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Proteasome-dependent, ubiquitin-independent degradation of Daxx by the viral pp71 protein in human cytomegalovirus-infected cells

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Abstract

The cellular Daxx protein represses human cytomegalovirus (HCMV) gene expression from the major immediate early promoter. HCMV prevents Daxx-mediated silencing during lytic infection by delivering the viral pp71 tegument protein to the nucleus, where pp71 binds to and induces the proteasomal degradation of Daxx. In this study, we show that a functional ubiquitin pathway is not required for the proteasomal degradation of the endogenous Daxx protein by tegument-delivered pp71 in HCMV-infected cells, demonstrating that the pp71-mediated degradation of Daxx occurs through a proteasome-dependent, ubiquitin-independent pathway.

Keywords: Daxx; HCMV; pp71; UL82; Ubiquitin-independent degradation; Proteasome

Introduction

Human cytomegalovirus (HCMV) is an important human pathogen that can cause severe disease in newborns infected in utero and in immunocompromised or immunosuppressed patients (Mocarski and Courcelle, 2001). Transcription of the HCMV genome is temporally regulated in a coordinated cascade that consists of immediate-early (IE), early (E), and late (L) gene expression (Wathen et al., 1981). The major immediate early promoter (MIEP) directs the production of the IE1 and IE2 proteins that are critical for initiating the lytic replication cycle of the virus (Greaves and Mocarski, 1998; Marchini et al., 2001). At the very start of infection, the viral pp71 protein is required for efficient transcription from the MIEP, as shown by the inability of recombinant viruses lacking pp71 to initiate IE gene expression and replicate after low multiplicity infections (MOIs) (Bresnahan and Shenk, 2000; Cantrell and Bresnahan, 2005).

pp71 is incorporated into the tegument layer of HCMV virions (Nowak et al., 1984), is delivered to cells immediately upon infection, and localizes to sub-nuclear structures called promyelocytic leukemia nuclear bodies (PML-NBs; also called PODs, for PML oncogenic domains; or ND10, for nuclear domain 10) (Hofmann et al., 2002; Ishov et al., 2002). At PML-NBs, pp71 interacts with Daxx (Hofmann et al., 2002; Ishov et al., 2002), a cellular repressor that associates with DNA binding transcription factors and recruits histone deacetylases (HDACs) to targeted promoters to silence transcription (Hollenbach et al., 2002; Li et al., 2000). Recombinant viruses in which the wild type pp71 gene is replaced with either of two mutant alleles whose protein products are unable to bind Daxx (Hofmann et al., 2002) have the same phenotype as a pp71-null virus (Cantrell and Bresnahan, 2005), indicating that the ability of pp71 to associate with Daxx is critical to its function as a facilitator of IE gene expression at the very start of a lytic infection. Although initial studies concluded that pp71 and Daxx cooperate to activate the MIEP (Hofmann et al., 2002), it is now clear from multiple lines of evidence that Daxx actually silences the MIEP, and that pp71 relieves this repression (Cantrell and Bresnahan, 2006; Preston and Nicholl, 2006; Saffert and Kalejta, 2006; Woodhall et al., 2006). For example, Daxx overexpression inhibits HCMV IE gene expression and replication, and knockdown of Daxx protein levels using RNA interference results in the loss of transcriptionally-repressive, and the gain of transcriptionally-active chromatin structure at

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the MIEP, increases expression from the MIEP, and rescues the growth defect of the pp71-null virus (Cantrell and Bresnahan, 2006; Preston and Nicholl, 2006; Saffert and Kalejta, 2006; Woodhall et al., 2006).

We discovered the mechanism through which pp71 facilitates IE gene expression at the start of lytic HCMV infections: pp71 mediates the proteasomal degradation of Daxx, an event that is required for IE gene expression in cells infected with HCMV at low multiplicities (Saffert and Kalejta, 2006). pp71 also interacts with and induces the degradation of the retinoblastoma (Rb) family member proteins, resulting in quiescent cells entering the cell cycle (Kalejta et al., 2003; Kalejta and Shenk, 2003a). Interestingly, the pp71-mediated degradation of the Rb proteins occurs through a proteasome-dependent, ubiquitin-independent pathway (Kalejta and Shenk, 2003b).

The ubiquitin–proteasome system is the primary intracellular machinery responsible for elimination of unfolded proteins and for the selective destruction of regulatory proteins involved in a wide range of cellular processes (Glickman and Ciechanover, 2002). Substrates are normally targeted to the 26S proteasome via polyubiquitination on internal lysine residues. Polyubiquitin chains mediate the binding of targeted proteins to the proteasome and assist in their unfolding, but are removed from the substrate prior to proteasomal degradation. Thus, the actual substrate for proteasomal degradation is a partially denatured, non-ubiquitinated protein. Purified 20S and 26S proteasomes can degrade nonubiquitinated, denatured substrates in vitro (Bercovich et al., 1989; Dick et al., 1994; Driscoll and Goldberg, 1989), indicating that, if a protein can be delivered to the proteasome in a denatured or partially unfolded state, ubiquitination may not be required for its degradation. In fact, there are now several examples of proteasome-dependent, ubiquitin-independent degradation of proteins in vivo (Orlowski and Wilk, 2003), including the pp71-mediated degradation of the Rb family proteins (Kalejta and Shenk, 2003b).

Here we show that, like the Rb proteins, pp71-mediates Daxx degradation in a proteasome-dependent, ubiquitin-independent manner. Additionally, we make the important advances of establishing that the proteasome-dependent, ubiquitin-independent degradation of an endogenous substrate by tegument-delivered pp71 takes place in HCMV-infected cells, and of simultaneously demonstrating proteasome-dependence and ubiquitin-independence.

Results and discussion

Mouse ts20 cells have a temperature-sensitive E1 ubiquitin-activating enzyme and are often used in experiments to determine if a degradation process requires polyubiquitination (Chowdary et al., 1994). At the restrictive temperature in ts20 cells, ubiquitin monomers cannot be activated, polyubiquitin chains are not synthesized, and proteins degraded by the ubiquitin–proteasome pathway, such as p53, are stabilized. To determine if the pp71-mediated degradation of Daxx is ubiquitin-independent, we monitored the steady state level of Daxx in ts20 cells at both the permissive (35 °C) and restrictive (39 °C) temperatures. ts20 cells cultured at 35 °C were transduced with a recombinant adenovirus (rAD) that expresses pp71 (Fig. 1A). At the time of transduction, the indicated cultures were shifted to 39 °C, and lysates were harvested from all cultures 24 h later and analyzed by Western blot. Mock-infected cells served as a control. We found that at both the permissive and non-permissive temperatures, the steady state level of the endogenous Daxx protein was decreased in ts20 cells (Fig. 1A), indicating that the pp71-mediated degradation of Daxx occurs through a ubiquitin-independent mechanism. Analysis of tubulin confirmed equal protein loading, and the stabilization at 39 °C of p53, a short-lived cellular protein that is degraded by the proteasome after it is polyubiquitinated (Maki et al., 1996), demonstrated that culturing these cells at the restrictive temperature inhibited ubiquitin-mediated proteolysis (Fig. 1A).

In control experiments, we found that a pp71 mutant unable to bind Daxx (Hofmann et al., 2002) did not induce Daxx degradation (Fig. 1B). Furthermore, the HCMV pp65 protein, and the cellular E2F-1 protein also failed to induce Daxx degradation in ts20 cells (data not shown). This corroborates our previous results in human fibroblasts (Saffert and Kalejta, 2006), and demonstrates specificity in Daxx degradation for a functional pp71 protein. In total, these experiments show that exogenously expressed pp71 does not require a functional ubiquitination system to mediate the degradation of Daxx.

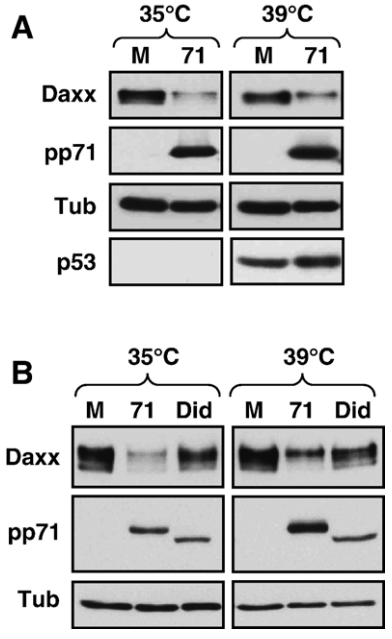

Fig. 1. HCMV pp71 induces Daxx degradation in cells unable to conjugate ubiquitin. (A) ts20 cells maintained at the indicated temperature were mock transduced (M) or transduced at 20,000 particles per cell (ppc) with a recombinant adenovirus (rAD) that expresses pp71 (71). Lysates harvested at 24 h post transduction (hpt) were analyzed by Western blot. Tub, tubulin. (B) ts20 cells maintained at the indicated temperature were mock transduced (M) or transduced at 20,000 particles per cell (ppc) with a recombinant adenovirus (rAD) that expresses wild type pp71 (71) or a pp71 mutant with a deletion in the Daxx interaction domain (Did) that fails to bind Daxx (Did 2–3). Lysates harvested at 24 h post transduction (hpt) were analyzed by Western blot.

Importantly, we wanted to determine if pp71 mediates ubiquitin-independent degradation of Daxx in HCMV-infected cells. Although HCMV cannot establish a lytic infection in mouse cells, the virus can enter and synthesize IE proteins (Lafemina and Hayward, 1988). Because Daxx degradation is required for IE gene expression in HCMV-infected human fibroblasts (Saffert and Kalejta, 2006), and because pp71 expressed from an rAD was able to induce the degradation of Daxx in ts20 cells (Fig. 1), we predicted that HCMV tegument-delivered pp71 would also degrade Daxx in these cells. To test this, ts20 cells were pre-incubated at either 35 °C or 39 °C for 18 h and then mock infected or infected with either a pp71-null (ADsubUL82) (Bresnahan and Shenk, 2000) or wild type HCMV-AD169, or with stocks of each of these viruses that had been exposed to ultraviolet (UV) light. Such exposure damages the viral genome and prevents viral gene expression, but does not interfere with the function of tegument proteins delivered directly to the cell upon viral entry. Note that the pp71-null virus was passaged once through non-complementing fibroblasts at high titer as described (Bresnahan and Shenk, 2000), and thus did not contain pp71 in the tegument. Cell lysates were harvested at 6 h post infection (hpi) and assayed for Daxx, pp71, IE1, pp65, p53, and tubulin by Western blotting. Monitoring pp71 was used to distinguish between the mutant and wild type virus, IE1 to observe viral gene expression, and pp65 was analyzed to confirm equal viral entry.

We found that wild type HCMV (both the live virus and the UV-inactivated virus) induced the degradation of endogenous Daxx at both temperatures, but the pp71-null virus did not (Fig. 2). Viral IE gene expression was detected only when cells were infected with wild type virus, and not during infection with UV-inactivated virus or with pp71-null virus. This experiment reveals many details of the pp71-mediated degradation of Daxx. It shows that viral gene expression is not required (UV-inactivated virus still degrades Daxx), but that tegument-delivered pp71 is required (pp71-null virions, both live and UV-inactivated, fail to induce Daxx degradation). It also shows that viral IE gene expression is regulated in non-permissive mouse ts20 cells in a similar manner as in fully permissive human fibroblasts because IE1 production is not observed in the absence of pp71 (in cells infected with the pp71-null virus). Most importantly, this experiment shows that the pp71-mediated degradation of Daxx in HCMV-infected ts20 cells does not require a functional ubiquitination system.

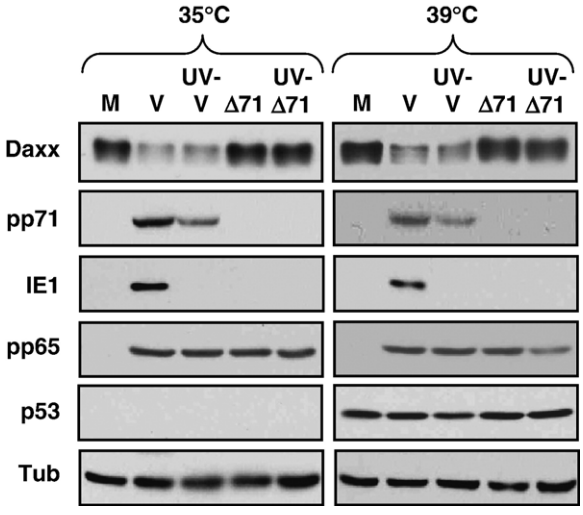

Fig. 2. pp71-mediated degradation of Daxx in HCMV-infected cells does not require a functional ubiquitination system. ts20 cells pre-incubated at the indicated temperature for 18 h were mock-infected (M) or infected with either wild type HCMV (V), UV-inactivated wild type HCMV (UV-V), a pp71-null HCMV (Δ71), or a UV-inactivated pp71-null HCMV (UV-Δ71) at an MOI of 6. Lysates harvested at 6 h post infection (hpi) were analyzed by Western blot.

To confirm that the reduction in the level of endogenous Daxx in HCMV-infected ts20 cells is due to proteasomal degradation, cultures were treated with DMSO, the proteasome inhibitor lactacystin, or the calpain inhibitor E64 prior to infection with HCMV. Lysates were harvested at 6 hpi and analyzed by Western blot. We found that Daxx was stabilized in the presence of lactacystin, but not by E64 or the solvent in which both inhibitors were dissolved, DMSO (Fig. 3A), demonstrating that the degradation of endogenous Daxx by tegument-delivered pp71 in HCMV-infected cells requires proteasome function. Furthermore, we confirmed that proteasome function is required for HCMV-induced Daxx degradation at the restrictive temperature, where ubiquitin-dependence is monitored. ts20 cells cultured at either 35 °C or 39 °C for 16 h were pre-treated with either DMSO or lactacystin as described above, then either mock-infected or infected with HCMV. After 6h, lysates were collected and assayed by Western blot. We found that at 39 °C where polyubiquitination is prevented (evidenced again by p53 stabilization), Daxx was still degraded upon HCMV infection, but Daxx degradation could be inhibited by lactacystin (Fig. 3B). As expected, lactacystin also stabilized p53 at 35 °C (Fig. 3B). This analysis demonstrates that, under conditions where HCMV-infection induces the ubiquitin-independent degradation of Daxx, proteasome function is still required for Daxx degradation.

This work demonstrates that the pp71-mediated degradation of Daxx occurs without a functional ubiquitin system while still requiring proteasome function, thus it is proteasome-dependent and ubiquitin-independent. While our previous experiments first demonstrated that ectopically expressed Rb family member proteins could be degraded by co-transfected pp71 in a proteasome-dependent, ubiquitin-independent manner (Kalejta and Shenk, 2003b), the work shown here demonstrates that HCMV tegument-delivered pp71 functions in a similar manner with another of its substrates, Daxx. This study is more physiologically relevant because it assayed HCMV-infected cells, monitored the levels of the endogenous Daxx protein, and simultaneously illustrated proteasome-dependence and ubiquitin-independence. Demonstrating ubiquitin-independent Daxx degradation during a productive lytic HCMV infection must await the development of tools to efficiently inactivate the ubiquitin conjugation system in cells fully permissive for HCMV replication such as primary human fibroblasts.

It is presently unclear if the more common ubiquitin-dependent pathway could substitute for the ubiquitin-independent degradation mechanism utilized by pp71. However, the presence of a deubiquitinating protease within the HCMV

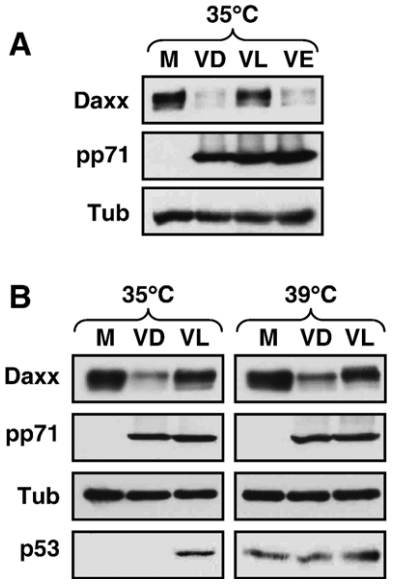

Fig. 3. pp71-mediated degradation of Daxx occurs through a proteasome-dependent, ubiquitin-independent pathway. (A) ts20 cells at the indicated temperature were treated with DMSO (D), lactacystin (L), or E64 (E) for 90 min. The cells were then mock-infected (M) or infected with HCMV (V) at an MOI of 6 in the presence of the drugs. Lysates harvested at 6 hpi were analyzed by Western blot. (B) ts20 cells cultured at the indicated temperature for 16 h were treated with either DMSO (D) or lactacystin (L) for 90 min at the same temperature. The cells were then mock-infected (M) or infected with HCMV (V) at an MOI of 6. Lysates harvested at 6 hpi were analyzed by Western blot.

virion (Wang et al., 2006) could indicate that ubiquitin-independent protein degradation may be favored in HCMV-infected cells. Finally, because Daxx degradation is required for IE gene expression at the start of a low multiplicity HCMV lytic infection (Saffert and Kalejta, 2006), and because ubiquitin-independent degradation is a rare event in un-infected cells, we suggest that drugs that target this novel degradation reaction to inhibit the pp71-mediated degradation of Daxx may have strong antiviral activity with limited toxicity.

Materials and methods

Cells and viruses

The mouse ts20 cell line, which contains a temperature-sensitive E1 ubiquitin activating enzyme was maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% (vol/vol) fetal bovine serum (Gemini), 100 U/ml penicillin, and 100 μg/ml streptomycin plus 0.292 mg/ml glutamine (Gibco), in a 5% CO_2 atmosphere at 35 °C (the permissive temperature); the restrictive temperature is 39 °C. The wild-type virus strain employed was AD169 (HCMV). The HCMV pp71-null (ADsubUL82) (Bresnahan and Shenk, 2000) virus has been described previously. Inactivation of virions by ultraviolet light was achieved by exposing viral stocks to the 254 nm light source at 0.12 J/cm^2 for 2 min in a UV Stratalinker 2400 (Stratagene). For infection, the medium was removed from confluent cells which were then incubated with virus at 37 °C. One hour later, the viral inoculum was removed and the old medium was returned to the cells. The recombinant adenoviruses were previously described (Saffert and Kalejta, 2006). Transductions were performed at the particle per cell (ppc) ratio indicated in the figure legends.

Inhibitors and antibodies

Lactacystin (20 μM) or E64 (50 μM) (Calbiochem) dissolved in dimethyl sulfoxide (DMSO) were added for 90 min prior to infection with HCMV. Antibodies to the following proteins were from commercial sources: Daxx (D7810 from Sigma), hemagglutinin (HA; Ha.11; Covance), tubulin (DM 1A; Sigma), pp65 (1025; Rumbaugh-Goodwin Institute). Antibodies against pp71 (2H10-9), IE1 (1B12) and p53 (421) have been previously described (Kalejta et al., 2003). Secondary horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies were from Chemicon.

Western blots

Cells were lysed in radioimmunoprecipitation assay buffer as described previously (Kalejta et al., 2003). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immobilized on Optitran membranes (Schleicher & Schuell). 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 in 1% milk–TBST, and the blots were developed with the ECL enhanced chemiluminescence system (Amersham).

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