Interaction of Epstein-Barr Virus Latent Membrane Protein 1 with SCF<sub>HOS/β-TrCP</sub> E3 Ubiquitin Ligase Regulates Extent of NF-κB Activation*

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The Epstein-Barr virus latent membrane protein 1 (LMP1) is pivotal in the transforming activity of this virus. We found that the common LMP1-95-8 variant interacts with Homologue of Slimb (HOS), a receptor for the SCF<sub>HOS/β-TrCP</sub> ubiquitin-protein isopeptide ligase (E3) via one canonical and one cryptic HOS recognition site. These sites are mutated or deleted in the tumor-derived LMP1-Cao variant, which did not bind to HOS. Mutations within these sites on LMP1-95-8 abrogated HOS binding and increased transforming activity of LMP1. HOS did not regulate stability of LMP1-95-8 unless it was mutated to bear additional lysine residues near the cryptic motif. LMP1 proteins that could not bind to HOS exhibited an increased ability to induce IκB degradation and NF-κB-mediated transcription without further increase in activation of IκB kinases. Expression of LMP1-95-8 reduced the levels of endogenous HOS available to interact with phosphorylated IκBα. Degradation of IκBα and dose dependence of NF-κB activation by LMP1-95-8 were promoted by co-expression of HOS. Our data suggest that LMP1-95-8 is a pseudo-substrate of SCF<sub>HOS/β-TrCP</sub> E3 ubiquitin ligase and that interaction between LMP1 and HOS restricts the extent of LMP1-induced NF-κB signaling. We discuss the potential role of this mechanism in transforming and cytostatic effects of LMP1 variants in cells and Epstein-Barr virus-associated tumors.

The latent membrane protein 1 (LMP1) is a transmembrane signaling protein encoded by the BNLF1 gene of Epstein-Barr virus (EBV). LMP1 plays a pivotal role in immortalization and proliferation of human B-lymphocytes infected with EBV, which is associated with a wide spectrum of human malignancies (reviewed in Ref. 1). LMP1 resembles a viral oncopogene by virtue of its capability to transform rodent cells in vitro and to induce tumorigenesis in transgenic mice (2–4).

LMP1 elicits its effects by constitutively engaging multiple signaling pathways, including those that activate NF-κB, jun/ AP-1, ATF2, signal transducers and activators of transcription, and Sma and Mad-related protein transcription factors, which, in turn, mediate pleiotropic events in cells infected with EBV or transfected with LMP1 vector (5–8). Although the specific contributions of many signaling cascades to the oncogenic properties of LMP1 remain to be elucidated, activation of the NF-κB pathway is linked to LMP1-induced immortalization of human primary B-lymphocytes (6, 9, 10) and tumorigenic transformation of cultured rodent cells (11, 12).

NF-κB, a dimeric transcription factor, activates transcription of a large number of target genes that play key roles in cell growth and death, as well as in immune and inflammatory responses. NF-κB activity is frequently increased in human tumors; it is essential for growth and survival of tumor cells. Cells tightly regulate activation of NF-κB by expression of a family of inhibitory proteins (IκB). In the absence of stimuli, IκB binds tightly to NF-κB and masks the domains responsible for NF-κB nuclear localization and DNA binding (13, 14).

LMP1 induces the NF-κB pathway by mimicking a constitutively activated tumor necrosis factor receptor (15, 16). LMP1 interacts with tumor necrosis receptor-associated factor and tumor necrosis receptor-associated death domain adapter proteins and activates IκB kinases (IKK) (10, 17). Activated IKK phosphorylates IκB on serines within the DSGXXS “destruction motif” (13). Specifically, phosphorylated IκB is recognized by WD40 domains of ubiquitin ligase receptors, which belong to the β-TrCP/Fbw1 subfamily of F box proteins, including β-TrCP1 (also termed FWD1/Fbw1a (18–21)) and β-TrCP2 (also termed HOS/Fbw1b). β-TrCP proteins interact with Skp1, Cullin1, and Roc1/Rbx1 proteins to form SCFβ-TrCP/HOS E3 ubiquitin protein ligases (reviewed in Ref. 22). These E3 ligases ubiquitinate IκB at lysines located 9–10 amino acids proximal to the destruction motif (23). 26 S proteasome-dependent degradation of ubiquitinated IκB relieves inhibition of NF-κB-dependent transcription (13).

An increased capacity of some natural LMP1 isolates from tumors to induce NF-κB activity is thought to be linked to their higher efficacy in transforming rodent cells in vitro compared with a canonical B95-8 variant of LMP1 (LMP1-95-8) (24, 25). These highly transforming LMP1 proteins (including LMP1-Cao, 1510, and C15) bear numerous point mutations and a 30-bp deletion in the C-terminal domain (26–28). Whereas the...
30-bp deletion increases transforming activity of LMP1-95-8 (29) and is frequently found in EBV-associated tumors (30), the mechanisms underlying either increased tumorigenicity or augmented induction of NF-κB by these LMP1 variants remain unknown.

Surprisingly, in transfected cells, when LMP1-95-8 is expressed at very high levels, it inhibits NF-κB activity (31, 32) and causes cytostatic and/or cytotoxic effects, indicating a role of the tight regulation of LMP1-95-8 levels in the maintenance of EBV latency (33–35). This inhibition is not observed upon expressing LMP1-Cao (33, 36), despite the fact that LMP1-Cao protein is more stable than LMP1-95-8 and accumulates to a greater extent in cells (37).

In human cells, LMP1-95-8 protein undergoes rapid degradation, which depends on the ubiquitin-proteasome pathway (38). However, E3 ubiquitin ligase(s) responsible for ubiquitination of LMP1 have not been identified as yet. We have noted that the C-terminal domain of LMP1-95-8 contains a potential β-TrCP recognition motif (DSGHE) and that this motif is mutated in LMP1-Cao. In this study, we investigated a potential role of SCF<sub>β-TrCP</sub> E3 ubiquitin ligases in degradation of LMP1. We found that LMP1-95-8, but not LMP1-Cao, interacted with β-TrCP/HOS. Although SCF<sub>HOS</sub> ligase did not regulate LMP1 stability, the association between LMP1-95-8 and the SCF<sub>HOS</sub> complex affected the availability of HOS to interact with phosphorylated IκB as well as IκB degradation and NF-κB activation. These data provide an insight into mechanisms underlying differences between LMP1-95-8 and highly tumorigenic LMP1 variants in their ability to activate NF-κB signaling.

MATERIALS AND METHODS

Plasmids—pSG5 vector-based constructs for expression of LMP1-95-8 and LMP1-Cao (39) were kindly provided by F. Graesser (Homburg, Germany). Constructs for expression of Cullin1, Skp1, Roc1, hemagglutinin-tagged HOS and HOS<sup>AF</sup> (40, 41), as well as κB- (42) and jun2-driven luciferase reporters (43) (generous gift of M. Karin and H. van Dam) were described previously. Histidine-tagged ubiquitin expression vector (44) was kindly provided by D. Bohmme. Site-directed mutagenesis on the pSG5-LMP1-95-8 backbone was carried out using a QuickChange Site-directed Mutagenesis kit (Stratagene) to create the following substitution mutants: G212S, S350A, S366T, G212S/S350A, G212S/S366T, G212S/S366T/0.1% SDS in phosphate-buffered saline) supplemented with 10% calf serum and antibiotics under similar conditions. Transfection of 293T cells grown in Dulbecco's modified Eagle's medium in the presence of 10% serum and antibiotics was performed using a lipofection reagent (Promega kit). Expression of recombinant SCF<sub>HOS</sub> complex components (HOS-HA, Cullin1, Skp1, and Roc1) in 293T cells and their purification were carried out as described previously (45).

**Cells, Transfection, and Cell Lysate Preparation**—293T cells were grown in Dulbecco’s modified Eagle’s medium in the presence of 10% fetal bovine serum and antibiotics at 37 °C and 5% CO<sub>2</sub>. Rat1a cells were maintained in Dulbecco’s modified Eagle’s medium in the presence of 10% calf serum and antibiotics under similar conditions. Transfections were performed according to the calcium phosphate procedure or with the aid of LipofectAMINE Plus (Invitrogen). Cells were harvested and lysed with RIPA buffer (0.5% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS in phosphate-buffered saline) supplemented with inhibitors of phosphatases (20 mM NaF and 1 mM Na<sub>2</sub>VO<sub>4</sub>) and a “mixture” of protease inhibitors (Sigma). Lysates were cleared by centrifugation at 16,000 × g for 30 min at 4 °C, and protein concentrations were determined using Bradford reagent (Pierce). pBSV-β-galactosidase (Pierce), pBSV-κB-luciferase vector was added to the transfection mixture to normalize the transfection efficiency on the basis of β-galactosidase activity (measured with a Promega kit). Expression of recombinant SCF<sub>β-TrCP/HOS</sub> complex components (HOS-HA, Cullin1, Skp1, and Roc1) in 293T cells and their purification were carried out as described previously (45).

**Monoclonal antibodies**—Monoclonal antibodies against HA tag (Roche Applied Science), LMP1 (CS1-4, Dako), IKKγ (Pharmingen), β-catenin (Transduction Laboratories), IKKα and IkBα (Santa Cruz Biotechnology), and α-tubulin (Sigma) as well as Cullin1 polyclonal antibody (NeoMarkers) were purchased. Polyclonal antibody against HOS (HOS-N) was described earlier (46). Pulse-chase analysis was performed as described previously (40). Briefly, 293T cells grown on 100-mm dishes and transfected with indicated plasmids were starved in medium lacking methionine and cysteine, metabolically labeled with a 35S-labeled methionine/cysteine mixture (PerkinElmer Life Sciences), and chased with medium containing 2 mM unlabeled methionine and cysteine for various time points. Cells were harvested, and LMP1 proteins were immunoprecipitated by Cullin1 antibody, separated by SDS-PAGE, and analyzed by autoradiography. Results were quantified using a Storm 860 Imager (Amersham Biosciences). An IKK immunoprotein assay was carried out with IKKα antibody as described elsewhere (47). Immunoprecipitation and immunoblotting procedures were described earlier. Data were analyzed using Scion Image Software (version Beta 4.0.2).

**Luciferase Assays**—Luciferase assays in the cells plated in 24-well plates and co-transfected with either κB- or jun2-driven luciferase reporter constructs and pBSV-β-gal plasmid were performed using the appropriate kit (Promega) as described earlier (40). Luciferase activity was normalized with respect to β-galactosidase activity.

**Foci Formation Assay**—Rat1a cells were transfected with LMP1 expressing constructs (0.5 μg) and pBabe-puro (3.5 μg) and grown for 14 days in the presence of puromycin (2 μg/ml, Sigma). The appearance of foci of transformed cells characterized by lack of contact inhibition was observed 14 days after transfection, and the number of foci was scored in a double blind manner.

**Measurement of Substrate-free HOS Levels**—In vivo-derived phosphorylated peptide (KKERLLDDILKPDSGLDPMKDE, where pS is phosphoserine) and its non-phosphorylated counterpart were synthesized by the Protein Chemistry Core Laboratory of Baylor College of Medicine (Houston, TX). Peptides were covalently coupled with beads using an AminoLink kit (Pierce), and the beads were washed and incubated with 1 mg of lysates from 293T cells transfected with LMP1 constructs for 1 h at 2 °C. After extensive washes with ice-cold phosphate-buffered saline supplemented with Nonidet P-40 (0.1%), the proteins were eluted with Laemmli sample buffer, separated by SDS-PAGE, and analyzed by immunoblotting with HOS-N antibody.

**RESULTS**

**LMP1-95-8 but Not LMP1-Cao Interacts with HOS**—We sought to investigate whether LMP1 is a target for SCF<sub>β-TrCP/HOS</sub> E3 ubiquitin ligases. Both β-TrCP1 and HOS motifs are found within the cytoplasmic C-terminal domain of LMP1-95-9 (Fig. 1C), which, unlike β-TrCP1, is primarily localized in the cytoplasm (49, 50). LMP1-95-8 protein expressed in 293T cells interacted with immobilized recombinant SCF<sub>HOS</sub> complex in vitro (Fig. 1A). Co-expression of LMP1-95-8 and HOS-HA in 293T cells fol-

**FIG. 1.** HOS interacts with LMP1 in vitro and in cells. A, binding of LMP1-95-8 (from the lysates of 293T cells transfected with LMP1 or empty vector) to SCF<sub>β-TrCP/HOS</sub> complex immunopurified with HA antibody and immobilized on protein A beads. Immunoblotting analysis with LMP1 antibody (upper panel) and Cullin1 antibody (lower panel) is shown. B, co-immunoprecipitation (IP) of HOS-HA and LMP1 proteins was observed in 293T cells. Immunoblotting (IB) analyses with HA and LMP1 antibodies are depicted. C, comparison of canonical HOS recognition sites in HOS substrates and in LMP1 proteins. Potentially phosphorylated serines are shaded, and mutation within LMP1-Cao is underlined.
Fig. 2. Interaction of HOS with LMP1-95-8 requires both canonical and cryptic sites. A, co-immunoprecipitation (IP) of HOS-HA and LMP1-95-8 and its mutant proteins expressed in 293T cells. Immunoblotting (IB) analyses with HA and LMP1 antibodies are depicted. B, comparison of LMP1-Cao and LMP1-95-8 proteins within the cryptic HOS recognition site. Putative phosphoserenes are light-shaded, and prolines that may participate in polypeptide chain turns (see “Discussion”) are underlined.

Fig. 3. The rate of proteolysis of LMP1-95-8 depends neither on the ability of LMP1-95-8 to bind HOS nor on HOS activities. A, pulse-chase analysis of LMP1 proteins co-expressed in 293T cells with the indicated HOS-HA-expressing plasmids. Autoradiographs of 35S-labeled and immunoprecipitated LMP1 proteins separated by SDS-PAGE are shown. B, the levels of HA-tagged HOS (left panel) and β-catenin (β-cat, right panel) proteins at time point “0” of the pulse-chase analysis (shown in A) were assessed by immunoblotting with the relevant antibodies. M, mock; WT, wild type; NS, nonspecific band.

LMP1-Cao protein, which cannot bind HOS (Fig. 1B), is more stable than LMP1-95-8 (37), we were prompted to investigate the role of HOS in the regulation of LMP1 protein stability. To this end, we compared the rate of proteolysis of LMP1-95-8 with its Triple mutant, which exhibited no binding to HOS (Fig. 2A). Contrary to our expectations, pulse-chase analysis of LMP1 proteins showed that the half-life of the Triple mutant was no longer than that of LMP1-95-8 (Fig. 3A). This result indicates that abrogation of HOS binding is not sufficient for stabilization of LMP1 proteins. Furthermore, LMP1-95-8 protein was not stabilized by co-expression of the dominant negative mutant HOS345 (Fig. 3A). This mutant, which was shown to abrogate degradation of known HOS/β-TrCP substrates (40, 41), was evidently expressed under the conditions of our experiments, resulting in accumulation of β-catenin (Fig. 3B). In some experiments we observed a modest stabilization of Triple-LMP1 protein in cells overexpressing HOS; the significance of these observations remains unclear.

In addition, although SCF<sub>HOS</sub> could be successfully bound to LMP1-95-8 (Fig. 1A), we failed to ubiquitinate this protein in vitro under conditions that enabled efficient ubiquitination of IκBα. Taken together, these results suggest that despite the ability of LMP1-95-8 to interact with SCF<sub>HOS</sub> E3 ubiquitin ligase, LMP1-95-8 is not a genuine substrate for this E3 ligase. These findings are in line with conclusions of another group that the C-terminal domain of LMP1-Cao does not contribute to its increased stability (37).

LMP1-95-8 Is a Pseudo-substrate of SCF<sub>HOS</sub> Because of the Absence of Ubiquitin-Acceptor Lysines—The recently solved SCF<sub>βTrCP1</sub> structure predicts that efficiency of ubiquitination by this ligase depends on the location of the ubiquitin-accepting lysine(s) relative to the destruction motif (52). LMP1-95-8 contains a single lysine residue (Lys-330) that is not essential for its ubiquitination (38). Lack of convenient ubiquitin-conjugation site(s) in proximity to HOS-binding sequences similar to Lys-21/22 within IκBα (23) or Lys-19 within β-catenin (52) may explain why LMP1-95-8 is not a bona fide substrate for SCF<sub>HOS</sub>. To investigate this possibility, we substituted two amino acids positioned at a similar spacing from the cryptic

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*a* W. Tang, V. Spiegelman, and S. Fuchs, unpublished data.
Figure 4. Introduction of two lysine residues near the cryptic HOS recognition domain converts LMP1-95-8 into a rapidly degrading protein whose proteolysis is regulated by HOS. A, pulse-chase analysis of LMP1 proteins expressed in 293T cells. Autoradiographs of \(^{35}S\)-labeled and immunoprecipitated LMP1 proteins separated by SDS-PAGE are shown. B, effect of dominant negative HOS expression on the rate of proteolysis of LMP1 mutants carrying double lysines measured by pulse-chase analysis in 293T cells as described in A. C, in vivo ubiquitination of LMP1 proteins in 293T cells transfected with indicated plasmids was assessed by immunoblotting (IB) LMP1 proteins in the eluates from the nickel-nitrilotriacetic acid column (ubiquitinated proteins, upper panel) and in whole cell lysates (middle panel) with LMP1 antibody. Immunoblotting analysis of HOS\(^{AF}\)-HA protein with HA antibody (lower panel) is also shown. D, average half-life (T\(_{1/2}\)) of LMP1 proteins (in minutes) co-expressed with the dominant negative HOS or empty vector as calculated from four independent experiments (in t test: *, \(p < 0.005\) versus LMP1-95-8+pCDNA3; **, \(p < 0.01\) versus LMP1-95-8-KK+pCDNA3).

HOS recognition site within LMP1-95-8 with two lysine residues and compared the rates of degradation of LMP-95-8 and of this 95-8-KK mutant (L339K/M340K). Pulse-chase analysis showed that introduction of the lysines noticeably decreased the half-life of the mutant LMP1 protein (Fig. 4, A and D). Furthermore, inhibition of HOS activities by co-expressing HOS\(^{AF}\) protein stabilized 95-8-KK (Fig. 4, B and D). This result indicates that introduction of lysines in proximity to the HOS cryptic recognition site creates a protein whose degradation is regulated by SCF\(^{HOS}\).

To confirm the role of HOS in controlling stability of 95-8-KK, we introduced the L339K/M340K substitution mutation into the background of the Triple mutant, which cannot bind HOS (Fig. 2A). The resulting Triple-KK mutant protein exhibited a half-life similar to that of LMP1-95-8, and the stability of Triple-KK was not increased by co-expression of HOS\(^{AF}\) (Fig. 4, B and D).

To corroborate our hypothesis that an increase in the rate of ubiquitination of 95-8-KK results in destabilization of this protein, we carried out in vivo ubiquitination experiments. Cotransfection of LMP1-95-8 with hexahistidine-tagged ubiquitin in 293T cells followed by purification of ubiquitinated proteins under stringent denaturing conditions (44) revealed that LMP1 is covalently conjugated with ubiquitin in vivo under these conditions (Fig. 4C). Compared with the LMP1-95-8 protein, the 95-8-KK mutant exhibited a noticeably higher extent of ubiquitination (Fig. 4C, lane 5 versus lane 3), and this increase was inhibited by co-expression of HOS\(^{2N}\) (lane 6 versus lane 5).

Mutation of HOS recognition sites in the Triple-KK protein abrogated the effect of introducing lysines (Fig. 4C, lane 7). In addition, the dominant negative HOS\(^{AF}\) mutant did not inhibit ubiquitination of either LMP1-95-8 or Triple-KK proteins. These results indicate that introduction of lysines into an LMP1 protein capable of binding to HOS converts the protein into a substrate for HOS-dependent ubiquitination. In all, our evidence suggests that LMP1-95-8 is a pseudo-substrate of the SCF\(^{E3}\) ubiquitin ligase. Although this ligase is capable of binding to LMP1-95-8, it does not undergo ubiquitination, most likely because of the lack of conveniently located lysine residues that would serve as ubiquitin acceptor sites.

The Ability of LMP1-95-8 to Interact with HOS Affects Transforming and Signaling Activities of LMP1—We compared the ability of LMP1-95-8 and LMP1-Cao to induce in vitro transformation in Rat1a fibroblasts. Consistent with data published previously (25, 29), transforming activity of LMP1-Cao was significantly higher than that of LMP1-95-8 (Fig. 5A). As LMP1-mediated transformation is known to depend on gene dosage (2), we initially attributed higher efficiency of LMP1-Cao solely to its greater stability (37) and abundance (Fig. 1B). However, the Triple mutant of LMP1-95-8, which was expressed at levels comparable with those of LMP1-95-8 (Fig. 2A), was also significantly more active in transforming Rat1a cells than LMP1-95-8 (Fig. 5A). This finding indicates that sequences within the canonical and cryptic degradation motifs of LMP1-95-8, which determine its ability to interact with HOS, may play a role in the outcome of LMP1-mediated transformation.

LMP1 expression initiates a number of signaling pathways, including induction of stress-activated protein kinases (reviewed in Refs. 7 and 8), that phosphorylate c-Jun and ATF2 transcription factors and activate the jun2 regulatory element within the promoter of the c-jun gene (43). We used jun2-luciferase reporter to compare the ability of LMP1-95-8, Triple mutant, and LMP1-Cao to induce this signal transduction pathway. All three LMP1 constructs induced comparable levels of jun2-driven luciferase in a dose-dependent manner (Fig. 5B). This result suggests that the ability of LMP1 to induce stress-activated protein kinase signaling is unlikely to confer variability in transforming activities on LMP1-95-8 and its Triple mutant or LMP1-Cao.

Activation of NF-\(\kappa\)B-dependent transcription is a hallmark of LMP1-mediated signaling that plays a key role in LMP1-mediated cell transformation (6, 9, 10). Whereas low doses of the LMP1-95-8 construct readily induced the activity of NF-\(\kappa\)B-driven luciferase reporter, no further activation was achieved by higher doses (Fig. 5C). Moreover, although increasing concentrations of LMP1-95-8 produced less NF-\(\kappa\)B activation compared with low doses, LMP1-Cao activated NF-\(\kappa\)B in a dose-dependent manner (Fig. 5C). These results are consistent with earlier observations of Johnson et al. (36) and more recent evidence from other groups (31, 32) confirming the biphasic curve of NF-\(\kappa\)B activation mediated by LMP1-95-8. Interestingly, the Triple mutant activated NF-\(\kappa\)B in a dose-dependent manner similar to LMP1-Cao (Fig. 5C). This result indicates that mutations in HOS recognition sites of LMP1-95-8 abrogate its ability to inhibit NF-\(\kappa\)B activation at high doses. Taken together, these data imply that binding of HOS to LMP1 may play a role in regulating the extent of LMP1-induced activation of NF-\(\kappa\)B signaling without affecting the stress-activated protein kinase pathway.

LMP1 Proteins Lacking HOS Binding Accelerate \(\kappa\)Ba Degradation without Additional Induction of \(\kappa\)B Kinases—Activation of NF-\(\kappa\)B by LMP1 relies on induction of the \(\kappa\)B kinases...
kinase assays to investigate the ability of high doses of LMP1 proteins to induce IKK activity. Expression of all three LMP1 proteins activated the endogenous IKK complex to a similar extent (Fig. 6A). This result indicates that an advanced ability of Triple or LMP1-Cao to activate NF-κB (compared with LMP1-95-8, Fig. 5C) can hardly be attributed to differences in induction of IKK activity.

We next examined degradation of endogenous IκBα in cells transfected with high doses of LMP1 constructs and treated with cycloheximide to prevent de novo protein synthesis. In the absence of LMP1 proteins, hardly any IκBα degradation was observed within 20 min of cycloheximide treatment, whereas noticeable degradation was detected in cells expressing LMP1-95-8. At the same time, more robust IκBα degradation was observed in cells expressing either Triple or LMP1-Cao proteins (Fig. 6B). More efficient degradation of IκBα in cells expressing LMP1 proteins that cannot bind to HOS (Triple and LMP1-Cao) may explain why high doses of these proteins are better activators of NF-κB than LMP1-95-8 (Fig. 5C). Because all tested LMP1 proteins activated IKK to a similar extent (Fig. 6A), a limited ability of high doses of LMP1-95-8 to induce IκBα degradation (Fig. 6B) and NF-κB activation (Fig. 5C) must be attributed to a mechanism that acts downstream of IKK activation and IκB phosphorylation.

LMP1-95-8 Is Capable of Squelching Cellular HOS and Restricting NF-κB Activation.—We previously found that an overall abundance of β-TrCP/HOS proteins in cells is important for regulating activity of SCFβ-TrCP/HOS E3 ubiquitin ligases and NF-κB activation (41, 46, 53, 54). The function of β-TrCP proteins can easily be disrupted by forced expression of viral Vpu protein, which acts as a pseudo-substrate and squelches β-TrCP to prevent its binding to phosphorylated IκB (55, 56).

We sought to investigate whether LMP1 is capable of squelching HOS in a manner similar to that of Vpu. We assessed the levels of cellular HOS, which is not bound to its substrates and hence is capable of associating with immobilized IκBα-derived peptide phosphorylated at Ser-32/36. We found that whereas ~10% of total HOS levels from the cells transfected with empty vector were pulled down with this peptide (Fig. 7A, panel I, lane 1), no HOS binding was detected with peptide in which Ser-32/36 was not phosphorylated (Mock*). Expression of LMP1-Cao or Triple moderately reduced levels of substrate-free cellular HOS (Fig. 7A, panel I, lanes 3 and 4 versus lane 1). This reduction was not unexpected because both Triple and LMP1-Cao efficiently induced IKK (Fig. 6A) and therefore could be predicted to increase intracellular levels of HOS substrate (phosphorylated IκB). Under the same conditions, expression of LMP1-95-8 strikingly decreased the levels of substrate-free HOS below the limits of detection of our assay (Fig. 7A, panel I, lane 2 versus lane 1). As IKK activity was induced by all tested LMP1 proteins to a comparable extent (Fig. 6A) and neither of the tested LMP1 proteins affected the overall levels of HOS (Fig. 7A, panel II), our results suggest that expression of LMP1-95-8 decreases substrate-free HOS levels in cells via HOS-LMP1-95-8 association.

HOS is required for phosphorylation-dependent IκB degradation and NF-κB activities (40, 57). It is conceivable that interaction of exogenously overexpressed LMP1-95-8 with endogenous HOS may reduce availability of HOS for IκBα, resulting in delayed IκBα degradation (Fig. 6B) and limited NF-κB activation (Fig. 5C). To examine this possibility, we attempted to reverse the effects of high doses of LMP1-95-8 on IκBα degradation and NF-κB activation by co-expressing HOS. Expression of HOS alone did not stimulate either IκBα degradation (Fig. 7B) or NF-κB activation (data not shown). However, co-expression of HOS with LMP1-95-8 was sufficient to
accelerate the rate of IκBα degradation by LMP1-95-8 (Fig. 7B) and to restore the dose dependence of LMP1-95-8-mediated NF-κB activation (Fig. 7C). In all, these data suggest that LMP1-95-8 (but not Triple or LMP1-Cao) is capable of squelching endogenous cellular HOS, thus preventing its interaction with phosphorylated IκBα and, in turn, inhibiting the following IκBα degradation and NF-κB activation.

**DISCUSSION**

In this study, we found that LMP1-95-8 is a pseudo-substrate for SCF^HOS^ E3 ubiquitin ligase and that LMP1-95-8 is capable of squelching HOS and restricting the extent of NF-κB signaling. LMP1-95-8 binds to HOS in a manner that depends on the integrity of both canonical and cryptic degradation motifs, which are located in the C-terminal domain of LMP1-95-8 (Figs. 1 and 2). This binding does not affect protein stability of LMP1-95-8, most likely because LMP1-95-8 lacks ubiquitin-acceptor lysines located within reach of the SCF^HOS^ E3 ligase catalytic center (Figs. 3 and 4). When expressed at high doses in cells, LMP1-95-8 is capable of squelching HOS and preventing its interaction with phosphorylated IκBα (Fig. 7). This squelching is likely to contribute to a limited ability of LMP1-95-8 to induce degradation of IκBα (Fig. 6) and activation of NF-κB (Fig. 5) in comparison with LMP1-Cao protein, which does not bind to HOS.

Technical problems with the quality of a few available antibody reagents against β-TrCP1 precluded us from conclusively assessing whether LMP1 is also capable of squelching this protein. Nevertheless, considering the important role of β-TrCP1 in ubiquitination of IκBα (48, 58) together with our data showing that LMP1-95-8 is capable of inhibiting IκBα degradation (Fig. 6) and activation of NF-κB (Fig. 5) in comparison with LMP1-Cao protein, which does not bind to HOS.
tion Vpu prevents β-TrCP proteins from interacting with phosphorylated IκB, thus inhibiting NF-κB activity and contributing to the human immunodeficiency virus type 1-induced apoptosis (55, 56).

β-TrCP1 and HOS are known to bind to phosphorylated substrates (22), and it remains to be investigated whether serines 211, 215, 350, and 366 within LMP1-95-8 are phosphorylated in cells. If such phosphorylation is indeed essential for the interaction of LMP1 with HOS, it is expected that the ability of LMP1 to squelch endogenous HOS and restrict the extent of NF-κB signaling in various types of cells will depend on the specific activities of the relevant protein kinase(s) in these cells.

The interaction of LMP1-95-8 with HOS is mediated by both the canonical DSGXXS motif and a more distal sequence within the C terminus of LMP1-95-8, and mutation of both sites is needed to abolish HOS-LMP1 binding (Fig. 2). At present, it is not clear how the non-canonical motif would bind HOS/β-TrCP. One possibility is that this cryptic motif is formed by phosphorylated serines 350 and 366, whereas two proline residues between those serines may enable the twisting of the polypeptide chain, which would allow presenting putative phosphoserines to HOS/β-TrCP (Fig. 2B). Seven WD40 domains of β-TrCP form a funnel-like structure with a channel that accommodates phosphorylated serines, which constitute a destruction motif within a substrate (52). The existence of additional twists of polypeptide chain between Ser-350 and Ser-366 within the non-canonical DSG(X)₄S motif of LMP1 (shaded in Fig. 2B) may contribute to positioning of both phosphoserines to fit this channel. The existence of an extended cryptic degradation motif in LMP1 seems unusual, although β-TrCP/HOS substrates harboring spacers longer than three amino acids between phosphoserines have been reported (50, 59).

It is also unclear why Thr306 in the 366 position would prevent recognition of non-canonical sequence by HOS/β-TrCP. We observed that S366T substitution alone caused a larger decrease in HOS binding than G212S mutation (Fig. 2A), indicating that Ser-366 may exert phosphorylation-independent effects on interactions between LMP1 and HOS. Further studies will identify structural determinants allowing formation of the HOS recognition sites on LMP1 as well as determine whether the efficiency of HOS binding may differ between canonical and cryptic sites.

LMP1-95-8 contains a single lysine (Lys-330) that plays no role in LMP1 ubiquitination (38). Recent structural studies indicate that SCF E3 ligases complexed with a phosphorylated substrate, and recruited E2 ubiquitin-conjugating enzymes exhibit a fairly rigid conformation, suggesting that ubiquitin-accepting lysine residues within a substrate should be optimally aligned toward E2 (60–62). This positioning model predicts an optimal spacing between a degradation motif and a lysine residue as a key characteristic of a bona fide SCF substrate. Indeed, efficient ubiquitination of β-catenin-derived phosphopeptide by SCFβ-TrCP requires such specific spacing (52). Our data showing that LMP1-95-8 stability is not regulated by SCFHOS E3 ubiquitin ligase unless lysine residues are introduced at optimal distance from non-canonical HOS recognition sites within LMP1 are in agreement with this positioning model.

Expressing high levels of LMP1-95-8, but not of LMP1-Cao, is known to limit NF-κB activation and elicit cytostatic effects (31, 32). Whereas squelching of HOS by LMP1-95-8 is apparently involved in restricting the extent of NF-κB signaling, further studies are required to determine whether binding of HOS by LMP1 plays a role in mediating the cytostatic effects of LMP1. Our data indicate that squelching HOS/β-TrCP by LMP1-95-8 decreases the rate of IκB degradation (Figs. 6 and 7). On the other hand, such squelching may also diminish NF-κB signaling by preventing appropriate processing of NF-κB precursors that depend on SCFHOβ-TrCP (50, 51). Furthermore, in EBV-infected cells, depletion of free HOS/β-TrCP proteins by LMP1 may also impede proteolysis of other substrates of HOS/β-TrCP, including such key regulators of the cell cycle as Wee1A (40) and Em1. Em1 stabilization in cells (63) and in β-TrCP1 knockout mice (48) may lead to defects in cell division. It is conceivable that HOS squelching mediated by degradation motifs within the C-terminal domain of LMP1-95-8 may contribute to its cytostatic and cytotoxic effects in concert with the mechanisms conferred by the transmembrane domain of LMP1-95-8 (31, 32, 35). These mechanisms are thought to play key roles in regulating the levels of LMP1 tolerated by infected cells as well as in supporting EBV persistence within infected cells (64).

Although future studies are needed to determine whether the ability of LMP1-95-8 to squelch endogenous HOS plays a role in limiting tumorigenic properties of this LMP1 variant, extensive epidemiological data refer to LMP1 mutations expected to abolish the ability of LMP1 to interact with HOS. In addition to 30-bp deletions, these mutations may include D349A substitution (65). Similar substitution within the DGSXXS motif in IκBo abrogated the ability of IκBo to recruit β-TrCP/HOS (66). Furthermore, substitutions of Ser-366 (such as S366T, S366A, and S366N) are observed more frequently in LMP1 isolates from EBV-associated tumors than in LMP1 from peripheral blood lymphocytes from apparently healthy individuals (30, 39, 65, 67). G212S mutation may not be as common as substitution of Ser-366 (65, 67). We did not find this substitution in many German or Russian LMP1 isolates that contained the S366T mutation. On the other hand, none of the described cases has revealed the presence of the LMP1-95-8 identical isolate in tumor tissue of any EBV-associated disease (39). These data imply that, in tumorigenesis, LMP1 is under selective pressure to diminish its ability to interact with HOS and thus to abandon restrictions on activating NF-κB signaling. The existence of such selection would predispose EBV-containing tumor cells to accumulate mutated LMP1 proteins, and this phenomenon is indeed frequently observed in EBV-associated tumors that preferentially contain LMP1 variants with either Cao-like deletions or mutations in positions 212 and 366 (30, 69, 70). Further detailed studies will determine the role of LMP1 mutations that affect the LMP1-HOS interaction in the pathogenesis of EBV-associated malignancies.

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