The Specificities of Kaposi's Sarcoma-Associated Herpesvirus-Encoded E3 Ubiquitin Ligases Are Determined by the Positions of Lysine or Cysteine Residues within the Intracytoplasmic Domains of Their Targets[▽]

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Kaposi's sarcoma-associated herpesvirus encodes two homologous E3 ligases, MIR1 and MIR2, that mediate the ubiquitination and subsequent downregulation of several cell surface proteins, and in particular major histocompatibility complex class I (MHC-I) molecules. We have previously shown that, in addition to lysine ubiquitination, MIR1 has the unique ability of transferring ubiquitin onto MHC-I molecules lacking available lysine residues, in a cysteine-dependent manner. Here we report that MIR1 activity is maximal when either a lysine or cysteine residue is placed approximately 15 amino acids away from the transmembrane domain, whereas MIR2 preferentially targets residues, including cysteines, that are closer to the transmembrane domain. Thus MIR1 and -2 can distinguish their substrates based on the position of the lysine or cysteine residues, suggesting that these proteins have evolved to target different sets of surface molecules. These results indicate that the position of target residues within a substrate is an essential determinant of E3 ubiquitin ligase specificity.

Ubiquitination is a posttranslational modification that regulates many fundamental cellular processes and is therefore a common target for manipulation by viruses (9). Kaposi's sarcoma-associated herpesvirus encodes two homologous E3 ligases, modulator of immune recognition 1 (MIR1) and MIR2, that catalyze the ubiquitination of several cell surface proteins (5, 12). These substrates include major histocompatibility complex class I (MHC-I) molecules, proteins that are critical for recognition of virally infected cells by the immune system. After ubiquitination at the cell surface by MIR1 and -2, MHC-I molecules undergo internalization from the cell surface and degradation in the lysosome (5, 7, 12, 14). E3 ubiquitin ligases catalyze the transfer of ubiquitin from an E2 enzyme to a lysine residue (or the N terminus) on the substrate (18). Accordingly, lysine residues in the intracytoplasmic domains of MHC-I molecules have been shown to be critical for downregulation by the MIR proteins (3, 7, 8, 10, 15).

In addition to this well-characterized pathway, we have previously shown that MIR1 is able to downregulate substrates lacking lysine residues in their intracytoplasmic domains (3). The MHC-I allele HLA.B7 that had its two intracytoplasmic lysines mutated to arginines (HLA.B7 2R) was downregulated by MIR1 to the same extent as wild-type (wt) HLA.B7 (3). Removal of lysine-less HLA.B7 from the cell surface was dependent on the presence of a single cysteine residue in the intracytoplasmic domain. Furthermore, in the presence of MIR1, lysine-less HLA.B7 2R was ubiquitinated, and the bond

between ubiquitin and HLA.B7 2R was labile under reducing conditions, consistent with a thioester bond, in contrast to the bond between ubiquitin and wt HLA.B7. Interestingly, despite its close homology to MIR1, MIR2 was not able to downregulate lysine-less HLA.B7 2R.

We performed a systematic study examining to what extent the position of lysine residues along the cytoplasmic tail of a substrate can affect its downregulation by ubiquitination. To our knowledge, such a systematic analysis has never been done. Moreover, to gain further insight into the mechanism by which cysteines control ubiquitination, we asked whether lysine- and cysteine-dependent ubiquitination had distinct positional constraints. To address this, either a single lysine or cysteine was substituted into various positions along the intracytoplasmic tail of HLA.B7. To avoid complications due to the presence of other amino acids, we used an HLA.B7 molecule composed of an artificial 36-amino-acid glycine/alanine intracytoplasmic tail. These constructs were generated by overlapping PCR as previously described (3) and expressed in BJAB cells by retroviral transduction. Surface expression of each individual HLA.B7 construct in the presence of MIR1 was quantified by flow cytometry using a R-phycoerythrin-conjugated anti-HLA.B7 monoclonal antibody (Chemicon). Downregulation of both lysine- and cysteine-containing tails displayed similar patterns where lysine and cysteine residues positioned close to the transmembrane region were less permissive for downregulation (Fig. 1). MIR1 reached peak activity when the lysine or cysteine was proximal to 15 amino acids away from the transmembrane region. However, a cysteine permitted less downregulation than a lysine at similar positions away from the peak activity. Thus, there is a similar structural constraint on lysine- and cysteine-mediated downregulation by MIR1, although for optimal downregulation, the position of the cysteine is more

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FIG. 1. The position of lysine or cysteine residues within the intracytoplasmic tail of HLA.B7 is an essential determinant of MIR activity. (A) BJAB cells stably expressing HLA.B7 with either one lysine or one cysteine at various positions within an artificial glycine/alanine tail were transduced with retroviral vectors expressing MIR1-EGFP or MIR2-EGFP. Downregulation (*n*-fold) was determined for each HLA.B7 molecule by quantifying surface expression in the presence (GFP⁺) or absence (GFP⁻) of the MIR protein using flow cytometry. (B) BJAB cells stably expressing MIR1 as well as HLA.B7 2R were transfected with either MIR1-EGFP, MIR2-EGFP, or EGFP alone. Surface HLA.B7 expression was analyzed by flow cytometry. Transfected cells are represented by the shaded histogram.

restricted. The same set of HLA.B7 constructs with lysines at different positions was quantified for cell surface expression in the presence of MIR2. Unexpectedly, we observed that MIR2 has a distinct preference for positions closer to the transmembrane region than those of MIR1 (Fig. 1A). Positioning the lysine further from the transmembrane region dramatically decreased downregulation by MIR2. This result indicates that,

even on the same substrate, the optimal position of the ubiquitination target residue can differ from one E3 ubiquitin ligase to another. In our previous study, we found that the lysine-less HLA.B7 2R molecule was not downregulated by MIR2 (3), suggesting that MIR2 was not able to perform lysine-independent, cysteine-dependent ubiquitination. However, in that construct,

FIG. 2. Two regions encoded within MIR1 are essential for downregulation of lysine-less HLA.B7 2R. (A) BJAB cells stably expressing wt HLA.B7 or HLA.B7 2R were transfected with chimeric mutants that combine regions of MIR1 and MIR2, and surface expression of HLA.B7 was measured by flow cytometry. (B) Additional chimeras were created in which various portions of the MIR2 N and C termini were placed on a MIR1 background. By determining which chimeras downregulate surface wt HLA.B7 and HLA.B7 2R, one region each within the N and C termini were identified. The alignments of these regions between MIR1 and -2 are shown in panel C.

the cysteine is 17 amino acids away from the transmembrane region, a position that, according to our present results, is not favorable for MIR2-mediated ubiquitination. Thus, it is possible that MIR2 cannot downregulate HLA.B7 2R because its only cysteine is too far away from the transmembrane domain. To address this possibility, we used a previously described HLA.B7 mutant (HLA.B7 RC) with no lysine but with a cysteine located eight amino acids from the transmembrane domain (3). Although MIR2-mediated downregulation of HLA.B7 2R was imperceptible, the repositioning of the cysteine conferred some degree of downregulation (data not shown). Altogether, these experiments suggest that MIR2 recognizes HLA.B7 2R as a substrate but does not downregulate it due to the accessibility of the cysteine. To confirm that MIR2 has the ability to perform cysteine-dependent downregulation, we examined the effect of MIR2 expression on the surface level of the previously used HLA.B7 constructs that have cysteines

at various positions along a glycine/alanine intracytoplasmic tail. We found that MIR2 could lead to a significant downregulation only when a cysteine is 10 to 15 amino acids from the transmembrane region (Fig. 1A). Altogether, these experiments suggest that MIR2, by binding its substrates without promoting its ubiquitination, could act as a dominant-negative inhibitor of MIR1. To test this hypothesis, MIR2 was overexpressed by transient transfection in BJAB cells stably expressing HLA.B7 2R and MIR1. Overexpression of MIR2 restored a high level of HLA.B7 2R at the cell surface compared to the enhanced green fluorescent protein (EGFP) control, in contrast to overexpression of additional MIR1, which led to increased downregulation (Fig. 1B). To identify regions of MIR1 and MIR2 that are responsible for these positional constraints, we generated chimeric mutants of MIR1 and MIR2 by overlapping PCR and examined their ability to downregulate HLA.B7 2R (Fig. 2A). All chi-

meras, except MIR2 with the transmembrane region replaced by MIR1 (chimera 2-1-2), were able to fully downregulate wt HLA.B7, indicating that these constructs are functional (Fig. 2A). Chimeras in which either the N-terminal or the C-terminal portion of MIR1 was replaced by the homologous region of MIR2 (chimeras 1-1-2 and 2-1-1) were unable to downregulate HLA.B7 2R. In contrast, MIR2, with both its N- and C-terminal regions replaced by that of MIR1 (chimera 1-2-1), was able to downregulate HLA.B7 2R. Further chimeras were created on a MIR1 backbone to identify the minimal regions in the N and C termini of MIR1 that were necessary for downregulation of HLA.B7 2R (Fig. 2B). Analysis of these chimeras allowed the identification of 12 amino acids in the N terminus and 7 amino acids in the C terminus of MIR1 that are required for HLA.B7 2R downregulation; the sequences of MIR1 and -2 within these regions are compared in Fig. 2C. To confirm the significance of these two regions, we replaced the homologous region of MIR2 with these two regions that are encoded by MIR1 (chimera 2-1-2-1-2). Chimera 2-1-2-1-2 displayed significant downregulation of HLA.B7 2R (Fig. 3A). In the converse experiment, a MIR1 mutant with the two regions replaced by those of MIR2 (chimera 1-2-1-2-1) was greatly impaired in its ability to downregulate HLA.B7 2R but not wt HLA.B7 (Fig. 3A). To determine if these two regions are involved in determining the favorable location of the target cysteine, cell surface expression of HLA.B7 constructs encoding cysteines at different locations in an artificial intracytoplasmic tail (same constructs as in Fig. 1) were quantified in the presence of chimera 2-1-2-1-2. Although chimera 2-1-2-1-2 has a MIR2 backbone, it downregulated HLA.B7 molecules with cysteines in positions that are more favorable for downregulation by MIR1 (Fig. 3B). These results suggest that the amino acids identified contribute to MIR1's preference for the position of the target residue.

E3 ligases are frequently viewed as molecular scaffolds that catalyze ubiquitination by bringing the substrate and E2 within close proximity. In the case of the MIRs, association with substrates occurs through transmembrane-transmembrane interactions (7, 19). In addition to the requirement for close proximity, our results indicate that the position of the target residue is an essential determinant of substrate specificity as well. Thus, the MIR proteins might interact with many membrane-associated proteins but downregulate only the ones that have a correctly positioned lysine or cysteine. This raises the possibility that an E3-ubiquitin ligase, by binding to a particular protein without ubiquitinating it, could act as a dominant-negative mutant, as shown here for the MIR proteins (Fig. 1B). Since MIR1 and MIR2 are expressed concomitantly during viral replication, it becomes important to test if MIR2 can exert an effect on the downregulation of a substrate specific to MIR1 or vice versa during an infection (2, 5, 6, 11–13, 15). Similarly, the recently identified MARCH proteins, cellular E3 ligases that are homologous to the MIR proteins, have an overlapping substrate specificity (1). Moreover, we have observed that several members of the MARCH family can be expressed in the same cell and that their relative expression can vary depending on external stimuli (data not shown). It is thus tempting to speculate that these E3 ligases might compete with each other for substrates and act as dominant-negative inhibitors as part of regulatory mechanisms.

FIG. 3. Two regions of the MIR proteins determine the optimal position of the cysteine in the substrate. (A) The two regions of MIR1 identified above were substituted onto MIR2-EGFP to create chimera 2-1-2-1-2. Similarly, the two regions of MIR1 were replaced by the homologous region of MIR2 to create chimera 1-2-1-2-1 fused to EGFP. BJAB cells stably expressing HLA.B7 2R were transfected with vectors expressing either the chimeras or the MIR proteins, and the extent of MHC-I downregulation was assessed by fluorescence-activated cell sorting. (B) BJAB cells stably expressing the HLA.B7 mutants containing cysteines at various positions were transduced with a retroviral vector expressing the chimera 2-1-2-1-2-EGFP and analyzed by flow cytometry as described for Fig. 1.

We identified regions within the MIRs that control the position of the substrate residues that are targeted for ubiquitination. Surprisingly, the positional constraint is not determined by the RING finger domain but instead by two regions located close to the transmembrane domains on the N- and C-terminal regions of the MIRs. This suggests a model where these two regions interact with each other and, by doing so, orient the E2 to target a specific region of the substrate (Fig. 4). To our knowledge, this is a novel aspect that has not been

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