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Cutting Edge: Selective Role of Ubiquitin in MHC Class I Antigen Presentation

Lan Huang, Julie M. Marvin,1 Nia Tatsis,2 and Laurence C. Eisenlohr

The importance of ubiquitination in MHC class I-restricted Ag processing remains unclear. To address this issue, we overexpressed wild-type and dominant-negative lysineless forms of ubiquitin (Ub) in mammalian cells using an inducible vaccinia virus system. Overexpression of the lysineless Ub nearly abrogated polyubiquitination and potently inhibited epitope presentation from a cytosolic N-end rule substrate as well as endoplasmic reticulum (ER)-targeted model Ags. These trends were location dependent; redirecting cytosolic Ag to the ER rendered presentation lysineless Ub-sensitive, whereas retargeting exocytic Ag to the cytosol had the inverse effect. This dichotomy was further underscored by small interfering RNA knockdown of the ER-associated Ub ligase Hrd1. Thus, Ub-dependent degradation appears to play a major role in the MHC class I-restricted processing of ER-targeted proteins and a more restricted role in the processing of cytosolic proteins. The Journal of Immunology, 2011, 186: 1904–1908.

The multicatalytic proteasome is involved in most cytosolic proteolysis, and increasing evidence indicates its indispensable role in production of most MHC class I-restricted antigenic peptides (1). The 26S proteasome is comprised of the 20S catalytic core capped at both ends with 19S regulator complexes that bind and unfold polyubiquitinated substrates (2). Although ubiquitin (Ub)-mediated degradation is critical for cellular viability and was originally considered to be the only means of proteasomal destruction, it is now generally accepted that a substantial portion of intracellular proteolysis is Ub independent (3). Other modifications have been shown to target substrates to the 26S proteasome (4, 5), and self-directed delivery to the 20S core has been described (6). In our own system, we observed that a large fragment of influenza nucleoprotein (NP) is efficiently presented even after every lysine, the target of conventional ubiquitination, had been substituted (7).

Most insights into the ubiquitination pathway have been gained with yeast- and cell-free systems. It remains challenging to study protein ubiquitination in higher eukaryotes in part because selective inhibitors of the pathway are unavailable. Use of a temperature-sensitive ubiquitination mutant in class I-processing studies has led to conflicting results (8, 9), possibly because a fraction of the temperature-sensitive protein (the E1 activating enzyme) is functional at the restrictive temperature (10). This approach is further complicated by the existence of a second E1 enzyme that may have redundant activity (11).

Based upon the rapid kinetics with which MHC class I-restricted peptides can be generated (12) and the increasingly apparent diversity of proteasome-based degradation, we have proposed a model in which nascent cytosolic polypeptides that fail to be engaged by the intracellular folding machinery are delivered to the 20S proteasome for immediate destruction (13). We also speculated that this might not apply to endoplasmic reticulum (ER)-targeted Ags because they are subjected to ER-associated degradation (ERAD), which generally entails a quality control decision followed by retrotranslocation, polyubiquitination, and degradation by the 26S proteasome (14). To explore these issues, we generated a vaccinia virus (VV) recombinant that conditionally expresses high levels of a dominant-negative lysineless Ub (UbK0), thereby interfering with polyubiquitination.

Materials and Methods

VV recombinants

Wild-type Ub (UbWT) and UbK0 genes (a generous gift from Dr. Bruce E. Clurman, Fred Hutchinson Cancer Research Center) with hemagglutinin (HA) epitope tags at the N terminus were constructed by standard PCR methods. Isopropyl β-D-thiogalactopyranoside (IPTG)-inducible VV recombinants were generated as described (15). Other Ag-expressing VVs have been described (16–18) and are shown in Fig. 1A.

Viral infection

Cells were infected with inducible VV recombinants for 1 h and then incubated in complete medium throughout the remainder of the assay. IPTG (Calbiochem) was used at a final concentration of 2 mM during the entire period of incubation.

The online version of this article contains supplemental material.

Abbreviations used in this article: ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; HA, hemagglutinin; IB, immunoblotting; IPTG, isopropyl β-D-thiogalactopyranoside; MFI, mean fluorescence intensity; m/S, minigene construct, expressing the minimal SBIF4KL epitope; NP, nucleoprotein; siRNA, small interfering RNA; TM, transmembrane; Ub, ubiquitin; UbK0, lysineless ubiquitin; UbWT, wild-type ubiquitin; VV, vaccinia virus.

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met/Cys. The degradation of Ub-Arg-cytoNP protein was determined by immunoprecipitation andautoradiography. S, OVA257–264, SP, signal peptide.

UbWT or UbK0 virus and Ub-Arg-cytoNP virus, all at 3 PFU/cell for 7 h in the presence of IPTG. The infected cells were then metabolically labeled with [35S]IPTG-induced expression of HA-tagged Ub.

expressing UbWT, UbK0, or no insert (control) at 3 PFU/cell in the presence or absence of IPTG for 12 h. IB analyses with anti-HA Ab were performed to detect

citric acid, 0.061 M Na2HPO4, 0.15 M NaCl [pH 3]) at 5.5 h postinfection epitope generation, the infected cells were treated with citrate buffer (0.13 M

to remove surface MHC class I peptides and cultured for an additional 12 h before flow cytometry analysis.

Immunoblotting analysis
Laemmli SDS-PAGE and transfer to nitrocellulose membrane were performed as previously described (19). The protein bands were blotted with the primary Abs including anti-HA (Roche), anti-polyubiquitinated proteins (FK1; Bio- mol), anti-β-actin (Sigma-Aldrich), and anti-Hrd1 (Abgent). The secondary Abs were HRP-conjugated anti-mouse IgG (HA), anti-mouse IgM (FK1), and anti-rabbit IgG (β-actin, Hrd1), respectively.

Pulse-chase assay
Metabolic labeling and immunoprecipitation were performed as previously described (20). Gels were dried and exposed to a Storage Phosphor Screen (Molecular Dynamics). Signal intensities were visualized on the Storm 860 fluorescent scanner and quantified using ImageQuant software (Molecular Dynamics).

RNA interference
 Knockdown of Hrd1 was carried out with a pre-designed small interfering RNA (siRNA) targeting Hrd1 or a control siRNA (Ambion) using Lipofectamine RNAiMax (Invitrogen) at a final concentration of 10 nM.

Flow cytometry
OVA257–264 epitope presentation was assessed by surface staining with 25D1.16 Ab specific for the K\(^{b}\)/OVA257–264 complex as previously described (16). The mean fluorescent intensity (MFI) of OVA257–264 presentation was normalized by MFI from the control virus without Ag expression. The inhibitory effect of Hrd1 siRNA on OVA257–264 presentation was determined by referencing to normalized MFI from UbWT infection. The inhibitory effect was calculated as the ratio of the MFI of OVA257–264 presentation in the presence of Hrd1 siRNA to the MFI of OVA257–264 presentation in the absence of Hrd1 siRNA.

Statistics
Statistical analysis and graphing was performed with GraphPad Prism software (GraphPad). Comparisons between groups were made using ANOVA with Tukey’s posttest analysis.

Results
Generation of inducible VV recombinants expressing UbWT and mutant Ub

The design of the dominant interfering UbK0 construct is depicted in Supplemental Fig. 1A. The key feature is substitution of each Lys residue (seven in total) with Arg, thereby eliminating the possibility of Ub branching beyond the point of UbK0 addition. In addition, a triple HA-tag was added to the N terminus of both constructs for detection of free and conjugated forms. Given the naturally high levels of Ub, we anticipated the need for excessive UbK0 expression. Initially, we used the conventional (constitutive) VV expression system but repeatedly failed to recover virus. This was likely due to toxicity of constitutive UbK0 expression, leading to premature death of the cells used for propagation of the virus. We therefore shifted to a tightly controlled conditional lac operon-based VV system (15). Induction of UbWT and UbK0 with the anticipated expression kinetics was confirmed by immunoblotting (IB) using an anti-HA Ab (Fig. 1B, Supplemental Fig. 1B). Subsequently, L-Kb cells were infected with different doses of virus for 12 h, and general protein ubiquitination was examined by IB using the FK1 Ab specific to polyubiquitinated conjugates. HA-tagged UbWT had limited impact even at a high dose, suggesting that endogenous Ub is at or near saturating levels. In contrast, polyubiquitination was potently abrogated by infection with UbK0 viruses ranging from 1–10 PFU/cell (Supplemental Fig. 1C). Reduction in polyubiquitination was easily detected at 8 h postinfection and appeared nearly complete by 12 h (Fig. 1C). Indeed, this approach may underestimate impact because the remaining bands could be residual species formed prior to the onset of effective UbK0 levels.

Proteins with large hydrophobic, basic, or acidic side chains at their N termini undergo rapid Ub-dependent degradation

![FIGURE 1](http://www.jimmunol.org/)

**A.** SIINFEKL-expressing model Ags. Residues 1–12 of NP are essential for nuclear localization. This segment was removed from NP, creating cytoNP (17), to simplify interpretation of the results. B. L-Kc cells were infected with VV expressing UbWT, UbK0, or no insert (control) at 3 PFU/cell in the presence or absence of IPTG for 12 h. IB analyses with anti-HA Ab were performed to detect IPTG-induced expression of HA-tagged Ub. C. L-Kc cells were infected with UbWT or UbK0 virus at 3 PFU/cell in the presence of IPTG. At the indicated times postinfection, cell lysates were subjected to 8% SDS-PAGE and blotted with FK1 Ab to detect protein polyubiquitination. D. L-Kc cells were coinfected with UbWT or UbK0 virus and Ub-Arg-cytoNP virus, all at 3 PFU/cell for 7 h in the presence of IPTG. The infected cells were then metabolically labeled with \([35S]\)Met/Cys. The degradation of Ub-Arg-cytoNP protein was determined by immunoprecipitation and autoradiography. S, OVA257–264. SP, signal peptide.
(the N-end rule) (21). We tested the impact of UbK0 expression on an N-end rule substrate, Ub-Arg-cytoNP (16), in which the Ub moiety is cotranslationally removed by Ub hydrolase and the resulting N-terminal Arg provides the degradation motif (22). Pulse-chase analyses demonstrated that Ub-Arg-cytoNP is very rapidly degraded in cells coexpressing UbWT (Fig. 1D). In contrast, equivalent coinfection with UbK0 resulted in marked accumulation of Ub-Arg-cytoNP at the zero time point due to turnover of substrate during the 10-min pulse (Supplemental Fig. 2) and a substantially slower degradation rate during the 10-min chase. Although we did not completely prevent degradation of this exceptionally unstable substrate, subsequent experiments demonstrated that this level of inhibition was more than sufficient for our purposes.

**Impact of UbK0 expression on the presentation of the same epitope from two different protein contexts**

Having validated the UbK0 system in biochemical assays, we assessed the impact of UbK0 expression on specific epitope presentation from defined Ags. All constructs tested were engineered to contain the H-2Kb-restricted OVA257–264 (SIINFEKL) epitope for which presentation can be quantified with the H-2Kb/OVA257–264-specific Ab 25.D1.16 (23). A cytosolically targeted version of influenza nucleoprotein, cytoNP, was used as a model cytosolic Ag, and human IL-2Rα chain (Tac) was used as a model exocytic Ag. The N-end rule substrate, Ub-Arg-cytoNP, served as a positive control, and a minigene construct, expressing the minimal SIINFEKL epitope (m/S), which does not require processing for presentation, was used as a negative control. Because cells cannot be sequentially infected with VV due to a potent interference effect (24), L-Kb cells were coinfected with Ag- and UbWT/UbK0-expressing viruses and then treated with citrate buffer 5.5 h postinfection to remove any peptides generated before effective concentrations of viral Ub had been reached. Following acid elution, the cells were incubated for an additional 12 h prior to assessment of surface Kb/OVA257–264 complexes. OVA257–264 presentation from Ub-Arg-cytoNP was markedly reduced by overexpression of UbK0 (Fig. 2A), indicating the ability of overexpressed UbK0 to block Ub-dependent Ag presentation. In contrast, UbK0 had limited impact on OVA257–264 presentation of the m/S construct. Interestingly, results with the two model Ags were similarly disparate. UbK0 had little impact on OVA257–264 presentation from the model cytosolic Ag cytoNP. This is consistent with a previous report that presentation of a stable

![FIGURE 2.](http://www.jimmunol.org/)

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![FIGURE 3.](http://www.jimmunol.org/)

UbK0 selectively inhibits presentation from the ER-targeted Tac Ag. A. Inhibitory effects of UbK0 on OVA257–264 presentation from different model Ags (see Supplemental Fig. 3 for representative primary data). Each symbol within a group represents an individual experiment, and the horizontal bars indicate the means and SD. Values of \( p \) were generated through the ANOVA test. B. L-Kb cells were treated with the indicated doses of epoxomicin for 15 min and then infected with virus expressing the model Ag at 3 PFU/cell for 5 h. OVA257–264 presentation was assessed by staining of surface OVA257–264/Kb complexes with 25.D1.16 Ab. Data are representative of three independent experiments.

![FIGURE 4.](http://www.jimmunol.org/)

Selective involvement of Ub E3 ligase Hrd1 in MHC class I-restricted presentation of ER-targeted Ags. L-Kb cells were treated with Hrd1 siRNA. A. Total cell lysates were subjected to IB using anti-Hrd1 Ab. B. Inhibitory effects of 72 h Hrd1 siRNA treatment on OVA257–264 presentation from different model Ags (see Supplemental Fig. 6 for representative primary data). A proteasome-independent m/S under the control of an 18-bp hairpin structure to limit expression level (m/S-HP18) (18), was used as a negative control. Tac (ATM) is a secreted version of Tac generated by deletion of the TM domain. Data were assessed as described for Fig. 2A.
cytosolic protein is not influenced by the temperature-sensitive E1 mutation (25). In contrast, UbK0 expression strongly inhibited presentation from the stable exocytic model Ag Tac.

A possible explanation for the results in Fig. 2A is that presentation of Tac is simply more proteasome-dependent than cytoNP. Tripeptidyl peptidase II, for example, has been reported to substitute for the proteasome in some cases, and its activity is not known to be regulated by Ub (26). To address this, aliquots of L-Kb cells were pretreated with various doses of the proteasome-specific inhibitor epoxomicin and then infected with the cytoNP-, Tac-, and mI/S-expressing viruses. Whereas the minigene construct was only marginally inhibited, dose responses of cytoNP and Tac were very similar (Fig. 2B). This outcome was independent of UbK0 coexpression (Supplemental Fig. 4), indicating that loss of polyubiquitination does not reorient Ag to a proteasome-independent pathway. Thus, despite equal proteasome dependence, the ER-targeted and cytosolic Ags are highly disparate with respect to polyUb dependence.

Altered Ub-dependence following retargeting of the model Ags

To investigate whether subcellular location provides the basis for the disparity in dependence upon polyubiquitination, we used a construct (ER–NP) in which cytoNP was directed to the exocytic compartment by attachment of an influenza HA-derived signal peptide to its N terminus (17). Presentation of OVA257–264 from ER–NP was as sensitive to UbK0 as the Tac Ag (Fig. 3).

Likewise, we removed the signal peptide from the Tac construct, which forced delivery to the cytosol, at the same time removing the transmembrane (TM) domain, which we have ascertained to constitute a degradation signal (Huang et al., submitted and revised for publication). In contrast to the parent protein, presentation of OVA257–264 from this cytosolic version, cytoTac (ΔTM), is essentially polyUb independent (Fig. 3). Thus, subcellular location appears to be a major determinant of polyUb-dependent Ag presentation.

Involvement of Ub ligase Hrd1 in presentation of ER-targeted proteins

ER-targeted proteins that fail quality control are usually dislocated to the cytosol and degraded by the proteasome in a process known as ERAD (27). Covalent Ub conjugation via the ER-situated Hrd1 E3 Ub ligase appears to be required in many cases for the dislocation step (28). To explore further the distinction between cytosolic and ER-targeted Ags, we examined the role of Hrd1 in the processing of our model substrates. A Hrd1-specific siRNA knockdown strategy was validated by IB analysis (Fig. 4A). Consistent with the results shown in Figs. 2 and 3, suppression of Hrd1 by siRNA inhibited presentation of ER-targeted Ags, whereas presentation of cytosolic Ags was, if anything, slightly enhanced (Fig. 4B). Similar results were obtained with two additional Hrd1-specific siRNA sequences (Supplemental Fig. 7). This result provides further support for a selective role of polyubiquitination in the generation of MHC class I peptides.

Discussion

The Ub-independent processing of the cytosolic proteins is consistent with the model we have proposed in which a fraction of the nascent cytosolic protein pool fails to be intercepted by the cellular folding machinery (the unfolded cohort) and is immediately degraded by the 20S proteasome (13). This is distinct from misfolded and senescent cytosolic proteins that are subjected to more prolonged conventional quality control decisions and polyUb targeting to the 26S proteasome. In contrast, processing of ER-targeted substrates may generally involve polyubiquitination and delivery to the 26S proteasome, a consequence of the topology and the processes that are involved in ERAD (14). Further supporting this notion is our recent observation that processing of ER-targeted versus cytosolic substrates is markedly prolonged, resulting in much lower peak levels of epitope at the cell surface (Huang et al., submitted and revised for publication). The impact of Hrd1 knockdown argues that the effect of UbK0 on ER-targeted substrates is direct rather than through effects on other cellular components. It seems virtually certain that, with cytosolic Ags, there will be exceptions to this generality because cases of Ub-independent ERAD have been described (29). Additionally, it is important to note that our experiments were performed in the context of VV infection, a potent inducer of the immunoproteasome (1), which degrades polyubiquitinated species more efficiently than the constitutive proteasome (30). Although such conditions are clearly relevant, it remains to be seen whether Ag processing in uninfected cells demonstrates a different dependence upon polyubiquitination.

Any cellular protein is heterogeneous in terms of folding state, location, and degree of modification. Thus, many proteins can be degraded via both Ub-dependent and -independent mechanisms (3). This will likely be the case for the processing of some Ags, but the decided partitioning of our model Ags with the positive and negative constructs suggests minimal heterogeneity in this respect.

The existence of such functionally distinct pathways may have important immunological consequences. In addition to the quantitative and temporal aspects in epitope production that have been eluded to, we have previously noted qualitative differences in processing when the same protein is targeted to the cytosol versus ER (17), perhaps a result of targeting to different proteasomal species. These differences could play a major role in determining those MHC class I-restricted epitopes within a complex Ag that are immunodominant and those that are most protective, both major considerations in rational vaccine design.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Characterization of inducible VV-based expression system. 

A, Schematic representation of HA-tagged UbWT and UbK0. B, L-Kb cells were infected with UbWT or UbK0 virus at 3 PFU/cell in the presence of IPTG. At the indicated times post infection, cell lysates were subjected to 15% SDS-PAGE and blotted with anti-HA Ab to detect the kinetics of Ub expression. Lower UbK0 levels were expected since only one UbK0 will be able to bind to a target Lys, while complete polyubiquitination is possible with UbWT. C, L-Kb cells were infected with UbWT or UbK0 virus at the indicated dose for 12 hours in the presence of IPTG. Lysates from infected cells were analyzed by immunoblotting for polyubiquitination using FK1 Ab.
Supplemental Figure 2. Greater recovery of Ub-Arg-cytoNP by UbK0 over-expression following a 10 minute metabolic pulse vs. a 5 minute pulse, as expected for a rapidly degraded protein. A, L-Kb cells were co-infected with UbWT or UbK0 virus, along with Ub-Arg-cytoNP virus at 3 PFU/cell each for 7 hours in the presence of IPTG. The infected cells were then metabolically labeled with [35S] Met/Cys for 5 min or 10 min. The amount of Ub-Arg-cytoNP protein was determined by immunoprecipitation and autoradiography. B, Phosphoroimager quantification of relative amounts of Ub-Arg-cytoNP.
Supplemental Figure 3. Representative antigen presentation from L-K\textsuperscript{b} cells co-infected with VV expressing the indicated model antigen and VV expressing UbWT or UbK0. See Figure 2A for the compiled data from multiple experiments.
Supplemental Figure 4. Role of proteasome in OVA\textsubscript{257-264} presentation under conditions of UbK0 overexpression. L-K\textsuperscript{b} cells were treated with 3 μM epoxomicin or DMSO for 15 min prior to infection with VV expressing the indicated model antigen and VV expressing UbWT or UbK0 (3 PFU/cell each virus). Six hours later, cells were treated with citrate buffer, 3 μM epoxomicin or DMSO for 15 min, and then cultured for an additional 10 hours before flow cytometry analysis with 25D1.16 Ab. IPTG was used at 2 mM during the entire period of virus infection. The “minigene” construct (m/S) was used as a negative control because it does not require proteasomal processing for presentation.
Supplemental Figure 5. Subcellular localization of Tac-based antigens. A, Confocal immunomicroscopy of HeLa cells infected with VV recombinants expressing the indicated variants of Tac. Anti-HA and anti-BiP (an ER marker) Abs were used for primary staining. B, Surface expression of Tac Ags in L-K^b cells infected with the indicated viruses for 5 hours. Flow cytometry, staining with anti-Tac Ab (7G7B6). C, Endoglycosidase H (Endo H) assay. L-K^b cells were infected with the indicated viruses for 5 hours. Cell lysates were treated with Endo H and analyzed by immunoblotting with
anti-HA Ab. Results indicate delivery of Tac and Tac (ΔTM) to the ER and delivery of cytoTac to the cytosol.
Supplemental Figure 6. Representative antigen presentation from L-K\textsuperscript{b} cells transfected with siRNA for 72 hours and then infected with Ag-expressing VVs for 5 hours before flow analysis with 25D1.16 Ab. See Figure 4\textit{B} for the compiled data from multiple experiments.
Supplemental Figure 7. Confirmation of Hrd1 siRNA impact. 

A, Knockdown of Hrd1 in L-Kb cells transfected with Hrd1 siRNA for 72 hours, detected by immunoblotting with anti-Hrd1 Ab.

B, OVA257-264 presentation from L-Kb cells treated separately with three different Hrd1-specific siRNA oligos or control siRNA for 72 hours and then infected with Tac-expressing VV for 5 hours before flow analysis with 25D1.16 Ab.