

Ubiquitination of serine, threonine, or lysine residues on the cytoplasmic tail can induce ERAD of MHC-I by viral E3 ligase mK3

Xiaoli Wang,¹ Roger A. Herr,¹ Wei-Jen Chua,¹ Lonnie Lybarger,² Emmanuel J.H.J. Wiertz,³ and Ted H. Hansen¹

¹Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110

²Department of Cell Biology and Anatomy, University of Arizona Health Sciences Center, Tucson, AZ 85724

³Department of Medical Microbiology, Leiden University Medical Center, 2300 RC Leiden, Netherlands

The mechanism by which substrates for endoplasmic reticulum-associated degradation are retrotranslocated to the cytosol remains largely unknown, although ubiquitination is known to play a key role. The mouse γ -herpesvirus protein mK3 is a viral RING-CH-type E3 ligase that specifically targets nascent major histocompatibility complex I heavy chain (HC) for degradation, thus blocking the immune detection of virus-infected cells. To address the question of how HC is retrotranslocated

and what role mK3 ligase plays in this action, we investigated ubiquitin conjugation sites on HC using mutagenesis and biochemistry approaches. In total, our data demonstrate that mK3-mediated ubiquitination can occur via serine, threonine, or lysine residues on the HC tail, each of which is sufficient to induce the rapid degradation of HC. Given that mK3 has numerous cellular and viral homologues, it will be of considerable interest to determine the pervasiveness of this novel mechanism of ubiquitination.

Introduction

ER-associated degradation (ERAD) is an important component of ER quality control whereby unwanted proteins that are misfolded, misassembled, or metabolically excessive are recognized and returned to the cytosol by a process called retrotranslocation or dislocation (Hampton, 2002; Jarosch et al., 2002a; Tsai et al., 2002; Meusser et al., 2005). Once exposed to the cytosol, ERAD-targeted proteins are ubiquitinated and subsequently degraded by the cytosolic proteasome. The fact that dysfunction in ERAD causes human diseases (McCracken and Brodsky, 2003) and many viral proteins hijack this pathway to evade detection by the immune system (Ploegh, 1998; Lybarger et al., 2005) highlights its importance.

Since ERAD was first appreciated over a decade ago, several key players have been identified, particularly from a study of yeast (Meusser et al., 2005). However, our knowledge of how ERAD substrates are specifically recognized and extracted from the ER lumen remains incomplete. Highly relevant to this question, recent studies have demonstrated that distinct protein complexes are formed at the ER membrane that are involved in the

recognition, ubiquitination, and extraction of specific substrate classes (Carvalho et al., 2006; Denic et al., 2006). Although only a few have been implicated in ERAD, ubiquitin (Ub) E3 ligases clearly play a central role in the organization of different ER membrane complexes involved in ERAD of distinct substrate classes. For example, yeast E3 ligase Hrd1p/Der3p is a key component of a core membrane complex that processes substrates with luminal lesions, the so-called ERAD-L pathway. This core complex includes membrane protein Hrd3p (Vashist and Ng, 2004; Carvalho et al., 2006; Denic et al., 2006) that recruits luminal folding sensor Yos9p (Bhamidipati et al., 2005; Kim et al., 2005) as well as the membrane protein Ubx2p that recruits the cytosolic cdc48 ATPase complex (Schuberth and Buchberger, 2005). On the other hand, Doa10p, another well-characterized yeast E3 ligase implicated in ERAD, is a key and central component of a core membrane complex that processes ERAD substrates with lesions in their cytoplasmic domains, a so-called ERAD-C pathway (Vashist and Ng, 2004; Carvalho et al., 2006). This Doa10p complex includes Ubc7 and its membrane anchor Cue1 as well as cdc48 and its co-factors. However, the specific factors that are capable of recognizing the defect in the cytoplasmic tail of a substrate have not been defined. Nevertheless, substrate ubiquitination as specifically rendered by the E3 ligase is required for both pathways to completely remove the ERAD target from the ER by the cdc48

Correspondence to Ted H. Hansen: hansen@pathology.wustl.edu

Abbreviations used in this paper: 2ME, 2-mercaptoethanol; endo H, endoglycosidase H; ERAD, ER-associated degradation; HC, heavy chain; KSHV, Kaposi's sarcoma-associated herpesvirus; MHC, major histocompatibility complex; TAP, transporter associated with antigen processing; TMB, thrombin; Ub, ubiquitin; wt, wild type.

ATPase complex (Biederer et al., 1997; Jarosch et al., 2002b; Flierman et al., 2003). Within this basic framework of how different substrates are targeted for ERAD, several critical questions remain. For example, in the context of each pathway, (1) how do E3 ligases impose substrate specificity, and (2) at which step of ERAD does substrate ubiquitination occur?

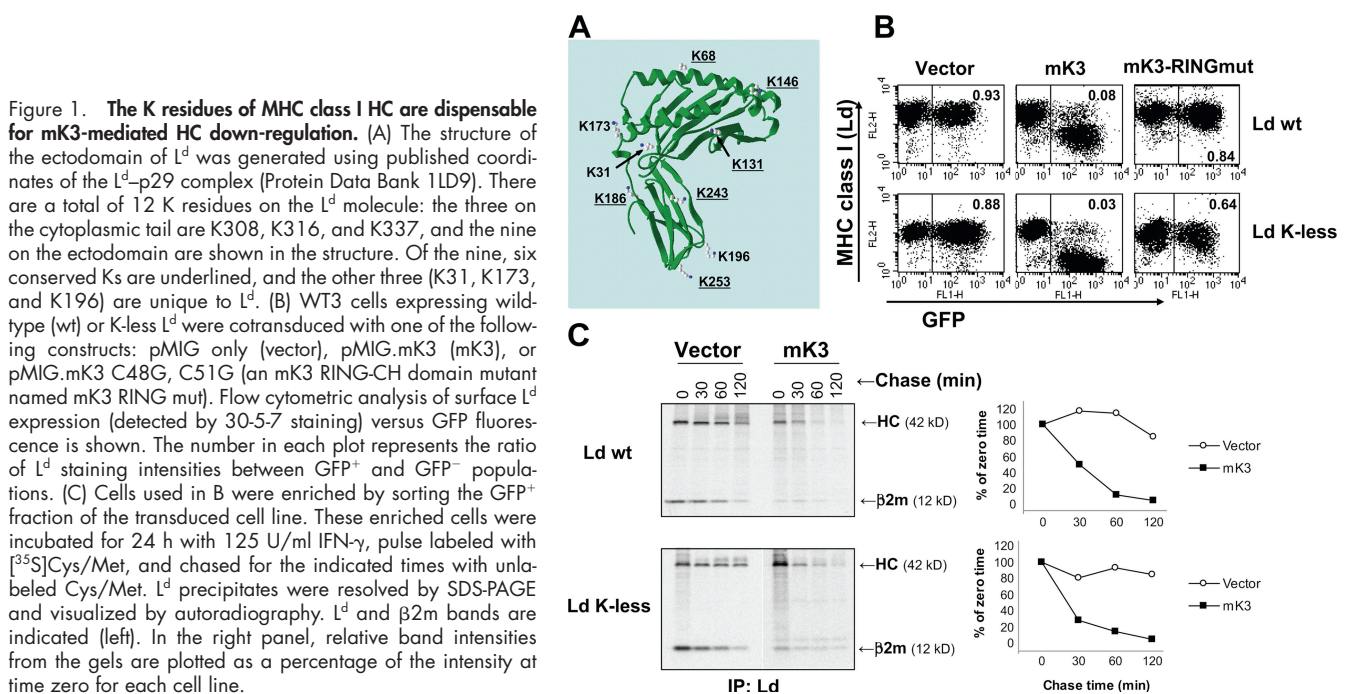
Whether different pathways defined in yeast such as the ERAD-L and ERAD-C pathways are conserved in mammals is not well established. However, the fact that most components of ERAD defined in yeast have functional homologues in mammals suggests evolutionary conservation. In agreement with this hypothesis, ER membrane core complexes, including E3 ligases that link ERAD substrates to ubiquitination and extraction machinery, have been defined in human cell studies (Lilley and Ploegh, 2005; Ye et al., 2005). However, the mammalian ERAD mechanism is clearly more complex. For example, three Der1p homologues have been defined in mammals, which are designated as Derlin1, 2, and 3. Derlin1 but not Derlin2 plays a central role in ERAD of major histocompatibility complex (MHC) class I heavy chain (HC) by human cytomegalovirus protein US11 (Lilley and Ploegh, 2004; Ye et al., 2004). In contrast, both Derlin2 and 3 are associated with EDEM (ER degradation-enhancing α -mannosidase-like protein) and p97 (cdc48 in yeast) and are functionally required for ERAD of NHK (null Hong Kong), a misfolded glycosylated luminal protein in the ER (Oda et al., 2006).

Higher eukaryotic cells presumably have many additional E3 ligases participating in ERAD compared with yeast. For example, mammals have a homologue of the yeast RING-H2-type E3 ligase Hrd1p called HRD-1 (Kaneko et al., 2002; Nadav et al., 2003; Kikkert et al., 2004). However, mammals have an additional RING-H2-type E3 ligase not found in yeast called gp78 (Fang et al., 2001; Liang et al., 2003). Interestingly, both HRD1 and gp78 are found in the same multiprotein ER membrane complex containing Derlin1 and p97 (Ye et al., 2005).

Whether they are responsible for distinct subsets of ERAD substrates or share the same substrates is not yet clear. Furthermore, multiple lines of evidence indicate that US2 and US11, two human cytomegalovirus-encoded immune evasion proteins, use distinct ERAD pathways to target HC for ERAD (Lilley and Ploegh, 2005; Hassink et al., 2006). However, neither HRD1 nor gp78 is required for US2- or US11-induced ERAD, suggesting that they may recruit novel E3s (Lilley and Ploegh, 2005; Ye et al., 2005). Collectively, these findings clearly suggest that mammals have evolved very specific and highly regulated ERAD pathways servicing distinct substrates.

Immune evasion protein mK3 is a viral E3 ligase encoded by mouse γ -herpesvirus 68 (Virgin et al., 1997). It was previously shown that mK3 specifically targets nascent HC for rapid degradation in an ubiquitination-proteasome-dependent manner (Boname and Stevenson, 2001; Yu et al., 2002). The RING-CH domain presumably conferring mK3 ligase activity is highly conserved among members of the K3 family, which includes a group of viral proteins encoded by herpesviruses and poxviruses (Coscoy and Ganem, 2003; Lehner et al., 2005). Cellular K3 homologues have also been detected, although their physiological function remains to be defined (Lehner et al., 2005). Interestingly, one of the cellular K3 homologues in human named MARCH VI (TEB4) has been recently identified as a homologue of Doa10p based on its ER localization and conserved cytosolic RING-CH domain with E3 ligase activity (Bartee et al., 2004; Hassink et al., 2005; Kreft et al., 2006). However, whether MARCH VI is a functional homologue of Doa10p remains to be determined.

We previously demonstrated that the specific recognition of HC by mK3 required its association with transporter associated with antigen processing (TAP; Yu et al., 2002; Lybarger et al., 2003), a transporter of peptides into the ER lumen (Pamer and Cresswell, 1998). mK3 is also physically associated with p97 and Derlin1, suggesting that mK3 exploits a physiological



ERAD pathway (Wang et al., 2006). Curiously, polyubiquitinated HCs detected in the presence of mK3 were membrane bound, but lysine (K) residues in the HC tail were not required for mK3-induced degradation (Wang et al., 2005). Based on these observations and similar findings with other ERAD substrates, a partial dislocation model was proposed whereby ectodomain residues of the substrate are ubiquitinated after exposure to the cytosol (Meusser and Sommer, 2004; Hassink et al., 2006). Recent findings also mandated that N-terminal or non-K forms of ubiquitination be considered (for review see Ciechanover and Ben Saadon, 2004; Cadwell and Coscoy, 2005).

In this study, we present unequivocal evidence that the cytoplasmic tail of HC is directly ubiquitinated in the presence of mK3 and that S or T residues are sufficient to induce the ubiquitination and rapid degradation of HC. These findings implicate a novel chemical mechanism of substrate ubiquitination via ester linkages. Mechanistically, our findings demonstrate that mK3-induced ubiquitination does not require partial dislocation of the N terminus of HC. Alternatively, our data support a model whereby mK3 substrates are dislocated via their C terminus after tail ubiquitination. These combined observations explain how the mK3 ligase can function as a central component of a core ER membrane complex constructed for the specific targeting of HC for ERAD.

Results

mK3-mediated MHC class I ubiquitination and rapid degradation are independent of luminal Ks

How ERAD substrates, especially ER membrane proteins, are extracted from the ER membrane remains elusive, although it has been known for a long time that ubiquitination of substrates is pivotal. We previously demonstrated that mK3 induces polyubiquitination and rapid degradation of HC with K-less tails (Wang et al., 2005). Given the fact that all of the functional ubiquitination components or domains, including mK3, are located outside of the ER, this finding and similar findings with other ERAD substrates (Shamu et al., 1999; Yu and Kopito, 1999; Meusser and Sommer, 2004) raised the possibility that K residues in the HC ectodomain were being ubiquitinated. The presumption is that ERAD substrates can be partially dislocated to the cytosol before ubiquitination. If this is the case for mK3-induced ERAD of HC, identifying which K residues in the ectodomain of HC are ubiquitinated would lend strong support to a partial dislocation model.

To determine whether and which K residues on the HC are required for ubiquitination, we took a mutagenesis approach using L^d as a prototypic MHC class I HC. As shown in Fig. 1 A, a total of nine Ks were replaced by arginines (Rs) singly or in groups based on HC domain structure. Because our previous study had shown that tail Ks were dispensable for mK3-mediated Ub conjugation and rapid degradation of HC (Wang et al., 2005), all ectodomain K mutations were introduced on a K-less tail template. Surface expression and TAP association were confirmed for all mutants to rule out gross misfolding. We next coexpressed each L^d K mutant with mK3 to test its capacity to

be mK3 regulated. The results were unequivocal and unexpected. None of the individual mutations, groups of mutations, or even totally K-less L^d were found to have a substantial impact on mK3-mediated HC down-regulation (unpublished data). For example, in the presence of mK3, surface K-less L^d was reduced >25-fold (Fig. 1 B) and rapidly degraded with kinetics similar to wild-type (wt) L^d (Fig. 1 C). Thus, mK3 down-regulation of HC is clearly not compromised by the absence of K residues in the substrate.

Down-regulation of K-less HC requires the Ub-proteasome pathway

To establish physiological relevance, we next tested whether the degradation of K-less HC was also dependent on Ub conjugation and proteasome activity. As expected, the surface down-regulation

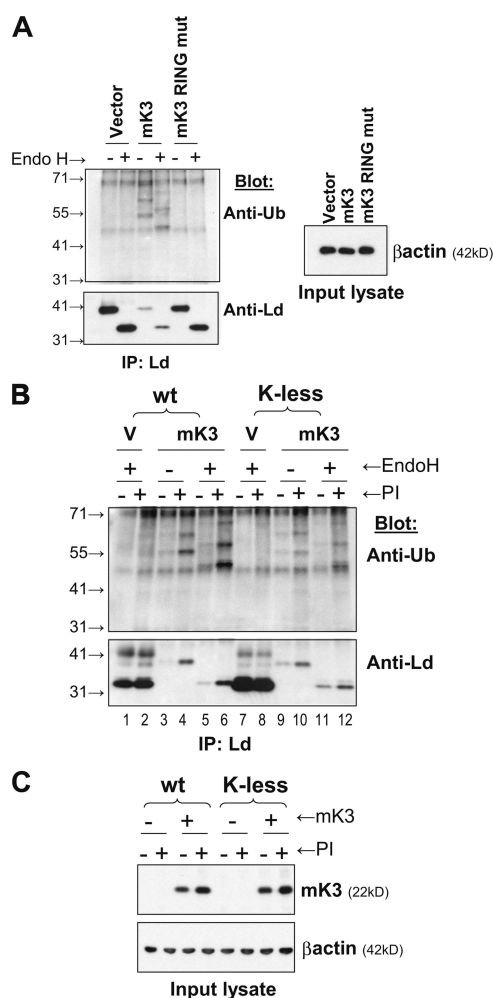


Figure 2. Down-regulation of completely K-less L^d is ubiquitination-proteasome dependent. (A) L^d HCs were immunoprecipitated with mAbs (30-5-7 and 64-3-7) from NP-40 lysates of the cell lines used in Fig. 1 B. Precipitates with or without endo H treatment were separated by SDS-PAGE and blotted for Ub and L^d HC. In the right panel, the β -actin blot was included as a loading control. (B) After incubation for 2 h with 5 μ M/ml of the proteasome inhibitor (PI) clasto-lactacystin β -lactone, cells expressing wt L^d or K-less L^d (\pm mK3) were harvested and NP-40 lysed. L^d HCs were precipitated, treated, or mock treated with endo H and were blotted with the indicated antibodies. (C) Lysates from B were blotted for mK3 (rabbit anti-mK3). The β -actin blot was included to control for equal loading.

and the polyubiquitination of K-less L^d were both detected only in the presence of wt mK3 but not the RING mutant (C48G, C51G mutations in its RING-CH domain; Figs. 1 B and 2 A, respectively). More importantly, the appearance of polyubiquitinated forms of K-less L^d in the presence of mK3 correlated with a lower steady-state level of unconjugated molecules. In contrast, in cells expressing the mK3 RING mutant, K-less L^d molecules were not Ub conjugated and were dramatically stabilized (Fig. 2 A). Thus, similar to wt L^d, down-regulation of K-less L^d was dependent on the polyubiquitination of HC facilitated by mK3. Moreover, compared with wt L^d, K-less L^d showed a strikingly similar pattern of ubiquitination (Fig. 2 B, lanes 9–12 vs. lanes 3–6). When treated with proteasome inhibitor, the polyubiquitinated conjugates of K-less L^d accumulated (Fig. 2 B, top; lanes 10 and 12 vs. lanes 9 and 11, respectively), and unconjugated K-less L^d was stabilized in cells expressing mK3 (Fig. 2 B, bottom; lanes 10 and 12 vs. lanes 9 and 11, respectively). These combined findings demonstrate that degradation of K-less HC, like wt HC, is ubiquitination and proteasome dependent. The implication of the aforementioned findings is that there must be sites other than the ε-NH₂ group of K on HC for mK3-induced ubiquitination. As recently reviewed, a few recent studies have suggested that the first Ub moiety can be fused linearly to the α-NH₂ group on the N-terminal residue of select substrates (for review see Ciechanover and Ben Saadon, 2004).

Fusion of a Ub moiety on the N terminus of HC does not induce ERAD of HC in the absence of mK3

To test the likelihood and consequences of the N-terminal ubiquitination of HC by mK3, four different Ub/HC fusion proteins

were made. More specifically, fusion proteins with either a wt or K-less Ub moiety were attached to the N terminus of either wt or K-less L^d. For these fusion proteins, the G76 residue of Ub was replaced with valine to avoid detection by deubiquitinating enzymes (Johnson et al., 1995; Baker, 1996; Dantuma et al., 2000). When expressed in WT3 cells, these fusion proteins (1) were glycosylated with N-linked endoglycosidase H (endo H)–sensitive glycans, (2) assembled normally with β2m, and (3) attained native class I folding based on their detection with conformation-dependent mAb. Thus, these fusion proteins clearly have the appropriate topology in the ER. Our rationale for this approach was the prediction that adding a K-less Ub to the N terminus might be unfavorable for polyubiquitination and ERAD based on the findings that the addition of large N-terminal tags (e.g., 6× myc or GFP) to the substrate prevented the degradation of proteins known to be capable of N-terminal ubiquitination (Ciechanover, 2005). Alternatively, fusion of a wt Ub moiety on HC might facilitate ERAD because it should be readily polyubiquitinated if or when it gains access to the cytosol. The rationale of making fusion proteins with K-less L^d as well as wt L^d was to determine whether the removal of Ks might promote N-terminal ubiquitination.

To test these possible scenarios, a pulse-chase experiment was conducted with cells expressing each of the four different fusion proteins to compare their relative stability in the presence or absence of mK3. As shown in Fig. 3 A, in the absence of mK3, both wt and K-less Ub/L^d fusion proteins were quite stable throughout the chase time (lanes 1–4; and not depicted). This demonstrates that N-terminal ubiquitination of HC alone is not sufficient to trigger a rapid degradation. A likely explanation is that the N terminus of HC does not gain access to the cytosol

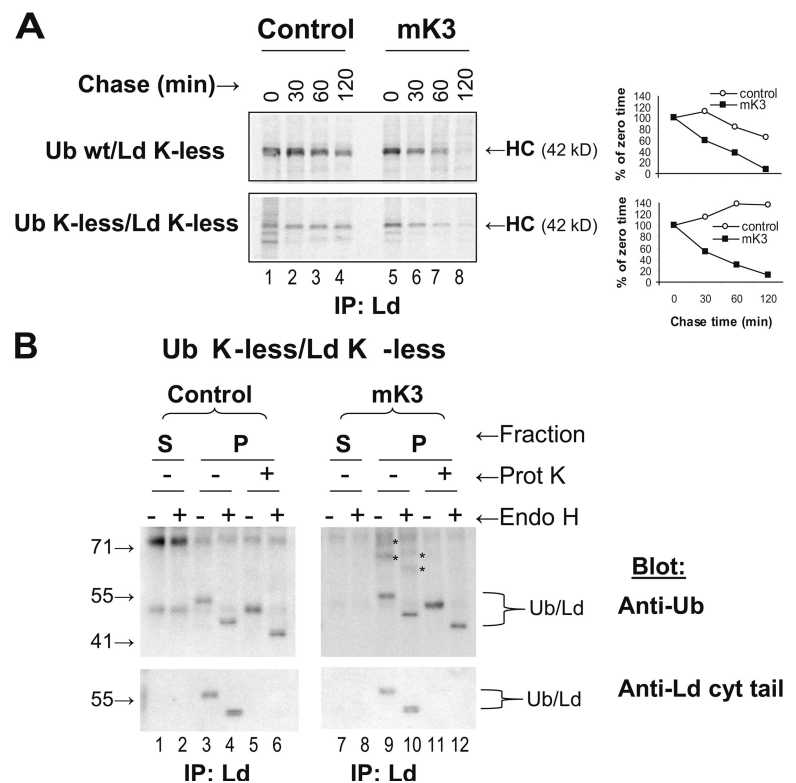


Figure 3. Ubiquitination and degradation of Ub/L^d fusion proteins in the presence of mK3. (A) After incubation for 24 h with 125 U/ml IFN-γ, WT3 cells expressing Ub wt/L^d K-less or Ub K-less/L^d K-less (±mK3) were pulse labeled with [³⁵S]Cys/Met for 15 min and chased for the indicated times. Ub/L^d fusion molecules were precipitated by anti-L^d mAbs. Precipitates then were resolved by SDS-PAGE and visualized by autoradiography (left). Relative band intensities from these gels were plotted as a percentage of the intensity at time zero for each line (right). (B) Cells used in A were Dounce homogenized. The homogenate was then subjected to serial centrifugations from 1,000 to 100,000 g. After digestion (or mock digestion) with 10 μg/ml proteinase K for 20 min on ice, Ub/L^d molecules were precipitated from NP-40 lysates of 100,000 g pellet (P) or supernatant (S) and analyzed by immunoblotting using anti-Ub mAb or rabbit anti-L^d cytoplasmic tail (cyt tail) antibodies. Polyubiquitinated forms are indicated by asterisks.

in the absence of mK3. However, in the presence of mK3, both wt Ub and K-less Ub/L^d fusion proteins were rapidly degraded (Fig. 3 A, lanes 5–8; and not depicted). More strikingly, all of these fusion proteins displayed similar ubiquitination patterns in cells with mK3 (unpublished data). These results argue against an N-terminal Ub conjugation. However, how these fusion proteins were polyubiquitinated, especially the one with K-less Ub fused to K-less L^d, remained unknown.

To solve this puzzle, a proteinase K assay was adopted to determine the site of the polyubiquitinated portion of completely K-less Ub/L^d fusion proteins. It was formally possible that the N terminus of K-less Ub/K-less L^d was polyubiquitinated after partial dislocation. If this was the case, the cytosolic-exposed N termini of fusion proteins as well as their poly-Ub forms would be sensitive to proteinase K digestion. To test this possibility, the ER fraction from cells expressing K-less Ub/K-less L^d was isolated by ultracentrifugation. The isolated ER fraction was then subjected to proteinase K digestion followed by immunoprecipitation of HC and blotting for Ub. As expected, Ub/L^d fusion HCs were observed only in the pellet fraction but not in the supernatant (Fig. 3 B), indicating that the Ub/L^d fusion protein is ER membrane bound, thus validating the ER isolation. Again, the poly-Ub bands were observed associated with HC precipitates in mK3-expressing cells but not in the cells without mK3 (Fig. 3 B, top; lanes 9 and 10 vs. lanes 3 and 4; poly-Ub forms are demarcated with asterisks). Also as expected, the polyubiquitinated and unconjugated HC bands were comparably sensitive to endo H, demonstrating that mK3 was modifying ER membrane-bound newly synthesized fusion proteins (Fig. 3 B, lane 10 vs. lane 9).

Upon digestion of the membrane fraction by proteinase K, domains exposed to the cytosol should be cleaved. Indeed, when treated with proteinase K, the K-less Ub/K-less L^d fusion protein was slightly reduced in size, resulting in a faster gel migration (Fig. 3 B, Ub blot; lanes 5 and 6 vs. lanes 3 and 4 and lanes 11 and 12 vs. lanes 9 and 10, respectively). Furthermore, the proteinase K-treated fusion protein was only detected by an antibody against Ub but not by an antibody to the L^d cytoplasmic tail (Fig. 3 B, lanes 11 and 12). Thus, proteinase K cleaved the HC tail of the fusion protein, demonstrating that it was in the cytosol, whereas the N-terminal Ub moiety was undisturbed, suggesting that it resided in the lumen. More strikingly, the poly-Ub conjugation bands also disappeared when the tail was cleaved by proteinase K (Fig. 3 B, Ub blot; lanes 11 and 12 vs. lanes 9 and 10), suggesting that the cytoplasmic tail of Ub/L^d fusion protein is the site of poly-Ub chain formation induced by mK3.

The cytoplasmic tail of HC is the site of Ub conjugation mediated by mK3

To rule out the possibility that the fusion proteins used an alternative pathway, a similar experiment was conducted with cells expressing either wt or K-less L^d. Again, we observed that poly-Ub forms of both wt and K-less L^d are ER membrane associated and disappear when the HC tail is removed (Fig. 4 A and not depicted), suggesting that for both Ub/L^d fusion protein and wt L^d, the cytoplasmic tail is the site of Ub conjugation. Although we

considered it unlikely, the possibility remained that proteinase K was removing poly-Ub from the N terminus of L^d or Ub/L^d that occurred after partial dislocation to the cytosol. If this was the case, the cytoplasmic tail would not have to be the site of ubiquitination. To further demonstrate that the tail is the site of ubiquitination, a thrombin (TMB) cleavage site, LVPRGG, was engineered into the tail of L^d right after the basic cluster KRRRNT proximal to the transmembrane domain. The K residue in this basic cluster was replaced by R. This molecule (L^d TMB) was normally glycosylated, β2m assembled, and folded when expressed in WT3 cells (unpublished data). In the presence of mK3, L^d TMB molecules were polyubiquitinated in a pattern similar to L^d (Fig. 4 B).

Lysates from cells expressing mK3 and wt L^d or L^d TMB were immunoprecipitated with antibodies specific for the luminal domain of L^d followed by TMB treatment. As expected, in the

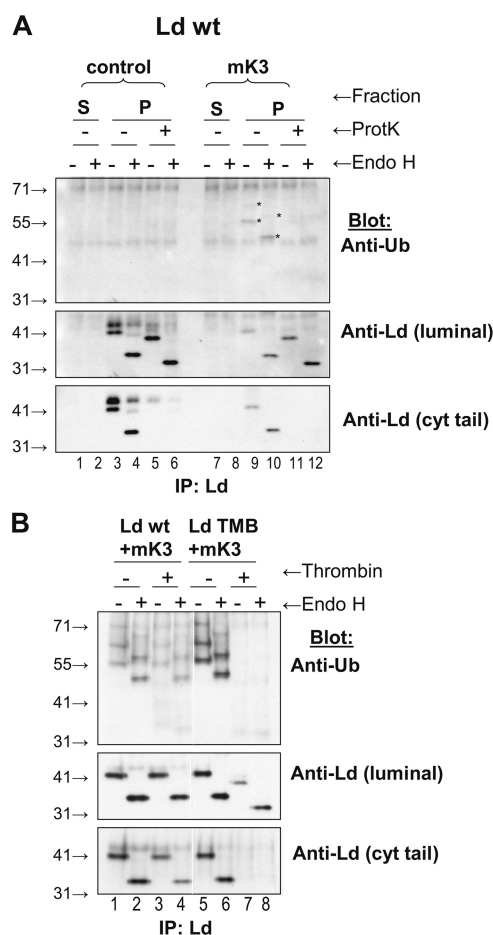


Figure 4. Polyubiquitinated L^d HCs were no longer detectable when the cytoplasmic tail of HC was removed. (A) Similar procedures as described in Fig. 3 B were used for analysis of the cells expressing wt L^d (±mK3). Precipitates of L^d HCs were immunoblotted by anti-Ub mAb, anti-L^d luminal mAbs (64-3-7), and anti-L^d cytoplasmic tail antibodies. Polyubiquitinated forms are indicated by asterisks. (B) L^d HCs were precipitated from NP-40 lysates of the cells coexpressing mK3 and wt L^d or mK3 and L^d TMB (wt L^d with a K308R mutation and an engineered TMB cleavage site in its cytoplasmic tail; the tail sequence of this molecule is VMRRRNTLVPRGGKGDYALAPGSQSSEMSLRDCKA, with the TMB cleavage site in bold). After incubation with TMB at room temperature for 3 h and then at 4°C overnight, the precipitates were analyzed by immunoblotting using the indicated antibodies.

presence of TMB, only the tails of L^d TMB molecules and not wt L^d were cleaved, as demonstrated by their faster migration and by blotting with the tail-specific antibody (Fig. 4 B, lanes 7 and 8 vs. lanes 5 and 6). In comparison, wt L^d molecules were resistant to the TMB treatment (Fig. 4 B, lanes 3 and 4 vs. lanes 1 and 2), indicating a specific and successful TMB cleavage. More importantly, along with the cleavage of the tail of L^d, the polyubiquitinated forms of L^d TMB also disappeared (Fig. 4 B, Ub blot; lanes 7 and 8). Because TMB treatment should not affect N-terminal polyubiquitination, these experiments along with the proteinase K experiments clearly defined the HC tail and not the N terminus as the site of ubiquitination. Importantly, the same observations were obtained with proteinase K–treated K-less L^d or tail K-less L^d TMB (unpublished data). Thus, the clear implication was that ubiquitination of HC by mK3 likely occurs on non-K residues. Notably, it was recently reported that the ubiquitination of class I HC induced by MIR1 (also called kK3), an E3 ligase of Kaposi’s sarcoma-associated herpesvirus (KSHV), requires a C residue in the cytoplasmic tail of MHC-I molecules (Cadwell and Coscoy, 2005).

Cysteine-conjugated ubiquitination is not required for mK3-mediated down-regulation of HC

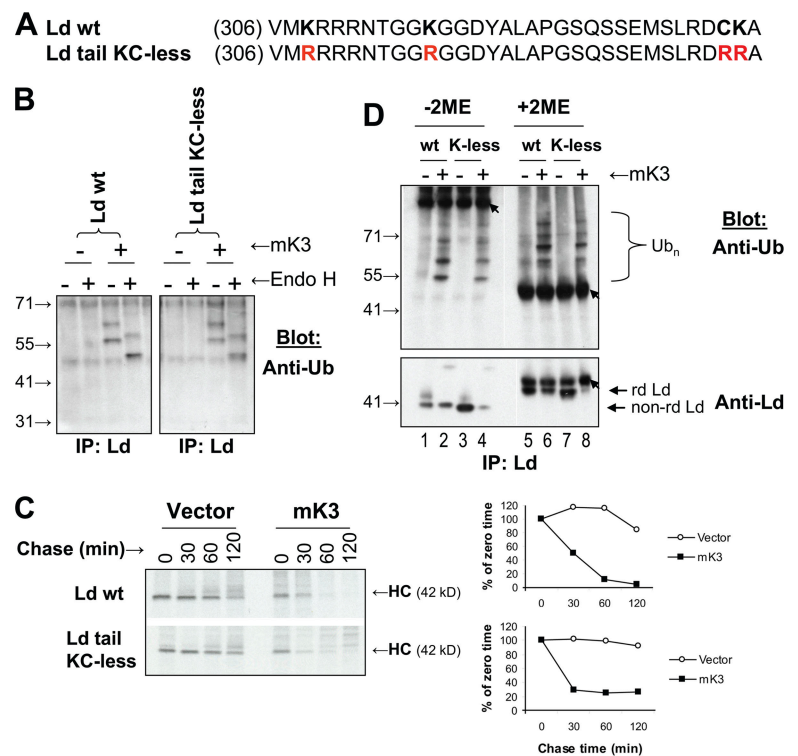
To test whether the mK3-induced ubiquitination of HC, like the MIR1-induced ubiquitination of HC, is dependent on a C residue in the tail of HC, a new L^d mutant was made. The L^d tail has a single C at position 336 that is conserved among most mouse HCs. Thus, to test the importance of the tail C residue, a C336S point mutation was introduced onto a tail K-less L^d template (Fig. 5 A). WT3 cells were cotransduced with this construct, termed L^d tail KC-less, and mK3. In the presence of mK3, this

KC-less mutant displayed prominent polyubiquitination (Fig. 5 B) and rapid degradation (Fig. 5 C) similar to wt L^d. Thus, unlike MIR1, a C residue in the HC tail is not required for HC to be a substrate for mK3-induced ubiquitination and degradation. To extend this finding and rule out the possibility that C residues in the HC ectodomain are required for mK3 ubiquitination, L^d HC molecules were immunoprecipitated and eluted in the presence of the thioester-reducing agent 2-mercaptoethanol (2ME; Fig. 5 D). It should be noted that this is the same treatment used to disrupt the aforementioned ubiquitination of HC by KSHV protein MIR1 (Cadwell and Coscoy, 2005). Dissociation of the Ig to heavy and light chains (Fig. 5 D, lanes 1–4 vs. lanes 5–8; indicated by arrows) was an internal control demonstrating the effectiveness of the reduction. However, comparison of the sample with and without 2ME treatment showed no reduction in the levels of polyubiquitination of wt or K-less L^d. These combined mutagenesis and chemical findings demonstrate that mK3-induced polyubiquitination does not require C residues in its HC substrate.

Direct ubiquitination of an S or T residue on the HC tail is sufficient to induce rapid degradation

Although it was predicted that they would have insufficient stability, T and S residues have hydroxyl groups that could potentially form ester bonds with Ub (Yu and Kopito, 1999; for review see Ciechanover and Ben Saadon, 2004). To test this hypothesis, we made a construct without any of the four potential residues in the tail for Ub conjugation. In total, three Ks, 4 Ss, 1 T, and 1 C in the tail of L^d were mutated (sequence shown in Fig. 6 A), but the ectodomain and transmembrane portion of wt L^d were unchanged. The resulting construct, termed KCST-less

Figure 5. The C residue in the tail of L^d HC is not required for its ubiquitination and rapid degradation mediated by mK3. (A) Sequence alignment of the cytoplasmic tails of wt L^d and a tail KC-less mutant is shown with wt residues in bold and substituted residues in red. (B) Polyubiquitination of L^d HCs in the cells expressing wt L^d or tail KC-less L^d (\pm mK3) was examined by immunoprecipitation of L^d HCs from NP-40 lysates (\pm endo H treatment) and immunoblotting for Ub. (C) After incubation for 24 h with 125 U/ml IFN- γ , cells used in B were pulse labeled with [³⁵S]Cys/Met for 15 min and chased for the indicated times. L^d HCs were precipitated, resolved, and visualized as described in Fig. 1 C. Relative band intensities from the gels are plotted as a percentage of the intensity at time zero for each line and are shown beside the gel photos. (D) After incubation with 60 μ M MG132 for 2 h, wt L^d HCs or K-less L^d HCs were precipitated by anti-L^d mAbs and eluted in the presence or absence of 5% 2ME at pH 8.0. Precipitates then were analyzed by immunoblotting for Ub and L^d HC. Ig bands are indicated by arrows; nonreduced or reduced (non-rd or rd) L^d HC bands and poly-Ub forms (Ub_n) are also indicated.



L^d tail, was stably expressed in WT3 cells with and without mK3. The ubiquitination status as well as the steady-state level of this L^d mutant was examined. As shown in Fig. 6 B, polyubiquitination of this KCST-less mutant was undetectable compared with wt L^d (left; third and fourth lanes vs. first and second lanes), although both cell lines expressed similar amounts of mK3 (Fig. 6 B, right). Concomitant with substantially diminished polyubiquitination, the KCST-less mutant was considerably more stable than wt L^d (Fig. 6 D). To rule out the possibility that resistance of this mutant to mK3 is the result of impaired mK3 interaction, we demonstrated that the KCST-less mutant coimmunoprecipitated with mK3 similar to wt L^d (unpublished data). Thus, the removal of Ks and all residues with the potential to form ester bonds from the HC tail did not affect its ability to associate with mK3, only its ability to be ubiquitinated by mK3. The fact that the polyubiquitinated forms disappeared when KCST residues were removed from only the tail of L^d and not the rest of the protein corroborated the conclusion that the tail is the site of ubiquitination. Furthermore, the fact that the KC-less tail mutant was ubiquitinated by mK3 but the tail KCST-less was not clearly implicated S and/or T residues in mK3 function.

We next compared the ability of individual residues to restore mK3-induced ubiquitination in the KCST-less tail. For these comparisons, one K (K337), one C (C336), one S (S329), or one T (T313) was added back to the KCST-less tail mutant of L^d (Fig. 6 A). These locations were selected because they are highly conserved among HC alleles. Because the T313 residue is membrane proximal, whereas the other potential Ub sites were clustered toward the C terminus of the tail, we also constructed a T337 mutant. Strikingly, a single S or T residue near the C terminus of the HC tail resulted in substantial polyubiquitination similar to the polyubiquitination of a single K in the presence of mK3 (Fig. 6, B and C; left). In contrast, the single C residue near the C terminus or the membrane-proximal T residue only showed very weak or undetectable ubiquitination. These findings suggested that hydroxylated amino acids (either S or T) can be sites of Ub conjugation but that location toward the C terminus of the tail is critical (Fig. 6, B and C; left). Importantly, similar levels of mK3 were expressed in these cells (Fig. 6, B and C; right). Consistent with Ub conjugation, the S329, T337, and K337 mutants were more rapidly degraded in mK3-expressing cells compared with the KCST-less tail mutant (Fig. 6 D).

The fact that the 1S or 1T (337) tail was polyubiquitinated and degraded in the absence of K residues largely ruled out the likelihood that S or T residues are required for other modifications, such as phosphorylation (Meusser and Sommer, 2004). However, the possibility remained that the observed ubiquitination of a K-less ERAD substrate might be the result of the ubiquitination of adaptor proteins (Yu and Kopito, 1999; Hassink et al., 2006). To definitively demonstrate that the HC is directly ubiquitinated, precipitates of the 1K or 1S L^d proteins were boiled in 0.5% SDS and 10 mM DTT and reprecipitated with antibody to denatured L^d. As shown in Fig. 7 A, polyubiquitination of 1K or 1S tails was unaffected by denaturation, whereas β2m assembly was eliminated. This result demonstrated that

either hydroxylated or amide amino acid side chains can be directly Ub conjugated by mK3. If this conclusion is true, the linkage between the hydroxyl side chain of S and the C-terminal glycine residue of Ub would be an ester bond. To test the existence of this linkage, we compared the stability of the bonds between Ub and 1K or 1S HCs after exposure to mild alkaline hydrolysis. It is known that ester bonds are more labile under these conditions than amide bonds, such as those formed between K residues and Ub (Hershko et al., 1980; Greene, 1981). Indeed, the ubiquitinated forms of the 1S mutant were undetectable upon incubation with either 1 M hydroxylamine, pH 9.0, or 0.1 M NaOH (Fig. 7 B, top and bottom, respectively). Similarly, ubiquitinated forms of the 1T(337) mutant were also labile to

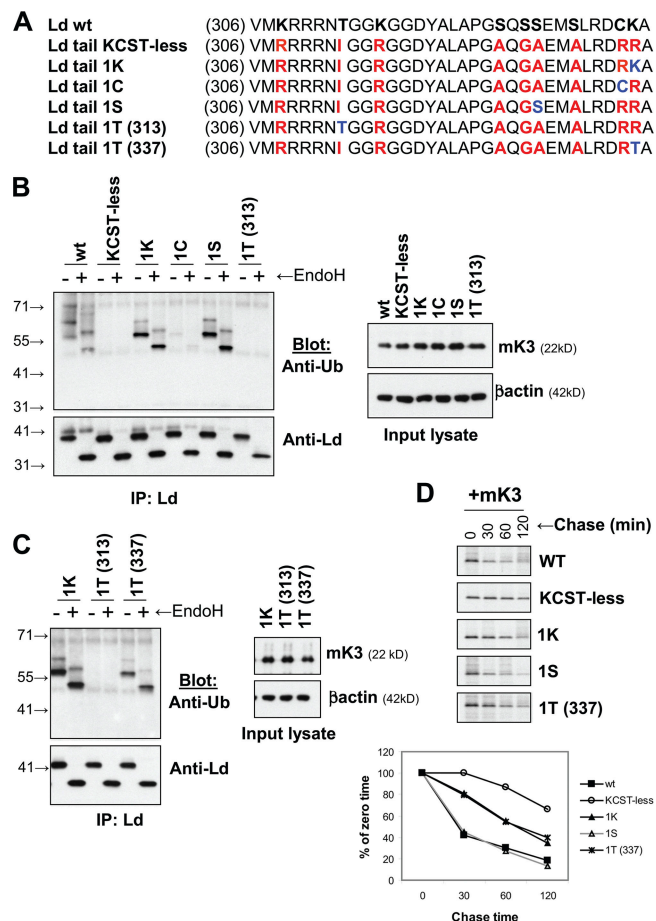


Figure 6. Ubiquitination of L^d by mK3 can occur on either S, T, or K residues on the tail of L^d. (A) Nomenclature and sequence alignment of the cytoplasmic tails of L^d tail mutants are shown with original wt residues in bold, substituted residues in red, and added back residues in blue. (B) WT3 cells stably coexpressing mK3 and one of the L^d tail mutants were NP-40 lysed. After precipitation of L^d, precipitates were immunoblotted for Ub and L^d (left). In the right panel, the β-actin and mK3 blots were included to show that a similar amount of input lysate of each line was used for immunoprecipitation and a similar amount of mK3 was expressed in each line. (C) Cells coexpressing mK3 and one of the L^d tail mutants 1K, 1T(313), or 1T(337) were lysed, immunoprecipitated, and blotted as described in B. (D) After incubation for 24 h with 125 U/ml IFN-γ, cells used in B and C were pulse labeled with [³⁵S]Cys/Met for 15 min and chased for the indicated times. L^d HCs were precipitated, resolved, and visualized as described in Fig. 1 C. Relative band intensities from the gels are plotted as a percentage of the intensity at time zero for each line and are shown beneath the gel photos.

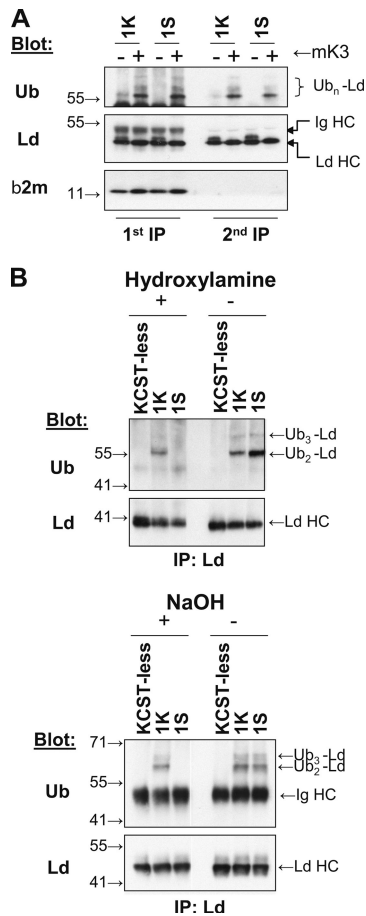


Figure 7. Evidence for the direct conjugation of Ub to an S residue of the L^d tail mediated by mK3. (A) L^d HCs were precipitated by anti-L^d mAbs 30-5-7 and 64-3-7 from NP-40 lysates of the cells expressing L^d tail 1K or L^d tail 1S mutant (\pm mK3). After incubation in 0.5% SDS and 10 mM DTT at room temperature for 10 min and boiling for 10 min, part of the elutes were reprecipitated by the conformation-independent anti-L^d mAb 64-3-7. Note that for this experiment, similar amounts of L^d were loaded for each sample. The first and second precipitates were immunoblotted for Ub, L^d, and β 2m. Ub-conjugated L^d forms (Ub_n-L^d) and unconjugated L^d HCs are indicated. Nonspecific staining of antibody heavy chain (Ig HC) is also indicated. (B) After incubation with 30 μ M MG132 for 3 h, the cells stably expressing L^d tail KCST-less, L^d tail 1K, or L^d tail 1S (\pm mK3) were lysed with NP-40. After precipitation of L^d, the precipitates were incubated with or without ($-/+$) 1 M sodium hydroxylamine, pH 9, for 4 h at 37°C (top) or with or without ($-/+$) 0.1 M NaOH for 2 h at 37°C (bottom). Then, the precipitates were subjected to SDS-PAGE and immunoblotting for Ub and L^d. Ub-conjugated L^d forms (Ub₂-L^d or Ub₃-L^d) and unconjugated L^d HCs are indicated. Because NaOH-treated samples were reduced by 2.5% 2ME before loading for SDS-PAGE, unconjugated and conjugated L^d HCs migrate slower than nonreduced sodium hydroxylamine-treated samples, and a disassociated Ig HC band can be seen in the blot.

mild alkaline treatment (unpublished data). As expected, the 1K mutant was stable to treatment with either reagent (Fig. 7 B). These data strongly support the hypothesis that mK3 is capable of facilitating the novel conjugation of ubiquitination to an S or T residue in the cytoplasmic tail of L^d via an ester linkage.

Discussion

The mechanism of how substrates are retrotranslocated or dislocated from ER membrane is still poorly understood. It is

known that ubiquitination plays a crucial role in this action, but when it happens and whether a direct Ub conjugation to the substrate is required are not clear. For soluble luminal substrates, mutations in the ubiquitination system result in substrates accumulating in the ER (Jarosch et al., 2002b), suggesting that the ubiquitination of substrates is required for their dislocation. However, because luminal substrates are separated from the ubiquitination apparatus by a membrane, the substrate ubiquitination obviously does not initiate their dislocation. On the other hand, for integral membrane proteins, one would think that its cytosolic portion might be the most convenient site for Ub conjugation, which could subsequently recruit Ub-binding protein and the ATPase complex required for complete extraction of the substrate. However, for several typical type I membrane protein substrates, such as orphan subunit TCR α and MHC-I HC in the presence of US2 or US11, K residues in the substrate's tail are dispensable for their dislocation (Shamu et al., 1999; Yu and Kopito, 1999; Furman et al., 2003). In the specific case of US2-induced ERAD of HC, internal K residues were found to be required for dislocation (Hassink et al., 2006). However, for the other aforementioned ERAD substrates, the replacement of all internal Ks on these ERAD substrates did not affect their dislocation, suggesting a non-K-mediated form of ubiquitination.

One possible explanation is N-terminal ubiquitination, in which the first Ub moiety is fused linearly to the α -NH₂ group of the substrate N terminus. This type of ubiquitination has been demonstrated for >12 proteins and, thus, provides an alternative mode of ubiquitination that may be particularly important for the regulation of proteins that have no accessible K residues (Ciechanover, 2005). In ERAD, however, such a process has not been well established, especially for soluble luminal substrates or type I membrane substrates with their N termini in the ER lumen. For these types of substrates, a partial dislocation of their N termini to the cytosolic side is presumably required before the addition of Ub because all of the functional ubiquitination components are located in the cytosol. Recently, Hassink et al. (2006) attached Ub to the N terminus of wt and K-less HCs and found that it did not accelerate their dislocation in the presence of US11. This finding suggests that N-terminal ubiquitination does not play a role in US11-initiated extraction of HCs from the ER membrane.

An alternative explanation for the dislocation of K-less membrane proteins is that an adaptor protein is ubiquitinated instead of direct ubiquitination of the substrate. Ubiquitination on an adaptor could link the associated substrate with the extraction machinery, resulting in complete extraction of the substrate. Interestingly, this mechanism is supported by recent studies of the dislocation of K-less HC by US11 (Hassink et al., 2006). However, the ultimate confirmation for such a model will have to await identification of the ubiquitinated adaptor protein. An important consideration regarding conclusions using K-less substrates is whether ubiquitination can occur via non-K residues. For example, it has been reported that MIR1 ligase (also called kK3) of KSHV can ubiquitinate C residues in the HC tail via thioester bonds, thus targeting it for degradation through an endocytic-lysosome pathway (Cadwell and Coscoy, 2005).

In the case of mK3-induced HC ubiquitination and degradation, we previously showed that the substitution of all Ks in the tail of MHC-I HC did not affect the ubiquitination status or the degradation of these molecules (Wang et al., 2005). Nevertheless, deletion of the tail abolished the ubiquitination of HC, leading to its stabilization in the ER (Wang et al., 2005). Indeed, a similar phenotype has also been found in the US11-induced turnover of MHC-I HC (Story et al., 1999; Barel et al., 2003). Our erroneous explanation for these observations was that the tail of HC is required for the initiation of dislocation but not for direct tail ubiquitination.

In the present study, a complete K-less L^d and a K-less Ub/K-less L^d fusion protein were found to be polyubiquitinated and rapidly degraded in the presence of mK3. This finding suggested that neither luminal Ks nor an α -NH₂ group of the N terminus of HC are essential Ub conjugation sites required by mK3. More importantly, we demonstrated that polyubiquitinated HCs become undetectable when the tail was removed by proteinase K digestion or via an engineered TMB cleavage site. These findings demonstrated that the HC tail is the site of ubiquitination. Of note, these observations were also made with wt L^d molecules, demonstrating that it is a normal process used by native protein and not merely a forced pathway used by substrates having no available Ks. Furthermore, we found that mutations of all K and C residues of the L^d tail did not affect mK3-induced ERAD. However, the additional removal of S and T residues in the tail abolished mK3-induced polyubiquitination and stabilized the HC. These findings suggested that one or both of the hydroxylated amino acids were sufficient for ubiquitination.

To extend these observations using a better defined approach, individual K, C, S, and T residues were reintroduced into L^d with a KCST-less tail. Remarkably, HCs with tails having only one S, T(337), or K were polyubiquitinated and were susceptible to ERAD in the presence of mK3. However, location was found to be important in that all efficacious ubiquitination sites were clustered toward the C terminus of the HC tail. In contrast, a tail with a membrane-proximal T residue displayed no detectable ubiquitination. S and T residues are potential phosphorylation sites that could be required to recruit a putative HC-associated protein that is ubiquitinated. To demonstrate that HC is itself ubiquitinated, HCs with single S or K in their tails were denatured and reprecipitated with a class I-specific mAb. In both cases, ubiquitinated HCs were still detected after treatment, demonstrating that Ub moieties can be covalently attached to either S, T, or K residues in the HC tail in the presence of mK3. In strong support of this conclusion, 2-ME that specifically disrupts thioester bonds formed with C residues had no effect on the Ub conjugation to S or K residues of the HC tail in the presence of mK3. Alternatively, mild alkaline hydrolysis with sodium hydroxylamine or NaOH, reagents that are effective in cleaving ester bonds and not amide bonds, removed detectable mK3-mediated ubiquitination via S or T but not K residues. Thus, in the presence of mK3, ester bonds are formed that covalently link Ub to S or T residues in the tail of the HC substrate, thereby defining a novel form of ubiquitination. It will be interesting to determine whether the ability to use S and T

as well as K residues reflects a unique, direct interaction of mK3 with the HC substrate or, alternatively, may involve distinct cellular components such as E2 conjugation enzymes or other necessary cofactors.

The ubiquitination pattern induced by mK3 is somewhat surprising. Even when the HC substrate has a wt tail, the Ub₂ and Ub₃ forms are predominant. It is noteworthy that Boname and Stevenson (2001) also detected HCs with predominantly Ub₂ and Ub₃ forms and few, if any, higher than Ub₃ in the presence of mK3. Because the proteasome requires Ub₄ to provide sufficient avidity for binding substrates for degradation (Thrower et al., 2000), we have considered the notion that multimers higher than Ub₃ may be unstable and, thus, difficult to detect in steady-state assays. Indeed, when proteasome inhibitor was used, Ub₄ and Ub₅ multimers can be detected (Figs. 2 B and 5 D). However, proteasome inhibitors result in only modest increases in forms higher than Ub₃, which is likely the result of the fact that the 2–3-h treatment does not appreciably affect steady-state levels of ubiquitination. Interestingly, tails containing a single S, T, or K residue displayed an even greater predominance of Ub₂ forms, although Ub₃ and higher multimers show up on longer exposure. In any case, HC tails with only one S, T, or K residue are viable ERAD substrates in the presence of mK3, implying they are polyubiquitinated to Ub₄. It is also interesting that in the presence of mK3, the minimum ubiquitination forms of HCs are Ub₂, regardless of whether it is a wt or tail 1S, 1T, or 1K substrate. This may imply that mK3 facilitates the transfer of a premade Ub₂ or Ub₃ chain from cognate E2 to the substrates.

The findings reported in this study clarify the molecular basis of mK3 substrate specificity. Our previous study failed to detect an HC-specific tail sequence required for mK3 interaction (Wang et al., 2004). The finding of alternative sites for mK3-mediated ubiquitination reported here is consistent with this conclusion. Interestingly, the primary binding partner for mK3 is TAP, the transporter responsible for dislocating proteasome-processed peptides into the ER lumen, and a mutagenesis study showed that it is the C terminus of mK3 that binds TAP (Wang et al., 2004). Most class I HC alleles require a physical association with TAP after assembly with β 2m and before the binding of high affinity peptide ligands (Grandea et al., 2000). These combined findings suggest a proximity model whereby the specific recognition of HC by mK3 is achieved by TAP binding to the mK3 C terminus, which then orients the N-terminal mK3 RING domain such that it can only interact with the HC tail. Thus, TAP is the key mK3 adaptor protein juxtaposing the mK3 RING domain with the HC tail to confer substrate specificity. The demonstration here of the importance of the location of ubiquitination sites within the tail lends strong support to this proximity model. This model provides a viable general strategy for allowing E3s to maintain their requisite substrate specificity while facilitating the ubiquitination of a highly polymorphic substrate such as HC. With the required juxtaposition of mK3 RING and HC tail, the necessary cellular ubiquitination components, including an appropriate E2, can then be recruited, resulting in ubiquitination of the HC tail. This core ER membrane complex centered on mK3 can also include Derlin1, although

it appears to have a redundant function (Wang et al., 2006; and unpublished data). ATPase p97 is also recruited to this mK3 core membrane complex, and this association clearly has an impact on mK3-induced ERAD (Wang et al., 2006).

The aforementioned findings suggest that the viral mK3 ligase assimilates a core membrane complex in mouse cells similar to ERAD complexes described in yeast. In this context, it is interesting to compare properties and outstanding questions regarding the mK3 membrane complex with the aforementioned yeast Doa10p (ERAD-C) and Hrd1p (ERAD-L) complexes (Vashist and Ng, 2004; Carvalho et al., 2006; Denic et al., 2006). HC tail ubiquitination by mK3 suggests that it mimics an ERAD-C pathway induced by substrates with a cytosolic lesion (Vashist and Ng, 2004; Carvalho et al., 2006), and ERAD-C does not require Der1p, as mK3 does not require the Der1p homologue Derlin1 (Wang et al., 2006; and unpublished data). Models such as ERAD-C and the mK3 model would not require the partial dislocation of substrate before ubiquitination, and polyubiquitination of the tail could be the initiating signal for dislocation. It is intriguing to speculate that mK3 may also be a component of the dislocation channel, if indeed one is required for this pathway. In the context of an ERAD-L-like model, it is unclear whether molecular interactions are required in the ER lumen to promote substrate dislocation. Although mK3 has only 12 amino acids in the ER lumen, it could disrupt transmembrane interactions between proteins within the core membrane complex, and this disruption could induce ER protein folding sensors (chaperones) to bind the ectodomain of HC and facilitate dislocation. Defining the molecular composition of the dislocation pore and the signals that initiate substrate dislocation are key questions for any of these Ub ligase-induced membrane complexes involved in distinct ERAD pathways. In any case, findings with viral ligase mK3 suggest that it can assemble a unique core ER membrane complex that specifically detects MHC class I HCs and targets them for ERAD.

In summary, we report that the mK3 ligase directly ubiquitinates its HC substrate at tail S, T, or K residues. This observation obviates the need for the partial dislocation of HC before ubiquitination, suggesting a vectorial exit from the ER. It will be interesting to determine whether other ERAD pathways involving transmembrane protein substrates might also involve tail ubiquitination using non-K residues. Furthermore, the fact that mK3 has numerous viral (including MIR1) and cellular homologues makes it attractive to speculate that other ubiquitination-regulated processes use similar nonconventional methods of Ub conjugation.

Materials and methods

Cell lines and flow cytometry

Mouse B6/WT3 (WT3, *H-2^d*) cells were described previously (Wang et al., 2004). 293T cells (DuBridgely et al., 1987) were used for the production of ecotropic retrovirus. All cells were maintained in complete RPMI 1640 with 10% FCS (HyClone) as described previously (Lybarger et al., 2003). Retrovirus-containing supernatants were produced using the Vpack vector system (Stratagene) with transient transfection of 293T cells. Cells transduced by the pMIN-containing virus were enriched by genetic selection, whereas GFP⁺ cells from pMIG-transduced lines were enriched by cell sorting. Where indicated, cells were cultured for 24 h with 125 U/ml of mouse

IFN- γ (Biosource International) and for 2–3 h with 30–60 μ M of the proteasome inhibitor MG132 (Boston Biochem) or 5 μ M clasto-lactacystin β -lactone (Calbiochem) before harvesting with trypsin-EDTA. All flow cytometric analyses were performed as previously described (Lybarger et al., 2003).

DNA constructs

Two retroviral expression vectors, pMSCV.IRES.GFP (pMIG) and pMSCV.IRES.neo (pMIN; Wang et al., 2004), were used to express mK3 and L^d constructs, respectively. mK3 sequence was obtained by PCR amplification of the K3 gene from a γ HV68 subclone (Virgin et al., 1997). Both mK3 and L^d mutants were generated by site-directed mutagenesis (Stratagene). Ub/L^d fusion constructs were made by overlapping PCR, wherein the uncleavable wt and K-less Ub sequences (wt Ub and K-less Ub, with replacement of glycine 76 to valine) were PCR amplified from the constructs pLZRS-Ub G76V-HLA.A2 and pLZRS-Ub_{K_R} G76V-HLA.A2 (Hassink et al., 2006). The correct sequences for all of the constructs were confirmed by DNA sequencing.

Antibodies

Rabbit anti-mK3, Ub antibodies, β -actin (AC-74) antibodies, and mAbs 30-5-7 and 64-3-7 to folded and open forms of MHC class I L^d were previously described (Lybarger et al., 2003). Antibodies (Ra20873) to the cytoplasmic tail of L^d were produced in rabbits immunized with the cytoplasmic tail peptide (Smith et al., 1995).

Immunoprecipitation and immunoblots

Immunoprecipitation and immunoblotting were conducted as previously described (Lybarger et al., 2003). In brief, cells were lysed in PBS buffer containing 1% NP-40, 20 mM iodoacetamide (Sigma-Aldrich), and Complete mini protease inhibitors (Roche) or 0.4 mM PMSF (Sigma-Aldrich). Postnuclear lysates were incubated with protein A-Sepharose beads (Sigma-Aldrich) and antibodies. After washing beads four times with PBS/iodoacetamide buffer containing 0.15% NP-40, immunoprecipitates were eluted from protein A by boiling for 3 min in lithium dodecyl sulfate sample buffer (Invitrogen). For endo H treatment, immunoprecipitates were eluted in 10 mM Tris-HCl, pH 6.8, with 0.5% SDS, and the elutes then were mixed with an equal volume of 100 mM sodium acetate, pH 5.4, and incubated with 1 μ U endo H (MP Biomedicals) at 37°C for 2 h. Immunoblotting was performed after SDS-PAGE separation of precipitated proteins or cell lysates as previously described (Yu et al., 2002). Specific proteins were visualized by chemiluminescence using the ECL system (GE Healthcare).

Metabolic labeling and pulse chase

After 30 min of preincubation in Cys- and Met-free medium (MEM-Earle's with 5% dialyzed FCS), cells were pulse labeled with Express [³⁵S]Cys/Met labeling mix (Perkin Elmer) at 150 μ Ci/ml for 10–15 min. Chase was initiated by the addition of an excess of unlabeled Cys/Met (5 mM each). Immunoprecipitation was performed as described in the previous section. Samples were subjected to SDS-PAGE, and gels were treated with Amplify (GE Healthcare), dried, and exposed to BioMax-MR film (Kodak).

ER isolation and proteinase K digestion

Cells were incubated in cold hypotonic extraction buffer containing 10 mM Hepes, pH 7.8, 25 mM KCl, and 1 mM EGTA for 20 min and were resuspended in isotonic extraction buffer containing 10 mM Hepes, pH 7.8, 250 mM sucrose, 25 mM KCl, and 1 mM EGTA before they were broken with 20 strokes of Dounce homogenizer on ice. The homogenate was then subjected to serial centrifugations at 1,000 g for 10 min, 10,000 g for 10 min, and 100,000 g for 60 min, which were all performed at 4°C. The 100,000 g pellet was resuspended in PBS with 20 mM iodoacetamide. Two aliquots of the suspension were incubated on ice with or without 10 μ g/ml proteinase K (Invitrogen) for 20 min. After the digestion was stopped by 2 mM PMSF, NP-40 was added to a final concentration of 1% to disrupt the membranes. PMSF and NP-40 were also added into 100,000 g supernatant fractions. Subsequent immunoprecipitation and immunoblotting were performed as described above in the Immunoprecipitation and immunoblots section.

Sodium hydroxylamine and sodium hydroxide treatment

Immunoprecipitates were boiled in 0.5% SDS for 3 min followed by incubation in either 1 M sodium hydroxylamine, pH 9, for 4 h at 37°C or sodium hydroxide (0.1 M NaOH) for 2 h at 37°C. Mock-treated samples were incubated with PBS. Before SDS-PAGE analysis, the sodium hydroxylamine-treated samples were dialyzed against PBS overnight at 4°C using 3500 MWCO MINI dialysis units (Pierce Chemical Co.).

We thank the monitoring editor, Dr. David Ron, for his suggestions of chemical approaches to distinguish ester from amide bonds.

This work was supported by National Institutes of Health grants AI19687 (to T.H. Hansen and X. Wang) and AI60723 (to L. Lybarger) and by Dutch Cancer Society grant UL 2005-3259 (To E.J.H.J. Wiertz).

Submitted: 13 November 2006

Accepted: 18 April 2007

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