Degradation of the Epstein-Barr Virus Latent Membrane Protein 1 (LMP1) by the Ubiquitin-Proteasome Pathway

TARGETING VIA UBIQUITINATION OF THE N-TERMINAL RESIDUE*

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The latent membrane protein 1 (LMP1) of the Epstein-Barr virus is a constitutively active receptor essential for B lymphocyte transformation by the Epstein-Barr virus. It is a short-lived protein, but the proteolytic pathway involved in its degradation is not known. The ubiquitin pathway is a major system for specific protein degradation in eukaryotes. Most plasma membrane substrates of the pathway are internalized upon ubiquitination and delivered for degradation in the lysosome/vacuole. Here we show that LMP1 is a substrate of the ubiquitin pathway and is ubiquitinated both in vitro and in vivo. However, in contrast to other plasma membrane substrates of the ubiquitin system, it is degraded mostly by the proteasome and not by lysosomes. Degradation is independent of the single Lys residue of the protein; a lysine-less mutant LMP1 is degraded in a ubiquitin- and proteasome-dependent manner similar to the wild type protein. Degradation of both wild type and lysine-less protein is sensitive to fusion of a Myc tag to the N terminus of LMP1. In addition, deletion of as few as 12 N-terminal amino acid residues stabilizes the protein. These findings suggest that the first event in LMP1 degradation is attachment of ubiquitin to the N-terminal residue of the protein. We present evidence suggesting that phosphorylation is also required for degradation of LMP1.

EBV, a human herpesvirus, causes lymphoproliferative dis-

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The abbreviations used are: EBV, Epstein-Barr virus; E1, ubiquitin-activating enzyme; E2, ubiquitin-carrier protein or ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; LMP1, latent membrane protein; WT LMP1 (19). Interestingly, most of the stabilizing mutations involve deletions in the N-terminal domain (Ref. 19; see also under “Discussion”). The proteolytic machinery that is responsible for LMP1 degradation has not been identified yet.

Many studies implicate the ubiquitin pathway in the degradation of various short-lived regulatory proteins. It is involved in processing and proteolysis of many cellular proteins including, for example, cell cycle regulators, oncoproteins and tumor suppressors, transcriptional activators, endoplasmic reticulum membrane proteins and cell surface receptors (20, 21). Formation of ubiquitin conjugates of a specific protein requires the sequential action of three enzymes: the ubiquitin-activating enzyme (E1), one of several ubiquitin-carrier proteins or ubiquitin-conjugating enzymes (E2s), and a member of the ubiquitin-protein ligase family (E3). E3 catalyzes the last step in

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the conjugation process, covalent attachment of ubiquitin to the substrate, and therefore plays an essential role in specific substrate recognition. Following transfer of the first ubiquitin moiety to the target protein, a polyubiquitin chain is synthesized by repetitive transfer of additional activated ubiquitin residues. The multi-ubiquitin chain is typically anchored to an e-NH₂ group of one or more internal lysine residues (20). There is no consensus as to the specificity of the internal Lys residues that are targeted by ubiquitin. In some cases, distinct lysines are required, whereas in others there is little or no specificity. Signal-induced degradation of IkBa involves two particular Lys residues, 21 and 22 (22), and in Gen4, lysine residues in the vicinity of a specific PEST degradation signal serve as ubiquitin attachment sites (23). In a different case, that of the ε chain of the T cell receptor, degradation of the protein is independent of any particular Lys residue and proceeds as long as one residue is present in the cytosolic tail of the molecule (24). In sharp contrast, ubiquitination of the transcription factor MyoD does not require any Lys residue. The protein is targeted to the proteasome following initial fusion of a ubiquitin moiety to the N-terminal ε-NH₂ group succeeded by synthesis of a poly-ubiquitin chain attached to this moiety (25). Interestingly, a mutant lysine-less T cell receptor ε chain is also degraded by the proteasome, a process that depends on an intact ubiquitin system. However, a role for direct ubiquitination of the substrate, as well as identification of potential ubiquitination site(s), has not been discerned (26). Similarly, ubiquitin-mediated endocytosis and degradation of the growth hormone receptor also proceeds in the absence of any Lys residue (27). The inability to identify ubiquitin adducts of the two receptors lead to the hypothesis that ubiquitination of another, yet to be identified, factor plays a role in the endocytic process.

In most cases, ubiquitination serves as a signal, targeting the substrate protein for degradation by the 26 S proteasome (20). It has been shown for several membrane proteins, including the yeast Ste2p (28) and the platelet-derived growth factor receptor (30), that ubiquitination leads to degradation in the vacuole/lysosome. Although most known plasma membrane substrates of the ubiquitin system are degraded in the lysosome/vacuole, a few exceptions have been reported, in which proteasome inhibitors had a significant effect on degradation. One such example is the platelet-derived growth factor receptor (30), another is the Met tyrosine kinase receptor (31).

Here we report that LMP1 is a substrate of the ubiquitin pathway that is targeted, following ubiquitination, to degradation by the proteasome. Attachment of the first ubiquitin moiety is not dependent on the single lysine residue at position 330 and appears to involve the α-amino group at the N-terminal residue.

**EXPERIMENTAL PROCEDURES**

**Materials**

Materials for SDS-PAGE and Bradford reagent were from Bio-Rad. Redvive PRO-MIX (containing l-[35S]methionine and l-[35S]cysteine) for metabolic labeling, as well as prestained molecular weight markers and immobilized protein A, were obtained from Amersham Pharmacia Biotech. Tissue culture sera and media were purchased from Biological Emek, Israel) or from Sigma. S12 mono-Cys mixture prior to disruption. The supernatant was layered on a 0.9 M sucrose cushion and centrifuged at 10,000 g for 10 min in a Beckman ultracentrifuge (model SW 28). 20 μg M1323 or 10 μl lactacystin; 20 μM Cl or 0.2 mM chelator were present throughout the experiment. Following incubation, the labeling amino acid was removed, and cells were further incubated for the indicated time periods in a complete medium ("chase"). Cells were harvested and lysed on ice, and the labeled protein was immunoprecipitated with S12 anti-LMP1 antibody followed by immunoblotting of the immunocomplex by protein A-Sepharose. Samples were analyzed by SDS-PAGE (10%), and proteins were visualized by PhosphorImager (Fuji, Japan).

**Detection of Ubiquitin Conjugates in Cells**—COS 7/5 cells were transiently transfected with either WT or K330R LMP1 and incubated with or without MG132 for 2 h. Cells were lysed under special conditions to prevent deubiquitination (35). Equal amounts of protein, as determined by the Bradford method (36), were immunoprecipitated using anti-LMP1 monoclonal antibody, and the samples were resolved on SDS-PAGE and transferred to nitrocellulose membranes. The membrane was incubated with a rabbit anti-ubiquitin antibody, followed by horseradish peroxidase-conjugated goat anti-rabbit serum (Sigma). SuperSignal chemiluminescent substrate (Pierce) was used to visualize proteins. When indicated, membranes were stripped and reprobed with anti-LMP1.

**Preparation of Crude Membrane Fraction**—EA-B1 cells or COS7/5 cells expressing LMP1 were collected and incubated at 0 °C for 10 min in hypotonic buffer (10 mM HEPES-KOH, pH 7.4,10 mM KCl, 1.5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM dithiothyroate), and disrupted using a tight-fitting Dounce homogenizer. Lysates were pelleted at 1000 × g for 10 min at 4 °C. The supernatant was layered over a 0.9 M sucrose cushion and centrifuged at 10,000 × g for 60 min at 4 °C. Pellets were resuspended in a buffer containing 10 mM Tris–HCl, pH 7.4, and 1 mM EDTA and used immediately for in vitro ubiquitination assays. When indicated, cell were labeled with [35S]Met/Cys mixture prior to disruption.

**Preparation and Fractionation of HeLa Cell Lysate**—HeLa cells were lysed, and high speed supernatant was prepared as described (37). The lystate was fractionated on DEAE-cellulose to unadsorbed material (Fraction I) and high salt eluate (Fraction II), as described before (38).

In Vitro Conjugation and Degradation Assays—Conjugation and degradation assays in cell-free systems were performed as described elsewhere (25, 37). Briefly, reaction mixtures contained in a final volume of 25 μl: 150 μg of whole cell lysate protein or 50 μg of fraction II protein, 5 μg of ubiquitin, and crude membrane fraction (20 μg of protein for degradation and 60 μg of protein for conjugation assays) from LMP1 transfected cells. Reaction was carried out in the presence of 0.5 mM

**Methods**

**Plasmids and Expression of LMP1**—For expression in mammalian cells, WT LMP1 cDNA was subcloned into the EcoRI site of the pc1-neo vector (Promega). For expression of Myc-tagged LMP1, the cDNA was lifted using polymerase chain reaction with an EcoRI-N terminus primer on the 5′ end, and 3′ commercial primer (NEB) on the 3′ end. The resulting fragment was digested with EcoRI and ligated into frame into pc522MT. Point mutations in LMP1 were generated by site-directed mutagenesis using the QuikChange™ kit (Stratagene). Deletion of the first 12 (∆N12) or 24 (∆N24) N-terminal amino acid residues of LMP1 was carried out using polymerase chain reaction and specific primers. Polymerase chain reaction products were digested with EcoRI and ligated into pc1-neo vector. Sequences of all constructs were confirmed using either a manual (Amersham Pharmacia Biotech) or automatic (ABI 310) sequencing systems.

**Cell Lines and Transfections**—The EBV-transformed lymphoblastoid cell line EA-B1 stably expressing LMP1 was grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum throughout. NS0 cells were transfected with WT LMP1 mutant using electroporation with Bio-Rad gene pulser. CHO-E36 and ts20 E1 mutant cells were grown at 32 °C in a minimal essential medium supplemented with 10% fetal calf serum as described (34). Cells were not transfected with LMP1 using the Fugene™ kit.

**Metabolic Labeling and Immunoprecipitation**—Cells were incubated in the presence of Met/Cys-deficient Dulbecco’s modified Eagle’s medium for 1 h, and [35S]labeled Met/Cys mixture (~200 μCi/ml) was added for an additional h. Proteasome and lysosome inhibitors were added during the labeling period ("pulse"); 20 μM MG132 or 10 μl lactacystin; 20 μM Leu-Leu-L-Leucinal (MG132), lactacystin, and protease inhibitors were present throughout the experiment. Following incubation, the labeling amino acid was removed, and cells were further incubated for the indicated time periods in a complete medium ("chase"). Cells were harvested and lysed on ice, and the labeled protein was immunoprecipitated with S12 anti-LMP1 antibody followed by immunoblotting of the immunocomplex by protein A-Sepharose. Samples were analyzed by SDS-PAGE (10%), and proteins were visualized by PhosphorImager (Fuji, Japan).

**Ubiquitin-Mediated Degradation of LMP1**

- Hexokinase and Fugene™ 6 transfection reagent from Pierce.
- Oligonucleotides were synthesized by Biotechnology General (Rehovot, Israel). All other reagents used were of high analytical grade.
ATP and an ATP-regenerating system (10 mM phosphocreatine and 5 μg of phosphocreatine kinase) or ATPγS (5 mM) as indicated. For depletion of ATP, 0.5 μg of hexokinase and 10 mM 2-deoxyglucose were added. Conjugation assays contained in addition 0.5 μg of the isopeptidase inhibitor ubiquitin aldehyde (39) and 1 μg of okadaic acid. Degradation reactions were carried out at 37 °C for 3 h, whereas conjugation assays were carried out at 30 °C for 30 min. Reactions were terminated by the addition of 8 μl of 4-fold sample buffer and were resolved by SDS-PAGE (10%). LMP1 was detected by phosphorimaging or Western blot analysis.

Immunofluorescence—Cells were plated on chamber slides coated with fibronectin and incubated for 1 h at 37 °C. Slides were washed twice with PBS and fixed with 2% paraformaldehyde. Fixation was stopped with three washes of 50 mM NH4Cl, and the cells were either permeabilized for 10 min in 0.05% Nonidet P-40 or incubated for the same amount of time in PBS. Cells were incubated with the primary antibody S12 for 1 h, washed excessively either in PBS/0.01% saponin/0.25% gelatin (permeabilized cells) or in PBS (nonpermeabilized cells), incubated with Rhodamine-labeled goat anti-mouse antibody (Jackson Laboratory), and washed in the same manner. Fluorescence was detected using a fluorescent microscope (Axioskop 2; Carl Zeiss).

RESULTS

LMP1 Is a Short-lived Protein In Vivo and Its Degradation Requires Metabolic Energy—The half-life of LMP1 ranges between 1.5–7 h (17–19). To assess the stability of the protein in our system, we performed a time course study in EA-B1 and COS cells. In addition, because the ubiquitin pathway is ATP-dependent, we tested the effect of ATP depletion on LMP1 degradation in cells. As shown in Fig. 1, the half-life of the protein in EA-B1 B cells is ~120 min. A similar result was obtained in COS7/5 cells (data not shown). When Antimycin A was added to deplete ATP, degradation of LMP1 was severely impaired, and the half-life time increased to approximately 5 h, demonstrating clearly the need for ATP in the proteolytic process.

LMP1 Is Degraded in a Proteasome-dependent Manner—Because LMP1 is a short-lived protein that is degraded in an ATP-dependent manner, we wanted to test whether, like many other short-lived proteins, it is subject to proteasomal degradation. To identify the system involved in degradation of LMP1, we monitored the effect of either the proteasomal inhibitors MG132 and lactacystin or the lysosomal inhibitors NH4Cl and chloroquine on the degradation of LMP1. As can be seen in Fig. 2A, MG132 completely blocked degradation of LMP1. A similar result was obtained with lactacystin (Fig. 2B). In striking contrast, NH4Cl and chloroquine had a small effect on the degradation of LMP1 (Fig. 2C). We concluded that LMP1 was degraded mostly by the proteasome.

LMP1 Is Ubiquitinated and Its Degradation Requires a Functional E1—All known proteasome substrates, except for one established case, that of ornithine decarboxylase (ODC) (see “Discussion”), must be first tagged by a polyubiquitin chain, marking them for degradation. A direct proof that LMP1 is processed via the ubiquitin pathway came from an in vivo conjugation experiment. LMP1 isolated from cells is clearly ubiquitinated, and the abundance of the conjugated species is greatly enhanced when proteasomal degradation was blocked.
by MG132 further corroborating the notion that the protein is targeted by the proteosome (Fig. 3A). Further evidence that an intact ubiquitination machinery is essential for degradation of LMP1 came from experiments in ts20 cells harboring a mutant temperature-sensitive E1. As can be seen in Fig. 3B, in the parent E36 cell, LMP1 was degraded efficiently at both the permissive and nonpermissive temperatures. In contrast, in ts20 cells, degradation proceeded at a normal rate at the permissive temperature but was essentially arrested at the nonpermissive temperature, thus demonstrating that a functional E1 is essential for degradation of LMP1.

A Large Proportion of LMP1 Is Localized to the Plasma Membrane—Previous reports showed that LMP1 is localized in patches on the plasma membrane of EBV immortalized lymphoblastoid cell lines (40). Because most known membrane proteins that are ubiquitinated are targeted to the lysosome/vacuole, the finding that LMP1 is degraded by the proteasome was unexpected. Therefore, it was important to verify the localization of LMP1 in our cells at steady state. Toward that end, we examined the localization of LMP1 in nonpermeabilized and permeabilized EA-B1 and COS7/5 cells. In nonpermeabilized cells (Fig. 4A), LMP1 can be clearly seen on the cell surface in clusters. In permeabilized cells (Fig. 4B), as expected, some of the protein is intracellular, and the staining pattern suggests endoplasmic reticulum localization. Still, it is clear that a large proportion of the protein is localized on the cell surface.

Reconstitution of a Cell-free System for Ubiquitination and Degradation of LMP1 and the Possible Role of Phosphorylation—To study in more detail the mechanisms involved in ubiquitin-mediated degradation of LMP1, we established a reconstituted cell-free proteolytic system. To use as native-like a protein as possible, we used as a substrate a crude-membrane fraction from LMP1-transfected COS7/5 cells. This ensured a substrate that is correctly folded and appropriately inserted into the cell membrane. As shown in Fig. 5A, when LMP1 was incubated in the presence of crude HeLa cell extract (as a source for ubiquitin system enzymes) and ATP, high molecular weight conjugates were generated. The high molecular weight species were identified as ubiquitinated forms of LMP1 by subjecting the LMP1-specific immunoprecipitates to Western blot analysis with anti-ubiquitin serum. To directly demonstrate ubiquitin-dependent degradation of LMP1, we incubated the substrate-containing crude membrane fraction from
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**Fig. 5.** ATP-dependent ubiquitination and degradation of LMP1 in a cell-free reconstituted system. A, ATP-dependent ubiquitination of LMP1 in vitro. Crude membrane fraction prepared from COS 7/5 cells transiently expressing LMP1 was used as a substrate in an in vitro conjugation (Conj.) assay. Membranes were incubated in the presence of crude HeLa cell extract in the absence or presence of ATP as described under “Experimental Procedures.” LMP1 was isolated using immunoprecipitation and SDS-PAGE. LMP1-ubiquitin adducts were identified using Western blot analysis with an anti-ubiquitin antibody (upper panel). Bottom panel, membranes were stripped and reprobed with anti-LMP1 antibody. Mock denotes cells transfected with an empty vector. B, ATP-dependent degradation of LMP1 in vitro. Crude membrane fraction from EA-B1 cells was used as a substrate in an in vitro degradation assay as described under “Experimental Procedures.” Membranes were incubated in the presence of HeLa cell Fraction II, and ubiquitin (Ub) and ATP were added as indicated.

**Fig. 6.** Conjugation and degradation of LMP1 in vitro probably requires phosphorylation. A, ATP but not ATPγS promotes conjugation of LMP1 in vitro. COS7/5 cells expressing LMP1 were labeled for 2 h with [35S]Met/Cys. A crude membrane fraction was prepared, and an in vitro conjugation (Conj.) assay was carried out as described under “Experimental Procedures.” LMP1 was immunoprecipitated and visualized by phosphorimaging following SDS-PAGE. Lane 1, reaction mixture was incubated on ice. Lanes 2–4, reaction mixtures were incubated at 30 °C. B, efficient degradation of LMP1 in vitro requires okadaic acid. LMP1-containing membranes were incubated with HeLa cell extract in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of ATP, and in the presence (lanes 1–3) or absence (lane 4) of okadaic acid (OKA). LMP1 was identified using Western blot analysis as described under “Experimental Procedures.”

A Lysine-less Mutant LMP1 (K330R) Is Degraded by the Ubiquitin System—Typically, the polyubiquitin chain is anchored to an ε-NH₂ group of one or more internal lysine residues. LMP1 has a single lysine residue at position 330, and testing its role in targeting the protein for degradation was the obvious next step. We replaced this lysine residue with arginine. To our surprise, K330R-LMP1 was degraded quite efficiently, albeit at a somewhat slower rate compared with the WT protein (Fig. 7A). To study the potential role of the ubiquitin system in the degradation of K330R-LMP1, we demonstrated that it is ubiquitinated in vitro. Furthermore, conjugation was enhanced in the presence of a proteasome inhibitor (Fig. 7B). When we subjected crude membranes from K330R-LMP1-expressing cells to an in vitro degradation assay, the mutant protein was efficiently degraded, and degradation was sensitive to inhibition by lactacystin (Fig. 7C). Lastly, we tested the requirement for a functional E1 in K330R-LMP1 degradation. Similar to WT LMP1 (Fig. 3B), K330R-LMP1 was rapidly degraded in ts20 cells at the permissive temperature. In contrast, this degradation was severely inhibited at the nonpermissive temperature (Fig. 7D), demonstrating the need for an intact E1 for the proteolytic process. Taken together, these three independent observations demonstrate that, similar to the WT protein, K330R-LMP1, is degraded in a ubiquitin- and proteasome-dependent manner.

Degradation of Lysine-less LMP1 Is Most Likely Mediated by N-terminal Ubiquitination—If conjugation of K330R-LMP1 does not occur on an internal lysine, what then is the mechanism for ubiquitination of the mutant protein? We tested the possibility that the first ubiquitin molecule is attached to the free amino group on the N-terminal residue of the protein. We
fused a Myc tag to the amino end of LMP1 and asked whether the tagged protein will be as efficiently degraded as the non-tagged protein. As can be seen in Fig. 8A, Myc-tagged WT LMP1 (that contains Lys-330) was significantly stabilized compared with nontagged protein. Not surprisingly, in a similar experiment using K330R-LMP1, degradation of the tagged protein was completely blocked (Fig. 8B; see "Discussion"). Attachment of the Myc tag to the C-terminal end of the LMP1 did not have any stabilizing effect (data not shown). The effect of the Myc tag is probably due to blocking of an essential recognition motif at the N-terminal region of the molecule. To demonstrate directly that the N-terminal domain is essential for recognition and subsequent degradation of the protein, we deleted the first 12 or 24 N-terminal residues. As can be clearly seen in Fig. 9, the truncated species are stable. The identity of the lower molecular weight protein observed in the ΔN12-LMP1 (Fig. 9B) is not known. It may be due to cleavage of the mature protein. Taken together, these data point at that area as crucial for ubiquitination and subsequent degradation of LMP1.

It was important to show that the substitution of the single Lys residue and the manipulations of the N-terminal domain did not affect the membrane expression of LMP1. As can be clearly seen in Fig. 10, similar to the WT protein, K330R LMP1 is expressed in patches on the plasma membrane (compare with Fig. 4A). The N-terminally Myc-tagged protein shows an identical distribution. The ΔN12-LMP1 mutant was also efficiently expressed at the plasma membrane, although some of it was in a more diffuse pattern.

DISCUSSION
Ubiquitin-mediated degradation of multiple key regulatory proteins is involved in the regulation of many basic cellular processes. Here we show that LMP1 is a substrate of the ubiquitin system. It is degraded in an ATP-dependent manner (Fig. 1). In the ubiquitin system, ATP is required for both ubiquitin conjugation and assembly of the proteasome, and ATP dependence has become an important hallmark for proteins that traverse the ubiquitin system. More direct examination of the role the ubiquitin system in the degradation of LMP1 revealed that the process is mediated by the proteasome;
inhibitors of the proteasomal but not of lysosomal function stabilized the protein (Fig. 2). Furthermore, inhibition of the proteasome is accompanied by accumulation of high molecular weight LMP1-ubiquitin adducts (Fig. 3A), and degradation of LMP1 requires a functional E1 (Fig. 3B). All these experiments strongly suggest that the ubiquitin system is involved in the degradation of LMP1; however, they do not prove unequivocally that direct ubiquitination of LMP1 is required for its targeting. For example, although the ubiquitin system is required for endocytosis and lysosomal targeting of the growth hormone receptor, the observed ubiquitination of the receptor may not be required (27). Conjugation of another, yet unknown, factor, is perhaps required for ubiquitin-mediated targeting of the receptor. However, it should be emphasized that unlike the case of a lysine-less growth hormone receptor that is not conjugated, a lysine-less LMP1 is strongly conjugated in vivo. Reconstitution of a cell free conjugation (Fig. 5A) and degradation (Fig. 5B) system further corroborated the notion that ubiquitination serves as an essential intermediate step in the proteolytic process. An interesting observation involves the possible signaling by phosphorylation in rendering the protein susceptible for degradation. LMP1 is a phosphoprotein and has been shown previously to be phosphorylated primarily on serine but also on some threonine residues (17). In several substrates of the ubiquitin pathway, IxBa, G1 cyclins, and the CDK inhibitor p27Kip1, for example, phosphorylation has been shown to be essential for targeting of the protein for degradation (20). We have shown that unlike ATP, the nonhydrolyzable analog ATPγS cannot promote conjugation of LMP1 (Fig. 6A). Because ATPγS can be used as an energy source by E1, substrates that are not signaled for conjugation by phosphorylation can be conjugated in the presence of ATPγS as an energy source (41). In contrast, substrates that are targeted following phosphorylation, such as IxBa, are not conjugated in the presence of ATPγS that cannot serve as a donor of a phosphate group. In addition, degradation was inefficient in the absence of okadaic acid, a broad range phosphatase inhibitor (Fig. 6B), further supporting a potential role for phosphorylation in targeting LMP1. Serine 313 and threonine 324 have been found to be two major phosphorylation sites of LMP1 (42), and so we replaced both residues with alanine in an attempt to affect degradation of LMP1. We could not detect any significant difference in the half-life of the mutants compared with WT LMP1 (data not shown) and concluded that other phosphorylation sites may be involved.

To analyze further the degradation signal of LMP1, we replaced the single lysine residue at position 330 with arginine, expecting to see a major stabilization effect. To our surprise, the resulting mutant, K330R-LMP1, was degraded quite efficiently, albeit at a somewhat slower rate compared with WT LMP1 (Fig. 7A). Additionally, K330R-LMP1 was ubiquitinated efficiently in vivo (Fig. 7B), and its degradation required a functional E1 (Fig. 7D), similar to WT LMP1 (Fig. 3B). Degradation of K330R-LMP1 in the cell free system was mediated by the proteasome (Fig. 7C). Because no lysine residues were available, we suspected that the first ubiquitin residue is attached to the free N-terminal group, as is the case for MyoD (25). Because in most substrates of the ubiquitin pathway internal lysines serve as target for ubiquitination, attachment to the N-terminal residue is probably sequence- and protein-specific. We therefore altered the N-terminus of both WT LMP1 and K330R-LMP1 by fusing it with a Myc tag. For both forms of LMP1, tagging resulted in major stabilization of the protein (Fig. 8). This stabilization effect is even more striking when one takes into account the existence of several lysine residues on the tag. We believe that the small difference in stability between Myc-WT LMP1 and Myc-K330R-LMP1 stems from the single internal lysine residue in LMP1, which may allow residual ubiquitination. Similar results were obtained also when analyzing the degradation of MyoD (25). The Myc tag can affect stability of a protein that is targeted for degradation following ubiquitination of the N-terminal residue by blocking the access of ubiquitin, the ligase, or both, to a specific ubiquitination site and/or recognition motif at the N-terminal domain. To test directly the role of the N-terminal domain in degradation of LMP1, we deleted the first 12 or 24 N-terminal amino acid residues. As can be seen in Fig. 9, deletion of as few as 12 amino acids was sufficient to stabilize the protein. Further supporting the notion that the degradation signal resides within the N-terminal domain is the finding that the steady state level of Δ6–17 and Δ6–24 LMP1s are significantly higher compared with that of the WT protein (40). It should be noted that Myc...
tagging and deletion of the N-terminal domain had little or no effect on membrane expression of LMP1 (Fig. 10), demonstrating that the stabilization of the protein by the tag or the deletion is due solely to blocking N-terminal ubiquitination rather than to intracellular expression of the proteins in a site from which they cannot be degraded. Interestingly, deletion of the first 12 amino acid residues of LMP resulted in a somewhat more diffuse expression of the protein, suggesting that these amino acids are important for patching. Retaining this putative signal in the Myc-tagged protein maintains the WT-like patchy membrane distribution while stabilizing the protein. It should be noted that similar membrane distribution was also observed for Δ6–17 and Δ6–24 LMP1s (40). We could not find sequence homology or a common motif between the N-terminal regions of LMP1 and MyoD (see above and below), but such functional motives or epitopes could form at the folded protein level rather than at the primary sequence. Future identification of the E3 ligase will be necessary to resolve the question of whether the N-terminal region serves also as a recognition site for the ligase. It should be emphasized again that N-terminal ubiquitination is different from the recognition via the N-end rule where the protein is recognized via the N-terminal residue, but conjugation still occurs on internal lysines.

At least three other examples where a lysine-less mutant is degraded by the proteasome have been reported. The first is the transcription factor MyoD, which has been firmly shown to be ubiquitinated on the N-terminal amino group (25). The second case involves the degradation of unassembled T cell receptor α chain (TCRα) (26). A recent study shows that a lysine-less mutant is still conjugated in vitro, although much less efficiently than the WT protein, and that this mutant is degraded in a ubiquitin- and proteasome-dependent manner (26). The researchers speculated that ubiquitination, possibly at the N-terminal residue, plays an important role in translocation of the protein from the endoplasmic reticulum and in its targeting for degradation. However, they did not rule out the possibility that ubiquitination of an as yet unidentified protein factor may be involved in dislocation of TCRα from the endoplasmic reticulum in trans. In that respect, TCRα may resemble the growth hormone receptor (Refs. 27 and 35 and see above). A third case involves the Cdk inhibitor p21Cip1 (43). Here, the protein undergoes ubiquitination, but the authors suggested that this modification is not necessary for degradation, and similar to ODC, the protein is targeted by the proteasome in a process that does not require ubiquitination. Thus, it appears that the three cases represent three distinct targeting mechanisms. The case of LMP1 appears to fall within the MyoD “class.”

The identity of the LMP1 conjugating enzymes is still not known. Interestingly, it has been recently shown that a PY motif at the N-terminal region of LMP2A, an EBV protein involved in down-regulation of B cell receptor signaling function, associates with WW domains characteristic to the HECT (homologous to the E6-AP C-terminal domain) family of ubiquitin ligases, E3s (44). Direct association of LMP2A has been demonstrated with several members of the HECT family such as Nedd4, AIP4, Itchy, and WW2/AIP2. Sequence analysis of LMP1 does not show however any PY motives.

The requirement for tight control of the level of LMP1 is still enigmatic. The finding that long-lived species fail to effect the phenotypic changes observed for WT LMP1 (19) is rather surprising, because one expects that accumulation of the protein should augment its biological activity. Many cell surface receptors involved in cell signaling, the EGF receptor for example (45), are short-lived and targeted by the ubiquitin system. The finding that LMP1 shares mechanisms used by the tumor necrosis factor/CD40 receptor family involved in cell signaling (14, 15) may provide us with a direction for further studies.

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