Ubiquitination on Nonlysine Residues by a Viral E3 Ubiquitin Ligase

Ken Cadwell and Laurent Coscosy*

Ubiquitination controls a broad range of cellular functions. The last step of the ubiquitination pathway is regulated by enzyme type 3 (E3) ubiquitin ligases. E3 enzymes are responsible for substrate specificity and catalyze the formation of an isopeptide bond between a lysine residue of the substrate (or the N terminus of the substrate) and ubiquitin. MIR1 and MIR2 are two E3 ubiquitin ligases encoded by Kaposi’s sarcoma–associated herpesvirus that mediate the ubiquitination of major histocompatibility complex class I (MHC I) molecules and subsequent internalization. Here, we found that MIR1, but not MIR2, promoted down-regulation of MHC I molecules lacking lysine residues in their intracytoplasmic domain. In the presence of MIR1, these MHC I molecules were ubiquitinated, and their association with ubiquitin was sensitive to β2-microglobulin, unlike lysine-ubiquitin bonds. This form of ubiquitination required a cysteine residue in the intracytoplasmic tail of MHC I molecules. An MHC I molecule containing a single cysteine residue in an artificial glycine and alanine intracytoplasmic domain was endocytosed and degraded in the presence of MIR1. Thus, ubiquitination can occur on proteins lacking accessible lysines or an accessible N terminus.

References and Notes

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Supporting Online Material

www.sciencemag.org/cgi/content/full/309/5731/125/DC1
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Ubiquitination controls a broad range of cellular functions. The last step of the ubiquitination pathway is regulated by enzyme type 3 (E3) ubiquitin ligases. E3 enzymes are responsible for substrate specificity and catalyze the formation of an isopeptide bond between a lysine residue of the substrate (or the N terminus of the substrate) and ubiquitin. MIR1 and MIR2 are two E3 ubiquitin ligases encoded by Kaposi’s sarcoma–associated herpesvirus that mediate the ubiquitination of major histocompatibility complex class I (MHC I) molecules and subsequent internalization. Here, we found that MIR1, but not MIR2, promoted down-regulation of MHC I molecules lacking lysine residues in their intracytoplasmic domain. In the presence of MIR1, these MHC I molecules were ubiquitinated, and their association with ubiquitin was sensitive to β2-microglobulin, unlike lysine-ubiquitin bonds. This form of ubiquitination required a cysteine residue in the intracytoplasmic tail of MHC I molecules. An MHC I molecule containing a single cysteine residue in an artificial glycine and alanine intracytoplasmic domain was endocytosed and degraded in the presence of MIR1. Thus, ubiquitination can occur on proteins lacking accessible lysines or an accessible N terminus.
ligases (3, 7), E3 ubiquitin ligases function as adaptors to facilitate positioning and transfer of ubiquitin (Ub) from an E2 enzyme directly onto the E3-bound substrate (1). The nature of the bond between Ub and its substrate has been well characterized: The Ub C-terminal glycine carboxy group forms an isopeptide bond with the ε-amino group of lysine residues or, less commonly, with the amino group at the N terminus of the substrate protein (8). MIR proteins recruit E2 enzymes with their N-terminal RING-CH domain (3). Either direct or indirect interactions between the transmembranes of the MIRs and MHC I molecules ultimately lead to the ubiquitination of lysine residues present in the MHC I intracytoplasmic tail (3, 9). Ubiquitinated molecules are then endocytosed and degraded by the lysosome (3, 7, 10–12). Mutating all the lysines to arginines in the intracytoplasmic domain of HLA.B7 (henceforth referred to as the HLA.B7 2R mutant or lysineless HLA.B7) abolishes internalization mediated by MIR2 (3).

However, in the presence of MIR1, the cell surface expression of both wild-type (wt) HLA.B7 and HLA.B7 2R was strongly down-regulated, even in cells expressing low levels of MIR1 (Fig. 1). In contrast, even high levels of MIR2 (Fig. 1) did not induce HLA.B7 2R down-regulation. Thus, the MIR1 protein can mediate the down-regulation of MHC I molecules lacking lysines. Similar results were observed in HeLa cells, suggesting that HLA.B7 2R down-regulation by MIR1 is not restricted to B cells. In the presence of MIR1, HLA.B7 2R molecules are endocytosed, translocated toward the lysosome, and degraded, which is similar to the effects of MIR1 on HLA.B7 wt molecules.

To test whether a particular motif encoded in the intracytoplasmic domain of HLA.B7 2R was required for MIR1-mediated down-regulation, we generated a set of HLA.B7 2R molecules lacking different parts of the intracytoplasmic domain (Fig. 2A) and tested their susceptibility to MIR1-mediated down-regulation. Deletion of the last seven amino acids in HLA.B7 2R did not prevent down-regulation (HLA.B7 ΔC), whereas further truncations (constructs HLA.B7 ΔΔ, ΔA, and ΔB) inhibited internalization (Fig. 2C). Thus, a critical determinant for MIR1-mediated down-regulation is encoded in the last seven residues of HLA.B7 ΔC.

Although we observed down-regulation of lysineless HLA.B7 by MIR1, a lysine-less HLA.A2 molecule is not down-regulated by MIR1 (7). Within the region identified above, HLA.B7 encodes a cysteine in the same position that HLA.A2 encodes a serine (Fig. 2B). We generated a HLA.B7 mutant lacking both cysteine and lysine in its cytoplasmic tail, which we call HLA.B7 2RS (Fig. 2B). Mutation of the cysteine decreased the extent of MIR1-mediated endocytosis (Fig. 2D). We then introduced by mutagenesis a cysteine into the intracytoplasmic tail of HLA.A2 in which all the intracytoplasmic lysines were previously mutated (Fig. 2B). HLA.A2 without lysines (HLA.A2 3R) was slightly down-regulated in the presence of MIR1, and introduction of the cysteine in HLA.A2 3R (HLA.A2 3RC) allowed full down-regulation (Fig. 2E). We also substituted the last arginine residue of HLA.B7 2RS (HLA.B7 2R without lysines or cysteines) with a cysteine and observed that this mutant was as susceptible as HLA.B7 wt to MIR1-mediated down-regulation (Fig. 2, B and D). This strongly suggested that the cysteine was not acting within a linear motif. Thus, in addition to the lysine- and Ub-dependent pathway, MIR1 can down-regulate surface molecules in a lysine-independent manner through a process that requires a cysteine in the intracytoplasmic tail of the target molecule. Other determinants may also be important, because HLA.B7 2RS and HLA.A2 3R, neither of which contains lysines or cysteines, are both partially down-regulated (Fig. 2, D and E).

To further demonstrate that a single cysteine was sufficient to promote MIR1-mediated down-regulation, we replaced the intracytoplasmic tail of HLA.B7 by a stretch of glycine and alanine residues (GA stretch) (Fig. 3A). To this GA stretch, we added each of the 20 amino acids at position X (Fig. 3B). As expected, the GA stretch did not allow down-regulation, whereas the presence of a lysine did. Thus, lysine is sufficient to promote ubiquitination-mediated down-regulation independent of surrounding motifs. The same phenotype was observed in the presence of a cysteine (Fig. 3B). None of the other amino acids lead to down-regulation in the presence of MIR1 (Fig. 3B), except serine. The extent of the down-regulation in the presence of a serine was modest but highly reproducible. This is consistent with the fact that HLA.B7 2R (which does not have lysines but has a cysteine) is strongly down-regulated, whereas HLA.B7 2RS (no lysines but cysteine) is partially down-regulated (Fig. 2D).

Indeed, HLA.B7 2RS contains nine serine residues in its cytoplasmic tail. Overall, it appears

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*To whom correspondence should be addressed.
E-mail: ltcoscoy@berkeley.edu

Department of Molecular and Cell Biology, 142 Life Sciences Addition Room 3200, Berkeley, CA 94720, USA.

**Fig. 1.** MIR1, but not MIR2, down-regulates the MHC I allele HLA.B7 in the absence of intracytoplasmic lysines. BJAB cells stably expressing wt HLA.B7 or the HLA.B7 2R mutant lacking the two intracytoplasmic lysines were transiently transfected with a vector expressing MIR1 or MIR2 fused to enhanced green fluorescent protein (EGFP). Cells were stained with a phycoerythrin-conjugated monoclonal antibody against HLA.B7 and analyzed by flow cytometry.
that MHC I molecules can be down-regulated independently of lysines, in a cysteine-dependent (and possibly serine-dependent) fashion.

We examined the possibility that lysineless MHC I molecules could be ubiquitinated in the presence of MIR1. We used the hamster CHO cell line, which is permissive for MIR1-mediated down-regulation and does not express endogenous human MHC I molecules. We stably transduced CHO cells with the different HLA.B7 constructs and MIR1. After selection, human MHC I heavy chains were specifically immunoprecipitated, and their ubiquitination status was analyzed. No ubiquitinated forms were observed in the absence of MIR1, or when MIR1 was coexpressed with the HLA.B7 construct lacking almost all its intracytoplasmic domain, HLA.B7Δ (Fig. 2A). However, in cells expressing MIR1, ubiquitination of HLA.B7wt and, to a lesser extent, HLA.B7 2R (no lysines) was readily detectable, because it produced a characteristic heterogeneous array. In addition, a small but detectable degree of ubiquitination was observed in HLA.B7 2RS (no lysines or cysteine), consistent with the lower level of down-regulation observed (Fig. 2B). Thus, a residue other than lysine was being ubiquitinated by MIR1.

We next examined the possibility that this cysteine was the ubiquitination site for HLA.B7 2R. We immunoprecipitated wt HLA.B7, as well as HLA.B7 2R, from CHO cells expressing MIR1, and we incubated these immunoprecipitates in the presence of β2-mercaptoethanol at pH 11 in order to break potential thiol-ester bonds (cysteine-Ub bond) but not isopeptide bonds (lysine-Ub bond). Ubiquitination of HLA.B7 2R, but not HLA.B7 wt, was completely eliminated by this treatment (Fig. 4, B and C). Similarly, treatment drastically diminished the cysteine-Ub bond from the E2 enzyme UBE2E3 (fig. S1).

Altogether, our results show that in the absence of lysine, HLA.B7 molecules are ubiquitinated in a cysteine-dependent manner. Moreover, the bond between ubiquitin and the lysineless HLA.B7 shares the same chemical property as the bond between ubiquitin and E2s, which strongly suggests that cysteine is the ubiquitin-attachment site for HLA.B7 2R. Direct visualization of the cysteine-ubiquitin bond by mass spectrometry is hindered by the small amount of ubiquitinated molecules available for purification.

The foregoing shows that the side chain of residues other than lysine can serve as receptors for substrate ubiquitination. It is puzzling that, although ubiquitination has been extensively studied, in particular using large-scale proteomic, such a modification has never been observed in the past. A thiol-ester bond (cysteine-ubiquitin) is more labile than an isopeptide bond (lysine-ubiquitin), which certainly hinders its detection. This may explain why the level of ubiquitination detected with HLA.B7 2R is not as robust as the one observed with HLA.B7 wt (Fig. 4A). In addition, we believe that this form of ubiquitination might be restricted to a subfamily of E3 ubiquitin ligases, such as the MIR1 E3 ubiquitin ligase family (MIR1 and its homologs) (13) (SOM text and fig. S2). The regulation processes mediated by ubiquitination may be more complex because nonlysine residues are also targets of ubiquitination. For example, the number of potential substrates could be extended to molecules that do not contain accessible lysines or an accessible N terminus, and/or transient ubiquitination of substrates may occur, because thiol-ester bonds (Ub-cysteine) are more labile than isopeptide bonds (Ub-lysine). It will be important to determine whether this alternate form of ubiquitination requires the same cellular cofactors as the ones involved in lysine ubiquitination.

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**Fig. 2.** An intracytoplasmic cysteine residue is critical for lysine-independent down-regulation by MIR1. (A) Polymerase chain reaction (PCR) mutagenesis was used to create serial deletion mutants in the intracytoplasmic tail of HLA.B7 2R. (B) We generated several mutants within the intracytoplasmic region of HLA.B7 and HLA.A2 so as to analyze the requirement of the cysteine residue unique to HLA.B7. (C) BJAB cells stably expressing the mutants in (A) were transiently transfected with a construct expressing MIR1-EGFP and analyzed for surface expression of HLA.B7 by flow cytometry. (D) BJAB cells stably expressing MIR1-EGFP along with the various HLA.B7 mutants were analyzed for surface HLA.B7 expression by flow cytometry. (E) HEK cells stably expressing MIR1-EGFP and various HLA.A2 mutants were analyzed for surface HLA.A2 expression. No down-regulation is indicated by cells stably expressing HLA.A2 and EGFP alone.

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![Diagram](https://www.sciencemag.org/science)
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A

<table>
<thead>
<tr>
<th>Transmembrane</th>
<th>Intracytoplasmic Tail</th>
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<tr>
<td>B7 AG X</td>
<td>VVIGAVAAVMCAAAAAAGGGAAGXAG</td>
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B

Fig. 3. One lysine or cysteine residue is sufficient to promote down-regulation of HLA.B7 by MIR1. (A) Amino acid sequence of the intracytoplasmic tail of HLA.B7 mutants where the tail has been replaced by a random GA stretch. Each of the 20 amino acids was substituted at position X. (B) BJAB cells stably expressing the HLA.B7 GA mutants were transfected with MIR1-EGFP and an EGFP control, then analyzed for surface expression of HLA.B7 using flow cytometry.

Fig. 4. A novel form of ubiquitination is detectable on HLA.B7 2R. (A) Lysates from CHO cells stably expressing wt HLA.B7, HLA.B7 2R, HLA.B7 2RS, and HLA.B7 Δ with or without stable expression of MIR1 were used in an immunoprecipitation reaction. The reaction was carried out using the antibody against human MHC I w6/32 (which recognizes only properly folded human MHC I molecules), and ubiquitinated species were detected by Western blot with an antibody against ubiquitin. (B) Lysates from CHO cells stably expressing wt HLA.B7 or HLA.B7 2R with MIR1 were immunoprecipitated with an antibody against MHC I, eluted in the presence of the reducing agent β-mercaptoethanol at either pH 8 or pH 11, and analyzed by Western blot using an antibody against ubiquitin. (C) WT HLA.B7 and HLA.B7 2R were immunoprecipitated as above, and the presence of HLA.B7 was determined by staining with the antibody against human MHC I, HC10.
CORRECTIONS & CLARIFICATIONS

ERRATUM

Post date 13 January 2006

Reports: “Ubiquitination on nonlysine residues by a viral E3 ubiquitin ligase” by K. Cadwell and L. Coscoy (1 July 2005, p. 127). In Fig. 3B, the top left panel was inadvertently duplicated and printed a second time as the top right panel. The correct version of the top right panel of Fig. 3B appears here. The conclusions of the paper are not affected by this error.
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