Daxx-Mediated Accumulation of Human Cytomegalovirus Tegument Protein pp71 at ND10 Facilitates Initiation of Viral Infection at These Nuclear Domains

Alexander M. Ishov, Olga V. Vladimirova, and Gerd G. Maul*

The Wistar Institute, Philadelphia, Pennsylvania 19104

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Human cytomegalovirus (HCMV) starts immediate-early transcription at nuclear domains 10 (ND10), forming a highly dynamic immediate transcript environment at this nuclear site. The reason for this spatial correlation remains enigmatic, and the mechanism for induction of transcription at ND10 is unknown. We investigated whether tegument-based transactivators are involved in the specific intranuclear location of HCMV. Here, we demonstrate that the HCMV transactivator tegument protein pp71 accumulates at ND10 before the production of immediate-early proteins. Intracellular trafficking of pp71 is facilitated through binding to a coiled-coil region of Daxx. The C-terminal domain of Daxx then interacts with SUMO-modified PML, resulting in the deposition of pp71 at ND10. In Daxx-deficient cells, pp71 does not accumulate at ND10, proving in vivo the necessity of Daxx for pp71 deposition. Also, HCMV forms immediate transcript environments at sites other than ND10 in Daxx-deficient cells, and so does the HCMV pp71 knockout mutant UL82−/− in normal cells. This result strongly suggests that pp71 and Daxx are essential for HCMV transcription at ND10. Lack of Daxx had the effect of reducing the infection rate. We conclude that the tegument transactivator pp71 facilitates viral genome deposition and transcription at ND10, possibly priming HCMV for more efficient productive infection.

Nuclear domains 10 (ND10), also called PML nuclear bodies or PML oncocytic domains (PODs), represent intranuclear accumulations of several proteins, including the transcription repressors PML, Sp100, HP1, and Daxx. Within the highly specialized intranuclear architecture, ND10 presumably act as nuclear depots to maintain the homeostatic balance through controlled recruitment and release of proteins (reviewed in references 24 and 27). PML is the key component for ND10 maintenance and is responsible for the recruitment of other proteins to ND10. Daxx is recruited to ND10 from condensed chromatin through interaction with PML modified by a small ubiquitin-like modifier (SUMO-1) (15). Human Daxx was originally discovered as a DNA-binding protein (17) and has been reported to be involved in several cellular processes, including apoptosis, transcription regulation, and embryo development (reviewed in reference 26). The growing list of Daxx-interacting partners suggests that Daxx acts as a protein modulator in numerous cellular activities, while its accumulation at ND10 appears to regulate the availability of soluble Daxx for these processes.

At least three ND10 proteins, PML, Sp100, and Daxx, are upregulated by interferon (7, 9, 10, 33), suggesting the potential involvement of ND10 in the antiviral cellular response. Indeed, studies have demonstrated that several DNA viruses start their synthetic processes in the immediate vicinity of ND10 (reviewed in reference 23). Input viral DNA accumulates at ND10, followed by transcription and replication juxtaposed to ND10. The structure of ND10 becomes modified in a virus-specific manner through the action of immediate-early (IE) proteins. Thus, human cytomegalovirus (HCMV) forms a highly dynamic immediate transcript environment (ITE) during the IE stage of infection as it begins transcription juxtaposed to ND10 (16). The HCMV IE1 protein accumulates at ND10 and eventually disperses the structure (1, 18, 34), possibly through direct interaction with PML (1), while the IE2 protein accumulates juxtaposed to a subpopulation of ND10 where HCMV starts transcription, thus providing an ITE marker (16). This high spatial-temporal correlation suggests that ND10 functionally influence viral infection, although the reasons for this relationship and the consequences for virus and cell remain unclear. To further understand the early association between ND10 and viruses, we examined the processes that occur before the initiation of viral transcription, focusing on the association of HCMV tegument transactivators with ND10.

During infection, the viral envelope fuses with the cell membrane, delivering tegument proteins located between the membrane envelope and capsid into the cell. While the assembly and overall function of viral tegument are not well understood, several studies have characterized transactivation functions of proteins within this structure. The HCMV tegument protein pp71, the product of the UL82 gene, activates a major IE promoter and other early promoters of this virus (5, 6, 22). Moreover, pp71 can activate a number of heterologous promoters, both in the context of transient transfection and upon infection by a herpes simplex virus type 1 (HSV-1) mutant expressing pp71 (13). The latter finding led to the suggestion that pp71 may be the functional counterpart of HSV-1 tegument protein VP16, which activates the IE genes of this virus through interaction with a number of cellular proteins, including Oct-1 (reviewed in...
reference 30). Transient transfection experiments also demonstrated that pp71 accumulates in the nucleolus (11) and dramatically enhances the infectivity of HCMV DNA, indicating that this tegument protein activates IE viral transcription before production of HCMV IE transactivators in the context of infection (2). Growth of an HCMV mutant that does not express pp71 is severely restricted at a low multiplicity of infection (MOI), further pointing to the transactivation function of this tegument protein during the IE stage of infection (5).

In the present study, we show that pp71 accumulates at ND10 upon HCMV infection and before production of IE proteins. Interaction with Daxx and subsequent binding of Daxx to SUMO-modified PML brings pp71 to ND10. In Daxx+/− cells, pp71 cannot accumulate at ND10 and ITE is not associated with ND10. Moreover, an HCMV pp71 knockout mutant UL82 cannot form ITE at ND10. Our findings suggest that intracellular accumulation of the transactivator pp71 can prime cells for more efficient infection and may in part explain initiation of HCMV IE transcription at ND10.

MATERIALS AND METHODS

Cells, growth conditions, inhibitors, and virus infection. WI38 human fibroblasts, COS-7 cells, MPEF mouse T antigen-immortalized fibroblasts, and PML−/− mouse T antigen-immortalized fibroblasts have been described (15). Daxx+/− mouse T antigen-immortalized fibroblasts were produced as described below. All cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1% antibiotics and grown at 37°C in a humidified 5% CO2 atmosphere. For immunohistochemical staining, cells were grown on round cover slips in 24-well plates (Corning Glass, Inc.) until approximately 80% confluent before fixation. Two days after plating, cells were infected with HCMV (Towne strain) or HCMV UL82−/− (5) at a multiplicity of 1 per 10 cells, resulting in 10% infected cells, as determined by staining with IE2 antibody. ES cells from a correctly targeted clone were cocultivated with FVB × FVB blastocysts and transferred into pseudopregnant FVB foster mothers. Two resulting high-grade chimeras (70% and 90% of agouti color) were mated to FVB/N females. Germ line transmission of the targeted Daxx allele was predicted by black eye color and agouti coat color in F2 and confirmed by PCR analysis with the primers GenDaxx227Up (5′-GGCGTTCGAGGAGAGGCAGG-3′) and NcoI (located in the 5′ promoter region of the neo gene cassette), 5′-GGCGAGGCCAGAGGCACACTGTGTTAGC-3′.

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Expression plasmids and transfection. IE1 and IE2 expression plasmids and the pETDaxx wild-type and deletion series have been described (15); pcDNApp71FLAG expresses pp71 fused at the N terminus with the Flag epitope (kindly provided by B. Plachter); pHP1FLAG, which encodes HPlIIs fused with the Flag epitope (31), was obtained from F. Rauscher (The Wistar Institute). Transfection was carried out with PolyFect transfection reagent (Qiagen) according to the manufacturer’s recommendations.

Immunoprecipitation assays. For immunoprecipitation analysis, COS-7 nuclear extracts were prepared from 105 cells (per 100-mm plate) cotransfected 20 h before with pET or one of the pETDaxx plasmids and either pcDNApp71FLAG or pHP1FLAG. Cells were washed twice, collected in phosphate-buffered saline, and incubated for 20 min in NLB–400 mM NaCl supplemented with protease inhibitors (1 mM EDTA plus 100 μg/ml phenylmethylsulfonyl fluoride [PMSF], 10 μg/ml aprotinin, and 10 μg/ml leupeptin per ml). Nuclear extracts were obtained, brought to 150 mM NaCl with NLB–400 mM NaCl, and incubated for 1 h at 4°C with anti-Flag-M2 antibody-conjugated beads (ProBond resin; Invitrogen). Beads were washed three times with NLB–150 mM NaCl buffer and boiled in 2× protein sample buffer. The eluted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 4 to 12% polyacrylamide gradient gels, transferred to a nylon membrane, and detected with rabbit anti-Flag antibodies (Clontech) diluted 1:300. Anti-Flag M2 monoclonal antibody (Sigma) was used to detect Flag-tagged pp71 and HPlIIs in input and immunoprecipitated samples.

RESULTS

HCMV tegument protein pp71 accumulates at ND10 upon infection. Several DNA viruses, including adenovirus type 5, HSV-1, simian virus 40, and HCMV, begin their transcription and replication in the vicinity of ND10 (reviewed in reference 23). HCMV induces a highly dynamic ITE in the infected nuclei that includes the initial positioning of the viral genome at ND10, where it begins to transcribe. Later, two IE HCMV proteins, IE1 and IE2, accumulate at ND10, and IE1 finally disperses ND10 (16). We analyzed the infectious process before initiation of viral transcription, asking specifically whether the viral tegument proteins locate to ND10.

Immunolocalization of HCMV tegument protein pp71 revealed accumulation of this polypeptide in the nucleolus already at 1.5 h postinfection, before production of IE2 proteins (Fig. 1a to d). In addition to the cytoplasmic and dispersed nucleoplasmic localization, pp71 also accumu-
FIG. 1. Localization of pp71 during HCMV infection. Human primary WI38 fibroblasts were infected at 0.1 MOI with HCMV (a to h and m to t) or with UV-inactivated HCMV (i to l) for 1.5 h (a to d) or 3 h (e to t) and triple stained for pp71 with monoclonal antibody CMV355 (b, f, j, and r), for IE2 with polyclonal rabbit antibody (c, g, k, o, and s), and for ND10 with human antibody 1745 reacting with the ND10-associated proteins Sp100 and PML (d, h, l, p, and t). Merged images demonstrate colocalization of pp71 and ND10 at 1.5 h postinfection, before IE proteins are visible (a), at 3 h postinfection, when only some cells start producing IE proteins (c), and upon infection by UV-inactivated virus (i). Another HCMV tegument protein, pp65, does not accumulate at ND10 (m to p). Details of pp71 accumulation are presented at the enlarged sections (q to t). IE2 accumulated juxtaposed to pp71/ND10 accumulations (q, arrow).
lated in ND10-like domains (Fig. 1b). Staining for ND10 revealed colocalization, indicating that pp71 is positioned at ND10 very early during infection (Fig. 1a, b, and d). At 3 h postinfection, pp71 remained localized at ND10, and IE2 protein also began to accumulate there (Fig. 1e to h). pp71 was located at ND10 in a majority of cells of the monolayer (Fig. 1f), while IE2 was visible in substantially fewer cells (Fig. 1g) at this low MOI, potentially reflecting the large number of viral particles that still can load cells with pp71.

Both the early timing of pp71 visualization (pp71 is expressed at the early and late but not at the immediate-early phase of infection [11]) and the high percentage of pp71-positive cells indicate the tegument origin of the protein. In cells infected with UV-inactivated HCMV, pp71 still accumulated at ND10, but IE2 was not detected (Fig. 1i to l), confirming that the pp71 accumulated in infected cells is the tegument protein. Another HCMV tegument protein, pp65, was homogeneously distributed in the nucleoplasm at 3 h postinfection, but did not accumulate at ND10, demonstrating the specificity of pp71 accumulation at ND10 (Fig. 1m to p).

The dynamics of pp71 and IE2 accumulation relative to ND10 is presented at higher magnification in Fig. 1q to t; pp71 co-localizes with ND10 in the lower cell; in the upper left cell, IE2 is accumulated juxtaposed to pp71/ND10 accumulations (arrow), and several ND10 are destroyed; in the upper right cell, all ND10 are destroyed and pp71 is almost homogeneously distributed in the nucleoplasm, with some low accumulations with IE2.

**Daxx mediates intracellular trafficking of pp71.** We examined the potential role of two ND10-associated proteins, PML and Daxx, in mediating pp71 accumulation at ND10 with the respective knockout mouse cells. Although mouse cells are not permissive for productive HCMV infection, they support IE events of infection. Unlike in human cells, HCMV cannot destroy ND10 in mouse cells (Ishov et al., unpublished data); they therefore remain as markers. When control MPEF were infected with HCMV, pp71 accumulated at ND10 (Fig. 2a, yellow domains in nucleoplasm resulting from colocalization of Daxx red and pp71 green signals), as in human cells. pp71 also remained visible in the cytoplasm and/or at the cell membrane as a component of the virion.

PML is important for ND10 assembly (15), suggesting the necessity of this ND10 protein for pp71 intranuclear accumulation. To test this possibility, we used PML−/− cells, in which ND10 are not formed and all ND10-associated proteins are located in alternative nucleoplasmic compartments. Daxx, for example, is accumulated at condensed chromatin in this cell line (15) (Fig. 2b, red for Daxx). When PML−/− cells were infected with HCMV, pp71 was detected homogeneously distributed in the nucleoplasm (Fig. 2b). These findings indicate that in the absence of the PML-based structure, pp71 did not accumulate at specific sites.

To test the potential effect of Daxx on pp71 accumulation at ND10, we derived a Daxx−/− cell line (see Materials and Methods) from a Daxx knockout mouse (unpublished data), infected the cells with HCMV, and probed for pp71 and PML. The absence of Daxx did not influence the ND10 structure itself, as observed by PML localization (Fig. 2c, PML in red); however, pp71 was not detected at ND10 but still accumulated in the nucleoplasm (Fig. 2c, pp71 in green). The punctate pattern of pp71 outside nuclei (Fig. 2a to c) likely represents pp71 inside adsorbed viral particles. The absence of pp71 accumulation at ND10 in Daxx−/− cells points to the role of Daxx in pp71 intracellular trafficking and accumulation at ND10.

**pp71 interacts with Daxx.** pp71 has recently been shown to interact with Daxx in a yeast two-hybrid system (H. Hofmann, H. Sindre, and T. Stamminger, Abstr. 25th Int. Herpesvirus Workshop, abstr. 10.03, 2000). To verify those data in mammalian cells, we performed immunoprecipitation experiments with COS-7 cells cotransfected with plasmid pcDNApp71 FLAG, expressing pp71-Flag, and plasmids expressing Daxx or several truncated forms of this protein fused with GFP-NLS (see reference 15 for plasmid details). Full-length Daxx interacted with pp71 (Fig. 3a, lane 5), as did all but the shortest N-terminal Daxx mutant (amino acids 1 to 142; Fig. 3a, lane 1), although to a lesser extent than the wild-type protein. These data map the interaction between Daxx and pp71 to amino acids 142 to 290.

The carboxyl-terminal region (amino acids 624 to 740), previously shown to interact with several proteins, including SUMO-modified PML (reviewed in reference 26), did not interact with pp71 (Fig. 3a, lane 6). These findings suggest that Daxx has an additional protein interaction domain located in its middle region, potentially a predicted coiled-coil domain that is located between amino acids 181 and 218. Negative controls demonstrated the specificity of the pp71-Daxx interaction (Fig. 3a, lanes 8 and 9) and the inability of GFP-NLS alone to interact with pp71 (Fig. 3a, lane 7).

**Formation of HCMV immediate transcript environment at ND10 is promoted by pp71.** Next we decided to test whether ND10 accumulation of pp71 is essential for the initiation of HCMV transcription and the formation of the HCMV immediate transcript environment at ND10. We demonstrated previously that IE2 accumulates exclusively beside the subpopulation of ND10 where HCMV transcribes (16) and thus can be used as a spatial marker for the ITE. Upon HCMV infection of mouse fibroblast cell lines, ND10 are not modified, and most IE2 domains are located juxtaposed or partially overlapping ND10 (Fig. 2d), indicating that HCMV forms an ITE at ND10 as in human cells. However, analysis of HCMV-infected Daxx−/− cells (Fig. 2f) revealed almost no spatial correlation between ND10 and IE2, indicating that HCMV does not transcribe at ND10 in Daxx−/− cells.

In HCMV-infected PML−/− cells, IE2 domains were often located juxtaposed to cloud-like accumulations of Daxx (Fig. 2e, upper and left cells) that did not correspond to condensed chromatin accumulations of Daxx specific for the PML−/− cells. Such Daxx accumulations near active transcription sites may indicate an unknown function of Daxx during virus infection. To further test whether HCMV transcription at ND10 is the consequence of pp71 accumulation at this domain, we used pp71-deficient HCMV mutant UL82−/− (3). While almost all IE2 domains are located adjacent to ND10 in HCMV-infected human fibroblasts (Fig. 2g), there was no correlation between IE2 and ND10 in HCMV UL82−/−-infected human (Fig. 2h) or mouse (Fig. 2i) fibroblasts. These data further point to the requirement for pp71 in the formation of the viral ITE at ND10.

Assessment of the consequences resulting from position-
ing the HCMV ITE at sites different from ND10 was hampered by the nonpermissiveness of mouse fibroblasts for productive HCMV infection. We therefore could not compare virus yield after infection in Daxx$^{-/-}$ and MPEF cells. However, we could quantify the number of IE2-positive cells upon HCMV infection in both cell types. Counting 500 cells 5 h after infection at 0.1 or 0.05 MOI, the number of IE2-positive cells in the Daxx$^{-/-}$ cell line is reduced twofold compared with MPEF for both MOIs tested. Combined with the necessity for Daxx for ITE formation at ND10, this suggests that the positional correlation between ND10 and sites of HCMV transcription has functional relevance.
DISCUSSION

Several groups have recently reported a spatial-temporal correlation between the nucleoplasmic structure ND10 and sites of viral synthesis during infection (reviewed in reference 23). However, the reasons for this phenomenon are unclear, and the question remains whether this correlation is advantageous for the virus or the host. Whereas the correlation of viral transcript and replication domains with ND10 suggests a helper function of this structure, at least three ND10-associated proteins, PML, Sp100, and Daxx (7, 9, 10, 33), are up-regulated by interferon, and PML, Daxx, HP1, and Sp100 are considered transcriptional repressors. Thus, ND10 may function as one of the last points of a cell’s defense against viruses by repressing viral input at these locations. Moreover, most DNA viruses induce ND10 disassembly during infection.

Before transcription can begin, the IE promoters must be transactivated by viral tegument proteins which are released into the cell. In the present study, we found that the HCMV tegument transactivator protein pp71 accumulates at ND10, the site of HCMV IE transcription, immediately after infection. This observation led us to investigate in detail the intracellular trafficking properties of this viral tegument polypeptide and the consequences of pp71 accumulation at ND10 for IE events of HCMV infection.

Mechanism of pp71 intracellular trafficking. Accumulation of pp71 at ND10 suggests a critical role for some of the ND10 proteins, potentially SUMO-modified PML or Daxx, in intracellular deposition of pp71. Our previous study of ND10 dynamics (15) indicated that PML is at the center of ND10 assembly, since ND10 are not formed in PML−/−/− cells and all ND10 proteins have an alternative nucleoplasmic localization, e.g., Daxx in PML−/−/− cells is concentrated at condensed chromatin. Concomitantly, pp71 in infected PML−/−/− cells is dispersed in the nucleoplasm, indicating again that PML is required for the assembly of the site of pp71 accumulation, ND10. The absence of pp71 accumulation at ND10 upon infection in Daxx−/−/− cells indicates that Daxx is essential for pp71 deposition at this structure, even in the presence of intact ND10. Direct interaction between pp71 and Daxx as determined by communoprecipitation experiments confirmed this idea.

Surprisingly, the region of pp71 interaction was mapped to amino acids 142 to 290 of the Daxx molecule, while all other reported Daxx-binding proteins, including CENP-C, Pax3, and Fas, interact with the carboxyl end of the Daxx molecule (12, 29, 36). Thus, Daxx has at least two regions of protein interaction. We showed previously that Daxx itself is positioned at ND10 through interaction of the carboxyl end of the molecule and SUMO-modified PML (15), as confirmed by others (19, 20, 37). From these observations follows the idea that both Daxx and PML are involved in intracellular trafficking of pp71 to ND10, which is facilitated through binding of pp71 with the coiled-coil-containing region of Daxx and subsequently mediated through interaction between the carboxyl end of Daxx and SUMO-modified PML. Hence, we present the first evidence of viral protein accumulation at ND10 through a cascade of interactions whereby pp71 is accumulated at this structure through interaction with Daxx, which is itself recruited to ND10 through direct interaction with SUMO-modified PML.
pp71 and HCMV infection. pp71 accumulated at ND10 even at low HCMV input, before initiation of IE events, and even in the absence of viral transcription after UV treatment of HCMV input, confirming the tegument origin of pp71 at this stage of infection. pp71 can activate a number of HCMV promoters, including the major immediate-early promoter (22, 35), and several heterogeneous promoters, including those of HSV-1 and adenovirus (13). Moreover, pp71 is required for HCMV infection following viral DNA transfection and after infection with UL82Δ/Δ (pp71 deletion mutant of HCMV) (2, 4, 5). Previously, we reported that only those HCMV input genomes positioned at ND10 started transcription and formed ITEs at these nuclear domains. Here we observed that ND10 is also the site of accumulation of pp71, the transactivator of HCMV IE promoters that penetrates cells at the time of infection as the tegument protein. Together, the data suggest that the tegument-derived pp71 can transactivate viruses positioned at ND10 before production of HCMV IE transactivators, as IE1 and IE2.

High concentrations of pp71 at ND10 may explain the initiation of viral transcription at these domains. In Daxx−/− cells, pp71 is not accumulated at ND10, nor are ITEs of HCMV (as determined by IE2 staining) formed there. Moreover, the HCMV UL82 deletion mutant virus, which is deficient in pp71 expression, forms ITEs mostly unassociated with ND10. Based on the collective data, we conclude that pp71 accumulation at ND10 provides transactivation advantages for HCMV input genomes at these nuclear domains. The observed twofold reduction in the number of IE2-positive Daxx−/− cells compared to the wild-type cells might be due, at least in part, to the inability of pp71 to accumulate at high concentrations at ND10 in the absence of Daxx.

Numerous studies have variously described Daxx functions in apoptosis and transcription regulation. Daxx has been identified as a repressor of basal and activated transcription (12, 20, 21), and repression of several genes through direct interaction between Daxx and activators such as Ets-1 and Pax3 has been reported (12, 21). The mechanism of Daxx-mediated repression has been suggested to involve recruitment of histone deacetylases to chromatin interaction between Daxx and these enzymes (20), although other investigators reported no release from Daxx repression activity in the presence of the histone deacetylase inhibitor trichostatin A (12). The transcription repressor function of Daxx has been implicated in interferon-induced apoptosis of lymphoid progenitors concomitant with increased accumulation of Daxx at ND10 (9). Our model of pp71 intranuclear accumulation raises the possibility that the repression activity of Daxx is facilitated, at least partly, through the targeting of other Daxx-binding proteins from their site of action to ND10.

pp71 induces mild cell growth inhibition, as indicated by a 20% reduction in colony formation (32), and transactivates not only HCMV promoters but also several heterogeneous promoters (13). Thus, pp71 may prime cells towards more efficient infection as a transactivator of some cellular promoters. Presumably, pp71 can block cell cycle progression, as demonstrated for HCMV IE proteins (reviewed in reference 8). pp71 recruitment to ND10 through interaction with Daxx may downregulate the transactivation of cellular genes by this viral protein. Thus, Daxx may repress transactivation of pp71 as this protein does for other transcription regulators, potentially through a similar mechanism. On the other hand, accumulation of pp71 at ND10 may provide transcription advantages for viral input genomes at these nuclear domains. Further studies are needed to reconcile these discrepant possibilities.

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