Samples were then analyzed by flow cytometry to quantify the expression levels. In each histogram, background staining (secondary antibody alone) is indicated in dark gray; staining of untreated cells is indicated in light gray shaded peaks, and staining of low pH-treated cells is indicated in black. B, class I HC were denatured and immunoprecipitated (IP) from 3KO cells (+ class I HC and hβ₂m, or + class I HC and Tβ₂,m.B7.B7) ± mK3 using mAb 64-3-7. Precipitates were then treated with endoglycosidase H (Endo H), separated by SDS-PAGE, and blotted for ubiquitin (Ub) and class I HC.
(T134K (32, 33)) is resistant to mK3-dependent ubiquitination (18) (Fig. 1D). To circumvent this problem, we turned to β2m, the soluble light chain of class I molecules, reasoning that because it pairs with the class I HC, it might be in a favorable position for mK3-dependent ubiquitination, if it was engineered to contain transmembrane (TM) and cytosolic domains.

We reported previously that B7.2 does not associate with the peptide-loading complex and is not degraded by mK3 (21); this latter finding is confirmed here (Fig. 1E). However, earlier studies showed that the cytosolic tail of B7.2 could substitute for the endogenous class I HC tail and support ubiquitination by mK3, consistent with the strict requirement that mK3 substrates associate with the peptide-loading complex (21). We therefore generated a chimeric molecule consisting of full-length human β2m appended to the TM and cytosolic domains of mouse B7.2. Human β2m was used because it is capable of pairing with mouse class I HC, and it can be distinguished from any endogenous mouse β2m with a specific antibody (34, 35). The resulting transmembrane β2m chimeric molecule is denoted as Tβ2m.B7.B7 and is depicted in Fig. 1, B and C. The tripartite name indicates (in this case) the ectodomain (β2m), TM domain (B7.2), and cytosolic domain (B7.2).

**Tβ2m.B7.B7 Supports Class I and mK3 Function**—A functional transmembrane version of β2m was described previously in which the TM and cytosolic domains were derived from CD3ζ (36). Therefore, we expected that the Tβ2m.B7.B7 molecule would also perform the normal function of β2m and support mK3-mediated down-regulation of MHC class I HC. To determine whether this was the case, we expressed Tβ2m.B7.B7 in a murine embryo fibroblast cell line (designated 3KO) derived from mice deficient in MHC class I HC and β2m (18). In these cells, we could control the expression of MHC class I HC and β2m following stable retroviral transduction with vectors encoding various combinations of Tβ2m.B7.B7, class I HC (mouse H2-Ld in this case), and mK3. We also expressed normal (soluble) human β2m (denoted as hβ2m) in combination with class I HC and mK3 for comparative purposes. To test the structure and function of Tβ2m.B7.B7, the surface expression of both Tβ2m.B7.B7 and class I HC was evaluated under normal conditions and following treatment with low pH for 3 min, which causes MHC class I molecules to denature at the cell surface (37). In untreated cells, we found that the surface levels of fully conformed MHC class I (as detected with an antibody that recognizes β2m-associated class I HC) was comparable when class I HC is paired with hβ2m or Tβ2m.B7.B7 (Fig. 2A). Furthermore, when cells were treated with low pH, we observed that the surface level of conformed MHC class I molecules decreased, indicating that the treatment caused dissociation of the class I HC and β2m, as expected. Consistent with this result, low pH treatment resulted in a loss of surface hβ2m (following dissociation from the class I HC) but not of Tβ2m.B7.B7 because it is membrane-anchored. Importantly, mK3 ubiquitinated class I HC when paired with either version of β2m (hβ2m or Tβ2m.B7.B7; Fig. 2B). Taken together, these data reveal that Tβ2m.B7.B7 is analogous to hβ2m with regard to association with the class I HC and supporting mK3-mediated ubiquitination of class I HC.

**Tβ2m.B7.B7 Is Ubiquitinated in the Presence of mK3**—We initially examined the steady-state levels of TAP-1, tapasin, class I HC, and Tβ2m.B7.B7 (or hβ2m) in the presence or absence of mK3 (Fig. 3A). As anticipated, the steady-state levels of TAP-1 and tapasin were unaffected by mK3. It should be noted that, in some cell lines, mK3 expression can reduce the steady-state levels of TAP-1 and tapasin; however, this is not accompanied by detectable ubiquitination or rapid turnover (20) and was not evident in the cells used here. By contrast, the
steady-state levels of class I HC, Tβ2m.B7.B7, and hβ2m were markedly decreased in the presence of mK3. The loss of class I HC correlates with its ubiquitination by mK3 (see Fig. 2B); the loss of Tβ2m.B7.B7 and hβ2m could be due to direct ubiquitination or due to the loss of the class I HC leading to an indirect effect on either version of the light chain. To determine whether the loss of light chain is due to direct targeting of light chain by mK3 or is merely due to the loss of its binding partner (class I HC), we assessed ubiquitination of light chains in the presence of mK3. Immunoprecipitation and immunoblot of the total cellular pool of Tβ2m.B7.B7, but not soluble hβ2m, revealed a low basal level of ubiquitination that was independent of mK3 expression (data not shown). It should be noted that we often observe similar, low level ubiquitination of MHC class I heavy chains in normal cells lacking any viral ubiquitin ligases (see Fig. 5A, for example). This ubiquitination is curious, but it does not appear to influence the turnover of class I HC or Tβ2m.B7.B7, because both proteins are quite stable at the surface of cells lacking mK3. This is likely due to ubiquitination at the cell surface, because in the case of class I HC it occurs on endoglycosidase H-resistant glycoforms.

To clearly reveal any potential effects of mK3, we performed sequential immunoprecipitation to isolate TAP-associated light chains. This technique provides an additional advantage in that the secondary precipitation (for Tβ2m.B7.B7 or hβ2m) follows a denaturation step, thereby removing any associated proteins that may carry ubiquitin. Under these conditions, we found that Tβ2m.B7.B7 is not ubiquitinated in the absence of mK3. However, unlike hβ2m, TAP-bound Tβ2m.B7.B7 is ubiquitinated in cells that express mK3 (Fig. 3B). A ladder of ubiquitinated light chains in the presence of mK3 is also observed (Fig. 4A).
ubiquitin bands was observed with $T\beta_m$-$B7.B7$, which is characteristic of poly-ubiquitination and similar to what occurs with class I HC. This provides the first indication that mK3 is able to directly target the $T\beta_m$-$B7.B7$ molecule for ubiquitination, but not $h\beta_m$ which lacks the TM and cytosolic domains. In turn, these results demonstrate that a heterologous protein with no primary sequence relationship to class I HC can be ubiquitinated by mK3, if it is recruited into the peptide-loading complex, consistent with the adapter-mediated substrate selection model.

**Ubiquitination of $T\beta_m$-$B7.B7$ Leads to Rapid, Proteasome-dependent Degradation**—We next asked whether ubiquitination of $T\beta_2m.B7.B7$ leads to rapid degradation of $T\beta_2m.B7.B7$ in the presence of mK3. Pulse-chase metabolic labeling (with $^{35}$S) and immunoprecipitation experiments were performed, analyzing the turnover of class I HC and $T\beta_m$-$B7.B7$ in cells ± mK3. Data presented in Fig. 4A show that in the presence of mK3, $T\beta_m$-$B7.B7$ is degraded with kinetics that are similar to those observed for class I HC. In cells that express $h\beta_m$, there is little loss of its signal over time in the presence of mK3, even though class I HC is degraded in these same cells (Fig. 4B). This result, coupled with the lack of detectable ubiquitination (Fig. 3B), indicates that $h\beta_m$ is not a direct target of mK3 and that the steady-state decrease of $h\beta_m$ induced by mK3 (seen in Fig. 3A) is an indirect effect on $h\beta_m$ resulting from the loss of class I HC. Additionally, we determined whether the rapid degradation of $T\beta_m$-$B7.B7$ that we observed is proteasome-dependent. In cells treated with the proteasome inhibitor, MG132, during labeling and chase, increased levels of labeled $T\beta_m$-$B7.B7$ were recovered during chase as compared with untreated cells, and the extent of recovery was comparable with class I HC from these same cells (Fig. 4C). Collectively, these results reveal that $T\beta_m$-$B7.B7$ is ubiquitinated in the presence of mK3 and degraded by proteasomes with kinetics akin to those seen with class I HC.

**Features of $T\beta_m$-$B7.B7$ That Confer mK3 Sensitivity**—The findings presented above demonstrate that class I HC and $T\beta_m$-$B7.B7$ behave similarly, in terms of the downstream effects resulting from a functional interaction with mK3. In addition to entry of each molecule into the peptide-loading complex, does mK3 have similar structural and sequence requirements of each substrate? For class I HC, ubiquitination (and all downstream consequences thereof) requires a cytosolic tail that contains ubiquitin acceptor residues. In the case of mK3, lysine, serine, and threonine residues can be ubiquitinated on the class I HC (22); a similar finding (“non-lysine” ubiquitination) was reported for an mK3-related protein encoded by Kaposi sarcoma herpesvirus (38). To address this issue of sequence requirements for mK3-mediated ubiquitination of $T\beta_m$-$B7.B7$, we generated mutant forms of this chimeric molecule. First, we generated a truncated version of $T\beta_m$-$B7.B7$ ($T\beta_m$-$B7.B7$-$\Delta$Cyt), which contains only the first five amino acids of the cytosolic tail (CHRRP). $T\beta_m$-$B7.B7$-$\Delta$Cyt functionally mimicked $T\beta_m$-$B7.B7$ with respect to supporting class I HC expression; it ushered class I HC into the peptide-loading complex, supported high levels of surface expression in the 3KO cells, and permitted ubiquitination of class I HC (Fig. 5A, right panel and data not shown). However, $T\beta_m$-$B7.B7$-$\Delta$Cyt itself was resistant to ubiquitination and rapid degradation in the presence of mK3, suggesting that the cytosolic tail of $T\beta_m$-$B7.B7$ is the site of ubiquitination (Fig. 5, A, left panel, and B). This is analogous to class I HC lacking its cytosolic domain (HC-$\Delta$Cyt), which is also resistant to ubiquitination and rapid degradation (15, 18) (Fig. 5, C, right panel, and D). Note that the steady-state level of class I HC-$\Delta$Cyt drops in the presence of mK3, but this is not due to rapid degradation. Instead, the lack of light chain ($T\beta_m$-$B7.B7$) in these cells has an indirect effect on class I stability over time, similar to cells lacking any light chains (39).

To test the specific sequence requirements for ubiquitination of the cytosolic tail of $T\beta_m$-$B7.B7$, we generated and expressed $T\beta_m$-$B7.B7$-$K$-less, in which all five of the lysine residues in the cytosolic tail were mutated to arginine. When expressed in cells along with mK3, we observed that TAP-associated $T\beta_m$-$B7.B7$-$K$-less was ubiquitinated and rapidly degraded (Fig. 5E and data not shown). Thus, as is the case for class I HC, ubiquitination of $T\beta_m$-$B7.B7$ by mK3 is lysine-independent. It should be noted that the low level of mK3-independent ubiquitination of $T\beta_m$-$B7.B7$ mentioned previously was not seen with $T\beta_m$-$B7.B7$-$K$-less, indicating that this post-ER ubiquitination event is lysine-dependent, contrary to mK3-mediated ubiquitination (data not shown). To ascertain whether ubiquitination by mK3 of $T\beta_m$-$B7.B7$-$K$-less was occurring on serine or threonine residues, the cytosolic serine and threonine residues (seven total residues) were mutated to produce $T\beta_m$-$B7.B7$-$K$ST-less (see sequence in Fig. 1C). In the presence of mK3, this molecule was not detectably ubiquitinated (Fig. 5E), demonstrating that mK3 can, as determined for class I HC, ubiquiti-
nate non-lysine residues (serine or threonine) on $\text{T}^\beta_m$.B7.B7 (22). Overall, our results reveal that mK3-mediated ubiquitination and degradation of $\text{T}^\beta_m$.B7.B7 is analogous to the degradation of class I HC, yet $\text{T}^\beta_m$.B7.B7 bears no sequence similarity to class I HC. This supports the adapter model, where recruitment and “presentation” of the substrate (i.e., class I HC or $\text{T}^\beta_m$.B7.B7) to the E3 ligase are key determinants for ligase-mediated ubiquitination. Interestingly, there are additional substrate requirements imposed by the ligase itself; namely, the E3 ligase has requirements for specific ubiquitin-acceptor residues. For mK3, substrates must possess a cytosolic tail containing lysine, serine, or threonine residues.

Relative Position of $\text{T}^\beta_m$.B7.B7 in the Peptide-loading Complex Determines Its Susceptibility to mK3-dependent Ubiquitin Conjugation—The most straightforward interpretation of our results is that the precise location of molecules within the peptide-loading complex determines the accessibility of their cytosolic domains to the RING domain of mK3. Such a model then predicts that the reason tapasin, for example, is not detectably ubiquitinated or rapidly degraded by mK3 is its relative location. We tested this prediction by replacing the cytosolic tail of $\text{T}^\beta_m$.B7.B7 with the tail from mouse tapasin to create $\text{T}^\beta_m$.B7.Tpn. Fig. 6A shows a sequence comparison between these two cytosolic domains; no obvious sequence similarity is apparent. Nonetheless, when expressed in 3KO cells with mK3, TAP-associated $\text{T}^\beta_m$.B7.Tpn molecules are ubiquitinated (Fig. 6B, left panel). Thus, the cytosolic domain of tapasin is fully capable of supporting robust ubiquitination by mK3. This finding is consistent with the idea that tapasin is normally resistant to ubiquitination by mK3 because of its relative position within the peptide-loading complex.

Next, we tested this hypothesis more directly by creating an additional chimera designed to occupy the position in the peptide-loading complex normally assumed by tapasin. We generated transmembrane $\beta_m$ with the TM and cytosolic domains of mouse tapasin ($\text{T}^\beta_m$.Tpn.Tpn; see Fig. 6A), with the intention that this molecule should bind to TAP as though it were tapasin. This is based on the fact that the TM domain of tapasin is known to be sufficient for TAP association (40–42). Would $\text{T}^\beta_m$.Tpn.Tpn be resistant to mK3-dependent ubiquitination because of its different location within the peptide-loading complex? We first expressed $\text{T}^\beta_m$.Tpn.Tpn in 3KO cells ± mK3. Sequential immunoprecipitation of TAP-associated $\text{T}^\beta_m$.Tpn.Tpn molecules and immunoblot revealed the clear presence of a poly-ubiquitin ladder (Fig. 6B, right panel), a finding contrary to our prediction. However, in these cells that contain endogenous tapasin, the manner in which $\text{T}^\beta_m$.Tpn.Tpn associates with the peptide-loading complex is not clear; it could bind in place of tapasin and/or it could be paired with class I HC performing its normal “$\beta_m$ function.”

To remove this confounding issue of how $\text{T}^\beta_m$.Tpn.Tpn associates with the peptide-loading complex, we repeated these experiments in tapasin-deficient cells (fibroblasts derived from tapasin knock-out mice (24)). In this case, $\text{T}^\beta_m$.Tpn.Tpn can only associate with TAP as a “tapasin mimic” and not as “$\beta_m$” (Fig. 6C), because class I molecules do not enter into the peptide-loading complex in the absence of tapasin (43). We began by confirming that $\text{T}^\beta_m$.Tpn.Tpn could associate with TAP-1/2 in these tapasin-deficient cells. This was assessed by screening for an increase in TAP-1 protein levels caused by any stabilizing effects of $\text{T}^\beta_m$.Tpn.Tpn (as compared with tapasin). Expression of the $\text{T}^\beta_m$.Tpn.Tpn molecule, but not $\text{T}^\beta_m$.B7.Tpn (which is incapable of binding directly to TAP), led to an increase in the steady-state level of TAP-1 (Fig. 6D). This increase in TAP-1 levels caused by $\text{T}^\beta_m$.Tpn.Tpn was comparable with that obtained with full-length tapasin, and it indicates that $\text{T}^\beta_m$.Tpn.Tpn binds to TAP in a similar manner to tapasin (and not as $\beta_m$). TAP stabilization by $\text{T}^\beta_m$.Tpn.Tpn also led to an increase in mK3 levels, indicating that mK3 binds to the residual peptide-loading complex in these cells (Fig. 6D, lower panel).

Next, we asked whether TAP-bound $\text{T}^\beta_m$.Tpn.Tpn is ubiquitinated by mK3. Sequential immunoprecipitation of TAP-associated molecules from cells expressing $\text{T}^\beta_m$.Tpn.Tpn or $\text{T}^\beta_m$.B7.Tpn revealed that only $\text{T}^\beta_m$.Tpn.Tpn could be co-precipitated along with TAP-1/2 (Fig. 6E, left panel). This result was expected, because $\text{T}^\beta_m$.Tpn.Tpn can bind directly to TAP-1/2, whereas $\text{T}^\beta_m$.B7.Tpn is incapable of associating with TAP-1/2 in the absence of endogenous tapasin (which recruits the class I HC/$\beta_m$ dimers into the peptide-loading complex). Surprisingly, immunoblot revealed some ubiquitinated forms of $\text{T}^\beta_m$.Tpn.Tpn in the absence of mK3 (Fig. 6E, left panel). This same molecule ($\text{T}^\beta_m$.Tpn.Tpn) in 3KO cells did not show this level of basal ubiquitination in the absence of mK3 (Fig. 6B). Thus, $\text{T}^\beta_m$.Tpn.Tpn bound directly to TAP-1/2 is constitutively ubiquitinated by an unknown but mK3-independent mechanism. However, it is important to note that mK3 expression does not increase the levels of ubiquitination of TAP-associated $\text{T}^\beta_m$.Tpn.Tpn (Fig. 6E, left panel). This suggests that when $\text{T}^\beta_m$.Tpn.Tpn is bound to TAP-1/2 in the place of tapasin, it cannot be accessed by the RING-CH domain of mK3. By contrast, in tapasin-sufficient 3KO cells where $\text{T}^\beta_m$.Tpn.Tpn can potentially bind to the peptide-loading complex in association with the class I HC, it can be ubiquitinated by mK3. Indeed, when we restored tapasin expression in the tapasin-deficient cells, $\text{T}^\beta_m$.Tpn.Tpn now showed higher levels of ubiquitination in the presence of mK3 (Fig. 6E, right panel). This is likely due to tapasin recruiting class I HC/$\text{T}^\beta_m$.Tpn.Tpn dimers into the peptide-loading complex. Tapasin reconstitution also restored the association of $\text{T}^\beta_m$.B7.Tpn with the peptide-loading complex and mK3-dependent ubiquitination. Comparison of the fate of $\text{T}^\beta_m$.Tpn.Tpn between tapasin-sufficient and -deficient cells supports the proposal that location within the peptide-loading complex determines accessibility of the proteins to mK3. Indeed, we show that the very same protein ($\text{T}^\beta_m$.Tpn.Tpn) in these two cell types adopts distinct fates, sensitivity versus resistance to ubiquitination by mK3. Clearly, this cannot be accommodated by a simple “recognition sequence,” which is common to substrates of mK3, and instead requires a position-dependent substrate recruitment mechanism.

**DISCUSSION**

Using the mK3 system, we directly tested a model of substrate selectivity during ERAD wherein the complex of proteins in the ER membrane with which mK3 is associated (the pep-
FIGURE 6. Transmembrane β₂m is not ubiquitinated by mk3 when it binds to the peptide-loading complex in the place of tapasin. A, depiction and sequence comparison of the constructs used in these experiments. Potential ubiquitin-acceptor residues are indicated in red. The TM and cytosolic domains are shown. Tβ₂m.B7.Tpn contains the B7.2 TM domain and the cytosolic tail of tapasin. Tβ₂m.Tpn contains the TM and cytosolic domains of tapasin (upper panel). Schematic diagrams are shown of the potential arrangements of Tβ₂m.B7.Tpn or Tβ₂m.Tpn.Tpn in tapasin-sufficient 3KO cells (lower panel of A). B, Tβ₂m.B7.Tpn or Tβ₂m.Tpn.Tpn was expressed in 3KO cells (with class I HC) mK3. Sequential immunoprecipitation (IP) was performed; primary anti-TAP-1 precipitates were denatured, and the indicated transmembrane β₂m chimeric molecules were recovered by immunoprecipitation, followed by immunoblot for ubiquitin and β₂m.

C, depiction of the possible arrangements of the indicated molecules in tapasin-deficient cells (Tpn−/−). D, immunoblot for steady-state levels of peptide-loading complex proteins were examined in tapasin-deficient fibroblasts following transduction of the indicated constructs, including full-length tapasin. Asterisk indicates the specific band. E, sequential anti-TAP-1/anti-β₂m immunoprecipitations were performed from Tpn−/− cells expressing the indicated constructs. In some cases, full-length tapasin was transduced into the cells. Samples were blotted for ubiquitin and β₂m.
ERAD Substrate Recruitment by Adapter Proteins

tide-loading complex) serves to deliver substrates to the RING domain of mK3 for ubiquitination. We show that proteins with no sequence homology to class I HC (our Tβ₃m chimeras) can be targeted for ubiquitination if recruited into the peptide-loading complex. However, mK3 requires some core sequence features of its substrates (class I HC and Tβ₂m) to mediate ubiquitination. This indicates that substrate recruitment (adapter-mediated) positions potential target molecules in a favorable orientation with respect to the RING domain of the ligase, but that the E3 ligase has additional specific requirements for ubiquitination. Overall, contrary to the initial presumption that mK3 is a “single subunit” E3 ligase, it is clear that adapter molecules play an essential role in substrate recruitment.

This requirement for adapter (or cofactor) proteins in substrate recruitment is somewhat suggestive of multi-subunit E3 ligases. Probably the best understood multi-subunit E3 ligases are the Cullin-RING ligases (sometimes referred to as SCF ligases; Skp, cullin, and F-box protein) (44, 45). Structural analyses demonstrate that the spatial array of these proteins along the cullin scaffold serves to position the substrate such that specific lysine residues are placed in the immediate vicinity of the ligase subunit to permit ubiquitination of diverse targets (45–51). This is supported by the finding that F-box proteins can be engineered to interact with new proteins and target them for destruction by SCF ligases (52). Thus, analogous to what we demonstrate for mK3, proper recruitment of the substrate with respect to the RING domain of the ligase subunit is critical for selectivity. This mechanism permits the ubiquitination of disparate substrates, while simultaneously regulating/restricting ligase activity toward desired target proteins. However, the well ordered SCF complexes may represent the extreme end of the spectrum, whereas mK3 and other E3 ligases of the ER membrane may be more dynamic in their interactions with cofactors that facilitate substrate recruitment.

The peptide-loading complex clearly selects substrates for mK3-mediated ubiquitination, allowing only those proteins that associate in a similar orientation as class I HC to be ubiquitinated. However, mK3 must recognize the substrate to complete the targeted degradation; the substrate must possess a cytosolic tail and must have a ubiquitin acceptor residue (serine, threonine, or lysine). Indeed, using HC it was shown that ubiquitination occurs when the ubiquitin acceptor residue is located close to the C terminus and not when it is membrane-proximal (16, 22). Kaposi sarcoma herpesvirus encodes two ubiquitin ligases (kK5 and kK5) that act to down-regulate immune molecules at the cell surface, targeting them for lysosomal degradation. Data show that kK5 will optimally down-regulate a substrate when the ubiquitin acceptor residue (lysine or cysteine) is proximal to the transmembrane domain, and the converse is true for kK5 (53). Although the specific E3-imposed substrate requirements are unknown for most cellular ERAD E3 ligases, further studies will most likely reveal requirements specific to each E3, which would provide yet another checkpoint ensuring proper targeting.

Evidence for the pivotal role of adapter complexes in substrate recruitment by cellular ERAD E3 ligases is rapidly expanding. Probably the best characterized ERAD E3 is Hrd1 (also called Der3) involved primarily in the degradation of ER luminal and membrane substrates (54). There are some interesting parallels between Hrd1 and mK3, including the fact that Hrd1 has an obligatory binding partner, an ER-localized transmembrane protein, Hrd3 (SEL1L in mammals) that is required for stable expression of Hrd1 (55). Like all ERAD E3 ligases, the ligase domain of Hrd1 is cytosolic, and a mechanism must exist to expose luminal substrates to the ligase. In some cases, recognition of a potential substrate is mediated by ER resident lectins (5, 8). These lectins recognize modified N-linked glycans (1, 56) and include Yos9 in yeast and OS-9 and XTP3-B in mammals (57–60). These lectins interact with substrates either directly or via molecular chaperones (i.e. GRP94) and SEL1/Hrd3 (60). Thus, SEL1/Hrd3 bridges substrates and the ligase, highlighting the varied roles of adapter proteins in substrate selection.

Beyond ERAD, our findings with mK3 raise the question of whether adapter complexes are involved in substrate recruitment for other viral and cellular RING-CH proteins. It is notable that the list of molecules known to be targeted by the kK5 protein of Kaposi sarcoma herpesvirus continues to grow (61, 62), yet most of these substrates share no obvious sequence relationship. It seems plausible that a mechanism exists to localize kK5 with its substrates and position them so that the RING-CH domain of kK5 can ubiquitinate the cytosolic domains of these targets. In addition to viral E3 ligases, similar concepts may be invoked for substrate selection by endogenous cellular RING-CH molecules. The MARCH proteins were discovered recently by virtue of their similarity to the viral RING-CH proteins, and these E3 ligases have been implicated in many cellular processes, including antigen presentation by the immune system (12, 61). In the case of MARCH-I and MARCH-VIII, it has been demonstrated these proteins down-regulate the surface expression of multiple unrelated substrates, including MHC class II, CD86, and Fas (27, 63, 64). It will be interesting to see whether adapter complexes are utilized by these E3 ligases to recruit their substrates.

In summary, the mK3 system afforded the opportunity to probe the basis for substrate recruitment during ERAD. We find that the peptide-loading complex serves to deliver the class I HC to the RING domain of mK3 for ubiquitination. Because MHC class I heavy chains exhibit sequence variability in their cytosolic domains, this mechanism imparts specificity for class I without a requirement for direct binding by mK3 to each allelic class I gene product. This concept may help to explain the ability of other E3 ligases to target the degradation of multiple, seemingly unrelated substrates.

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