Ubiquitin-Independent Proteasomal Degradation of Tumor Suppressors by Human Cytomegalovirus pp71 Requires the 19S Regulatory Particle

Laura L. Winkler, Jiwon Hwang and Robert F. Kalejta


Updated information and services can be found at: http://jvi.asm.org/content/87/8/4665

These include:

**SUPPLEMENTAL MATERIAL**

Supplemental material

**REFERENCES**

This article cites 45 articles, 18 of which can be accessed free at: http://jvi.asm.org/content/87/8/4665#ref-list-1

**CONTENT ALERTS**

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), [more»](http://journals.asm.org/site/misc/reprints.xhtml)

Information about commercial reprint orders: [http://journals.asm.org/site/misc/reprints.xhtml](http://journals.asm.org/site/misc/reprints.xhtml)

To subscribe to another ASM Journal go to: [http://journals.asm.org/site/subscriptions/](http://journals.asm.org/site/subscriptions/)
The proteasome is responsible for the bulk of protein turnover within cells. Most substrates arrive at the proteasome poly-ubiquitinated, where the ubiquitin chains mediate proteasome binding but are then removed prior to substrate degradation by the proteasomal 20S catalytic core. The core particle can associate with activator complexes that modulate proteasome function (1). For example, the 19S regulatory particle (RP) associates with one or both ends of the 20S core to form the 26S proteasome species responsible for ubiquitin-mediated degradation events. Proteasomal activity is critical for cellular homeostasis, cell cycle progression, transcription, DNA repair, and dichotomously, both the success of viral infections and essential defenses against viral pathogens (2–6).

Proteasomal degradation of viral antigens to generate peptides displayed by major histocompatibility group (MHC) molecules is a well-documented part of adaptive immunity (4). Less well appreciated is the number of cellular intrinsic defense proteins targeted for proteasomal degradation by viral factors (7, 8). For example, human cytomegalovirus (HCMV) infections, which cause severe disease in immunocompromised, -suppressed, or -naive individuals, induce the degradation of several cellular transcriptional corepressors to create an environment conducive to productive, lytic infection. Within the tegument layer of its virion, HCMV packages the viral pp71 protein, which is introduced into cells immediately upon infection, traffics to the nucleus, and induces the degradation of BclAF1, Daxx, and the retinoblastoma (Rb) family members Rb, p107, and p130 (9–11). BclAF1 and Daxx degradation promotes viral immediate early (IE) gene expression. Rb family inactivation, which also occurs through phosphorylation by the virally encoded kinase UL97 (12), likely increases the efficiency of viral DNA replication. These pp71-dependent degradation events are prevented by pharmacologic inhibition of the 20S catalytic core, indicating that they are proteasomal processes. Other experimental evidence, however, indicates that these proteasomal degradation events occur without the usual requirement for substrate polyubiquitination (13, 14).

Ubiquitin-independent protein degradation has recently been associated with the PA28γ proteasomal activator (15–17). To define the proteasomal requirements for pp71-mediated protein degradation, we surveyed all known proteasome activators for potential roles during pp71-mediated Daxx degradation. Interestingly, we found that only the 19S RP was required for the pp71-mediated degradation of both Daxx and Rb. Our results place the 19S RP on a novel virally directed route of nonubiquitinated proteins to the proteasome for degradation that may represent a potential point for therapeutic inhibition of HCMV infection.

**MATERIALS AND METHODS**

**Cells, viruses, and assays.** Human foreskin fibroblasts (HFs), mouse embryonic fibroblasts (MEFs), and mouse B cells were cultured as previously described (11, 18–20). Virus strains AD169 and AdsubUL82 (pp71 null) were propagated, UV inactivated, and used to infect cells as previously described (11, 21). Infections with recombinant adenoviruses were performed as previously described (9). Proteins visualized on film were quantified with ImageQuant 5.2 software. Proteins visualized with the LI-COR Odyssey Fc imaging system and IRDyes (926-68170 and 827-08365) were quantified with LI-COR Image Studio software. In both cases, bands were normalized to those of loading controls and are reported as percentages of the results for their respective mock-infected samples. Statistical analyses utilized two-tailed paired t tests.

**Inhibitors and antibodies.** Leptomycin B (40 nM) (Calbiochem) was added 2 h prior to infection. Lactacystin (20 μM) (Calbiochem) and Gö6976 (250 nM) (Calbiochem) were added at the time of infection. Primary antibodies are listed in Table S1 in the supplemental material (9, 22). Secondary antibodies conjugated with horseradish peroxidase were used.
purchased from Chemicon (anti-mouse and -rabbit antibodies) or Santa Cruz (anti-goat antibody); those conjugated with Alexa Fluor 488 were from Molecular Probes. Immunoblots, immunofluorescence, and immunoprecipitations were completed as previously described (11, 23).

**Nuclear and cytoplasmic fractionation.** Cells were resuspended in a hypotonic buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl₂, 1.0 mM dithiothreitol [DTT], 0.2 mM phenylmethylsulfonyl fluoride [PMSF], and protease inhibitors) and then lysed with 0.25% NP-40. Nuclear (pellet) and cytoplasmic (supernatant) fractions were separated by centrifugation. Cytoplasmic fractions were subjected to five freeze-thaw cycles and a lysate-clearing centrifugation. Nuclear fractions were resuspended in extraction buffer (20 mM HEPES [pH 7.9], 0.45 M NaCl, 1.5 mM MgCl₂, 10 mM DTT, 0.2 mM PMSF, and 0.2 mM EDTA) and then treated as described above for the cytoplasmic fractions.

**RNA interference.** Reagents for RNA interference were purchased from Dharmacon. Sequences are listed in Table S1 in the supplemental material. For transient knockdowns, an equal number of HFs were transfected with 40 pmol (ubiquitin), 80 pmol (Rpn1 and PA28 isoforms) or 160 pmol (Rpt2 and Rpn11) small interfering RNA (siRNA)/10⁶ cells using Lonza nucleofection kits (VPI-1002; Lonza) following the manufacturer’s protocol and equally distributed among culture dishes. (Scrambled control siRNAs were added at concentrations that mimicked ubiquitin or proteasome subunit siRNA conditions in each experiment.) Fresh medium was added 24 h after transfection at the multiplicities of infection (MOIs) (calculated using scrambled control cell numbers) indicated in the figure legends. Stable PA200 knockdown 293 cells were generated by retroviral transduction as previously described (24) and transfected with a previously described mutant of pp71 (25).

**RESULTS**

Cytoplasmic proteasome activators are dispensable for the pp71-mediated ubiquitin-independent degradation of Daxx. pp71 degrades Daxx (Fig. 1A and B) even when ubiquitin levels are decreased by RNA interference to a sufficient level to stabilize p53 (Fig. 1A), a well-documented ubiquitin-dependent proteasomal substrate. Furthermore, pp71 degrades Daxx when cells are treated with leptomycin B (Fig. 1C), which inhibits the CRM1-mediated nuclear export of proteins like NF-[kappa]B (Fig. 1D). From this and previous data (13, 14), we conclude that pp71 degrades Daxx in a ubiquitin-independent manner within the nucleus, suggesting that exclusively cytoplasmic proteasome activators would not participate in this process. The known mammalian proteasome activators include the 19S RP (ubiquitin-dependent proteolysis), PA28αβ (the immunoproteasome), PA28γ (ubiquitin-in-
dependent proteolysis), and PA200 (spermatogenesis and DNA repair) (reviewed in reference 1). PA200 and PA28β are exclusively cytoplasmic in primary human fibroblast cells fully permissive for HCMV infection, whereas the other activators, as well as 20S cores, are found in both the nucleus and the cytoplasm (Fig. 2A). These localizations generally agree with those described in the published literature, where all subunits have been found in both the cytoplasm and the nucleus (26–30). The only real differences we found include the apparently exclusively cytoplasmic localization of PA28α/H9252 and PA200 in our experiments; however, longer exposures might have revealed some fraction of these proteins within the nucleus. Knockdown of PA28α/H9251 (Fig. 2B), PA28β/H9252 (Fig. 2B), or PA200 (Fig. 2C) failed to inhibit Daxx degradation by pp71, whereas treatment with lactacystin, a specific inhibitor of the 20S catalytic core, stabilized Daxx. These data indicate that the immunoproteasome (PA28αβ) and PA200 are not required for pp71-mediated Daxx proteasomal degradation. Note that PA200 knockdown experiments were performed in nonpermissive 293 cells, as we were unable to achieve detectable knockdown of this proteasome activator in fibroblasts.

**PA28γ is dispensable for pp71-mediated Daxx degradation.** While PA28γ mediates the ubiquitin-independent degradation of both cellular and viral targets (15–17, 31), knockdown of PA28γ failed to inhibit pp71-mediated degradation of Daxx, which was prevented by lactacystin (Fig. 2B). Likewise, infection with wild-type but not pp71-null HCMV induced Daxx degradation in embryonic fibroblasts derived from PA28α/H9253 (Fig. 2D and E) and PA28αβ/H9253 (Fig. 2D and F) knockout mice (PA200 knockout MEFs are not available and, thus, were not examined). In addition, PA28αβγ knockdown MEFs supported pp71-mediated Daxx degradation after transduction with a pp71-expressing recombinant adenovirus (Fig. 2G) but not one expressing the HCMV UL69 tegument protein. Thus, the proteasome activator previously implicated in ubiquitin-independent degradation, PA28γ, is dispensable for the pp71- and proteasome-mediated degradation of Daxx.
The 19S RP is required for ubiquitin-independent, pp71-mediated Daxx degradation. The remaining proteasome activator, the 19S RP, is required for ubiquitin-dependent proteolysis and can be biochemically divided into base and lid subcomplexes (32). The base consists of six ATPases that associate with the 20S core (Rpt1 to -6), two non-ATPase subunits (Rpn1 and Rpn2), and two ubiquitin receptors (Rpn10 and Rpn13), while the lid is comprised of eight subunits (Rpn3, -5, -6, -7, -8, -9, -11, and -12) (33–35). Depletion of certain 19S RP subunits, like Rpn1, can inhibit 19S RP function and stabilize substrates that are degraded by the 26S proteasome (15, 36). Daxx degradation during HCMV infection was inhibited in Rpn1-depleted cells where p53, a 26S proteasome substrate, was stabilized (Fig. 3A). Daxx was also stabilized by lactacystin treatment or depletion of the \( \alpha \)-H9252 chymotrypsinlike subunit of the 20S core. Importantly, under conditions where 19S RP-dependent substrates like p53 are stabilized in Rpn1-depleted cells, p21, a substrate that is constitutively degraded through a 19S RP-independent, 20S-dependent process (15), is still degraded (Fig. 3B), indicating that 19S RP subunit depletion does not affect all proteasomal degradation. At least three individual subunits of the 19S RP with independent functions (Rpn1, a structural scaffold [37], Rpt2, an ATPase [38], and Rpn11, a deubiquitinase [39]) are each required for pp71-mediated Daxx degradation (Fig. 3C, D, E, and F). Inhibition of 19S RP function by Rpn1 depletion or inhibition of 20S function by lactacystin does not impair the ability of pp71 to enter the nucleus (Fig. 4A and B) or associate with Daxx (Fig. 5). The punctate appearance of pp71 in cells either depleted of Rpn1 or treated with lactacystin likely results from its previously described localization to promyelocytic leukemia nuclear bodies in the absence of Daxx degradation, which, as shown above, is inhibited under these conditions. Thus, we conclude that the 26S proteasome is responsible for pp71-mediated Daxx degradation. At the start of HCMV infection, pp71 degrades not only Daxx but also the hypophosphorylated form of the tumor suppressor Daxx.
Rb (12) without its prior ubiquitination (14). Residual hypophosphorylated Rb not degraded by pp71 is later phosphorylated by UL97 (12). In cells transfected with a scrambled siRNA, pharmacological inhibition of UL97 with Gö6976 failed to stabilize Rb during HCMV infection, whereas proteasome inhibition did (Fig. 6A). This indicates that within the time frame of these experiments, the loss of hypophosphorylated Rb occurs through pp71-mediated degradation, not UL97-mediated phosphorylation. Similar to Daxx, Rb was degraded in control cells but remained near mock levels in HCMV-infected cells previously depleted of Rpn1 (Fig. 6A), Rpt2, or Rpn11 (Fig. 6B). In total, these results suggest that, although Daxx and Rb degradation by pp71 are ubiquitin-independent processes, degradation of these substrates during HCMV infection requires the 19S RP complex.

**DISCUSSION**

Viruses commandeer proteasome pathways to combat cellular immune defenses and promote viral replication (4, 7, 40). Most viral and cellular substrates are degraded through the canonical ubiquitin-dependent pathway where target substrates are covalently modified with ubiquitin molecules and delivered to the proteasome for degradation. There is, however, a growing realization that substrates can be degraded by the proteasome without prior ubiquitination (8, 41). Interestingly, the majority of substrates degraded in this manner, Rb (12) without its prior ubiquitination (14). Residual hypophosphorylated Rb not degraded by pp71 is later phosphorylated by UL97 (12). In cells transfected with a scrambled siRNA, pharmacological inhibition of UL97 with Gö6976 failed to stabilize Rb during HCMV infection, whereas proteasome inhibition did (Fig. 6A). This indicates that within the time frame of these experiments, the loss of hypophosphorylated Rb occurs through pp71-mediated degradation, not UL97-mediated phosphorylation. Similar to Daxx, Rb was degraded in control cells but remained near mock levels in HCMV-infected cells previously depleted of Rpn1 (Fig. 6A), Rpt2, or Rpn11 (Fig. 6B). In total, these results suggest that, although Daxx and Rb degradation by pp71 are ubiquitin-independent processes, degradation of these substrates during HCMV infection requires the 19S RP complex. **DISCUSSION**

Viruses commandeer proteasome pathways to combat cellular immune defenses and promote viral replication (4, 7, 40). Most viral and cellular substrates are degraded through the canonical ubiquitin-dependent pathway where target substrates are covalently modified with ubiquitin molecules and delivered to the proteasome for degradation. There is, however, a growing realization that substrates can be degraded by the proteasome without prior ubiquitination in uninfected and virus-infected cells (8, 41). Interestingly, the majority of substrates degraded in this manner, Rb (12) without its prior ubiquitination (14). Residual hypophosphorylated Rb not degraded by pp71 is later phosphorylated by UL97 (12). In cells transfected with a scrambled siRNA, pharmacological inhibition of UL97 with Gö6976 failed to stabilize Rb during HCMV infection, whereas proteasome inhibition did (Fig. 6A). This indicates that within the time frame of these experiments, the loss of hypophosphorylated Rb occurs through pp71-mediated degradation, not UL97-mediated phosphorylation. Similar to Daxx, Rb was degraded in control cells but remained near mock levels in HCMV-infected cells previously depleted of Rpn1 (Fig. 6A), Rpt2, or Rpn11 (Fig. 6B). In total, these results suggest that, although Daxx and Rb degradation by pp71 are ubiquitin-independent processes, degradation of these substrates during HCMV infection requires the 19S RP complex.

**DISCUSSION**

Viruses commandeer proteasome pathways to combat cellular immune defenses and promote viral replication (4, 7, 40). Most viral and cellular substrates are degraded through the canonical ubiquitin-dependent pathway where target substrates are covalently modified with ubiquitin molecules and delivered to the proteasome for degradation. There is, however, a growing realization that substrates can be degraded by the proteasome without prior ubiquitination in uninfected and virus-infected cells (8, 41). Interestingly, the majority of substrates degraded in this manner,
were analyzed with immunoblotting and quantified with ImageQuant. (B) HFs were infected (V) with HCMV at an MOI of 1. Lysates collected 3 h postinfection or infected (M) or infected (VL), or DMSO (V). Lysates harvested 9 h postinfection were analyzed with immunoblotting and quantified with ImageQuant. (C) FIG 6 The 19S RP is required for the degradation of Rb by pp71 during HCMV infection. (A) HFs were transfected with a scrambled control (Scr) or Rpn1-specific siRNA (R1.2) for 72 h and then mock infected (M) or infected (V) at an MOI of 1. Forty-eight hours prior to infection, cells were incubated in medium containing 0.1% serum. At the start of infection, cells were treated with G66976 (VG) (an inhibitor of UL97-mediated phosphorylation), lactacystin (VL), or DMSO (V). Lysates harvested 9 h postinfection were analyzed with immunoblotting and quantified with ImageQuant. (B) HFs were transfected with a scrambled control siRNA or one specific for the indicated protein (46). For Daxx, it is unclear whether or not faster-migrating bands represent unmodified proteins or, perhaps, one of the differentially spliced forms recently described (43). While Daxx sumoylation is stimulated by pp71, this posttranslational modification is not required for its degradation (44).

Here, we show that base and lid components of the 19S RP are required for pp71-mediated degradation of both Daxx and Rb, indicating that pp71 uses a proteasome species that is predominantly associated with ubiquitin-dependent degradation to degrade its substrates. The requirement for a 19S RP ATPase (Rpt2) might be expected, as substrate unfolding appears necessary for translocation into the narrow 20S channel harboring the protease active sites. As Rpn1 chaperones the assembly of an Rpt ATPase subcomplex and is required for the accumulation of Rpt2 (Fig. 3C) (37), its requirement is also not surprising. However, the requirement for Rpn1, a lid deubiquitinase, was unexpected. Perhaps the overall structure and/or function of the 19S RP is required for pp71-mediated degradation of Daxx and Rb.

How the 19S RP facilitates the ubiquitin-independent degradation of pp71 substrates is unknown. The 19S RP is also implicated in the ubiquitin-independent in vitro degradation of ornithine decarboxylase (ODC), as this is carried out by purified 26S but not 20S proteasomes (45) and is inhibited by the addition of polyubiquitin chains (46) that presumably compete with the substrate for 19S availability or function. Interestingly, we observed an enhancement of Daxx degradation during HCMV infection when ubiquitin was depleted (Fig. 1B). Perhaps depletion of the free-ubiquitin pool and a decrease in ubiquitinated substrates increased 26S proteasome availability for pp71-mediated degradation of Daxx. ODC degradation also requires a binding partner (antizyme) to unmask a cryptic degron within the carboxy terminus of the protein (46). In vivo degradation of a reporter fused to this degron required 19S RP function (36), but mechanistic features of this process have not been described. While pp71 could be playing an antizymelike role in the degradation of Daxx and Rb, degrons within these substrates have not been identified. Thus, the role for the 19S RP in the ubiquitin-independent degradation of antizyme and pp71 substrates remains to be defined.

ACKNOWLEDGMENTS

We thank Phil Balandyk (UW—Madison) for expert technical assistance, Ron Kopito (Stanford) for assistance with the PA200 experiments, and Martin Rechsteiner (University of Utah), Lance Barton (Austin College), and Barry Sleckman (Washington University) for generously providing materials.

This work was supported by National Institutes of Health grant AI074984 (to R.F.K.) and training grant T32 CA009135 (L.L.W.). R.F.K. is a Vilas Fellow and Burroughs Wellcome Fund Investigator in the Pathogenesis of Infectious Disease.

REFERENCES


