Human Cytomegalovirus Protein pp71 Induces Daxx SUMOylation

Jiwon Hwang and Robert F. Kalejta*

Institute for Molecular Virology and McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, Wisconsin 53706

Received 22 December 2008/Accepted 10 April 2009

Proteins that participate in a diverse array of cellular processes can be modified covalently and reversibly on lysine residues by the small ubiquitin-like modifier proteins termed SUMOs. In some instances, such modification profoundly affects protein function, but the biological significance of many SUMOylation events remains unknown. Protein SUMOylation is modulated during many viral infections. Here we demonstrate that the human cytomegalovirus (HCMV) pp71 protein promotes the SUMOylation of its cellular substrate, Daxx. A component of promyelocytic leukemia nuclear bodies, Daxx is a transcriptional corepressor that silences the expression of viral immediate-early (IE) genes at the start of both lytic and quiescent HCMV infections. pp71 is a tegument component delivered directly to cells by infecting HCMV virions. At the start of lytic infections, it travels to the nucleus and stimulates viral IE gene expression by displacing the chromatin remodeling protein ATRX from Daxx and by mediating Daxx degradation through a rare ubiquitin-independent, proteasome-dependent process. Here we report that pp71 also substantially increases the basal level of SUMOylated Daxx observed in cells. To date, consequences of Daxx SUMOylation have not been observed for cellular promoters, and we detected no qualitative change in viral IE gene expression in the absence of pp71-induced Daxx SUMOylation. Thus, while pp71 enhances the basal level of SUMOylated Daxx, the role that this modification plays in regulating Daxx activity in uninfected or HCMV-infected cells remains an enigma.

Human cytomegalovirus (HCMV) is an important human pathogen that causes severe disease in newborns infected in utero and in immunocompromised or immunosuppressed patients (29). Transcription of the HCMV genome during a productive, lytic infection is temporally regulated in a coordinated cascade that consists of immediate-early (IE), early (E), and late (L) gene expression (43). The major IE promoter (MIEP) directs the production of IE1 and IE2, two viral proteins that are critical for initiating lytic replication (9, 26). When latent HCMV infections are established, expression from the MIEP is silenced (39). The MIEP contains a myriad of binding sites for cellular transcriptional activators but is also tightly controlled by cellular transcriptional corepressors (27, 35, 42).

HCMV genomes entering the nucleus are targets of an intrinsic immune defense mediated by cellular proteins that localize to subnuclear structures called promyelocytic leukemia nuclear bodies (PML-NBs; also called PODs, for PML oncogenic domains, or ND10, for nuclear domain 10) (37). In addition to the PML protein itself, the transcriptional corepressor Daxx, a resident PML-NB protein, is a principal component of this intrinsic defense against HCMV and other viruses (35, 42). Daxx silences HCMV IE gene expression by inducing a transcriptionally inactive chromatin state around the MIEP. Additional cellular proteins, such as ATRX (alpha thalassemia/mental retardation syndrome X-linked) and histone deacetylases, cooperate with Daxx to silence HCMV gene expression through a mechanism that is not completely understood (6, 7, 25, 34, 37, 40, 41, 45).

Not surprisingly, HCMV has evolved a mechanism to efficiently neutralize the Daxx-mediated defense through the action of the viral pp71 protein. The pp71 protein is incorporated into the tegument layer of HCMV virions and thus is delivered to cells immediately upon infection (20, 32). In cells where productive lytic replication initiates, pp71 travels to the nucleus and localizes to PML-NBs, where it interacts with Daxx (14, 16), disrupts Daxx-ATRX complexes (25), and promotes Daxx degradation through an uncommon proteasome-dependent, ubiquitin-independent pathway (15). Interestingly, in cells where quiescent infections are established, tegument-delivered pp71 remains in the cytoplasm and the Daxx-mediated intrinsic defense remains intact (36). Thus, differential inactivation of this cellular intrinsic immune defense in specific cell types may be one of the many potential ways in which the eventual outcome (lytic or latent) of an HCMV infection event could be determined (10, 36).

Like many proteins that localize to PML-NBs, the small ubiquitin-like modifier SUMO is found covalently attached to a small subset of Daxx proteins (18, 23). Daxx also has a SUMO interaction motif (SIM) with which it binds to other SUMOylated proteins (23). The cellular SUMOylation machinery parallels that of ubiquitination reactions and includes an E1 activating enzyme (the heterodimer Aos1-Uba2), an E2 conjugating enzyme (Ubc9), and one of several E3 ligases that covalently attach SUMOs to lysine residues of targeted proteins. Protein SUMOylation can also be reversed by isopeptidases termed SENPs (sentrin/SUMO-specific proteases) (reviewed in reference 11). Thus, in addition to other protein modifications, SUMOylation is a dynamically regulated reversible posttranslational process that has the potential to regulate protein function. Indeed, there are documented cases where
SUMOylation affects protein activity, stability, or localization, and SUMOylation is emerging as an important regulatory event controlling gene expression (11, 28). However, many SUMOylation events have no or, more likely, still unrecognized effects on the modified proteins (28).

For example, SUMOylation of Daxx has no detectable effect on Daxx function. A Daxx mutant in which 15 carboxy-terminal lysine residues have been replaced with arginines (termed Daxx-15KR) is not detectably modified by SUMO (23) but still localizes to PML-NBs, binds to all but one of the same proteins with which wild-type Daxx interacts, and effectively represses transcription from the glucocorticoid promoter in a reporter assay (23). While SUMOylation of Daxx has as yet undocumented effects on protein function, the inability of Daxx to interact with SUMOylated proteins (by deletion of its SIM) severely inhibits the ability of the protein to repress transcription (23). Deletion of the SIM presumably prevents Daxx from recruiting chromatin remodeling enzymes to targeted promoters by preventing Daxx from interacting with the SUMOylated transcription factors bound there (23, 38).

In a testament to its importance, many viruses modulate the cellular SUMOylation pathway (3). Most often, viral infection inhibits the SUMOylation of cellular proteins, presumably to inactivate them and thus enhance viral replication. To date, a single example of a viral protein that enhances the SUMOylation of a cellular protein has been reported (30). Here we provide a second example of a viral protein that enhances the SUMOylation of a cellular protein, presumably to inactivate them and thus enhance viral replication. To date, we detected no changes in any early event mediated by Daxx or pp71 in HCMV-infected cells in the absence of pp71-induced Daxx SUMOylation. This newly recognized stimulation of Daxx SUMOylation by pp71 provides an additional opportunity to explore the role of SUMOylated Daxx in uninfected cells, as well as the enhancement of protein SUMOylation during viral infections.

**MATERIALS AND METHODS**

**Cells and viruses.** Human foreskin fibroblast (HF), C33A, HeLa, and N-tera2 (NT2) cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen), and THP-1 cells were cultured in RPMI 1640 medium (Invitrogen). All media were supplemented with 10% (vol/vol) fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin plus 0.292 mg/ml glutamine (Gibco). Cells were grown in a 5% CO2 atmosphere at 37°C. NT2 and THP-1 cells were differentiated as described previously (36). The wild-type virus strain employed was AD169 (HCMV). The HCMV pp71-null (ADsubUL2) virus has been described previously (5). For UV inactivation, virus stocks were placed on ice in a UV Stratalinker 2400 instrument (Stratagene) and exposed to a 254-nm light for 10 minutes.

**Inhibitors and antibodies.** Lactacystin (20 µM) dissolved in dimethyl sulfoxide was added for 60 min prior to infection with HCMV. N-Ethylmaleimide (NEM; Acros Organics) dissolved in ethanol and iodoacetamide (IAA; Acros Organics) dissolved in distilled water were added to lysis buffers. Antibodies from commercial sources were obtained for the following proteins: Daxx (D7810; Sigma), hemagglutinin (HA) (HA-11; Covance), FLAG (F3165; Sigma), tubulin (DM 1A; Sigma), pp65 (1025; Rumberg-Goodwin Institute), SUMO-1 (GMP; Zyomed), PML (H-238; Santa Cruz), and ATRX (H-300; Santa Cruz). Antibodies against pp71 (2H10-9 and IE233) and IE1 (1B12) have been described previously (37). Secondary horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies were obtained from Chemicon. Secondary antibodies for indirect immunofluorescence, conjugated with Alexa Fluor 488 (A-11029) or Alexa Fluor 546 (A-11030), were obtained from Molecular Probes.

**Transfection and IP.** For C33A cotransfection assays, cells were transfected with the indicated plasmids by the calcium phosphate method. The DNA precipitate was removed 12 h later, and cells were rinsed and cultured in 10% fetal bovine serum-containing Dulbecco’s modified Eagle’s medium for an additional 24 h before protein lysates were harvested. For immunoprecipitation (IP) to detect ubiquitin-like (Ub)-protein conjugation, cells were lysed in lysis buffer (20 mM Tris [pH 8.0], 250 mM NaCl, 3 mM EDTA, 10% glycerol, 1% sodium dodecyl sulfate [SDS], 0.5% NP-40, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 25 mM NaF, 10 µg of pepstatin A/ml, 10 µg of aprotonin/ml, 25 µg of leupeptin/ml, 25 µg of trypsin inhibitor/ml, 20 µM NEM, and 5 mM IAA) and then boiled for 20 min. All subsequent incubations were done at 4°C. Lysates were diluted in 0.1% SDS-containing lysis buffer and were preclarified by the addition of goat serum to a final concentration of 5% for 30 min prior to incubation with a preparation of heat-killed protein A-positive *Staphylococcus aureus* (Roche) for 30 min. The cleared lysates were incubated with the appropriate antibody for 4 h. Immune complexes were captured on protein A-agarose beads (Pharmacia) and washed six times with 0.1% SDS-containing lysis buffer. Bound proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by Western blotting. For IP to detect endogenous SUMOylated Daxx, HFs were used in RIP (radioimmunoprecipitation assay) buffer in the presence of protease inhibitors, as described previously (21), and then subjected to IP using a SUMO-1 antibody and visualized by Western blotting as described above.

**Western blots.** Cells were lysed in either 1% SDS lysis buffer, RIPA buffer (see above), or 0.5% NP-40 lysis buffer (20 mM Tris [pH 8.0], 250 mM NaCl, 3 mM EDTA, 10% glycerol, 0.5% NP-40) in the presence of protease inhibitors (see above). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and immobilized on Optitran membranes. Blots were blocked in 5% nonfat dry milk dissolved in TBST (10 mM Tris [pH 8.0], 150 mM NaCl, 0.05% Tween 20). Antibody incubations were done in 1% milk-TBST, and the blots were developed with an ECL enhanced chemiluminescence system (Amersham).

**Indirect immunofluorescence.** Cells were grown on glass coverslips and infected as described above. Coverslips were harvested and washed twice with phosphate-buffered saline (PBS; Gibco) prior to fixation with 1% paraformaldehyde in PBS and three washes with PBST (PBS plus 0.1% Triton X-100) for 5 min at room temperature in PBST plus 5% goat serum and 0.5% bovine serum albumin, followed by three washes with PBST for 5 min each. Coverslips were then washed twice with distilled water, incubated with Hoechst 33342 for 10 min, washed twice more with distilled water, and finally mounted with Fluoromount-G (Southern Biotech). Images were taken with a Zeiss microscope and camera (Axiovert 200 M).

**Generation of cell lines ectopically expressing Daxx.** Telomerase-immortalized HF (4) with reduced levels of Daxx (called shDaxx-HFs) were produced by transducing cells with a retrovirus (based on pUbiSuper from Oligo Engine) expressing a puromycin resistance gene and a short hairpin RNA (shRNA) specific to Daxx (36). Selection with puromycin produced a cell line with reduced levels of Daxx that complemented the replication of a pp71-null virus (R.T. Saffert and R. F. Kalejta, unpublished observations). Retroviruses based on the pRevTRE backbone that express wild-type HA-Daxx or HA-Daxx-15KR (generous gifts from H. M. Shih) and a hygromycin resistance gene were produced as previously described (36). Site-directed mutagenesis to alter the shRNA target sequence in Daxx (HA-tagged wild type and 15KR mutant), rendering the mRNAs resistant to RNA silencing, was also performed. Four sites (624, 627, 630, and 636) within the Daxx (HA-tagged wild type and 15KR mutant) were changed without affecting any amino acids to generate shRNA-resistant Daxx cDNAs, which were subsequently used to make pRevTRE-based recombinant retroviruses. To make cell populations expressing the indicated protein, shDaxx-HFs were transduced four to six times with the appropriate retrovirus, followed by drug selection.

**RESULTS**

HCMV pp71 induces the appearance of a posttranslationally modified form of Daxx. One mechanism through which pp71 stimulates HCMV IE gene expression is by inducing the
Western blots to monitor the steady-state level and electrophoretic mobility of Daxx in the presence and absence of pp71 to determine if pp71 induced a posttranslational modification of Daxx that could be required for Daxx degradation.

Ubl-modified proteins can be difficult to detect because they are often insoluble (they can partition to the pellet fraction of nondenatured lysates) and because Ubl modifications are efficiently removed in lysates by isopeptidases (11). To maximize our ability to detect a putative pp71-induced Ubl-modified form of Daxx, we initially prepared denatured lysates in buffer containing 1% SDS, with or without 20 mM NEM and 5 mM IAA, at 24 h posttransduction (hpt) and analyzed by Western blotting with the indicated antibodies. Short (top) and long (middle) exposures of the same blot are shown. Numbers on the left represent molecular weight standards in kilodaltons. Tubulin (Tub) served as a loading control.

The modified form of Daxx induced by pp71 migrated with an apparent molecular mass that was ~20 kDa larger than the primary species of Daxx (which we refer to as unmodified Daxx), and it was observed only when NEM and IAA were included in the lysis buffer (Fig. 1). While these isopeptidase inhibitors were required to detect pp71-induced modified Daxx, the analysis of denatured lysates was not. Modified Daxx was observed in the presence of pp71 when the soluble fractions of lysates prepared under nondenaturing conditions were analyzed (see Fig. S1A in the supplemental material). Thus, all subsequent lysates were prepared in nondenaturing buffers (except where specifically indicated) containing NEM and IAA.

The pp71-induced modification of Daxx was also observed during a productive lytic infection of HF with HCMV. As a component of the viral tegument, pp71 is delivered to cells immediately upon infection. In fibroblasts, it migrates to the nucleus and degrades Daxx (Fig. 2A) (37). The pp71-induced modification of Daxx in HCMV-infected HF was observed prior to (Fig. 2A) and in the absence of (Fig. 2B) IE gene expression. HCMV virions exposed to UV light accrue mutations to their DNA genomes that prevent viral gene expression, but these UV-treated virions still deliver functional tegument proteins to cells and still induce Daxx degradation and modification (Fig. 2B) (37). Infection of HF with pp71-null viruses grown on Daxx-knockdown-complementing cells (shDaxx-HFs) that do not have pp71 protein in the tegument failed to induce Daxx degradation or modification (Fig. 2C) (37). Entry of the pp71-null virus was confirmed by observing the presence of the pp65 tegument protein in the infected cell lysates (Fig. 2C). The timing of the modification of Daxx during HCMV infection and its appearance independent of viral gene expression but dependent on virion-delivered pp71, combined with the ability of pp71 to induce Daxx modification when expressed alone in cells, allow us to conclude that pp71 is necessary and sufficient to induce the modification of Daxx that we observe at very early times during HCMV lytic infection of permissive fibroblasts.

Daxx modification induced by pp71 requires direct interaction of the two proteins. We reasoned that pp71 may induce
mediated modification of Daxx was restricted to permissive cells, we transduced several cell lines with rAd-pp71 and assayed for Daxx modification. In all cell lines tested, we were able to detect the pp71-induced modified form of Daxx (see Fig. S1B in the supplemental material), implying that either pp71 itself is sufficient to induce this modification or pp71 recruits a widely expressed cellular ligase to promote Daxx modification.

The modification of Daxx induced by pp71 is SUMOylation. We took a candidate approach to try to identify the modification of Daxx induced by pp71. Most proteins that localize to PML-NBs, including Daxx, are known to be modified covalently by SUMO. In HeLa cells, a small fraction of endogenous Daxx is SUMOylated (23), and we were able to identify two modified forms of Daxx in untreated HeLa cells (see Fig. S1B in the supplemental material) that presumably represent SUMO-modified Daxx. Interestingly, one of the modified forms accumulated to higher levels in the presence of pp71. This result prompted us to ask if pp71 induced Daxx SUMOylation.

We chose the human papillomavirus-negative cervical carcinoma cell line C33A for these experiments because these cells are easy to transfect, have undetectable levels of modified Daxx, and support robust Daxx modification upon pp71 expression (see Fig. S1B in the supplemental material). C33A cells were transfected with a series of expression plasmids, and denatured lysates (prepared in 1% SDS-containing lysis buffer) were analyzed by both Western blotting and IP followed by Western blotting. Denatured lysates were used to ensure that any detected SUMO proteins represented covalent modifications, not simply a noncovalent interaction.

Transfection of FLAG-tagged SUMO-1 revealed a low level of SUMOylation of cotransfected HA-Daxx (Fig. 4A, lane 1), both by observing a modified form of Daxx in the lysates by straight Western blotting (top panels) and by direct visualization of SUMOylated Daxx after IP with a FLAG antibody followed by Western blotting with a Daxx antibody (bottom panel). Coexpression of His-tagged pp71 substantially increased the levels of modified/SUMOylated Daxx observed by both techniques (Fig. 4A, lane 2). Coexpression of the SUMO-specific protease SENP-1 prevented both the low-level constitutive SUMOylation of Daxx (Fig. 4A, lane 3) and pp71-induced Daxx SUMOylation (Fig. 4A, lane 4). Western analysis of lysates with the FLAG antibody confirmed the general decrease in SUMOylated proteins upon SENP-1 overexpression and failed to reveal any detectable global stimulation of SUMOylation by pp71.

We also observed the modification of Daxx with the endogenous SUMO-1 protein in fully permissive fibroblasts either transduced with rAd-pp71 (Fig. 4B) or infected with HCMV (Fig. 4C). Lysates (prepared in RIPA lysis buffer) from transduced or infected cells were analyzed by IP with a SUMO-1 antibody followed by Western blotting with a Daxx antibody. In addition to identifying SUMOylated Daxx by this protocol, unmodified Daxx was also observed in anti-SUMO-1 IPs from transduced/infected cells as well as mock-treated cells, likely because Daxx interacts with numerous SUMOylated proteins through the SIM of Daxx (38). Interestingly, in some experiments, an increased level of coimmunoprecipitated unmodified Daxx was observed in cells expressing pp71. This could possibly
of infection, but Daxx reaccumulates at later times (see Fig. S2A in the supplemental material). In HCMV-infected hpi, while IE gene expression was not observed until 3 hpi (see radiation was detected at 1 hpi and increased between 2 and 6 substantially at later time points. In these experiments, Daxx deg- S2A in the supplemental material) (37). The temporal association of pp71-induced Daxx degradation and SUMOylation both during HCMV infection and in pp71-expressing cells prompted us to ask if pp71-induced SUMOylation of Daxx was required for the degradation of Daxx stimulated by pp71.

A mutant Daxx protein in which the 15 carboxy-terminal lysine residues were replaced with arginines (termed the 15KR mutant) is very inefficiently SUMOylated both in vitro and in transfected cells (23). Because pp71 augmented the normal SUMOylation of Daxx in HeLa cells, we asked if pp71 failed to induce the SUMOylation of the Daxx 15KR mutant. To diminish any potential influence of the endogenous protein in our studies, we generated cell lines by using telomerase life-extended HFs (4) in which the levels of endogenous Daxx were constitutively decreased (by transduction with a retrovirus expressing a shRNA specific to Daxx) and in which HA-tagged wild-type or HA-tagged 15KR Daxx was ectopically expressed (by subsequent transduction with a different retrovirus containing the appropriate Daxx transgene in which the shRNA binding site was mutated). This allowed for the combined residual expression of endogenous Daxx and the ectopic expression of HA-tagged Daxx to achieve a protein level that approximated that of wild-type cells (Fig. 5A) and that permitted faithful recruitment of both the residual endogenous Daxx and the ectopically expressed HA-tagged Daxx proteins to PML-NBs (Fig. 5B; see Fig. S3 in the supplemental material). Unfortunately, expression of the HA-tagged Daxx proteins was observed in only a fraction of the cells, with approximately 25% expressing wild-type HA-Daxx and 50% expressing HA-Daxx-15KR (data not shown). This prevented us from detecting pp71-induced HA-Daxx SUMOylation by Western blotting. However, pp71-induced SUMOylation of wild-type HA-Daxx, but not HA-Daxx-15KR, was observable in these cells by IP and Western blotting (Fig. 5C). Interestingly, pp71-induced degradation of both wild-type HA-Daxx and HA-Daxx-15KR was observed after infection of these cells with HCMV (Fig. 5D) or after transduction with rAd-pp71 (Fig. 5E). Thus, pp71-induced Daxx SUMOylation is not required for pp71-induced Daxx degradation.

SUMOylation of Daxx is not required for pp71-induced events at the start of HCMV lytic infections. IE gene expression after infection at a low multiplicity of infection (MOI) is blocked in the absence of pp71 (5) or when the ability of pp71 to degrade Daxx is prevented by the proteasome inhibitor lactacystin (37). However, at high MOIs, lactacystin fails to inhibit HCMV IE gene expression in the presence of pp71 (37). To determine if pp71-induced Daxx SUMOylation allows for viral IE gene expression at high MOIs when Daxx degrada-

Despite a temporal correlation, the stimulation of Daxx SUMOylation by pp71 is not required for pp71-induced Daxx degradation. Our initial experiments with HCMV-infected HFs demonstrated that pp71-induced Daxx SUMOylation preceded or was coincident with pp71-induced Daxx degradation (Fig. 2A), which itself precedes viral IE gene expression (Fig. 2A) (37). These results were confirmed in subsequent experiments that included the analysis of more time points (see Fig. S2A in the supplemental material). After HCMV infection, Daxx SUMOylation was detected as early as 1 h postinfection (hpi), was strongest between 2 and 4 hpi, and decreased substantially at later time points. In these experiments, Daxx degradation was detected at 1 hpi and increased between 2 and 6 hpi, while IE gene expression was not observed until 3 hpi (see Fig. S2A in the supplemental material). In HCMV-infected cells, pp71 induces Daxx degradation during the first few hours of infection, but Daxx reaccumulates at later times (see Fig.

reflect pp71-induced SUMOylation of Daxx-binding proteins or Daxx dimerization. From these experiments, we concluded that pp71 promotes the SUMOylation of Daxx.

FIG. 4. pp71 enhances Daxx SUMOylation. (A) C33A cells were transfected with expression plasmids for the indicated proteins. Twenty-four hours later, lysates were harvested in 1% SDS lysis buffer and subjected to direct Western blotting (WB) or to IP with an anti-FLAG antibody followed by Western blotting with an anti-HA antibody. See Materials and Methods for details. Tubulin (Tub) served as a loading control. (B) HFs were mock transduced (M) or transduced at 10,000 ppc with an rAd that expresses pp71 (71). Lysates harvested at 24 hpt with RIPA lysis buffer were subjected to direct Western blotting or to IP with an anti-SUMO-1 antibody followed by Western blotting with an anti-Daxx antibody. (C) HFs were mock infected (M) or infected with the wild-type AD169 strain of HCMV (V) at an MOI of 3. Lysates were analyzed by direct Western blotting or IP as described for panel B.
also expressed IE1. Although we detected a small decrease in IE1 gene expression in the presence of HA-Daxx-15KR compared to that in the presence of wild-type HA-Daxx, this change was not statistically significant (Fig. 6A).

Finally, a recent report demonstrated that pp71 disrupts a complex between Daxx and the cellular ATRX protein that participates in chromatin remodeling and transcriptional repression (25). ATRX is normally found localized at PML-NBs but is dispersed throughout the nucleus in the presence of pp71. The ability of pp71 to disperse ATRX may be independent of its ability to degrade Daxx, but nevertheless, it enhances IE gene expression upon HCMV infection (25). Using our wild-type HA-Daxx and HA-Daxx-15KR endogenous Daxx knockdown cell lines, we found that ATRX was recruited to PML-NBs by both wild-type HA-Daxx and SUMOylation-deficient HA-Daxx-15KR cells (Fig. 6B), as expected. Furthermore, we found that pp71 delivered as part of the tegument during HCMV infection was able to disperse ATRX from PML-NBs with similar efficiencies in the presence of wild-type HA-Daxx or HA-Daxx-15KR (Fig. 6C). In summary, pp71 promotes Daxx SUMOylation, but this induction of Daxx SUMOylation is not required for pp71-induced Daxx degradation or ATRX dispersal and, as such, appears to play either no or an insignificant role in the induction of IE gene expression during lytic HCMV infection by tegument-delivered pp71.

**DISCUSSION**

In our quest to define the uncommon proteasome-dependent, ubiquitin-independent mechanism used by pp71 to facilitate Daxx degradation, we observed a modified form of Daxx induced by pp71. This covalent modification was identified as also expressed IE1. Although we detected a small decrease in IE1 gene expression in the presence of HA-Daxx-15KR compared to that in the presence of wild-type HA-Daxx, this change was not statistically significant (Fig. 6A).

Finally, a recent report demonstrated that pp71 disrupts a complex between Daxx and the cellular ATRX protein that participates in chromatin remodeling and transcriptional repression (25). ATRX is normally found localized at PML-NBs but is dispersed throughout the nucleus in the presence of pp71. The ability of pp71 to disperse ATRX may be independent of its ability to degrade Daxx, but nevertheless, it enhances IE gene expression upon HCMV infection (25). Using our wild-type HA-Daxx and HA-Daxx-15KR endogenous Daxx knockdown cell lines, we found that ATRX was recruited to PML-NBs by both wild-type HA-Daxx and SUMOylation-deficient HA-Daxx-15KR cells (Fig. 6B), as expected. Furthermore, we found that pp71 delivered as part of the tegument during HCMV infection was able to disperse ATRX from PML-NBs with similar efficiencies in the presence of wild-type HA-Daxx or HA-Daxx-15KR (Fig. 6C). In summary, pp71 promotes Daxx SUMOylation, but this induction of Daxx SUMOylation is not required for pp71-induced Daxx degradation or ATRX dispersal and, as such, appears to play either no or an insignificant role in the induction of IE gene expression during lytic HCMV infection by tegument-delivered pp71.

**DISCUSSION**

In our quest to define the uncommon proteasome-dependent, ubiquitin-independent mechanism used by pp71 to facilitate Daxx degradation, we observed a modified form of Daxx induced by pp71. This covalent modification was identified as also expressed IE1. Although we detected a small decrease in IE1 gene expression in the presence of HA-Daxx-15KR compared to that in the presence of wild-type HA-Daxx, this change was not statistically significant (Fig. 6A).

Finally, a recent report demonstrated that pp71 disrupts a complex between Daxx and the cellular ATRX protein that participates in chromatin remodeling and transcriptional repression (25). ATRX is normally found localized at PML-NBs but is dispersed throughout the nucleus in the presence of pp71. The ability of pp71 to disperse ATRX may be independent of its ability to degrade Daxx, but nevertheless, it enhances IE gene expression upon HCMV infection (25). Using our wild-type HA-Daxx and HA-Daxx-15KR endogenous Daxx knockdown cell lines, we found that ATRX was recruited to PML-NBs by both wild-type HA-Daxx and SUMOylation-deficient HA-Daxx-15KR cells (Fig. 6B), as expected. Furthermore, we found that pp71 delivered as part of the tegument during HCMV infection was able to disperse ATRX from PML-NBs with similar efficiencies in the presence of wild-type HA-Daxx or HA-Daxx-15KR (Fig. 6C). In summary, pp71 promotes Daxx SUMOylation, but this induction of Daxx SUMOylation is not required for pp71-induced Daxx degradation or ATRX dispersal and, as such, appears to play either no or an insignificant role in the induction of IE gene expression during lytic HCMV infection by tegument-delivered pp71.

**DISCUSSION**

In our quest to define the uncommon proteasome-dependent, ubiquitin-independent mechanism used by pp71 to facilitate Daxx degradation, we observed a modified form of Daxx induced by pp71. This covalent modification was identified as also expressed IE1. Although we detected a small decrease in IE1 gene expression in the presence of HA-Daxx-15KR compared to that in the presence of wild-type HA-Daxx, this change was not statistically significant (Fig. 6A).

Finally, a recent report demonstrated that pp71 disrupts a complex between Daxx and the cellular ATRX protein that participates in chromatin remodeling and transcriptional repression (25). ATRX is normally found localized at PML-NBs but is dispersed throughout the nucleus in the presence of pp71. The ability of pp71 to disperse ATRX may be independent of its ability to degrade Daxx, but nevertheless, it enhances IE gene expression upon HCMV infection (25). Using our wild-type HA-Daxx and HA-Daxx-15KR endogenous Daxx knockdown cell lines, we found that ATRX was recruited to PML-NBs by both wild-type HA-Daxx and SUMOylation-deficient HA-Daxx-15KR cells (Fig. 6B), as expected. Furthermore, we found that pp71 delivered as part of the tegument during HCMV infection was able to disperse ATRX from PML-NBs with similar efficiencies in the presence of wild-type HA-Daxx or HA-Daxx-15KR (Fig. 6C). In summary, pp71 promotes Daxx SUMOylation, but this induction of Daxx SUMOylation is not required for pp71-induced Daxx degradation or ATRX dispersal and, as such, appears to play either no or an insignificant role in the induction of IE gene expression during lytic HCMV infection by tegument-delivered pp71.

**DISCUSSION**

In our quest to define the uncommon proteasome-dependent, ubiquitin-independent mechanism used by pp71 to facilitate Daxx degradation, we observed a modified form of Daxx induced by pp71. This covalent modification was identified as also expressed IE1. Although we detected a small decrease in IE1 gene expression in the presence of HA-Daxx-15KR compared to that in the presence of wild-type HA-Daxx, this change was not statistically significant (Fig. 6A).

Finally, a recent report demonstrated that pp71 disrupts a complex between Daxx and the cellular ATRX protein that participates in chromatin remodeling and transcriptional repression (25). ATRX is normally found localized at PML-NBs but is dispersed throughout the nucleus in the presence of pp71. The ability of pp71 to disperse ATRX may be independent of its ability to degrade Daxx, but nevertheless, it enhances IE gene expression upon HCMV infection (25). Using our wild-type HA-Daxx and HA-Daxx-15KR endogenous Daxx knockdown cell lines, we found that ATRX was recruited to PML-NBs by both wild-type HA-Daxx and SUMOylation-deficient HA-Daxx-15KR cells (Fig. 6B), as expected. Furthermore, we found that pp71 delivered as part of the tegument during HCMV infection was able to disperse ATRX from PML-NBs with similar efficiencies in the presence of wild-type HA-Daxx or HA-Daxx-15KR (Fig. 6C). In summary, pp71 promotes Daxx SUMOylation, but this induction of Daxx SUMOylation is not required for pp71-induced Daxx degradation or ATRX dispersal and, as such, appears to play either no or an insignificant role in the induction of IE gene expression during lytic HCMV infection by tegument-delivered pp71.
SUMOylation through its sensitivity to isopeptidases in general (NEM and IAA are required for its detection) (Fig. 1) and to the SUMO-specific protease SENP-1 in particular (Fig. 4A) and by its detection in denatured lysates (showing that the modification is covalent, not just an association through the SIM of Daxx). Furthermore, the pp71-induced Daxx modification was increased by ectopic overexpression of a tagged SUMO-1 construct (Fig. 4A). Finally, precipitation with a SUMO-1 antibody identified the pp71-induced modification of Daxx as SUMOylation in fully permissive fibroblasts without the overexpression of either Daxx or SUMO-1 (Fig. 4B and C). These results show that SUMO-1 can be conjugated to Daxx and that this conjugation can be enhanced by pp71. However, we have not excluded the possibility that other SUMO proteins (SUMO-2 and -3) also modify Daxx during HCMV infection.

Daxx is modified in uninfected cells predominantly with SUMO-1, although SUMO-2 can be conjugated to Daxx in vitro (23). While the modification site(s) has not been mapped definitively in uninfected or HCMV-infected cells, it is likely to be one or more of the carboxy-terminal 15 lysine residues because the Daxx-15KR mutant is not SUMOylated in either the absence or presence of pp71. Whether the Daxx SUMOylation sites are the same or different in uninfected and HCMV-infected cells is not known. SUMO is an 11-kDa protein. Because SUMOylated Daxx migrates with an apparent molecular mass that is approximately 20 kDa larger than that of the unmodified protein, it is likely that SUMOylated Daxx represents either two mono- or one di-SUMOylation event.

We have not determined the mechanism through which pp71 induces Daxx SUMOylation. However, because interaction between the two proteins is required for the pp71-induced SUMOylation of Daxx, we suggest that pp71 likely functions as an E3 SUMO ligase. A caveat to this hypothesis is that cells express only one E2 SUMO conjugating enzyme, Ubc9 (11, 28), and we have as yet been unable to detect a stable interaction between pp71 and Ubc9. It is unclear how pp71 could function as an E3 SUMO ligase if it does not interact with Ubc9. pp71-induced Daxx SUMOylation occurs only at pre-IE times during HCMV infection (Fig. 2A; see Fig. S2A in the supplemental material), even though pp71 interacts with the protein at later times (7), when Daxx reaccumulates to normal levels after early degradation by pp71 (37). It is not clear why pp71 is unable to induce Daxx SUMOylation at late times, but it apparently is not a result of PML-NB disruption during HCMV infection, because pp71-induced Daxx SUMOylation does not require localization to PML-NBs, as it occurs in the presence of the HCMV IE1 protein (Fig. 3C).

Determining if and how SUMOylation regulates protein function can be challenging because, for most proteins, including Daxx, the SUMO-modified form represents a vanishingly small minority of the total protein within a cell. Thus, for many proteins, roles for SUMOylation have not been determined. However, SUMOylation in general seems to be an important process, because transgenic mice lacking the gene for Ubc9 display embryonic lethality (28, 31). In cases where functional consequences of SUMOylation have been identified, various processes, such as transcriptional activation and repression, protein localization and stability, and perhaps most significantly, protein-protein interactions, are regulated by SUMOylation (11). Strong biological effects of SUMOylation are easier to reconcile with the low level of protein modification observed if SUMOylation is required only transiently (if there is a “memory” of past SUMOylation events), as postulated previously (11).

The consequences, if any, of Daxx SUMOylation in uninfected or HCMV-infected cells are unknown. In uninfected cells, a SUMOylation-deficient Daxx protein (termed Daxx-15KR) was essentially functionally indistinguishable from the wild-type protein (23). Daxx-15KR still localized to PML-NBs, bound to all but one of the proteins with which wild-type Daxx interacts, and repressed a reporter driven by the glucocorticoid promoter as well as wild-type Daxx. Similar to the situation for uninfected cells, we have as yet been unable to identify a functional significance for the pp71-induced SUMOylation of Daxx in HCMV-infected cells. pp71 was still able to disperse ATRX from PML-NBs populated mainly with Daxx-15KR, and this SUMOylation-deficient mutant protein was still subject to pp71-mediated degradation. We also detected no statistically significant differences in IE gene expression in the presence of Daxx-15KR. A caveat to all these experiments is that they were performed with knockdown (not knockout) permissive HFs that still express a low level of endogenous wild-type Daxx. A true test of the functional ramifications of pp71-induced Daxx SUMOylation would be to determine the phenotype of a recombinant virus expressing a pp71 mutant unable to induce Daxx SUMOylation. We are currently examining a panel of pp71 deletion and point mutants to identify such an allele. Additionally, although pp71-mediated Daxx SUMOylation may not effect IE gene expression, it may alter the ability of the protein to modulate cellular gene expression or apoptosis, processes regulated by Daxx in uninfected cells (24). Clearly, more work is needed to determine the significance of Daxx SUMOylation in uninfected and HCMV-infected cells.

Because viruses are genetically poor obligate parasites, it is generally considered that if a viral protein performs a function, that activity will somehow be important for replication or pathogenesis. Thus, we assume that the pp71-mediated SUMOylation of Daxx must have some functional significance. However, a provocative hypothesis is that Daxx SUMOylation after HCMV infection represents a cellular mechanistic attempt to inhibit pp71-mediated Daxx degradation. SUMOylation can act as an antagonist to ubiquitination by blocking lysine acceptor sites and thus can enhance protein stability. Such is the case for the inhibitory subunit of the NF-kB complex, the IκBα protein (8). Perhaps pp71, needing to degrade Daxx and facing a protein with its ubiquitin acceptor sites blocked by SUMOylation, evolved to degrade Daxx in a ubiquitin-independent manner. If this is true, Daxx SUMOylation during HCMV infection may have no functional consequences but may simply represent another attempt by a cellular defense to inhibit viral replication that has been thwarted by HCMV.

Finally, the pp71-induced SUMOylation of Daxx is something novel in that it is only the second example of a viral protein inducing the SUMOylation of a cellular substrate. The first example, the adenovirus E1B-55K-induced SUMOylation of p53, also has yet to have a defined functional consequence (30). Although these viral protein-induced SUMOylations of cellular proteins are unique, viral modulation of SUMOylation pathways is not. Many viral proteins are themselves known to
be SUMOylated as well as to prevent the SUMOylation (or enhance the de-SUMOylation) of cellular proteins (3). More work is needed to determine how enhanced and inhibited protein SUMOylation modulates viral infections and if EIB-55K, pp71, and perhaps the K-bZIP protein of Kaposi’s sarcoma-associated herpesvirus (17) represent the first examples of true viral E3 SUMO ligases.

ACKNOWLEDGMENTS

We thank Phil Balandry for expert technical assistance, Ryan Safert for comments on the manuscript, Hsiu-Ming Shih for the pcDNA3-HA-Daxx and 15KR plasmids, and Shannon Kenney for the SENP-1 plasmid.

This work was supported by NIH grants R56-AI064703 and R01-AI074984. R.F.K. is a Burroughs Welcome Fund Investigator in Pathogenesis.

REFERENCES


