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*J Immunol* 2010; 184:6978-6985; Prepublished online 12 May 2010; doi: 10.4049/jimmunol.0904154

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*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Stabilization of an E3 Ligase–E2–Ubiquitin Complex Increases Cell Surface MHC Class I Expression

Lidia M. Duncan, James A. Nathan, and Paul J. Lehner

The Kaposi’s sarcoma-associated herpesvirus-encoded ubiquitin E3 ligase K3 ubiquitinates cell-surface MHC class I molecules (MHC I), causing the internalization and degradation of MHC I via the endolysosomal pathway. K3 recruits the cellular E2 ubiquitin-conjugating enzyme Ubc13 to generate lysine-63–linked polyubiquitin chains on MHC I, leading to the clathrin-mediated endocytosis and lysosomal degradation of MHC I. In this study, we identify a ubiquitin isoleucine-44-alanine mutant (I44A) that inhibits K3-mediated downregulation of MHC I by preventing MHC I polyubiquitination. This E3-specific inhibition by I44A prevents dissociation of the MHC I–K3–Ubc13–ubiquitin complex, allows the in vivo visualization of a transient substrate–E3–E2–ubiquitin complex interaction, and highlights a potential substrate hierarchy between the different MHC I alleles downregulated by K3. The I44A mutant also increases cell-surface MHC I expression in control cells in the absence of K3, predicting the presence of an endogenous E3 ubiquitin ligase required for cell-surface MHC I regulation. The Journal of Immunology, 2010, 184: 6978–6985.

Major histocompatibility complex class I (MHC I) molecules bind peptides derived from endogenous and viral proteins for display at the cell surface to CTLs (1). Peptides destined for MHC I molecules are generated predominantly by the proteasome and delivered to the endoplasmic reticulum (ER) via the TAP transporter for loading onto MHC I molecules (1). The ER is therefore a major site of MHC I regulation, and the binding of high-affinity peptide causes MHC I to dissociate from the peptide loading complex and traffic through the secretory pathway to the cell surface. How MHC I molecules are regulated at the cell surface is less clear, but involves clathrin- and dynamin-independent, Arf6-mediated MHC I endocytosis and recycling (2, 3).

As part of their immune evasion strategy, many viruses encode one or more gene products that downregulate cell-surface MHC I and help evade CTL-mediated recognition of viral peptides. The identification of an increasing number of viral genes emphasizes the importance of the MHC I Ag presentation pathway in host defense and also provides valuable tools for analysis of this pathway. A role for ubiquitin in the regulation of cell-surface MHC I was suggested following the identification of the K3 gene family that ubiquinates MHC I and other critical immunoreceptors (4). We and others (5–7) showed that the K3 and K5 viral gene products from Kaposi’s sarcoma-associated herpesvirus (KSHV) downregulate cell-surface MHC I molecules. K3 is a viral ubiquitin RING-CH E3 ligase, which associates with MHC I at the plasma membrane, leading to the lysine-63–linked ubiquitination, internalization, and endolysosomal degradation of MHC I molecules (8). Lysine-63–linked polyubiquitin chains are recognized to play an increasingly important role in the endocytosis and sorting of plasma membrane proteins, such as the epidermal growth factor receptor (9), and there is some evidence for endogenous, ubiquitin-dependent regulation of cell-surface MHC molecules. The membrane-associated RING-CH (MARCH) E3 ligases are the cellular orthologs of the K3 family, and MARCH1 and MARCH8 regulate cell-surface MHC class II expression in dendritic cells in a ubiquitin-dependent manner (10). Exogenous expression of the related MARCH4 and MARCH9 causes downregulation of cell-surface MHC I, but evidence for a physiological role of these cellular ligases in MHC I regulation is lacking (11). However, the viral ligases are likely to have appropriated a cellular route of MHC I disposal rather than invented a new pathway, as highly conserved lysine residues are identified in the cytosolic tail of MHC I molecules.

All ubiquitination reactions begin with a highly conserved catalytic cascade in which ubiquitin is activated by one of the two ubiquitin-activating E1 enzymes and transferred to the active site cysteine of one of ~40 ubiquitin-conjugating enzymes (E2). The specificity of the ubiquitin reaction is conferred by the E2 ligase, which includes proteins of the RING and HECT families. The larger group of RING ligases does not itself bind ubiquitin, but associates with the ubiquitin-charged E2 and facilitates transfer of the activated ubiquitin from the E2 to a lysine residue on the target protein. Thus, the RING family of E3s is not itself catalytically active, but acts as a molecular scaffold to recruit specific E2s via their RING domain and helps orientate the E2 for ubiquitin transfer onto the substrate (12). Beyond monoubiquitination, ubiquitin can also form polyubiquitin conjugates via one or more of its seven lysine residues and its N-terminal. The interaction between the E3 ligase and its physiologically relevant E2-conjugating enzyme is critical to the outcome of the ubiquitin reaction. This interaction determines both the pattern of ubiquitin chains that are generated and by inference the biological outcome. Although E3–E2 interactions have been characterized in vitro, there are few studies on these interactions in vivo, mainly due to the low interaction affinity between the two binding partners.

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Received for publication December 28, 2009. Accepted for publication April 10, 2010.

This work was supported by the Wellcome Trust.

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Abbreviations used in this paper: β2-m, β2-microglobulin; ER, endoplasmic reticulum; I44A, isoleucine-44-alanine; K63R, lysine-63-arginine; KSHV, Kaposi’s sarcoma-associated herpesvirus; MARCH, membrane-associated RING-CH; MHC I, MHC class I; UEV, ubiquitin E2 variant; wtUb, wild-type ubiquitin.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0904154
Despite the importance of defining E2–E3 interactions, elucidating physiologically relevant E2–E3 pairings in vivo remains a major challenge. Active E2–E3 pairs do not stably associate due to weak binding (low micromolar range), a prerequisite to the dynamic catalytic process of the polyubiquitination reaction (13). Because the E1 and E3 enzyme bind the ubiquitin E2 enzyme at the same site, E2 loading with ubiquitin is an iterative process that requires dissociation from the cognate E3, reloading with ubiquitin, and subsequent reassociation (14). Much progress has been made toward the identification of potential E3–E2 pairs by in vitro enzyme pairing and yeast-two-hybrid approaches (15, 16), often in combination with structural studies to confirm binding (13). However, these approaches also have their limitations and may result in enzymatically nonfunctional E3–E2 pairs in vitro. For example, the Brcal-Bard1 E3 heterodimer stably binds Ubc7 in vitro, yet the complex is inactive for ubiquitin transfer (17, 18). Although the combination of structural and computational methods provides critical insight into the E3–E2 binding interfaces (19), in vivo data are required to understand the physiological effect of E3–E2 pairs on their substrates.

Lysine-48–linked chains typically signal proteasomal degradation, whereas the conjugation of lysine-63–linked polyubiquitin chains is associated with DNA repair, kinase signaling pathways, and receptor regulation (20). A role for lysine-63–linked polyubiquitination in receptor regulation was first demonstrated for the yeast plasma membrane protein uracil permease (21) and in mammalian cells for the downregulation of cell-surface MHC class I molecules by the K3 viral E3 ligase (8). K3 recruits the Ubc13 E2 enzyme, which is required for the generation of lysine-63–linked polyubiquitin chains on MHC I molecules, leading to their internalization and sorting through the endosomal pathway for lysosomal degradation. The endogenous downregulation and degradation of an increasing number of unrelated cell surface receptors, including the IFN-αR (22), the nerve growth factor receptor (23) the aquaporin receptor (24), and the prolactin receptor, involves a similar process (25).

The internalization of lysine-63 polyubiquitinated MHC I requires ubiquitin-binding proteins including the epsin endocytic adaptor, which contains three ubiquitin-interaction motifs. The majority of ubiquitin-interaction motifs bind ubiquitin via its hydrophobic patch, centered on the critical Ile44 residue (26). To investigate how this hydrophobic patch affects the K3-mediated downregulation of MHC I, we made an isoleucine-44-alanine (I44A) mutant in the identical vector to the previously used wild-type and lysine-63-arginine (K63R) ubiquitin mutant. A major advantage of these ubiquitin mutants is that they encode a C-terminal GFP, which is cotranslationally cleaved such that the expression level of exogenous ubiquitin is proportional to the level of GFP. In this study, we show that the I44A mutant induces a marked increase in cell-surface MHC I levels in HeLa-K3 as well as HeLa cells. This is due to a loss of ubiquitination and stabilization of the MHC I–K3–Ubc13 interaction, allowing visualization of this substrate–E3–E2 complex in vivo. This differential effect of I44A on HLA-A and HLA-B allotypes identifies a potential hierarchy of substrate binding.

Materials and Methods

Abs

The following Abs were used: mAb HC10 (anti-class I H chain), mAb w6/32 (anti-MHC I), mAb 4E (anti-HLA-B), mAb M2 (anti-FLAG) (Sigma-Aldrich, St. Louis, MO), mAb (anti-Ubc13) (Affinity, Nottingham, U.K.), mAb BB7.2 (anti-HLA-A2) (Serotec, Oxford, U.K.), mAb D9 (HLA-C specific) (a kind gift from Ashley Moffett, Department of Pathology, University of Cambridge, Cambridge, U.K.), 3D12 (eBioscience, San Diego, CA), mAb PUMO37 (anti–HLA-A28) (One Lambda, Canoga Park, CA), anti-IgG2b isotype-control (eBioscience), APC anti-human CD54 (BD Biosciences, San Jose, CA), mAb AF8 (anticalnexin) (a kind gift from M. Brenner, Harvard Medical School, Boston, MA), and mAb anti-6His tag (Qagen, Valencia, CA). Fluorescent secondary Abs were from Molecular Probes (Eugene, OR) and HRP-conjugated secondary Abs from Jackson ImmunoResearch Laboratories (West Grove, PA).

 Constructs and cell lines

The constructs and cell lines used were as described (8) except: pCDNA3 ZZ-Flag-wK3 was generated from pCDNA3 Flag-wK3 by insertion of the ZZ tag upstream of the N-terminal Flag tag. The HeLa-ZZ-K3 cell line was created by transfecting the pCDNA3 ZZ-Flag-wK3 construct into the parent cell line and selecting with neomycin. The cells were grown in RPMI 1640 supplemented with 10% FCS. The GFP-tagged wild-type and mutant ubiquitin constructs (wUb and UbK63R) were used as described (27) (a kind gift of Doug Gray, Ottawa Hospital Research Institute, University of Ottawa, Ottawa, Ontario, Canada). The UbI44A construct was generated by site-directed mutagenesis of the above 6His-wUb-GFP-pDQ268 using the QuickChange kit (Agilent Technologies, Stockport, U.K.). The sense oligonucleotide used for I44A mutation was UbI44A 5′-ATACGCAAGACTGCCCTTTGGCTGGAACGAC-3′.

Transient transfections

HeLa, HeLa-K3, and HeLa-K5 cells were transfected at 60% confluence in six-well plates with 2 μg per well using the TransIT-HeLa MONSTER transfection Kit (Mirus Bio, Madison, WI). The 293 cells were similarly transfected using 293 Transit reagent (Mirus Bio). Transfected cells were analyzed by flow cytometry 48 h following transfection.

Radiolabeling and immunoprecipitations

Cells were starved for 20 min in methionine, cysteine-free medium, labeled with [35S]methionine and [35S]cysteine for 10 min, and chased in media containing excess cold methionine and cysteine for the indicated time periods. Immunoprecipitations were performed as described (7). Samples were analyzed by SDS-PAGE (10% or 12%), processed for autoradiography with a Cyclone scanner (Packard Instrument, Meriden, CT) and analyzed with OptiQuant software (PerkinElmer, Waltham, MA). Visualization of ubiquitin bands required autoradiography for 7–21 d. Flow cytometric analysis was performed as described (7) with the mAbs w6/32 and anti-mouse secondary Ab as indicated in the figure legends.

Immunofluorescence and confocal microscopy

Cells were plated on coverslips and incubated with w6/32 class I mAb for 15 min. Soluble cellular proteins were removed and cells treated with 0.01% saponin in cytosolic buffer (25 mM HEPES-KOH [pH 7.4], 25 mM KCl, 2.5 mM magnesium acetate, 5 mM EGTA, 150 mM K-glutamate) for 5 s prior to fixation with 4% paraformaldehyde. Cells were then blocked with 0.2% BSA in PBS and probed with the desired Ab. The coverslips were washed with 0.2% BSA in PBS prior to mounting on slides. Slides were visualized with the LSM510 META confocal microscope (Zeiss, Oberkochen, Germany) and analyzed using Axiovision software (Zeiss).

Immunoblot analysis

Cells were lysed in 1% Triton X-100 in TBS plus PMSF and iodoacetamide on ice for 30 min. The postnuclear supernatants were incubated with IgG sepharose for 2 h at 4˚C, and the pellets were washed with 0.2% BSA in PBS prior to mounting on slides. Slides were visualized with the LMS510 META confocal microscope (Zeiss, Oberkochen, Germany) and analyzed using Axiovision software (Zeiss).

ZZ pulldown assays and immunoblotting

HeLa-ZZ-Flag-K3 and HeLa control cells (≥ 144A) were lysed on ice for 30 min at 106 cells/ml in either TBS/1% Triton x-100 (7) or RIPA buffer (50 mM Tris [pH 8], 0.5% sodium deoxycholate, 150 mM NaCl, 1.0% w/v IGEPAL CA-630, and 0.1% SDS), both containing 0.5 mM PMSF (Sigma-Aldrich) and 5 mM iodoacetamide (Sigma-Aldrich). The postnuclear supernatants were incubated with IgG sepharose for 2 h at 4˚C, and pellets were washed three times with the corresponding lysis buffers diluted 1:2 prior to SDS-PAGE and immunoblotting as above.
Live cell sorting

Live cells were labeled with w6/32 mAb at room temperature for 15 min, washed twice with PBS/2% FCS, labeled with anti-mouse Cy5 secondary Ab, and washed again as above prior to cell sorting by MoFlo Cell Sorter (Beckman Coulter, Miami, FL) for three fractions with no (−), low (+), and high GFP (+++) intensity combined with MHC I staining.

Results

Mutant I44A ubiquitin inhibits the K3 viral E3 ligase and predicts a role for ubiquitination in the steady state regulation of MHC I

The viral K3 ubiquitin E3 ligase downregulates cell-surface MHC I (Fig. 1A). We previously showed K3 generates lysine-63–linked polyubiquitin chains that signal the internalization and trafficking of MHC I through the endolysosomal pathway. Overexpression of a ubiquitin mutant (K63R), which is unable to make lysine-63–linked polyubiquitin chains and competes with endogenously expressed ubiquitin, shows a dose-dependent increase in MHC I, concomitant with the increase in GFP levels (Fig. 1A). However, a very different phenotype is observed with the I44A mutant. In contrast to the gradual increase in cell-surface MHC I seen with K63R, there is a sudden, stepwise increase in cell-surface MHC I, and even low levels of I44A expression completely rescue MHC I back to the cell surface. This dramatic phenotype suggested a block rather than the graduated inhibition seen with the K63R mutant (Fig. 1A). Because the resulting cell-surface MHC I levels are even higher than in normal HeLa cells, we examined how the ubiquitin mutants affected MHC I on control HeLa cells. In these cells, neither wtUb nor mutant K63R ubiquitin affected cell-surface MHC I. However, in the presence of the I44A mutant, cell-surface MHC I also increased, showing that the effect is not restricted to K3-expressing cells (Fig. 1B). The same phenotype was visualized by immunofluorescence confocal microscopy (Fig. 1C). Expression of the I44A ubiquitin mutant induced a dramatic increase in cell-surface MHC I in K3-expressing as well as control HeLa cells.

Inhibition by the I44A ubiquitin mutant is MHC I specific and does not affect all E3 ligases

KSHV also encodes K5, another viral E3 ligase that shares ∼40% homology with K3 and downregulates cell-surface MHC I as well as additional cell-surface proteins including the ICAM-1 adhesion molecule (28, 29). To determine the specificity of the I44A ubiquitin mutant, we examined its effect on cell-surface MHC I expression in K5-expressing HeLa cells. In contrast to the I44A-induced increase in cell-surface MHC I expression in HeLa-K3 and HeLa cells, even high levels of I44A had little effect on the K5-mediated downregulation of MHC I in HeLa-K5 cells, implying that I44A-mediated inhibition is selective (Fig. 2A). Further specificity was determined by examining the effect of I44A on ICAM-1 expression. The I44A mutant had little effect on cell-surface ICAM-1 expression in HeLa or HeLa-K3 cells or on the low ICAM-1 levels seen in HeLa-K5 cells, suggesting that the I44A mutant is both E3 and target specific (Fig. 2B).

The I44A ubiquitin mutant differentially affects cell-surface HLA-A, HLA-B, and HLA-C alleles

To determine whether I44A expression affects cell-surface MHC I in other cell types, we analyzed 293 cells transfected with the I44A

FIGURE 1. The I44A ubiquitin mutant increases cell-surface MHC I in HeLa-K3 and HeLa cells. A and B, Cytofluorometric analysis of MHC I expression in HeLa-K3 or HeLa cells following transient transfection with GFP-tagged wtUb, K63R-ubiquitin (UbK63R), or Ile44Ala-ubiquitin (UbI44A). Cells were stained with mAb w6/32. C, Wild-type HeLa and HeLa-K3 cells were transfected with His-UbI44A-GFP (first two panels) and stained with w6/32 mAb for MHC I (third two panels) and DAPI as a nuclear stain (second two panels) (scale bar, 10 μm).

FIGURE 2. The I44A ubiquitin mutant is K3 and MHC I specific. A, I44A expression does not affect cell-surface MHC I in HeLa-K5 cells. Cytofluorometric analysis of MHC I expression in HeLa-K3 or HeLa-K5 cells following transient transfection with GFP-tagged UbI44A or wtUb as control. Cells were labeled as in Fig. 1A. B, I44A expression does not affect cell-surface ICAM-1 levels. Cytofluorometric analysis of ICAM-1 expression in HeLa, HeLa-K5, and HeLa-K3 cells following transient transfection with GFP-tagged UbI44A or wtUb as control. Cells were labeled with APC-conjugated anti–ICAM-1 mAb.
The I44A ubiquitin mutant is MHC I allotype specific. A–C, I44A expression leads to upregulation of the cell-surface HLA-A allotype and decreased expression of HLA-B, HLA-C, and HLA-E. Cytofluorometric analysis of MHC I expression in 293 (A), HeLa-K3 (B), or HeLa (C) cells following transient transfection with GFP-tagged UbI44A. Cells were labeled with w6/32 Ab (total MHC I), anti–HLA-A2 mAb, anti–HLA-A28 mAb, anti–HLA-B mAb, anti–HLA-C, or anti–HLA-E mAb as indicated. The I44A ubiquitin mutant prevents K3-mediated degradation of MHC I and promotes HLA-A at the expense of HLA-B expression. A, K3-induced degradation of MHC I is inhibited by the I44A ubiquitin mutant. Cell-sorted HeLa-K3 cells (± I44A) were pulsed for 10 min with [35S]methionine and [35S]cysteine and chased for the times indicated. Triton X-100 lysates were immunoprecipitated with the anti-MHC I w6/32 mAb and protein A-agarose beads for 2 h and treated with Endo H to follow maturation of MHC I. Samples were analyzed by 10% SDS-PAGE and autoradiography. B, The I44A mutant delays MHC I maturation and promotes expression of HLA-A28 at the expense of HLA-B. Cell sorted HeLa cells (± I44A) were pulse labeled, immunoprecipitated with the anti-MHC I w6/32 mAb as in A, and analyzed by 12% SDS-PAGE. C, In the presence of I44A, expression of the HLA-B allele is dramatically reduced. To compare expression levels of the HLA-A and -B allele in the presence of I44A, HeLa cells (± I44A) were pulse chased for 15 min, immunoprecipitated with the anti-MHC I w6/32 mAb, anti–HLA-A28 mAb, and the anti–HLA-B mAb 4E, and analyzed as in A.

The I44A ubiquitin mutant prevents K3-mediated polyubiquitination of MHC I

Expression of the I44A mutant in HeLa-K3 cells increased cell-surface MHC I and by pulse-chase analysis was shown to prevent...
MHC I degradation. We therefore wanted to determine the effect of the I44A mutant on K3-mediated MHC I ubiquitination. We previously showed that immunoprecipitation of radiolabeled MHC I molecules from K3-expressing cells allows the visualization of higher m.w. ubiquitinated MHC I species (7). MHC I molecules were therefore immunoprecipitated from radiolabeled HeLa-K3 cells with the w6/32 mAb. In the presence of the I44A ubiquitin mutant, polyubiquitinated MHC I species were reduced (Fig. 5A). K3-associated, ubiquitinated MHC I molecules may also be visualized in radiolabeled cells by immunoprecipitating the K3 complex and reprecipitating MHC I-associated proteins (7). To further determine the extent that I44A inhibited K3-mediated ubiquitination, we radiolabeled two sorted cell populations (Fig. 5B). Hela-K3 cells were transfected with the I44A mutant and sorted for the GFP-positive (I44A-expressing, MHC I-high population) and GFP-negative (untransfected, MHC I-low population) (Fig. 5B, left panel). Following radiolabeling, FLAG-K3 was immunoprecipitated and the resulting complex reprecipitated with the MHC I H chain-specific mAb HC10, using HeLa cells as a control. Although polyubiquitinated MHC I was detected in Hela-K3 cells, these ubiquitinated MHC I species were completely lost in the presence of I44A (Fig. 5B, right upper panel). Furthermore, examination of the loading controls for the primary FLAG-K3 immunoprecipitation (10% of input) revealed the presence of associated MHC I H chains, not normally seen at this low level of K3 protein loading, suggesting an accumulation of MHC I on K3 in the presence of I44A and a potential block in the K3/MHC I complex (Fig. 5B, right lower panel).

Because the I44A ubiquitin mutant blocked K3-mediated MHC I ubiquitination, it was important to demonstrate that the I44A mutant could be incorporated into polyubiquitin chains and does not interfere with all polyubiquitin chain formation. This was already suggested by our observation that K5-mediated downregulation of MHC I is not blocked by the I44A mutant (Fig. 2A). We confirmed that HeLa-K5 cells transfected with I44A show normal polyubiquitinated MHC I chain formation (Fig. 5C), showing that polyubiquitin chain generation is not necessarily inhibited by the I44A mutant. To ensure that the I44A mutant does not interfere with the proteasomal pathway, total cell lysates from HeLa cells transiently transfected with the I44A mutant were examined for the formation of His-tagged polyubiquitin chains. High m.w. polyubiquitinated proteins were readily visualized with the His-specific Ab and showed a marked accumulation in the presence of proteasome inhibitors (Fig. 5D). Therefore, despite our findings that I44A blocks K3-mediated ubiquitination, in the presence of K5, the I44A mutant is readily incorporated into polyubiquitin chains, and I44A is also incorporated into polyubiquitin chains destined for proteasome-mediated degradation. Polyubiquitin chain generation is not generally inhibited by the I44A mutant.

The I44A ubiquitin mutant traps the otherwise transient MHC I–E2–E3 ligase complex

In the presence of the I44A mutant, K3-mediated MHC I ubiquitination is inhibited. RING E3 ligases, such as K3, do not themselves directly bind ubiquitin, but recruit a ubiquitin-charged E2 enzyme that promotes transfer of the ubiquitin to the substrate, in this case the MHC I H chain. As our previous studies have shown that K3 recruits the Ubc13 E2 enzyme to generate lysine-63–linked ubiquitin chains on the MHC I H chain (8), we reasoned that the I44A-induced ubiquitin block may involve the E2 Ubc13. To examine the effect of I44A on Ubc13, we sorted I44A-transfected HeLa-K3 cells into three populations (Fig. 6A, upper panel) representing: 1) untransfected GFP-negative, I44A-negative (−), MHC I-low cells; 2) GFP-intermediate, I44A intermediate (+), MHC I-low cells; and 3) GFP-high, I44A-high (++), MHC I-high cells. This allowed a direct comparison of cells expressing equivalent levels of GFP from the wild-type versus the I44A mutant-expressing population. Detergent cell lysates from these populations were analyzed for the presence of Ubc13 by immunoblotting (Fig. 6A, lower left panel) and reducing conditions (Fig. 6A, lower right panel). Ubc13 is predominantly detected at its predicted 16 kDa molecular mass, and under nonreducing conditions, the ubiquitin-charged Ubc13 at 24 kDa and the slightly larger His-tagged Ubc13 are readily visualized and disappear on reduction. In Hela-K3 cells expressing high yet comparable levels of exogenous wtUb and I44A ubiquitin, significantly more His-tagged Ubi44A was detected on Ubc13 than exogenous wtUb (Fig. 6A, lower panel, lane 3 versus lane 6), suggesting that the normal catalytic removal of I44A from Ubc13 is inhibited. In Hela-K3 cells expressing high levels of I44A, an additional 120-kDa band is also detected under nonreducing conditions and disappears upon reduction. Therefore, in the presence of I44A, an SDS-stable, reducible high m.w. complex containing the Ubc13 E2 enzyme is visualized.

To identify additional components present in this 120-kDa complex, epitope-tagged K3 was immunoprecipitated from Hela-K3 cells containing I44A under different detergent conditions.
Immunoprecipitated K3 and associated proteins were then visualized by immunoblotting. Following epitope-tagged K3 immunoprecipitation, high molecular mass bands at 120-kDa (Fig. 6Bi) were again detected and shown to contain K3 (Fig. 6Bi), MHC I H chain (Fig. 6Bii), Ubc13 (Fig. 6Biii), and ubiquitin (Fig. 6Biv). Upon addition of DTT to the sample preparation buffer, the high m.w. complex was again lost and the high molecular mass complex reduced into its monomeric components. Thus, the I44A ubiquitin mutant allows the in vivo visualization of a stable substrate–E3–E2–Ub complex.

**Discussion**

Expression of the I44A mutant ubiquitin caused a marked increase in cell-surface MHC I expression in HeLa-K3, HeLa, and HEK293 cells. This was predominantly due to an increase in the HLA-A allotype at the expense of the HLA-B allotype. In HeLa-K3 cells, expression of I44A correlates with a loss of MHC I ubiquitination and stabilization of a DTT-sensitive substrate–E3–E2–Ub complex. The detection of K3 in association with its cognate E2 (Ubc13), substrate (MHC I), and ubiquitin likely represents a transient intermediate complex, which is blocked and unable to dissociate due to the I44A mutant.

The key features in the I44A-mediated increase in surface MHC I are the loss of MHC I ubiquitination and the appearance of the DTT-sensitive substrate–E3–E2–Ub complex. In trying to understand these results, the most likely primary event is the trapping of the transient intermediate substrate–E3–E2–Ub complex due to an inability to ubiquitinate the MHC I substrate, resulting in the stabilization of the intermediary components at the cell surface. The resulting increase in cell-surface MHC I delays MHC I trafficking through the secretory pathway and causes a subsequent downregulation of the HLA-B allotype, which is likely to be a secondary event (see below).

Why does the I44A ubiquitin induce trapping of the substrate–E3–E2–Ub complex? I44A ubiquitin-charged Ubc13 clearly associates with K3, but the viral ligase appears unable to discharge the I44A ubiquitin, resulting in a stabilization of the substrate–E3–E2–Ub complex. Support for this model comes from the finding that the substrate–E3–E2–Ub interaction is only detectable under nonreducing conditions, and addition of a reducing agent dissociates the complex into its constituent components. Because the thioester bond linking I44A ubiquitin to Ubc13 is the only readily reducible bond in the complex, reduction of this thioester causes disintegration of the high m.w. complex.

More difficult to determine in vivo is why the I44A ubiquitin cannot be discharged. The generation of lysine-63–linked polyubiquitin chains requires formation of a heterodimer between Ubc13 and the inactive ubiquitin E2 variants (UEVs), MMS2 in the nucleus or its homolog UEV1a in the cytoplasm (30, 31). Although Ubc13 forms a thioester bond with the activated ubiquitin, also referred to as the donor ubiquitin, UEVs bind both Ubc13 and the ubiquitin on the substrate, the acceptor ubiquitin, in a noncovalent manner (19). UEVs then orientate the acceptor ubiquitin to the Ubc13 active site such that only lysine-63 is available for chain formation (32, 33). The UEV-acceptor ubiquitin interface is centered around the critical Ile44 residue (34), and mutations of this residue inhibit lysine-63–linked polyubiquitin chain formation in the yeast RAD6 pathway (35). Furthermore, the affinity of the UEV for Ubc13 may be enhanced by

**FIGURE 6.** The I44A ubiquitin mutant stabilizes the MHC I–E3–E2–Ub ligase complex. A, The Ubc13 E2-conjugating enzyme is detected in a 120-kDa reducible complex in the presence of the I44A ubiquitin mutant. I44A-transfected HeLa-K3 cells were labeled for surface MHC I with the w6/32 Ab, and cells were sorted into three populations representing untransfected GFP-negative (I44A-negative) cells (−); GFP-intermediate, (I44A-intermediate) MHC I low cells (+); and GFP-high, (I44A-high), MHC I high cells (++) prior to immunoblotting equivalent amounts of total lysates for Ubc13 under nonreducing and reducing conditions (lower panels). B, Ubc13, K3, MHC I, and I44A-Ub are components of the 120-kDa complex. HeLa-ZZ-Flag-K3 cells ± I44A were lysed in 1% Triton X-100/TBS or RIPA buffers and total cell lysates (Lys) were either directly immunoblotted for K3 (anti-Flag M2 Ab), MHC I (HC10 Ab), Ubc13, and 6HisI44A (anti-His Ab) or pulldowns performed with ZZ-FLAG-K3 (PD) and the resulting complex separated by SDS-PAGE and then probed for the same components. Calnexin was used as loading control.

**FIGURE 7.** Schematic model to show trapping of the MHC I–E3–E2–Ub complex. A, In the presence of wtUb, K3 recruits Ub-charged Ubc13 for transfer of the charged donor ubiquitin from Ubc13 to the acceptor ubiquitin. B, In the presence of the I44A mutant ubiquitin, Uev1a is unable to bind the I44 patch on the acceptor ubiquitin and orient its K63 for conjugation by Ubc13. Uev1a dissociates, and in the absence of polyubiquitination, the monoubiquitin on MHC I is likely to be removed by a deubiquitinating enzyme. As Ubc13 is unable to transfer the donor ubiquitin to an acceptor ubiquitin, it cannot dissociate from K3, stabilizing the otherwise transient MHC I–E3–E2–I44A complex.
synergistic noncovalent interactions between the UEV and the acceptor ubiquitin on the substrate (19). We speculate that the absence of I44 is likely to prevent the binding of the UEV complex to the acceptor ubiquitin of the catalytic complex and thus prevents Ubc13 from transferring its charged ubiquitin to MHC I. The E3–E2–Ub complex is therefore inactive and unable to polyubiquitinate (Fig. 7). Although Ubc13 is readily detectable in the high m.w. complex, we have been unable to detect UEV1a, the likely UEV binding partner. The predicted molecular mass of the identified complex components is 130 kDa, which includes ubiquitin-charged Ubc13 (26 kDa), MHC I H chain (43 kDa), β2-microglobulin (β2-m) (13 kDa), and K3 (48 kDa), and this correlates with the 120-kDa complex identified under nonreducing conditions and further suggests that UEVs are not present.

The identification of K3 associated with its cognate E2 is most unusual. The very transient nature of the interaction between E3 ligases and E2-conjugating enzymes means there are few data on E3–E2 pairing in vivo. In those few examples in which high-affinity E3–E2 binding has been visualized in vivo, the E3 RING domain does not contribute to formation of the complex. High-affinity binding between the Ubc2 E2 enzyme and the RING-E3 ligase of the N-end rule pathway, Ubr1, maps to a basic rich region, rather than the proximal RING domain (36). Similarly, gp78, an ER-resident E3 ligase, recruits its cognate E2, Ube2g2 via a specific high-affinity binding site that is distinct from the RING (37). Therefore, our knowledge on E3–E2 interactions facilitated by the RING domain is based on in vitro studies. RING domains are thought to either position the ubiquitin-charged E2 and facilitate the transfer of ubiquitin to substrates or function as allosteric activators of ubiquitin-charged E2s (12, 38). Because the E2 binding sites for RING fingers and the E1 enzyme overlap, an E2 which discharges its ubiquitin must dissociate from its cognate ligase prior to reloading with ubiquitin (14). The affinity of an ubiquitin-charged E2 for its E3 is therefore likely to be higher than the noncharged E2, and this is indeed the case as reported for the E2–E3 HsUbc2b–E3α pair, in which the affinity of the ubiquitin-charged E2 is 8.4 times higher than the noncharged E2 (39).

A surprising finding of this study is the I44A-induced, allotype-specific increase in MHC I, with the differential effect on the HLA-A and -B allotypes seen in both HeLa and HEK293 cells, as well as HLA-C and HLA-E in HeLa cells. Although K3 downregulates both HLA-A and HLA-B equally effectively, in the presence of I44A, only HLA-A is rescued to the cell surface, at the expense of HLA-B, which is undetectable. This allotype-specific phenotype may reflect an increased affinity of K3 for HLA-A over HLA-B, creating a substrate hierarchy, though the explanation may be more complex.

The tight control of cell-surface MHC I expression seen in most cell types reflects multiple levels of regulation including transcriptional, posttranslational, and peptide loading steps. For example, different MHC I allotypes vary in their genomic promoter regulatory elements (40) and MHC class I H chains compete for folding, independently of β2-m and peptide (41). In the ER, peptide loading of MHC I molecules on the TAP peptide loading complex is the major site of MHC I regulation, and competition between MHC I alleles for peptide loading on TAP is well described (42, 43). The accumulation of HLA-A molecules at the cell surface of I44A-expressing cells is likely to induce secondary effects on MHC I loading and trafficking through the secretory pathway, particularly on the peptide-loading complex. Radiolabeled pulse-chase analysis showed that in I44A-expressing HeLa cells, HLA-A28 transport through the secretory pathway is delayed, which may cause a backlog of MHC I proteins and increased competition for binding the peptide loading complex. Our inability to detect any HLA-B allotype, even in the presence of proteasome inhibitors, suggests further effects of the I44A mutant, which might also be secondary to the accumulation of cell-surface HLA-A or unrelated to the cell-surface phenotype. These possibilities are being pursued.

A critical control in these experiments was the lack of effect of I44A on K5-expressing cells. Unlike the effect seen with HeLa and HeLa-K3 cells, I44A had no effect on K5’s ability to ubiquitinate and downregulate MHC I (Fig. 4). Indeed, K5 is refractory to the I44A effect. This resistance of MHC I molecules to I44A in HeLa-K5 cells serves as a useful control for the increase in cell-surface MHC I seen in HeLa-K3 and HeLa cells. It shows that I44A neither blocks polyubiquitination in general nor lysine-63–linked polyubiquitination in particular, as the K5-mediated ubiquitination and downregulation of cell-surface MHC I is absolutely dependent on lysine-63–linked polyubiquitin chains (44). Furthermore, HLA-B is readily detected in I44A-expressing HeLa-K5 cells, supporting our assertion that the decreased HLA-B expression in HeLa-K3 and HeLa cells is secondary to the cell-surface block induced by I44A. Although we do not fully understand why I44A is able to block the K3 but not K5 viral ligase, it is likely to reflect the recruitment of different E2s or differences in E2 binding. Despite sharing MHC I as a common substrate, there are significant differences between K3 and K5 that may help explain K5’s protection against I44A. K3’s primary substrates are MHC I molecules, whereas K5 efficiently downregulates a number of immunoreceptors from the cell surface (11). K5 has other unusual properties. It promotes ubiquitination on cysteines as efficiently as lysine residues in the cytoplasmic tail of HLA-B7 (45) and generates mixed lysine-11 and lysine-63–linked polyubiquitin chains on MHC I. It may therefore recruit cellular E2s in addition to Ubc13 to catalyze the polyubiquitination and downregulation of MHC I.

Our data also provide insight into the regulation of MHC I cell-surface molecules in the absence of viral ligases. The increase in MHC I expression in I44A-expressing HeLa as well as HeLa-K3 cells implies the existence of an ubiquitin-mediated mechanism in the steady-state regulation of MHC I molecules at the plasma membrane. Assuming the same mechanism is occurring in HeLa as in HeLa-K3 cells, I44A may block the endogenous cellular ligase in an identical way to the inhibition seen in K3-expressing cells. Regulation of cell-surface MHC I by an endogenous cellular ligase is likely, as evolutionarily conserved lysine residues can be identified in the MHC I H chain cytosolic tail as far back as sharks. Ubiquitination may promote MHC I H chain internalization following peptide or β2-m dissociation at the cell surface. MARCH1 and -8, orthologs of K3 and K5, regulate cell-surface MHC class II (46), and MARCH orthologs or unrelated ligases may be involved in MHC I regulation. Although overexpression of two MARCH family members (MARCH4 and -9) causes MHC I downregulation (47, 48), a physiological role for these ligases in MHC I regulation in HeLa cells has not yet been demonstrated.

In summary, the I44A Ub mutant inhibits the K3 viral ligase, resulting in increased cell-surface MHC I expression. This has allowed visualization of an otherwise transient substrate–E3–E2–Ub complex in vivo, exposed an MHC I allotype hierarchy, and predicted the presence of a cellular E3 ligase involved in the steady state regulation of cell-surface MHC I.

Acknowledgments
We thank D. Gray (University of Ottawa, Ottawa, Ontario, Canada) for mutant ubiquitin-GFP constructs.

Disclosures
The authors have no financial conflicts of interest.


