Requirements for the Selective Degradation of Endoplasmic Reticulum-Resident Major Histocompatibility Complex Class I Proteins by the Viral Immune Evasion Molecule mK3

Xiaoli Wang,¹ Rose Connors,¹ Michael R. Harris,² Ted H. Hansen,¹
and Lonnie Lybarger^{3*}

Department of Pathology and Immunology, Washington University School of Medicine,¹ and Department of Pediatrics, St. Louis Children's Hospital,² St. Louis, Missouri, and Department of Cell Biology and Anatomy, University of Arizona Health Sciences Center, Tucson, Arizona³

Received 27 July 2004/Accepted 23 October 2004

Recent studies suggest that certain viral proteins co-opt endoplasmic reticulum (ER) degradation pathways to prevent the surface display of major histocompatibility complex class I molecules to the immune system. A novel example of such a molecule is the mK3 protein of gammaherpesvirus 68. mK3 belongs to an extensive family of structurally similar viral and cellular proteins that function as ubiquitin ligases using a conserved RING-CH domain. In the specific case of mK3, it selectively targets the rapid degradation of nascent class I heavy chains in the ER while they are associated with the class I peptide-loading complex (PLC). We present here evidence that the PLC imposes a relative proximity and/or orientation on the RING-CH domain of mK3 that is required for it to specifically target class I molecules for degradation. Furthermore, we demonstrate that full assembly of class I molecules with peptide is not a prerequisite for mK3-mediated degradation. Surprisingly, although the cytosolic tail of class I is required for rapid mK3-mediated degradation, we observed that a class I mutant lacking lysine residues in its cytosolic tail was ubiquitinated and degraded in the presence of mK3 in a manner indistinguishable from wild-type class I molecules. These findings are consistent with a “partial dislocation” model for turnover of ER proteins and define some common features of ER degradation pathways initiated by structurally distinct herpesvirus proteins.

A common strategy that viruses use to avoid elimination by the immune system is to inhibit recognition of virus-infected cells by CD8⁺ T lymphocytes through blockade of major histocompatibility complex (MHC) class I-restricted antigen presentation. This phenomenon has been documented for many viruses, representing diverse virus families, and examples can be found of pathogen-encoded molecules that target essentially every step of the class I antigen presentation pathway (47, 57, 67). Recently, a new family of molecules has been described that inhibits class I expression through ubiquitin-mediated processes (11, 15, 52). Genes encoding molecules of this type have been found in gammaherpesviruses and poxviruses, and they are characterized by (i) membrane association and a type III orientation (N and C termini in the cytosol), (ii) a conserved RING finger domain of the RING-CH subtype (56) located at the N terminus, and (iii) a C-terminal cytosolic tail that is highly divergent among family members. The best-characterized members of this family (referred to here as the K3 family) are the kK3 and kK5 molecules of Kaposi's sarcoma-associated herpesvirus (9, 23, 30), mK3 from gammaherpesvirus 68 (53), and M153R from myxoma virus (21, 40). Importantly, these molecules have been shown to contribute to the virulence of the respective pathogens. Deletion of the M153R

gene from myxoma virus resulted in substantially reduced lethality in rabbits (21). In addition, analysis of an mK3-deficient gammaherpesvirus 68 in mice showed a reduced frequency of latently infected cells in the spleen, and this reduction could be reversed by depletion of CD8⁺ T cells (54). Thus, mK3-related molecules can function in vivo to promote evasion of the immune response against viruses.

In the case of kK5 and M153R, the RING-CH domains of the respective proteins have been shown to possess ubiquitin ligase (E3) activity (12, 40). Similar activity has been assumed for the other family members as well, since the RING-CH domains are relatively well conserved, and ubiquitinated class I heavy chains have been observed in the presence of both kK3 and mK3. Furthermore, targeted mutation of the RING-CH domain in either molecule abolishes function and results in the disappearance of ubiquitinated class I heavy chains (5, 24, 42). Despite the similarities between K3 family members, significant differences have been noted. In terms of substrate specificity, all family members are capable of targeting class I molecules for ubiquitination and degradation. However, kK5 can also downregulate B7.2 and ICAM-1 (10, 29), and m153R also targets CD4 and CD95 (FAS) (21, 40). The most notable difference among K3 family molecules is the subcellular site of target degradation. For kK5, kK3, and M153R, ubiquitination of the target molecules (most likely in a post-endoplasmic reticulum [ER] compartment) (24) results in their enhanced endocytosis from the cell surface and degradation within lysosomes (9, 12, 30, 37, 42). Evidence indicates that these mole-

* Corresponding author. Mailing address: Department of Cell Biology and Anatomy, University of Arizona Health Sciences Center, 1501 North Campbell Ave., Rm. 4205, Tucson, AZ 85724. Phone: (520) 626-1044. Fax: (520) 626-6354. E-mail: lybarger@email.arizona.edu.

cules (kK5, kK3, and M153R) bind to target proteins via interactions between the transmembrane domains of both the K3 family molecule and its targets, such as class I (12, 24, 29, 30, 40). Upon binding, the RING-CH domain catalyzes ubiquitin addition to the cytosolic tails of target molecules. Indeed, for kK5, kK3, and M153R, lysine residues in the cytosolic tail of target molecules are essential for target ubiquitination and destruction (12, 24, 40).

The mK3 molecule of gammaherpesvirus 68, although related to other K3 family members in terms of domain organization, differs with respect to its site of action. Expression of mK3 results in decreased cell surface class I expression by targeting nascent class I molecules in the ER for degradation in a proteasome-dependent manner (5, 53, 69). In the presence of mK3, ER-resident class I heavy chains are ubiquitin conjugated; this requires an intact RING-CH domain within mK3 (5). A striking feature of mK3 is that its function is critically dependent on the MHC class I peptide-loading complex (PLC) (38), a group of ER-resident molecules that facilitate class I assembly (13, 20, 63). In fact, mK3 fails to degrade class I in the absence of the PLC molecules TAP-1, TAP-2, and tapasin (38, 60). Furthermore, mK3 associates with the PLC in the absence of class I heavy chains, and heavy-chain mutants that are incapable of PLC association are resistant to mK3-mediated rapid degradation (38). TAP-1 and TAP-2 appear to be the primary binding partners of mK3 and binding requires the C-terminal cytosolic tail of mK3 (4, 60). Recently, it was also observed that mK3 expression, in some cell types, could lead to a decrease in TAP/tapasin protein levels. This indicates that these molecules are degraded by an mK3-dependent process and this, in turn, affects the surface expression of a broader array of class I molecules than mK3 can directly target (4). The kinetics of TAP/tapasin turnover were much slower than those observed for class I heavy chains, suggesting that mK3-induced degradation of class I versus TAP/tapasin involves distinct mechanisms.

Based largely on analogies to kK3, kK5, and M153R it has been assumed that mK3 initiates the class I degradation pathway by catalyzing ubiquitin addition to specific lysine residues in the cytosolic tail of class I. Indeed, it was reported that D^b molecules lacking lysine residues in the cytosolic tail were not rapidly degraded in the presence of mK3 (5). Here, we have examined the factors that influence rapid degradation of class I heavy chains, considering the results in the context of the PLC. Our data demonstrate the following. (i) Unexpectedly, mK3 targeting of class I (L^d) for degradation involves a pathway that requires recognition of a cytosolic tail of the appropriate length but can proceed efficiently in the absence of lysine residues in the cytoplasmic tail. (ii) Despite the degeneracy in class I tail sequence requirement, this process remains specific for class I heavy chains in the PLC. (iii) Lastly, we provide evidence that full assembly (peptide binding) of class I is not a required step for mK3-mediated degradation. These results highlight differences between the mechanisms of target recognition and ubiquitination by mK3 versus other characterized K3 family members, which likely reflect the disparate cellular sites of substrate degradation. Instead, mK3 appears to utilize a unique strategy to shunt class I molecules into the ER-associated degradation pathway.

MATERIALS AND METHODS

Cell lines. B6/WT3 (*H-2^b*), tapasin-deficient murine embryo fibroblasts (Tpn^{-/-}, *H-2^b*), and L-cell fibroblasts (*H-2^k*) have been described (19, 48, 71). In addition, L-cell transfectants/transductants (L^d, L^d/B7.2, and H2-M3) and B6/WT3 transductants (L^d and L^d/B7.2) have been described (60). 293T cells (14) were used for production of ecotropic retrovirus (to transduce B6/WT3 and Tpn^{-/-} cells) and amphotropic retrovirus (to transduce L cells) vectors. All cells were maintained in complete RPMI 1640 (Invitrogen, Carlsbad, Calif.) supplemented with 10% fetal calf serum (HyClone, Logan, Utah) as described previously (38). Retrovirus vector-containing supernatants were produced as described previously (38) by using the Vpack vector system (Stratagene, La Jolla, Calif.) with transient transfection of 293T cells. After transduction, virus-infected cells were enriched by antibiotic selection or cell sorting, depending on the vector used (see below). With neomycin and hygromycin resistance vectors, cells were selected with 0.6 mg of Geneticin (Life Technologies, Gaithersburg, Md.) and 0.15 mg of hygromycin (Sigma, St. Louis, Mo.)/ml, respectively. When necessary, the green fluorescent protein-positive (GFP⁺) cells from GFP virus-transduced lines were enriched by cell sorting.

DNA constructs. Generation of the L^d/mouse B7.2 chimeric molecule and the monoclonal antibody (MAb) 64-3-7 epitope-tagged H2-M3 molecule has been reported (39, 60). Murine tapasin and mK3 expression vectors have been described (38). A RING-CH mutant of mK3 (C48G, C51G) that disrupts mK3 function (5) was constructed by site-directed mutagenesis. L^d Δcyt (cytosolic tail deletion) was created by PCR and consists of residues 1 to 307. The H2-M3.L^d construct was generated by overlap PCR to replace the cytosolic tail of H2-M3 with that from L^d; this construct consists of H2-M3 residues 1 to 304, followed by residues 308 to 338 of L^d. The tapasin.L^d construct was also generated by overlap PCR and replaces the cytosolic tail of tapasin with that from L^d; this construct consists of tapasin residues 1 to 417, followed by residues 308 to 338 of L^d. Site-specific mutants were generated using the QuikChange mutagenesis kit (Stratagene, La Jolla, Calif.) according to the manufacturer's instructions. The correct sequence for all of the constructs was confirmed by DNA sequence analysis. All constructs were expressed from one of the following bicistronic retrovirus vectors: pMSCV-IRES-GFP (pMIG; (59)), pMSCV-IRES-neomycin (pMIN), and pMSCV-IRES-hygromycin (pMIH). pMIN and pMIH were generated in our lab. These vectors permit the gene of interest and the gene for GFP, neomycin, or hygromycin resistance to be expressed from a single bicistronic mRNA. The specific vectors used for each construct are listed in the appropriate figure legends.

Antibodies and peptides. Hamster anti-mouse tapasin MAb 5D3 and rabbit antisera against C-terminal sequences of mK3 (residues 167 to 187), mouse TAP-1, and mouse tapasin have been described (38). Hamster MAb 130 recognizing H2-M3 (7) was a gift from Chyng-Ru Wang (University of Chicago). Anti-actin MAb (AC-74) was obtained from Sigma, and anti-ubiquitin MAb (PD41) was obtained from Santa Cruz Biotech (Santa Cruz, Calif.). MAbs 28-14-8 (44) and 15-5-5 (45) were used to detect D^b and D^k, respectively. MAb 64-3-7 is specific for the α1 domain of open forms (unassembled) of L^d or epitope-tagged class I heavy chains (51, 70). MAb 30-5-7 is specific for the α2 domain of folded forms of L^d (51). The H2-M3 binding peptide Fr38 (FMIVIL [22]) was synthesized by using an Applied Biosystems (Foster City, Calif.) model 432A synthesizer.

Flow cytometry. All flow cytometric analyses were performed by using a FAC-SCalibur (Becton Dickinson, San Jose, Calif.). The data were analyzed by using CellQuest software (Becton Dickinson). Staining was performed as described previously (69). Phycoerythrin-conjugated goat anti-mouse immunoglobulin G (IgG; BD Pharmingen, San Diego, Calif.) was used to visualize MHC class I expression. Phycoerythrin-conjugated goat anti-hamster immunoglobulin (BD Pharmingen) was used to visualize H2-M3 expression.

Immunoprecipitations and immunoblot. Coimmunoprecipitations were performed essentially as described previously (38) by using 1.0% digitonin (Wako, Richmond, Va.) in the lysis buffer (Tris-buffered saline [pH 7.4]). For immunoblot of cell lysates, cells were lysed in Tris-buffered saline containing 1.0% NP-40 (Sigma). Postnuclear lysates were mixed with LDS sample buffer (Invitrogen), and 2-mercaptoethanol (Sigma) was added to a final concentration of 1%. Immunoblot was performed after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation of precipitated proteins or cell lysates as described previously (69). Biotin-conjugated goat anti-mouse IgG or goat anti-hamster IgG (both from Caltag, San Francisco, Calif.) or donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, Pa.) was used as a secondary staining reagent, followed by streptavidin-horseradish peroxidase (Zymed Laboratories, San Francisco, Calif.). Specific proteins were visualized by chemiluminescence with the ECL System (Amersham Biosciences, Piscataway, N.J.). The protea-

FIG. 1. Class I heavy chains with a heterologous, lysine-less cytosolic tail are sensitive to mK3. *H-2^b* fibroblasts expressing L^d molecules with the cytosolic tail of murine B7.2 (60) and a mutant version in which all five tail lysines were converted to arginine (L^d/mB7.2 K→R) were transduced with the empty vector (pMIG; Vector) or pMIG.mK3. (A) Flow cytometric analysis for L^d expression (MAb 30-5-7) versus GFP (mK3). Numbers indicate the ratio of L^d staining between the GFP⁺ and GFP⁻ fractions in each histogram. (B) Newly synthesized proteins were pulse-labeled with ³⁵S-Met/Cys for 15 min and then chased for 60 min in an excess of unlabeled Met/Cys. L^d/mB7.2 molecules were precipitated with MAbs 30-5-7 and 64-3-7. Precipitates were separated by SDS-PAGE and subjected to autoradiography. (C) Anti-L^d/mB7.2 immunoprecipitations (IP) were performed with the indicated cell lines by using MAb 64-3-7. Cells were transduced with wild-type mK3 (mK3), GFP only vector (V), or mK3 RING-CH mutant (RING mut) that renders mK3 nonfunctional for ubiquitin conjugation. Precipitates (+ or - endo H treatment) were blotted for ubiquitin or L^d heavy chains.

some inhibitors MG132 (Calbiochem, San Diego, Calif.) and lactacystin (Alexis Biochemicals, San Diego, Calif.) were used as described in the relevant figure legends.

Metabolic labeling and/or pulse-chase. After ~45 min of preincubation in cysteine- and methionine-free medium (Cys/Met-free; Dulbecco's modified Eagle's medium with 5% dialyzed fetal calf serum), cells (at 10⁷ cells/ml) were pulse-labeled with Express ³⁵S-Cys/Met labeling mix (Perkin-Elmer Life Sciences, Boston, Mass.) at 300 μ Ci/ml for 10 to 20 min. Chase was initiated by the addition of an excess of unlabeled Cys/Met (5 mM each). Immunoprecipitation and endo- β -N-acetylglucosaminidase H (Endo H; ICN Pharmaceuticals, Costa Mesa, Calif.) treatment were performed as described previously (43). Samples were subjected to SDS-PAGE, and gels were treated with Amplify (Amersham), dried, and exposed to BioMax-MR film (Kodak, New Haven, Conn.).

RESULTS

MK3 substrates do not require cytosolic tail lysines for mK3-mediated rapid degradation. We demonstrated previously that chimeric molecules consisting of L^d with the cytosolic tail of either human or mouse B7.2 (CD86) were efficiently targeted for ubiquitination and degradation by mK3, even though there is very limited sequence similarity between these tails and the native L^d tail aside from the presence of multiple lysine residues (60). Unexpectedly, when all of the

lysine residues were mutated in the context of the L^d/B7.2 chimeras, there was no defect in their surface downregulation, ubiquitination, and degradation mediated by mK3 (but not a RING-CH mutant) in *H-2^b* or *H-2^k* fibroblasts (Fig. 1 and data not shown). This demonstrated that each of these processes could proceed efficiently even when the substrate was incapable of being ubiquitinated within the cytosolic tail, which is clearly not the case for other K3 family members (12, 24, 40). To extend these findings, we analyzed L^d with its natural tail but with all tail lysines mutated to arginine. When we compared L^d and the L^d K→R mutant expressed in L cells, both molecules were downregulated to similar degrees in the presence of mK3 (Fig. 2A). Likewise, pulse-chase labeling and immunoprecipitation revealed that both molecules were degraded with similar kinetics in mK3-expressing cells (Fig. 2B). Consistent with the degradation results, comparable amounts of ubiquitinated heavy chains were detected with both molecules. These ubiquitinated heavy chains were endo H sensitive, indicating that they had not trafficked beyond the mid-Golgi (Fig. 2D). For both molecules, treatment of cells with proteasome inhibitors could partially stabilize class I heavy chains

FIG. 2. L^d molecules lacking cytosolic tail lysine residues are sensitive to mK3. L cells ($H-2^k$) and $H-2^b$ fibroblasts (WT3) were stably transduced with L^d or L^d K→R (lacking all three tail lysines). Cells were then transduced with pMIG.mK3. For pulse-chase and immunoprecipitation (IP), mK3-expressing cells were enriched by sorting the GFP⁺ fraction of the transduced lines. (A) Flow cytometric analysis for L^d expression (MAb 30-5-7) versus GFP (mK3). Numbers indicate the ratio of L^d staining between the GFP⁺ and GFP⁻ fractions in each histogram. (B) Newly synthesized L^d molecules expressed in WT3 cells (+ or - mK3) were pulse-labeled with ³⁵S-Met/Cys for 15 min and chased for the indicated times. L^d heavy chains were precipitated with MAb 30-5-7 and 64-3-7, treated or mock treated with endo H, and subjected to SDS-PAGE and autoradiography. Relative band intensities from the gels (of samples not treated with endo H) were estimated by using NIH Image software, and these values are plotted as a percentage of the signal at time zero. (C) Pulse-chase and immunoprecipitation (IP) analysis was performed with L cells expressing L^d or L^d K→R (+ or - mK3), similar to above. Cells were pulse-labeled for 15 min and chased for the indicated times. L^d heavy chains were precipitated with MAb 30-5-7 and 64-3-7, treated or mock treated with endo H, and subjected to SDS-PAGE and autoradiography. (D) L cells expressing L^d or L^d K→R (+ or - mK3) were incubated for 2 h with proteasome inhibitors (PI) as described above prior to harvesting. L^d heavy chains were precipitated (MAb 64-3-7), treated or mock treated with endo H and then blotted with the indicated antibodies. (E) Anti- L^d immunoprecipitations (MAb 64-3-7) were performed with digitonin lysates from L cells expressing L^d or L^d K→R (+ or - mK3). Precipitates were separated by SDS-PAGE and blotted with the indicated antibodies.

(both unmodified and ubiquitin conjugated) in the presence of mK3 (Fig. 2C and D), a result consistent with previous findings (5, 69). Lastly, the L^d K→R mutant showed no defect in PLC association (Fig. 2E); this result was anticipated since the PLC is required for mK3 function. Thus, in these experiments, no detectable differences were observed between wild-type L^d and

L^d K→R, with respect to regulation mediated by mK3. Certainly, this is distinct from molecules such as kK3, kK5, and M153R and raises the interesting question of how mK3 is able to target the L^d K→R molecule for ubiquitination when the mK3 RING-CH domain resides on the opposite face of the ER membrane from the class I ectodomain. Our findings with the

FIG. 3. A cytosolic tail is required for mK3-mediated degradation of L^d. L cells were transduced with L^d or L^d with a deletion of the cytoplasmic tail (L^dΔcyt). These cells were subsequently transduced with pMIG (vector) or pMIG.mK3. (A) Flow cytometric analysis for L^d expression (MAB 30-5-7) versus GFP (mK3). Numbers indicate the ratio of L^d staining between the GFP⁺ and GFP⁻ fractions in each histogram. (B) L^d heavy chains were precipitated (MAB 64-3-7) from the indicated cell lines; pMIG vector only (V) or pMIG.mK3 (mK3). After endo H digestion, samples were blotted for ubiquitin and L^d. (C) L^d heavy chains were pulse-labeled for 15 min and chased for the indicated times. Heavy chains were precipitated with MABs 30-5-7 and 64-3-7, separated by SDS-PAGE, and subjected to autoradiography. (D) Digitonin lysates from the indicated cell lines were precipitated with MAB 64-3-7. These precipitates were then blotted with the antibodies listed on the right.

lysine-less class I tail do not exclude the possibility that the cytoplasmic tail of wild-type class I molecules is normally ubiquitinated by mK3. However, it is striking that the pattern and amount of ubiquitinated heavy chains and their turnover is indistinguishable in the presence or absence of lysine residues in the tail.

Tail-less class I heavy chains associate with the PLC and mK3 and yet are resistant to mK3. It was previously reported that a D^b construct in which the native cytosolic tail was replaced with a short epitope tag did not associate with mK3 and was not downregulated at the cell surface (5). This result could

be explained by either a failure of this construct to associate with TAP/tapasin or specific tail requirements for mK3-mediated regulation. To more precisely define the tail requirements for mK3-induced degradation of class I, an L^d mutant completely lacking the tail (L^dΔcyt) was generated. We failed to see substantial surface downregulation of this molecule compared to wild-type L^d, although there was a modest effect (Fig. 3A) that could involve an indirect influence of mK3 on class I expression (4). Importantly, there was no evidence for ubiquitination or turnover of the L^dΔcyt molecule (Fig. 3B and C). However, coimmunoprecipitation revealed that the L^dΔcyt

FIG. 4. Introduction of an additional class I gene into mK3-expressing cells reduces the activity of mK3 toward the endogenous class I molecules. L cells were transduced with the indicated L^d constructs. The resulting lines were subsequently transduced with mK3. In each histogram, staining of the parental line (without mK3) is indicated by the shaded peak and staining of the mK3 transductant is indicated by the heavy line. Cells were analyzed for L^d (MAb 30-5-7), K^k (MAb 11-4-1), and D^k (MAb 15-5-5).

mutant was associated with the PLC and mK3. Therefore, the cytosolic tail of L^d is not required for association with mK3, but the tail is necessary for rapid degradation of class I heavy chains. In addition, the length of the cytosolic tail may be an important factor determining recognition by mK3, since L^d with a truncated tail (containing the first 13 residues) exhibited an intermediate level of sensitivity to mK3 compared to wild-type L^d and $L^d \Delta$ cyt (not shown).

During our analysis of $L^d \Delta$ cyt, we noted that this molecule had an inhibitory effect on mK3-mediated regulation of the endogenous class I molecules. Specifically, when introduced into mK3-expressing L cells, it almost completely rescued cell surface expression of the endogenous class I molecules (Fig. 4). Mutation of a tapasin interaction site (^{134}Thr) (33, 46) on $L^d \Delta$ cyt restored full activity of mK3 toward the endogenous class I molecules, strongly suggesting that the inhibitory effects of the transduced $L^d \Delta$ cyt were due to competition for the PLC. This is further supported by the observation that wild-type L^d also has a similar, though less pronounced effect (Fig. 4). The reason that $L^d \Delta$ cyt is more potent in this regard than L^d could be due to the fact that L^d is turned over by mK3, resulting in lower steady-state levels, whereas $L^d \Delta$ cyt is not susceptible mK3-induced turnover. Thus, the presence of more class I molecules within the PLC, especially those that cannot serve as an mK3 target, prevents downregulation of normally sensitive molecules. Presumably, this is due to competition between class I molecules that reduces the amount of time they spend in the PLC and, thus, the window in which they can be targeted by mK3 for degradation. These results also help explain an earlier observation that K^k molecules are relatively refractory to mK3 (69). In that earlier study, as here, we showed that K^k surface expression was not affected by mK3 in L^d -expressing L cells. However, we now show that this was likely a result of PLC competition, since K^k is mK3 sensitive in the same cell line expressing the L^d T134K mutant (Fig. 4).

MK3 targeting of H2-M3: full assembly of class I molecules is not required for mK3-induced degradation. Although the aforementioned class I tail requirements of mK3 distinguish it from other K3 family members, they are reminiscent of class I degradation by human cytomegalovirus US2 and US11 proteins, which also induce rapid degradation of nascent class I from the ER (discussed in more detail below). For US2, multiple lines of evidence have shown that full assembly of class I heavy chains with β_2 -microglobulin and peptide is required for recognition by US2, leading to degradation (3, 16, 17, 62). In the case of mK3, it remains less clear whether full assembly of class I is similarly required. Even though PLC-associated class I molecules are the primary targets of mK3 (38), they could represent heavy-chain- β_2 -microglobulin heterodimers awaiting peptide or fully assembled class I molecules that have yet to dissociate from the PLC (34). Furthermore, mK3 can induce the turnover of class I molecules that are reactive with conformation-specific MAbs specific for fully assembled class I (5, 38). To rigorously determine whether full assembly was required for mK3 targeting of class I, we studied the effects of mK3 on class I molecules in a peptide-limiting background. In other words, we sought to determine whether peptide binding by class I is a requirement for mK3 recognition.

Since mK3 requires TAP-1 and TAP-2 proteins for its stable expression and function (4, 38, 60), we could not examine mK3-induced turnover of class I in TAP-deficient cells. Alternatively, we sought a system that mimics the effects of TAP deficiency but retains the entire PLC and mK3 in a functional state. To accommodate these requirements, we examined H2-M3 expression in normal cells. H2-M3 is a murine class Ib molecule that preferentially binds peptides that contain an N-terminal formylated Met (36). Given the paucity of appropriate peptides in cells under normal conditions, H2-M3 molecules remain within the ER in a peptide-receptive state after synthesis and mobilize to the cell surface only in the presence of formylated peptides (7). Importantly, H2-M3 associates with the PLC while awaiting peptides in the ER, much like class Ia molecules (8, 39). When we initially examined the effects of mK3 on H2-M3, we found that H2-M3 was completely resistant to degradation, even in the absence of a high-affinity peptide ligand (Fig. 5A and data not shown). However, H2-M3 possesses a relatively short cytosolic tail (KRRGAGER) by class Ia standards and mK3 targets must possess a tail for degradation (Fig. 3). To test the tail requirement in the context of mK3, we replaced the native tail of H2-M3 with that of L^d (H2-M3. L^d), which supports mK3 recognition. We then compared H2-M3 and H2-M3. L^d for sensitivity to mK3 in the presence or absence of an H2-M3 binding peptide (Fig. 5). Pulse-chase labeling and immunoprecipitation revealed that in the absence of exogenous peptides, both molecules remained within the ER (endo H sensitive) and were quite stable over the time course of the experiment (Fig. 5A, left panels). Thus, these cells lack sufficient quantities of endogenous peptides that are capable of binding to H2-M3 and inducing detectable maturation. When a specific peptide was added to the cells prior to labeling, assembly and maturation (ER-to-mid-Golgi transport) of both molecules was observed, as expected. In the presence of mK3, wild-type H2-M3 was completely stable, whereas H2-M3. L^d was essentially undetectable after the chase period (Fig. 5A, right panels). Most notably, degradation of

FIG. 5. MK3 targets the degradation of unassembled H2-M3 heavy chains possessing the cytosolic tail of L^d. Stable L-cell transductants were generated with H2-M3 or H2-M3.L^d constructs in the vector pMIN. Where indicated, these lines were transduced with pMIG.mK3 and the GFP⁺ cells were enriched by cell sorting. (A) Pulse-chase labeling of H2-M3 heavy chains, with (+) or without (-) the addition of an H2-M3 binding peptide (Fr38). Prior to labeling, cells were preincubated for 45 min in Met/Cys-free medium. Where indicated, Fr38 peptide was added to the medium during the preincubation period at a concentration of 10 μ M. Cells were labeled for 20 min and chased for the indicated times after the addition of chase medium (with 10 μ M peptide where appropriate). H2-M3 heavy chains were immunoprecipitated by using MAb 130, and all precipitates were digested with endo H. Samples were then visualized by SDS-PAGE and autoradiography. The migration of endo H-sensitive (S) and endo H-resistant (R) heavy chains is indicated. (B) Peptide-induced surface expression of H2-M3 versus H2-M3.L^d in the presence or absence of mK3. Cells were incubated overnight with or without 10 μ M Fr38 peptide prior to flow cytometric analysis. Thin lines indicate MAb130 staining of cells incubated without exogenous peptide. Heavy lines indicate MAb130 staining of cells incubated with peptide. Dotted lines indicated endogenous D^k (MAb 15-5-5) expression. Note that the level of peptide-induced M3 staining in the H2-M3.L^d plus mK3-expressing cells (heavy line in lower right histogram) is comparable to the parental L-cell line and represents predominantly staining of endogenous H2-M3 in this line, which is mK3 resistant (data not shown). However, the amount of endogenous H2-M3 expression is insufficient for detection in the pulse-chase labeling conditions used in panel A.

H2-M3.L^d occurred in the absence of exogenous binding peptides. Both H2-M3 molecules were detected in association with mK3 and the PLC (data not shown). These results demonstrate that full assembly of class I heterotrimers is not a strict requirement for mK3-induced degradation of H2-M3 heavy chains and, furthermore, confirm the importance of the cytosolic tail in this process. As mentioned above, the impact of peptide-binding by class Ia molecules on mK3 susceptibility has been difficult to address. However, there is no reason to assume that class Ia molecules would differ from H2-M3 in this regard, especially since they both show a dependence on the PLC for optimal peptide binding.

Tapasin with the L^d cytosolic tail is not a target of mK3: support for a model of mK3 target recognition where the

FIG. 6. Tapasin with the cytosolic tail of L^d is not rapidly degraded by mK3. Tapasin-deficient fibroblasts were transduced with pMIH.tapasin.L^d. This line was then transduced with pMIG.mK3. (A) Surface D^b expression (MAB 28-14-8) on the parental tapasin^{-/-} line (thin line), tapasin^{-/-} cells expressing tapasin.L^d (heavy line), and the tapasin.L^d-expressing line subsequently transduced with mK3 (dotted line). The shaded peak represents cells stained with secondary antibody alone as a control. (B) Immunoprecipitation with antisera against the indicated molecules in tapasin.L^d-expressing cells mK3, followed by anti-tapasin immunoblot. (C) Immunoblot of whole-cell lysates from tapasin^{-/-} cells or the same cells expressing tapasin.L^d with (+) or without mK3. Lysates were blotted with anti-tapasin or anti-actin as a loading control.

orientation of mK3 imposed by its interaction with the PLC determines its specificity for class I. MK3 is associated with the PLC, even in the absence of MHC class I, yet only class I heavy chains in the PLC are degraded with rapid kinetics (half-life of ≤ 30 min) (4, 38, 69). This could be due to a failure of mK3 to functionally interact with the cytosolic tail of tapasin or to other restrictions imposed on mK3 target recognition, such as the precise orientation or proximity of mK3 with respect to the other members of the PLC. We explored these possibilities by replacing the native tail of tapasin (25 residues in length) (18) with the cytosolic tail of L^d (31 residues) since we know that the L^d tail can support mK3-mediated degradation of class I molecules (see H2-M3 data). Expression of tapasin.L^d molecules in tapasin-deficient cells restored surface class I expression, indicating functionality of the chimeric tapasin (Fig. 6A). In the complete absence of tapasin, mK3 function is impaired (38), but in these tapasin.L^d-reconstituted cells, mK3 expression resulted in substantial class I downregulation.

lation. This demonstrated a functional interaction between mK3 and tapasin.L^d-containing loading complexes. Coimmunoprecipitation with anti-mK3 or anti-tapasin antisera revealed that mK3 was associated with the PLC in tapasin.L^d-expressing cells (Fig. 6B). Most significantly, we did not observe any decrease in the steady-state levels of tapasin.L^d (Fig. 6B and C) or in its turnover (data not shown) when mK3 was present. Collectively, these findings provide strong experimental support for a model wherein substrate (class I) recognition by mK3 is a function of the spatial organization of molecules within the loading complex (60). The binding of mK3 to TAP/tapasin may orient mK3 in such a way that it is only capable of interacting with a cytosolic tail on class I molecules within the PLC, although the specific sequence of that tail is not a critical factor. These results do not exclude the possibility that transmembrane domain (TM) sequences of class I, in addition to the tail, are also recognized by mK3 to confer specificity. However, our data with H2-M3 argue against this, since the TM domain of H2-M3 bears no homology to murine class Ia molecules and yet addition of only the cytosolic tail of L^d to H2-M3 renders it sensitive to mK3.

DISCUSSION

Comparison of class I targeting by mK3 versus other K3 family members and US2/11. The available evidence indicates that kK3, kK5, and M153R all bind directly to their substrates (such as class I) and catalyze ubiquitin addition to lysine residues in the cytoplasmic tail (12, 24, 40). This serves as a trigger to initiate endocytosis of ubiquitinated heavy chains from the cell surface, which are then routed to lysosomes for degradation. These processes likely invoke normal cellular pathways that utilize ubiquitin, especially mono-ubiquitin modification, to regulate cell surface molecule expression (25, 26). The mK3 molecule is clearly a member of the K3 family due to the RING-CH domain at its N terminus and its domain organization and membrane topology. In addition, we have observed that the RING-CH domain of mK3 does have ubiquitin ligase activity *in vitro* (X. Wang, unpublished result). However, unlike other characterized K3 family members, we show that lysine residues in the cytosolic tail of class I (L^d) are not a strict requirement for mK3 targeting. This difference may be a reflection of the distinct sites of action for mK3 versus kK3, kK5, and M153R. Indeed, the cellular machinery involved in degradation of class I in lysosomes (after removal from the cell surface) will likely be quite different from that involved in ER-associated degradation pathways initiated by mK3, and substrate requirements will likewise differ.

Our findings with L^d K→R contrast with a published report on D^b lacking cytosolic tail lysines, where heavy chains were still ubiquitinated but no heavy-chain turnover and surface downregulation was observed (5). The difference between these two studies is difficult to reconcile, but we have consistently observed a precise correlation between mK3-induced ubiquitination and rapid degradation of class I. Since D^b and L^d are identical in amino acid sequence from the α₃ domains through the C termini, it seems unlikely that the disparity results from sequence differences between these two class I molecules. The contrasting fates of D^b and L^d could reflect subtle but important differences between the cell lines used in

each study to express the respective constructs. Despite this, however, our results demonstrate that class I ubiquitination and turnover can proceed efficiently in the absence of cytosolic tail lysine residues.

The function of mK3 within the ER, as well as certain other features of mK3-mediated class I degradation, are similar to the human cytomegalovirus proteins US2 and US11. With US2/11, class I loss from the ER involves a process termed dislocation (61, 62). Dislocation is an ATP-dependent pathway that operates under normal conditions to purge the ER of subunits from unassembled or inappropriately assembled oligomeric protein complexes (32). In fact, unassembled class I heavy chains and the TCRα chain, for example, are degraded by this pathway (1, 27, 28, 58, 64, 68). It was recently shown that US11 initiates dislocation of class I by bridging it to a novel ER membrane protein called Derlin-1 (35, 66), which is the mammalian homolog of the yeast Der1, a protein that is required for ER-associated degradation (ERAD) (31). A dominant-negative form of Derlin-1 inhibited US11-mediated dislocation of class I (35) but did not affect US2-mediated dislocation, suggesting that other downstream effector molecules can also initiate the dislocation pathway. The current hypothesis is that Derlin-1 recruits substrates to a "dislocation pore." Once a substrate begins to emerge into the cytosol from the ER, it is ubiquitinated and then recognized by the cytosolic ATPase, p97 (VCP), and its associated cofactors which provide the mechanical force to extrude the substrate (32, 41). Indeed, p97 has been shown to associate with both US11 and US2 (6, 65).

This partial dislocation model for US2/11-mediated degradation (49, 50) is consistent with some of our findings with mK3, as evidenced by the following. (i) mK3 expression results in the appearance of mono-, di-, and tri-ubiquitinated heavy chains (5, 60; the present study), and this pattern is quite similar to that observed with US2 (16). (ii) Like mK3, US11 induces degradation of ER-resident class I molecules in a cytosolic tail-dependent, but lysine-independent fashion (50, 55). For US2, the role of the class I cytosolic tail and lysine residues therein remains controversial (2, 16, 55). (iii) Tail-less class I molecules inhibit the activity of mK3 toward endogenous class I molecules (Fig. 4), and a similar phenomenon has been reported for US2 and US11 (55).

Despite these similarities, notable differences exist between mK3 and US2/US11. Probably the most obvious difference is that US2 and US11 are not ubiquitin ligases and share no obvious sequence similarity to mK3. Further, mK3 and US2/11 act from opposite sides of the ER membrane and target different class I assembly intermediates. More specifically, mK3 predominantly targets incompletely assembled class I heavy chains for ubiquitination that is cytosolic RING-CH domain dependent. In contrast, US2 requires fully conformed class I molecules (3, 16, 17, 62), and an immunoglobulin fold in the ER lumenal domain of US2 binds at the junction of the class I peptide-binding platform and the α₃ domain (17). Less is known about the US11 association with class I, but it was predicted to have an immunoglobulin fold similar to US2. However, unlike US2, US11 appears to target multiple class I assembly intermediates (16, 61). Thus, even though mK3 and US2/11 all target nascent class I molecules, the specific mechanisms of substrate recognition and host cofactor molecules

involved in initiating the degradation pathway are likely to be distinct between each of these molecules. In regard to the initiation of US11-mediated turnover of class I, our preliminary data indicate that US11 (like mK3) can associate with class I molecules while they are bound to the PLC. However, US11 does not require TAP-1 or -2 proteins for class I regulation (Wang, unpublished).

For mK3, it is unclear whether the initial event in class I turnover is mK3-mediated ubiquitination of the class I tail. Clearly, ubiquitin acceptor sites (lysines) in the L^d tail are not a strict requirement for ubiquitination and degradation. This could reflect an alternate mechanism for the initiation of the degradation pathway where the class I ectodomain is first ubiquitinated after partial dislocation, similar to what has been suggested for US2/11 (49, 50). This could also reflect a "by-stander effect," where the cytosolic tail lysine-deficient mutants are sensitive because they are present in the PLC with wild-type class I molecules. In this case, wild-type molecules with lysine residues in the tail are necessary to initiate the dislocation pathway and the lysine-deficient molecules are brought along, rendering their ectodomains available for ubiquitination. Such a "collateral damage" model offers a possible explanation for the TAP/tapasin decrease seen in some mK3-expressing cells (4), which is less efficient than class I heavy-chain degradation. Regardless, mK3 utilizes a unique mechanism to initiate ERAD pathways to block class I-restricted antigen presentation as a means to evade the host immune system.

ACKNOWLEDGMENTS

We thank Herbert "Skip" Virgin IV for continued support of this project and for helpful comments on the manuscript. We also thank Andrey Shaw for review of the manuscript and for constructive suggestions.

This study was supported by National Institutes of Health grants ROIA119687 (to T.H.H.), ROIA1060723 (to L.L.), and T32AI07063 (to X.W.).

REFERENCES

- Albring, J., J. O. Koopmann, G. J. Hammerling, and F. Momburg. 2004. Retrotranslocation of MHC class I heavy chain from the endoplasmic reticulum to the cytosol is dependent on ATP supply to the ER lumen. *Mol. Immunol.* **40**:733–741.
- Barel, M. T., M. Rensing, N. Pizzato, D. van Leeuwen, P. Le Bouteiller, F. Lenfant, and E. J. H. J. Wiertz. 2003. Human cytomegalovirus-encoded US2 differentially affects surface expression of MHC class I locus products and targets membrane-bound, but not soluble HLA-G1 for degradation. *J. Immunol.* **171**:6757–6765.
- Blom, D., C. Hirsch, P. Stern, D. Tortorella, and H. L. Ploegh. 2004. A glycosylated type I membrane protein becomes cytosolic when peptide: N-glycanase is compromised. *EMBO J.* **23**:650–658.
- Boname, J. M., B. D. De Lima, P. J. Lehner, and P. G. Stevenson. 2004. Viral degradation of the MHC class I peptide-loading complex. *Immunity* **20**:305–317.
- Boname, J. M., and P. G. Stevenson. 2001. MHC class I ubiquitination by a viral PHD/LAP finger protein. *Immunity* **15**:627–636.
- Chevalier, M. S., and D. C. Johnson. 2003. Human cytomegalovirus US3 chimeras containing US2 cytosolic residues acquire major histocompatibility class I and II protein degradation properties. *J. Virol.* **77**:4731–4738.
- Chiu, N. M., T. Chun, M. Fay, M. Mandal, and C. R. Wang. 1999. The majority of H2-M3 is retained intracellularly in a peptide-receptive state and traffics to the cell surface in the presence of N-formylated peptides. *J. Exp. Med.* **190**:423–434.
- Chun, T., A. G. Granda III, L. Lybarger, J. Forman, L. Van Kaer, and C. R. Wang. 2001. Functional roles of TAP and tapasin in the assembly of M3-N-formylated peptide complexes. *J. Immunol.* **167**:1507–1514.
- Coscoy, L., and D. Ganem. 2000. Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. *Proc. Natl. Acad. Sci. USA* **97**:8051–8056.
- Coscoy, L., and D. Ganem. 2001. A viral protein that selectively downregulates ICAM-1 and B7-2 and modulates T cell costimulation. *J. Clin. Invest.* **107**:1599–1606.
- Coscoy, L., and D. Ganem. 2003. PHD domains and E3 ubiquitin ligases: viruses make the connection. *Trends Cell Biol.* **13**:7–12.
- Coscoy, L., D. J. Sanchez, and D. Ganem. 2001. A novel class of herpesvirus-encoded membrane-bound E3 ubiquitin ligases regulates endocytosis of proteins involved in immune recognition. *J. Cell Biol.* **155**:1265–1273.
- Cresswell, P. 2000. Intracellular surveillance: controlling the assembly of MHC class I-peptide complexes. *Traffic* **1**:301–305.
- DuBridge, R. B., P. Tang, H. C. Hsia, P. M. Leong, J. H. Miller, and M. P. Calos. 1987. Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. *Mol. Cell. Biol.* **7**:379–387.
- Fruh, K., E. Barte, K. Gouveia, and M. Mansouri. 2002. Immune evasion by a novel family of viral PHD/LAP-finger proteins of gamma-2 herpesviruses and poxviruses. *Virus Res.* **88**:55–69.
- Furman, M. H., J. Loureiro, H. L. Ploegh, and D. Tortorella. 2003. Ubiquitinylation of the cytosolic domain of a type I membrane protein is not required to initiate its dislocation from the endoplasmic reticulum. *J. Biol. Chem.* **278**:34804–34811.
- Gewurz, B. E., R. Gaudet, D. Tortorella, E. W. Wang, H. L. Ploegh, and D. C. Wiley. 2001. Antigen presentation subverted: structure of the human cytomegalovirus protein US2 bound to the class I molecule HLA-A2. *Proc. Natl. Acad. Sci. USA* **98**:6794–6799.
- Granda, A. G., III, P. G. Comber, S. E. Wenderfer, G. Schoenhals, K. Fruh, J. J. Monaco, and T. Spies. 1998. Sequence, linkage to H2-K, and function of mouse tapasin in MHC class I assembly. *Immunogenetics* **48**:260–265.
- Granda, A. G., III, T. N. Golovina, S. E. Hamilton, V. Sriram, T. Spies, R. R. Brutkiewicz, J. T. Harty, L. C. Eisenlohr, and L. Van Kaer. 2000. Impaired assembly yet normal trafficking of MHC class I molecules in tapasin mutant mice. *Immunity* **13**:213–222.
- Granda, A. G., III, and L. Van Kaer. 2001. Tapasin: an ER chaperone that controls MHC class I assembly with peptide. *Trends Immunol.* **22**:194–199.
- Guerin, J. L., J. Gelfi, S. Boullier, M. Delverdier, F. A. Bellanger, S. Bertagnoli, I. Drexler, G. Sutter, and F. Messud-Petit. 2002. Myxoma virus leukemia-associated protein is responsible for major histocompatibility complex class I and Fas-CD95 down-regulation and defines scrapins, a new group of surface cellular receptor abductor proteins. *J. Virol.* **76**:2912–2923.
- Gulden, P. H., P. Fischer III, N. E. Sherman, W. Wang, V. H. Engelhard, J. Shabanowitz, D. F. Hunt, and E. G. Pamer. 1996. A *Listeria monocytogenes* pentapeptide is presented to cytolytic T lymphocytes by the H2-M3 MHC class Ib molecule. *Immunity* **5**:73–79.
- Haque, M., K. Ueda, K. Nakano, Y. Hirata, C. Parravicini, M. Corbellino, and K. Yamanishi. 2001. Major histocompatibility complex class I molecules are down-regulated at the cell surface by the K5 protein encoded by Kaposi's sarcoma-associated herpesvirus/human herpesvirus-8. *J. Gen. Virol.* **82**:1175–1180.
- Hewitt, E. W., L. Duncan, D. Mufti, J. Baker, P. G. Stevenson, and P. J. Lehner. 2002. Ubiquitinylation of MHC class I by the K3 viral protein signals internalization and TSG101-dependent degradation. *EMBO J.* **21**:2418–2429.
- Hicke, L. 2001. Protein regulation by monoubiquitin. *Nat. Rev. Mol. Cell Biol.* **2**:195–201.
- Hicke, L., and R. Dunn. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu. Rev. Cell Dev. Biol.* **19**:141–172.
- Hughes, E. A., C. Hammond, and P. Cresswell. 1997. Misfolded major histocompatibility complex class I heavy chains are translocated into the cytoplasm and degraded by the proteasome. *Proc. Natl. Acad. Sci. USA* **94**:1896–1901.
- Huppa, J. B., and H. L. Ploegh. 1997. The alpha chain of the T-cell antigen receptor is degraded in the cytosol. *Immunity* **7**:113–122.
- Ishido, S., J. K. Choi, B. S. Lee, C. Wang, M. DeMaria, R. P. Johnson, G. B. Cohen, and J. U. Jung. 2000. Inhibition of natural killer cell-mediated cytotoxicity by Kaposi's sarcoma-associated herpesvirus K5 protein. *Immunity* **13**:365–374.
- Ishido, S., C. Wang, B. S. Lee, G. B. Cohen, and J. U. Jung. 2000. Down-regulation of major histocompatibility complex class I molecules by Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins. *J. Virol.* **74**:5300–5309.
- Knop, M., A. Finger, T. Braun, K. Hellmuth, and D. H. Wolf. 1996. Der1, a novel protein specifically required for endoplasmic reticulum degradation in yeast. *EMBO J.* **15**:753–763.
- Kostova, Z., and D. H. Wolf. 2003. For whom the bell tolls: protein quality control of the endoplasmic reticulum and the ubiquitin-proteasome connection. *EMBO J.* **22**:2309–2317.
- Lewis, J. W., A. Neisig, J. Neefjes, and T. Elliott. 1996. Point mutations in the alpha 2 domain of HLA-A2.1 define a functionally relevant interaction with TAP. *Curr. Biol.* **6**:873–883.
- Li, S., K. M. Paulsson, H. O. Sjogren, and P. Wang. 1999. Peptide-bound major histocompatibility complex class I molecules associate with tapasin before dissociation from transporter associated with antigen processing. *J. Biol. Chem.* **274**:8649–8654.
- Lilley, B. N., and H. L. Ploegh. 2004. A membrane protein required for dislocation of misfolded proteins from the ER. *Nature* **429**:834–840.

36. Lindahl, K. F., D. E. Byers, V. M. Dabhi, R. Hovik, E. P. Jones, G. P. Smith, C. R. Wang, H. Xiao, and M. Yoshino. 1997. H2-M3, a full-service class Ib histocompatibility antigen. *Annu. Rev. Immunol.* **15**:851–879.
37. Lorenzo, M. E., J. U. Jung, and H. L. Ploegh. 2002. Kaposi's sarcoma-associated herpesvirus K3 utilizes the ubiquitin-proteasome system in routing class major histocompatibility complexes to late endocytic compartments. *J. Virol.* **76**:5522–5531.
38. Lybarger, L., X. Wang, M. R. Harris, H. W. Virgin, and T. H. Hansen. 2003. Virus subversion of the MHC class I peptide-loading complex. *Immunity* **18**:121–130.
39. Lybarger, L., Y. Y. Yu, T. Chun, C. R. Wang, A. G. Grandea III, L. Van Kaer, and T. H. Hansen. 2001. Tapasin enhances peptide-induced expression of H2-M3 molecules, but is not required for the retention of open conformers. *J. Immunol.* **167**:2097–2105.
40. Mansouri, M., E. Bartee, K. Gouveia, B. T. Hovey Nerenberg, J. Barrett, L. Thomas, G. Thomas, G. McFadden, and K. Fruh. 2003. The PHD/LAP-domain protein M153R of myxomavirus is a ubiquitin ligase that induces the rapid internalization and lysosomal destruction of CD4. *J. Virol.* **77**:1427–1440.
41. McCracken, A. A., and J. L. Brodsky. 2003. Evolving questions and paradigm shifts in endoplasmic-reticulum-associated degradation (ERAD). *Bioessays* **25**:868–877.
42. Means, R. E., S. Ishido, X. Alvarez, and J. U. Jung. 2002. Multiple endocytic trafficking pathways of MHC class I molecules induced by a herpesvirus protein. *EMBO J.* **21**:1638–1649.
43. Miley, M. J., S. M. Truscott, Y. Y. Yu, S. Gilfillan, D. H. Fremont, T. H. Hansen, and L. Lybarger. 2003. Biochemical features of the MHC-related protein 1 consistent with an immunological function. *J. Immunol.* **170**:6090–6098.
44. Ozato, K., T. H. Hansen, and D. H. Sachs. 1980. Monoclonal antibodies to mouse MHC antigens. II. Antibodies to the H-2Ld antigen, the products of a third polymorphic locus of the mouse major histocompatibility complex. *J. Immunol.* **125**:2473–2477.
45. Ozato, K., N. Mayer, and D. H. Sachs. 1980. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. *J. Immunol.* **124**:533–540.
46. Peace-Brewer, A. L., L. G. Tussey, M. Matsui, G. Li, D. G. Quinn, and J. A. Frelinger. 1996. A point mutation in HLA-A*0201 results in failure to bind the TAP complex and to present virus-derived peptides to CTL. *Immunity* **4**:505–514.
47. Petersen, J. L., C. R. Morris, and J. C. Solheim. 2003. Virus evasion of MHC class I molecule presentation. *J. Immunol.* **171**:4473–4478.
48. Pretell, J., R. S. Greenfield, and S. S. Tevethia. 1979. Biology of simian virus 40 (SV40) transplantation antigen (TrAg). V. In vitro demonstration of SV40 TrAg in SV40-infected nonpermissive mouse cells by the lymphocyte mediated cytotoxicity assay. *Virology* **97**:32–41.
49. Shamu, C. E., D. Flierman, H. L. Ploegh, T. A. Rapoport, and V. Chau. 2001. Polyubiquitination is required for US11-dependent movement of MHC class I heavy chain from endoplasmic reticulum into cytosol. *Mol. Biol. Cell* **12**:2546–2555.
50. Shamu, C. E., C. M. Story, T. A. Rapoport, and H. L. Ploegh. 1999. The pathway of US11-dependent degradation of MHC class I heavy chains involves a ubiquitin-conjugated intermediate. *J. Cell Biol.* **147**:45–58.
51. Smith, J. D., N. B. Myers, J. Gorka, and T. H. Hansen. 1993. Model for the in vivo assembly of nascent Ld class I molecules and for the expression of unfolded Ld molecules at the cell surface. *J. Exp. Med.* **178**:2035–2046.
52. Stevenson, P. G., J. M. Boname, B. de Lima, and S. Efstathiou. 2002. A battle for survival: immune control and immune evasion in murine gamma-herpesvirus-68 infection. *Microbes Infect.* **4**:1177–1182.
53. Stevenson, P. G., S. Efstathiou, P. C. Doherty, and P. J. Lehner. 2000. Inhibition of MHC class I-restricted antigen presentation by gamma 2-herpesviruses. *Proc. Natl. Acad. Sci. USA* **97**:8455–8460.
54. Stevenson, P. G., J. S. May, X. G. Smith, S. Marques, H. Adler, U. H. Koszinowski, J. P. Simas, and S. Efstathiou. 2002. K3-mediated evasion of CD8⁺ T cells aids amplification of a latent gammaherpesvirus. *Nat. Immunol.* **3**:733–740.
55. Story, C. M., M. H. Furman, and H. L. Ploegh. 1999. The cytosolic tail of class I MHC heavy chain is required for its dislocation by the human cytomegalovirus US2 and US11 gene products. *Proc. Natl. Acad. Sci. USA* **96**:8516–8521.
56. Swanson, R., M. Locher, and M. Hochstrasser. 2001. A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Mat α 2 repressor degradation. *Genes Dev.* **15**:2660–2674.
57. Tortorella, D., B. E. Gewurz, M. H. Furman, D. J. Schust, and H. L. Ploegh. 2000. Viral subversion of the immune system. *Annu. Rev. Immunol.* **18**:861–926.
58. Tortorella, D., C. M. Story, J. B. Huppa, E. J. Wiertz, T. R. Jones, I. Bacik, J. R. Bennink, J. W. Yewdell, and H. L. Ploegh. 1998. Dislocation of type I membrane proteins from the ER to the cytosol is sensitive to changes in redox potential. *J. Cell Biol.* **142**:365–376.
59. Van Parijs, L., Y. Refaeli, J. D. Lord, B. H. Nelson, A. K. Abbas, and D. Baltimore. 1999. Uncoupling IL-2 signals that regulate T-cell proliferation, survival, and Fas-mediated activation-induced cell death. *Immunity* **11**:281–288.
60. Wang, X., L. Lybarger, R. Connors, M. Harris, and T. H. Hansen. 2004. Model for the interaction of gammaherpesvirus 68 RING-CH finger protein mK3 with MHC-I and the peptide-loading complex. *J. Virol.* **78**:8673–8686.
61. Wiertz, E. J., T. R. Jones, L. Sun, M. Bogoy, H. J. Geuze, and H. L. Ploegh. 1996. The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell* **84**:769–779.
62. Wiertz, E. J., D. Tortorella, M. Bogoy, J. Yu, W. Mothes, T. R. Jones, T. A. Rapoport, and H. L. Ploegh. 1996. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature* **384**:432–438.
63. Williams, A., C. A. Peh, and T. Elliott. 2002. The cell biology of MHC class I antigen presentation. *Tissue Antigens* **59**:3–17.
64. Yang, M., S. Omura, J. S. Bonifacio, and A. M. Weissman. 1998. Novel aspects of degradation of T cell receptor subunits from the endoplasmic reticulum (ER) in T cells: importance of oligosaccharide processing, ubiquitination, and proteasome-dependent removal from ER membranes. *J. Exp. Med.* **187**:835–846.
65. Ye, Y., H. H. Meyer, and T. A. Rapoport. 2001. The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature* **414**:652–656.
66. Ye, Y., Y. Shibata, C. Yun, D. Ron, and T. A. Rapoport. 2004. A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature* **429**:841–847.
67. Yewdell, J. W., and A. B. Hill. 2002. Viral interference with antigen presentation. *Nat. Immunol.* **3**:1019–1025.
68. Yu, H., G. Kaung, S. Kobayashi, and R. R. Kopito. 1997. Cytosolic degradation of T-cell receptor alpha chains by the proteasome. *J. Biol. Chem.* **272**:20800–20804.
69. Yu, Y. Y., M. R. Harris, L. Lybarger, L. A. Kimpler, N. B. Myers, H. W. Virgin, and T. H. Hansen. 2002. Physical association of the K3 protein of gamma-2 herpesvirus 68 with major histocompatibility complex class I molecules with impaired peptide and β_2 -microglobulin assembly. *J. Virol.* **76**:2796–2803.
70. Yu, Y. Y., N. B. Myers, C. M. Hilbert, M. R. Harris, G. K. Balendiran, and T. H. Hansen. 1999. Definition and transfer of a serological epitope specific for peptide-empty forms of MHC class I. *Int. Immunol.* **11**:1897–1906.
71. Yu, Y. Y., H. R. Turnquist, N. B. Myers, G. K. Balendiran, T. H. Hansen, and J. C. Solheim. 1999. An extensive region of an MHC class I alpha 2 domain loop influences interaction with the assembly complex. *J. Immunol.* **163**:4427–4433.