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Lysine-Independent Ubiquitination of Epstein–Barr Virus LMP2A

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Latent membrane protein 2A (LMP2A) of latent Epstein–Barr virus (EBV) specifically associates with HECT domain-containing Nedd4-family ubiquitin-protein ligases (E3s). Here we demonstrate that LMP2A is specifically ubiquitinated by the HECT domains of AIP4 and WWP2. Deletion and site-specific mutation of LMP2A indicates that LMP2A is ubiquitinated at its amino-terminus and is not ubiquitinated on lysine residues. LMP2A and LMP1, also encoded by EBV, are two of only four proteins that have been identified that are ubiquitinated at the amino-terminus, indicating that EBV may specifically target and utilize this host cell protein modification.

INTRODUCTION

Ubiquitination of proteins is a posttranslational modification in which a 76 amino acid polypeptide, ubiquitin, or a multiubiquitin chain, is attached to proteins tagging them for degradation (Hershko and Ciechanover, 1998). Ubiquitination is catalyzed by the sequential actions of the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3). The E1 first activates ubiquitin in an ATP-dependent reaction by forming a thioester bond at its active-site cysteine with the carboxy-terminus of ubiquitin. Activated ubiquitin is next transferred to an active site cysteine of the E2. The E3 is bound to the targeted substrate and catalyzes the covalent attachment of ubiquitin to the ϵ-amino group of a lysine residue of the substrate. The generation of multiubiquitin chains is mediated by the repeated attachment of ubiquitin onto a lysine residue of ubiquitin. E3s play an essential role in specific substrate recognition. They are defined as proteins or protein complexes that include RING-finger-containing E3s (Freemont, 2000) and HECT-domain containing E3s (Huibregtse *et al.,* 1995). The HECT family E3s are the only known E3s that form ubiquitin-thioester intermediates and directly catalyze substrate ubiquitination. Nedd4-family E3s belong to the HECT domain family and their binding specificity for target proteins is contained within a C2 domain and two to four repeats of a WW domain (Harvey and Kumar, 1999).

Ubiquitination commonly occurs within internal lysine residues; however, recent studies revealed that ubiquitin conjugation can occur at the α-amino group of amino-terminal residue. Substitution of all lysine residues in MyoD (Breitschopf *et al.,* 1998), LMP1 (Aviel *et al.,* 2000), and E7 (Reinstein *et al.,* 2000) does not affect significantly their conjugation and degradation in the ubiquitin pathway. In contrast, extension of amino-terminus by the addition of a few amino acids makes all the proteins stable, indicating the microenvironment of the amino-terminus is important for their ubiquitination and subsequent degradation. However, the mechanism of ubiquitin conjugation at amino-terminal residue, especially by specific E3s, is unclear.

Epstein-Barr virus (EBV) is a ubiquitous human oncogenic herpesvirus associated with numerous proliferative disorders, including Burkitt's lymphoma (BL), Hodgkin's disease (HD), AIDS-associated immunoblastic lymphoma, oral hairy leukoplakia (OHL), and nasopharyngeal carcinoma (NPC) (Kieff, 1996; Longnecker, 1998). B lymphocytes infected with EBV *in vitro* are immortalized and subsequently termed lymphoblastoid cell lines (LCLs). These EBV-transformed LCLs express a restricted set of latency-associated viral products, including six EBV nuclear antigens (EBNAs), three latent membrane proteins (LMP1, LMP2A, and LMP2B), two small RNAs (EBERs), and *Bam*HI A rightward transcripts (BARTs). In healthy individuals, EBV typically establishes a persistent latent infection in peripheral B lymphocytes. These latently infected cells express only LMP2A, EBNA1, and BARTs (Chen *et al.,* 1999; Thorley-Lawson, 2001). Further, LMP2A is expressed in tumor cells in several EBV-associated malignancies including NPC and HD (Kieff, 1996; Longnecker, 1998; Thorley-Lawson, 2001).

LMP2A is a 12 transmembrane-spanning integral membrane protein that localizes in lipid rafts in EBV-immortalized LCLs (Dykstra *et al.,* 2001; Longnecker, 1998). The 119 amino acid amino-terminal domain of LMP2A contains a variety of motifs involved in protein–

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protein interactions, including eight tyrosine residues and two PY motifs. LMP2A functions to down-regulate B cell receptor (BCR)-mediated signal transduction and viral activation from latency by recruitment and constitutive phosphorylation of protein-tyrosine kinases (PTKs) such as Lyn and Syk (Fruehling and Longnecker, 1997; Fruehling *et al.,* 1998; Miller *et al.,* 1995). LMP2A also acts to provide B cells with both a survival and a developmental signal *in vivo* (Caldwell *et al.,* 1998). Previously, we have shown that the level of LMP2A transcripts correlates with the severity of the LMP2A *in vivo* phenotype, suggesting that LMP2A function may be regulated by the amount of LMP2A (Caldwell *et al.,* 2000). Recently, we have shown that LMP2A specifically associates with Nedd4-family ubiquitin-protein ligases (E3s), including AIP4, WWP2, and Nedd4. This interaction is important in regulating protein stability and phosphorylation of LMP2A and LMP2A-associated proteins (Ikeda *et al.,* 2000; Winberg *et al.,* 2000). The studies in the current article demonstrate that LMP2A is ubiquitinated at the amino-terminus by Nedd4-family E3s and not at lysine residues.

RESULTS

LMP2A is ubiquitinated by the HECT-domain of Nedd4-family E3s

Our recent studies have shown that LMP2A specifically associates with Nedd4-family E3s, suggesting that LMP2A is directly conjugated by ubiquitin which targets it for degradation. To detect LMP2A ubiquitination in cells, we cotransfected the EBV-negative B lymphoma cell line BJAB with LMP2A, Nedd4-family E3s, and HA-tagged ubiquitin expression plasmids and identified ubiquitinated products by blotting anti-LMP2A immunoprecipitates with anti-HA antibody. To confirm the role of the binding of the Nedd4-family E3s AIP4 and WWP2 to LMP2A in LMP2A ubiquitination, AIP4 or WWP2 was cotransfected with the LMP2A PY mutant (PY1PY2) in which both the PY motifs (PPPPY) are mutated to PAAPY. This LMP2A-PY1PY2 mutant does not bind to the WW domains of Nedd4-family E3s *in vitro* and *in vivo* (Ikeda *et al.,* 2000, 2001). As shown in Fig. 1, AIP4 and WWP2 catalyzed the ubiquitination of wild-type LMP2A as observed by the presence of high molecular weight ladder bands of ubiquitin conjugates that were readily observed in LMP2A and HA-tagged ubiquitin transfected cells (Fig. 1, HA and LMP2A Blot, lanes 2 and 5). These same complexes were not observed when the cells were transfected only with the HA-tagged ubiquitin (Fig. 1, lanes 1 and 4) or when the LMP2A PY motifs are mutated (Fig. 1, HA Blot, lanes 3 and 6). A densitometric analysis revealed that the ubiquitination of PY mutant LMP2A was 38-fold less with AIP4 and 142 times less with WWP2, when compared to wild-type LMP2A, although mutant LMP2A expression was two times less than wild-type.

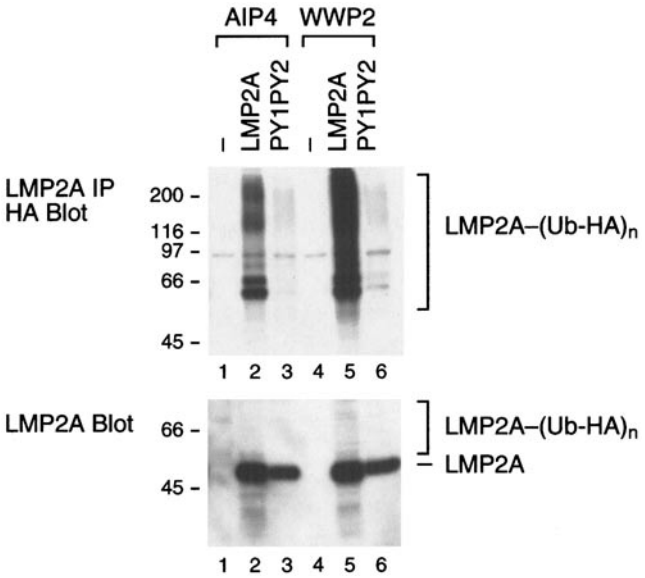

FIG. 1. Ubiquitination of LMP2A PY motif mutant. BJAB cells were transiently transfected with empty vector (lanes 1 and 4), LMP2A (lanes 2 and 5), or LMP2A PY mutant (lanes 3 and 6), with AIP4 (lanes 1 to 3), or WWP2 (lanes 4 to 6), and with HA-Ub (lanes 1 to 6). LMP2A immunoprecipitates were immunoblotted with anti-HA to detect ubiquitin conjugates and whole lysates were immunoblotted with anti-LMP2A to detect LMP2A expression. A densitometric analysis revealed that the ubiquitination of PY mutant LMP2A was 38-fold less with AIP4 and 142 times less with WWP2 when compared to wild-type LMP2A, although mutant LMP2A expression was two times less than wild-type. Protein standards are indicated in kDa.

The somewhat reduced level of LMP2A-PY1PY2 protein in the LMP2A blot when compared to wild-type LMP2A is a result of experimental variation likely due to differences in transfection efficiency since it was not routinely observed. These results indicate that LMP2A is ubiquitinated by exogenous E3s in BJAB cells and that the LMP2A PY motifs are required for the ubiquitination of LMP2A by AIP4 and WWP2.

Next, to confirm the role of Nedd4-family E3s in LMP2A ubiquitination, the active-site cysteine of the HECT domain, C823 in WWP2, was mutated to alanine to create a dominant-negative form WWP2. This mutant will retain binding to LMP2A since the WW domains within WWP2 will be unaltered and this is the region critical for binding of WWP2 to the LMP2A PY motifs (Ikeda *et al.,* 2000). When compared with WWP2, the dominant-negative C823A did not direct LMP2A ubiquitination (Fig. 2, HA and LMP2A Blot, compare lanes 3 and 4), indicating that the cysteine-to-alanine mutation completely blocked the activity of the WWP2 HECT domain. Interestingly, mutation of the WWP2 HECT domain resulted in a decrease in WWP2 ubiquitination (Fig. 2, FLAG Blot, compare lanes 3 and 4), suggesting that WWP2 may be susceptible to autoubiquitination which may be important for WWP2 degradation. Similar results were obtained using AIP4 and a corresponding mutation in the AIP4 HECT domain (data not shown).

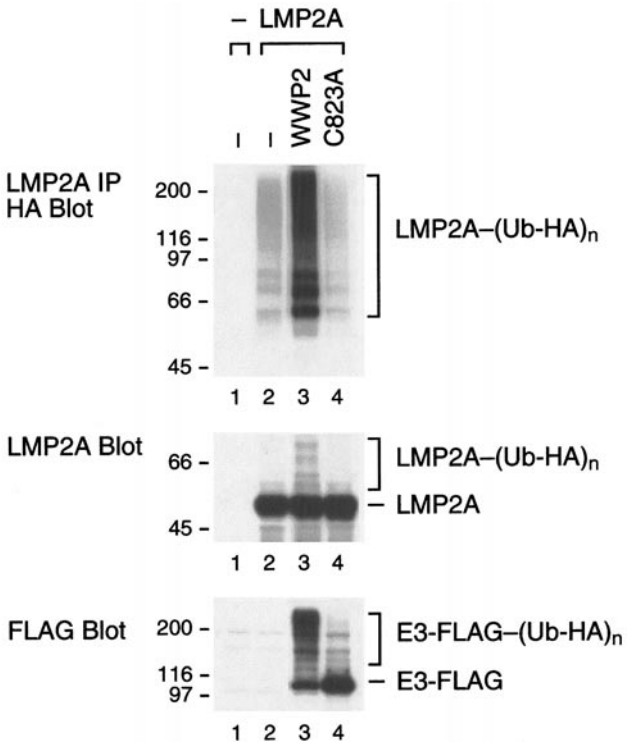

FIG. 2. LMP2A ubiquitination by wild-type and dominant-negative Nedd4-family E3s. BJAB cells were transiently transfected with empty vector (lane 1) or LMP2A (lanes 2 to 4), WWP2 (lane 3), or C823A (lane 4), and with HA-Ub (lanes 1 to 4). LMP2A immunoprecipitates were immunoblotted with anti-HA to detect ubiquitin conjugates and whole lysates were immunoblotted with anti-LMP2A to detect LMP2A or anti-FLAG to detect Nedd4-family E3 expression. Protein standards are indicated in kDa.

Lysine-less LMP2A mutants are ubiquitinated by Nedd4-family E3s

Target proteins are commonly ubiquitin-conjugated to internal lysine residues by specific E3s. The LMP2A amino acid sequence includes three lysine residues: K177 and K349 are located in cytosolic domain, and K381 is located in extracellular domain. To identify the sites responsible for the ubiquitination of LMP2A, deletion mutants were used in the transient ubiquitination assay. Both Δ312–497 and Δ168–365 mutants contain one lysine residue, while Δ168–497 does not contain any lysine residues. Surprisingly, all of the three deletion mutants, including the lysine-less Δ168–497 mutant, were ubiquitinated in the transient ubiquitination assay (Fig. 3, HA and LMP2A Blot, lanes 2–5). This clearly indicates that nonlysine residues are involved in LMP2A ubiquitination. To confirm the ubiquitination at nonlysine residue, we constructed a site-directed mutant in which all of lysine residues were changed to alanine (LMP2A Lys-less). Similar to the results of deletion mutants, Lys-less LMP2A was ubiquitinated by endogenous E3s such as wild-type LMP2A (Fig. 4, HA Blot, lanes 1 and 2). Moreover, Lys-less LMP2A ubiquitination by WWP2 was similar to that observed for wild-type LMP2A ubiquitination (Fig. 4, HA Blot, lanes 3 and 4). Therefore, we conclude that LMP2A is specifically ubiquitinated at nonlysine residues by Nedd4-family E3s.

The amino-terminal microenvironment of LMP2A is important for LMP2A ubiquitination

Although lysine is a common target site for ubiquitination, amino-terminal ubiquitination of MyoD, LMP1, and E7 have recently been reported. In each case, the attachment of a Myc-tag to the amino-terminus stabilized the proteins (Aviel *et al.,* 2000; Breitschopf *et al.,* 1998; Reinstein *et al.,* 2000). An amino-terminal deletion of LMP1 and E7 also stabilized the protein (Aviel *et al.,* 2000; Reinstein *et al.,* 2000). These results suggest that the microenvironment of amino-terminus is important for the amino-terminal ubiquitination. To examine the role of the LMP2A amino-terminal domain in ubiquitination, we constructed a series of amino-terminal mutants. The amino-terminus of LMP2A contains three potential ATG codons corresponding to amino acids M1, M6, and M9 of the reported LMP2A sequence (Sample *et al.,* 1989). This region is relatively hydrophobic and conserved in the

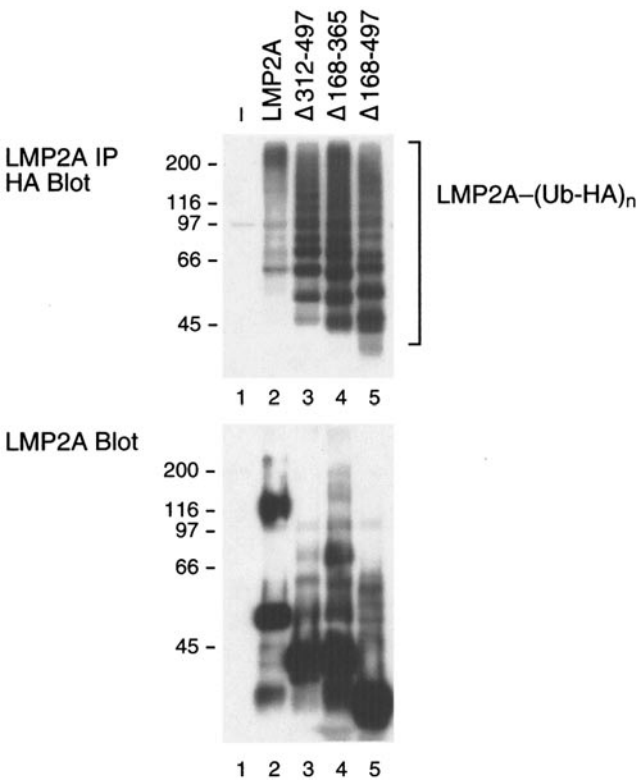

FIG. 3. Ubiquitination of LMP2A deletion mutants. BJAB cells were transiently transfected with empty vector (lane 1), LMP2A (lane 2), LMP2AΔ312–497 (lane 3), LMP2AΔ168–365 (lane 4), LMP2AΔ168–497 (lane 5), and with HA-Ub (lanes 1 to 6). LMP2A immunoprecipitates were immunoblotted with anti-HA to detect ubiquitin conjugates (top) and whole-cell lysates were immunoblotted with anti-LMP2A to detect LMP2A expression (bottom). Protein standards are indicated in kDa.

EBV homologues which infect monkeys and in different EBV clinical isolates (Busson *et al.,* 1995; Franken *et al.,* 1995; Rivailler *et al.,* 1999). Analysis of the EBV genomic nucleotide sequence indicates that the M1 and M6 sequences are perfectly matched to the Kozak sequence, whereas the sequence surrounding M9 partially matches the Kozak sequence (Kozak, 1986). Thus, each of the ATGs may function as a start codon. To identify the actual start codon for LMP2A, we constructed two expression plasmids containing cDNAs that would direct LMP2A synthesis starting from either M1 or M9. The LMP2A expression plasmid we used in Figs. 1 to 4 contains a cDNA starting from the M6 ATG. Moreover, a His-tag or a Myc-tag was inserted just after M1 to change the microenvironment of the LMP2A amino-terminus. These mutations were incorporated into the amino-terminus of the Lys-less LMP2A mutant to exclude the possibility of ubiquitination at lysine residues. Ubiquitination observed in M1-Lys-less LMP2A was similar to that in M6-Lys-less LMP2A (Fig. 5A, HA Blot, lanes 1 and 2). However, M9-Lys-less LMP2A demonstrated an increase in LMP2A ubiquitination compared with M1-Lys-less LMP2A and M6-Lys-less LMP2A (Fig. 5A, HA Blot, compare lanes 1 and 2 with lane 3). Interestingly, the addition of the hydrophobic and positively charged His-tag to the M1-Lys-less LMP2A resulted in LMP2A ubiquitination that was similar to the parental M1-Lys-less LMP2A (Fig. 5A, HA Blot, compare lanes 1 and 4). In contrast, the addition of hydrophilic Myc-tag caused an increase of LMP2A ubiquitination (Fig. 5A, HA Blot, compare lane 5 with lanes 1 and 4). Similar results were observed when each of these different LMP2A amino-terminal constructs were tested using wild-type LMP2A without the mutated lysines (data not shown). In addition, AIP4 and WWP2 transient expression specifically increased the ubiquitination of each of the LMP2A amino-terminal constructs (data not shown). These results indicate that the hydrophobic region of LMP2A amino-terminus can be targeted for ubiquitination by Nedd4-family E3s.

To understand the mechanism of LMP2A amino-terminal ubiquitination, we attempted to determine the site of initiation of LMP2A. To this end, the protein size of LMP2A from EBV transformed LCLs was compared with

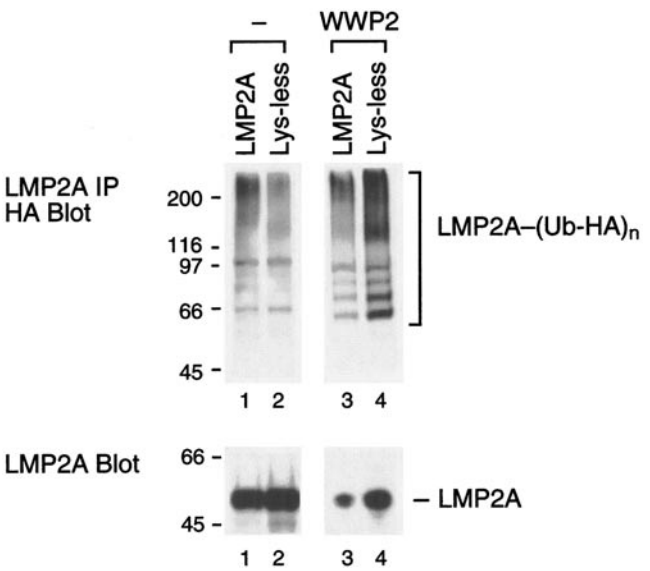

FIG. 4. Ubiquitination of LMP2A lysine-less mutant. BJAB cells were transiently transfected with LMP2A (lanes 1 and 3), or LMP2A Lys-less (lanes 2 and 4), with empty vector (lanes 1 and 2), or WWP2 (lanes 3 and 4), and with HA-Ub (lanes 1 to 4). LMP2A immunoprecipitates were immunoblotted with anti-HA to detect ubiquitin conjugates (top) and whole cell lysates were immunoblotted with anti-LMP2A to detect LMP2A expression (bottom). Protein standards are indicated in kDa.

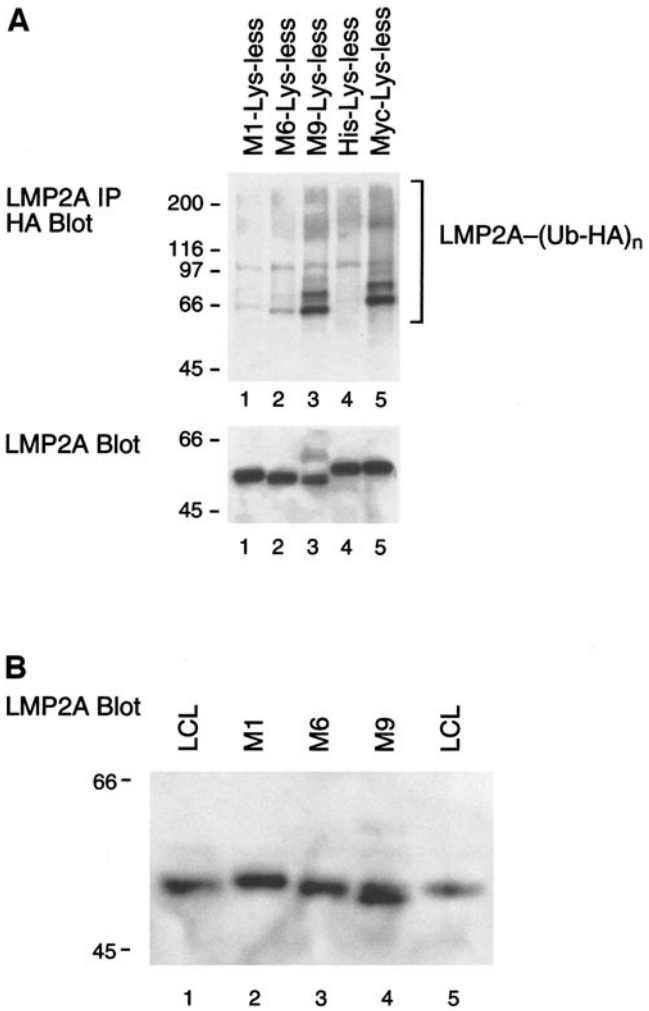

FIG. 5. Ubiquitination and molecular size of LMP2A amino-terminal mutants. (A) BJAB cells were transiently transfected with M1-Lys-less (lane 1), M6-Lys-less (lane 2), M9-Lys-less (lane 3), His-Lys-less (lane 4), or Myc-Lys-less (lane 5), and with HA-Ub (lanes 1 to 6). LMP2A immunoprecipitates were immunoblotted with anti-HA to detect ubiquitin conjugates and whole lysates were immunoblotted with anti-LMP2A to detect LMP2A expression. (B) Lysates of LCL (lanes 1 and 5), or BJAB transfectants of M1 (lane 2), M6 (lane 3), or M9 (lane 4) were immunoblotted with anti-LMP2A. Protein standards are indicated in kDa.

LMP2A produced in transfected cells on a large SDS–polyacrylamide gel. Figure 5B shows that LMP2A expressed from virus genome in EBV-transformed LCLs is of identical size to the protein expressed from LMP2A-M6 cDNA, indicating that the most abundant form of LMP2A expressed in EBV-transformed LCLs is initiated at M6 as long as the posttranslational modification of LMP2A is similar in both EBV-transformed LCLs and BJAB cells expressing LMP2A. LMP2A expressed in BJAB cells has been shown to be functional (Miller *et al.,* 1993). These results suggest the amino-terminus of LMP2A is important in the regulation of the amino-terminal ubiquitination of LMP2A.

DISCUSSION

Ubiquitin conjugation of proteins is strictly regulated by specific ubiquitin-protein ligase E3s. Here we show that LMP2A associates with and is mono-, di-, and polyubiquitinated by Nedd4-family E3s through the binding of the two LMP2A PY motifs with the WW domains contained in Nedd4-family E3s. We had previously shown that mutation of the LMP2A PY motifs caused loss of binding of the Nedd4-family E3s with LMP2A (Ikeda *et al.,* 2001). The mutation of the active site within the WWP2 HECT domain, a Nedd4-family E3, resulted in the inhibition of LMP2A ubiquitination, indicating that the HECT domain is required for LMP2A ubiquitination. In addition, we demonstrated that an LMP2A mutant in which the three lysines contained within LMP2A were mutated to alanine (lysine-less LMP2A) can be a substrate of Nedd4-family E3s similar to the wild-type LMP2A, indicating that nonlysine residues are the sites of LMP2A ubiquitin conjugation. Finally, we determined that the amino-terminal domain of LMP2A was critical for LMP2A ubiquitination. Taken together, we conclude that LMP2A is ubiquitinated at the amino group of amino-terminus by Nedd4 family E3s.

Recently, amino-terminal ubiquitination has been reported in studies of MyoD, LMP1, and E7, all of which are ubiquitinated in lysine-less mutants (Aviel *et al.,* 2000; Breitschopf *et al.,* 1998; Reinstein *et al.,* 2000). For all three proteins, deletion mutations and a Myc-tag insertion at amino-terminus blocked ubiquitination and their subsequent degradation. Similar to LMP2A, EBV-encoded LMP1 is a multiple membrane spanning protein that lacks a significant extracellular domain and both the amino- and the carboxy-terminus are localized within cytosol. LMP2A is targeted to the membrane rafts where it associates in characteristic patches (Longnecker and Kieff, 1990). LMP1 and LMP2A colocalize within these membrane patches in the EBV latent infected cells (Longnecker and Kieff, 1990). Having similar structure, it is of interest to note that both LMP1 and LMP2A are ubiquitinated at the amino-terminus, indicating that EBV may exploit this particular host cell modification, although LMP1 does not appear to use Nedd4-family E3s in its ubiquitination (Aviel *et al.,* 2000; Ikeda *et al.,* 2001). It is still unclear whether amino-terminal ubiquitination is a general modification for most proteins. This awaits the construction of mutations in other cellular and viral proteins in which all the lysines have been mutated to alternate amino acids. In addition, other modifications such as acetylation at the amino-terminus of proteins may prevent amino-terminal ubiquitination. Acetylation is the most common form of cotranslational chemical modification involving approximately 85% of all proteins (Driessen *et al.,* 1985). Further study is clearly warranted. In addition, the influence of phosphorylation for ubiquitination and degradation varies between LMP2A and LMP1. LMP1 is phosphorylated primarily on serine but also on threonine residues and this phosphorylation is required for LMP1 ubiquitination (Aviel *et al.,* 2000). LMP2A is highly tyrosine phosphorylated, but in contrast to LMP1, this phosphorylation is not required for LMP2A ubiquitination. Mutation of tyrosine 112 to phenylalanine in LMP2A results in a complete absence of LMP2A tyrosine phosphorylation (Fruehling *et al.,* 1998), and this mutant is ubiquitinated similar to wild-type LMP2A (data not shown).

We found that the predominant form of LMP2A expressed in EBV immortalized lymphocytes begins at methionine 6 in the LMP2A protein sequence as long as the posttranslational modification of LMP2A is similar in both LCLs and BJAB cells. This form of LMP2A, in comparison to LMP2A beginning at methionine 9 or a mutant form of LMP2A with a Myc-Tag inserted immediately after methionine 1, was not as highly ubiquitinated, indicating that the sequence context or microenvironment of the amino-terminal domain is important in the ubiquitination of LMP2A. By analyzing the amino acid sequence of amino-terminus, we found that LMP2A contains along with the methionine two additional hydrophobic amino acids at its amino-terminus. This hydrophobicity is conserved in different γ-herpesviruses (Franken *et al.,* 1995; Rivailler *et al.,* 1999) and EBV clinical isolates (Busson *et al.,* 1995). It is too short to span the plasma membrane; however, it may still be able to interact with the plasma membrane, another region of the LMP2A, another protein, or it may contain a specific signal required for LMP2A ubiquitination. Thus changes in this amino-terminal region of LMP2A, as shown in this study, may dramatically alter the amount of LMP2A ubiquitination.

Finally, a conformational change might also be required for the efficient LMP2A ubiquitination. A recent NMR study elucidating the tertiary structure of Nedd4 WW domain associating with ENaC PY motif peptide revealed that binding of Nedd4 WW domain leads a sharp β-turn at a leucine residue adjacent to the PY motif (Kanelis *et al.,* 2001), indicating that Nedd4 binding induces a conformational change in the substrate protein. LMP2A ubiquitination likely plays a role in modulating

the signaling capacity of LMP2A complexes within the plasma membrane of latently infected cells. We have previously shown that mutation of the LMP2A PY motifs, responsible for binding the Nedd4-family ubiquitin-protein ligases, results in a dramatic increase in the phosphorylation of signaling proteins involved in LMP2A signal transduction. Further investigation of the amino-terminal ubiquitination of LMP2A by the construction of mutants with altered ubiquitination may provide clues to the mechanism and functional significance of amino-terminal ubiquitination.

MATERIALS AND METHODS

Antibodies

Rat monoclonal antibody against LMP2A (14B7) was described previously (Fruehling *et al.,* 1996). Mouse monoclonal antibody against FLAG tag (M2) was from Sigma. Mouse monoclonal antibody against HA tag (HA. 11) was from Covance. All horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Amersham.

Plasmids

The wild-type LMP2A cDNA was subcloned into *Eco*RI-*Bgl*II site of the mammal expression vector pSG5. The PY1PY2, Lysine-less, M1, M6, M9, Myc, and His LMP2A expression constructs were made using standard cloning strategies as described previously (Ikeda *et al.,* 2000). Each construct contains the same upstream sequence from the initiating ATG along with a perfectly matched Kozak sequence to insure that each construct translates equivalent amounts of LMP2A. The FLAG-tagged AIP4 and WWP2 cDNA, subcloned into the mammal expression vector pcDNA3.1 Neo, was described previously (Ikeda *et al.,* 2000). C823A mutation at an active-site cysteine of the WWP2 HECT domain was also produced using a PCR-based strategy. HA-tagged ubiquitin (Ub-HA) expression plasmid was provided by Dr. D. Bohmann (Treier *et al.,* 1994).

Cell culture and transient transfections

BJAB is an EBV negative B lyphoma cell line obtained from ATCC (Rockville, MD). The generation of LCLs was described elsewhere (Fruehling *et al.,* 1996). All cell lines were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 1000 U/ml penicillin, and 1000 μg/ml streptomycin. BJAB cells (1×10^7 cells) were transiently transfected with Gene Pulser (Bio-Rad) at 210 V and 960 μF capacitance and were analyzed after 24 h.

Detection of ubiquitin conjugates

BJAB cells were transiently transfected with 20 μg of LMP2A expression plasmid, 10 μg of E3 expression plasmid, and 10 μg of HA-tagged ubiquitin expression plasmid. Following culture, the cells were harvested and lysed in 0.5 ml of 1× PBS containing 1% SDS to prevent deubiquitination and minimize nonspecific protein–protein association. After sonication, lysates were adjusted to 1 ml with 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% SDS, 0.25% deoxycholic acid, 10% glycerol, 10 mM NaF, 1 mM Na_3VO_4, 2mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, and 10 μg/ml leupeptin. Cleared lysates were incubated with the appropriate antibody for 1 h at 4°C. Immune complexes were captured with 20 μl of Protein A or G-Sepharose (Pharmacia) for 1 h at 4°C. Following three washes with lysis buffer, immunoprecipitated proteins were resuspended in 2× SDS–PAGE sample buffer. Protein samples were resolved by SDS–PAGE, transferred to Immobilon, and blocked with 4% skim milk at room temperature for 1 h. Membranes were then incubated in 4% skim milk in TBST (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) with primary antibody for 1 h, and then with appropriate secondary antibody for 1 h. The blot was visualized using ECL (Amersham).

ACKNOWLEDGMENTS

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