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Nuclear body formation and PML body remodeling by the human cytomegalovirus protein UL35

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ABSTRACT

The human cytomegalovirus (HCMV) UL35 gene encodes two proteins, UL35 and UL35a. Expression of UL35 in transfected cells results in the formation of UL35 nuclear bodies that associate with promyelocytic leukemia (PML) protein. PML forms the basis for PML nuclear bodies that are important for suppressing viral lytic gene expression. Given the important relationship between PML and viral infection, we have further investigated the association of UL35 with PML bodies. We demonstrate that UL35 bodies form independently of PML and subsequently recruit PML, Sp100 and Daxx. In contrast, UL35a did not form bodies; however, it could bind UL35 and inhibit the formation of UL35 bodies. The HCMV tegument protein pp71 promoted the formation of UL35 bodies and the cytoplasmic localization of UL35a. Similarly, UL35a shifted pp71 to the cytoplasm. These results indicate that the interplay between UL35, UL35a and pp71 affects their subcellular localization and likely their functions throughout infection.

Introduction

Human cytomegalovirus (HCMV) is a member of the beta herpesvirus subfamily and can establish both lytic and life-long latent infections in human hosts. In most healthy adults, HCMV infections are mild or asymptomatic. However, lytic HCMV replication can cause serious complications and even death in immunocompromised hosts, such as transplant recipients, people with AIDS and neonates (Crough and Khanna, 2009). Lytic replication involves the controlled and coordinated expression of viral genes generally divided into the immediate early (IE), early (E) and late (L) phases. In addition to this, pre-formed viral proteins contained within the tegument layer of the virion are delivered to newly infected cells and can modify the cellular environment and contribute to the initiation of IE gene expression during a pre-IE phase of the replication cycle (Kalejta, 2008). The major immediate early promoter (MIEP) controls the expression of the immediate early gene products, IE1 and IE2, which in turn coordinate the expression of early and late genes and drive the progression of lytic replication (Reeves, 2010; Sinclair and Sissons, 2006). Expression from the MIEP is complex with both viral and cellular factors regulating its activation and repression during latency and various stages of lytic replication (Kalejta, 2008; Sinclair, 2010). During initial infections, components of the virion tegument facilitate activation of the MIEP promoter (Kalejta, 2008). The most important and best characterized of these is the tegument protein pp71 (UL82) (Cantrell and Bresnahan, 2005; Saffert and Kalejta, 2006). In addition, two other tegument proteins, UL69 and UL35, have the ability to activate the MIEP in reporter assays and may behave similarly during infection (Liu and Biegalke, 2002; Winkler et al., 2000).

In order to suppress lytic infection, the host cell can inhibit viral gene expression through promyelocytic leukemia (PML) nuclear bodies (NBs). PML-NBs are dynamic, spherical, multi-protein nuclear complexes that require the PML protein for formation (Bernardi and Pandolfi, 2007). PML-NBs are involved in regulating a host of nuclear activities including apoptosis, gene expression, DNA repair and senescence (Bernardi and Pandolfi, 2007; Dellaire and Bazett-Jones, 2004). In addition, PML and the PML body components Sp100 and Daxx form part of an intrinsic and interferon-inducible antiviral response (Everett et al., 2006; Saffert and Kalejta, 2006; Tavalai et al., 2006). For herpes simplex virus-1 (HSV) and HCMV, the basis of this response is the silencing of viral IE gene expression through association with PML body components and the ability of these components (e.g., Daxx, ATRX, Sp100) to affect transcriptional regulation (Everett et al., 2006; Lukashchuk and Everett, 2010; Lukashchuk et al., 2008; Negorev et al., 2006; Saffert and Kalejta, 2006; Woodhall et al., 2006). For HSV infections, PML, Sp100 and Daxx become associated with viral genomes soon after delivery to the nucleus and, in the absence of the tegument protein ICP0, this association suppresses lytic replication (Everett and Murray, 2005; Everett et al., 2006). ICP0 also localizes to PML-associated genomes where it promotes the degradation of PML and Sp100, resulting in the disruption of PML-

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NBs and relief of repression (Chelbi-Alix and de The, 1999; Everett and Maul, 1994). Similar to HSV, incoming HCMV genomes become associated with PML early after infection, resulting in repression of the strong CMV IE promotor, possibly through histone modification of the MIEP promoter region (Ishov et al., 1997; Nitzsche et al., 2008; Reeves, 2010; Woodhall et al., 2006).

In order to counter the repressive effects of PML-NBs, HCMV encodes several proteins that have the ability to alter and disrupt PML NBs or the PML NB components that repress immediate early gene expression (Ahn and Hayward, 2000; Saffert and Kalejta, 2006; Salsman et al., 2008). The tegument protein pp71 plays an important part in this process by countering the repressive effects of Daxx and ATRX on the MIEP (Cantrell and Bresnahan, 2006; Lukashchuk et al., 2008; Preston and Nicholl, 2006). These effects are dependent on the interaction between Daxx and pp71, which facilitates the displacement of ATRX from genome-associated PML bodies, the modification of Daxx by SUMO and the degradation of Daxx by the proteasome (Hofmann et al., 2002; Hwang and Kalejta, 2007; Hwang and Kalejta, 2009; Lukashchuk et al., 2008; Saffert and Kalejta, 2006). Activation of the MIEP results in expression of IE1, which also localizes to PML-associated genomes and causes de-sumoylation of PML and dispersion of PML NBs, allowing for more efficient expression from the MIEP and replication to proceed (Ahn and Hayward, 1997; Ahn and Hayward, 2000; Korioth et al., 1996). In addition to pp71 and IE1, UL35 also associates with PML and pp71 appears to enhance this association (Schierling et al., 2004). Furthermore, UL35 acts cooperatively with pp71 to activate expression from the MIEP in reporter assays (Liu and Biegalke, 2002; Schierling et al., 2004), suggesting that UL35 might contribute to relieving PML-mediated repression of the MIEP.

The HCMV UL35 gene encodes two proteins, UL35 and UL35a, which are expressed at different times during infection (Liu and Biegalke, 2002). The 640 amino acids—long UL35 is produced late in infection, is packaged into progeny virions as a minor tegument component (Varnum et al., 2004), and is thus delivered pre-formed to newly infected cells. UL35a, which consists of amino acids 448–640 of UL35, is transcribed from a separate promoter and expressed during both the early and late phases of infection (Liu and Biegalke, 2002). Unlike UL35, UL35a does not appear to be packaged in the tegument of the virion (Liu and Biegalke, 2002; Varnum et al., 2004). In addition, UL35a can antagonize pp71-mediated activation of the MIEP, whereas UL35 enhances pp71-mediated MIEP activation (Liu and Biegalke, 2002; Schierling et al., 2004). Deletion of the UL35 gene results in loss of both UL35 and UL35a, and studies with the mutant virus demonstrate that the UL35 gene is essential at low MOI and results in delayed replication and some growth defects at higher MOI (Dunn et al., 2003; Schierling et al., 2005).

Given the importance of PML NBs for controlling viral replication, we have previously conducted a screen for viral proteins that alter or disrupt PML NBs (Salsman et al., 2008). Ninety-three proteins from HSV, HCMV and EBV were examined for their effects on PML NBs, identifying nineteen proteins that induce loss of the NBs and five proteins that localized with and altered PML NBs. UL35 was identified in the latter category and was observed to form ring-like structures that contained PML. Here we further explore the nature of these UL35 bodies, their association with PML and how the interplay between UL35, UL35a and pp71 affects these bodies as well as each protein's subcellular localization.

Results

HCMV UL35 forms nuclear bodies

Various localizations of UL35 have been reported in the literature including pan-nuclear localization of untagged UL35 (Schierling et al., 2004), nuclear with nuclear body (NB) localization for SPA-tagged UL35 (Salsman et al., 2008) and cytoplasmic localization for GFP-tagged UL35 (Liu and Biegalke, 2002). To help clarify these discrepancies, we generated plasmids that express UL35 with either no epitope tag (UL35-wt), a C-terminal triple FLAG tag (UL35-F) or a C-terminal sequential peptide affinity (SPA) tag (Zeghouf et al., 2004) consisting of a calmodulin binding peptide and a triple FLAG epitope tag (UL35-S). We also generated plasmids that express UL35a, the truncated form of UL35, with either no epitope tag (UL35a-wt), a FLAG tag (UL35a-F) or a SPA tag (UL35a-S). Western blots of cell lysates expressing these constructs confirmed their expected size and epitope tags and showed that all the UL35 and UL35a proteins reacted with the UL35 antiserum (Fig. 1A). Importantly, no UL35a sized bands were detected in UL35-transfected cell lysates, and therefore, the subcellular localizations seen in UL35-transfected cells represent differently localized UL35 and not a combination of UL35 and UL35a localizations.

By immunofluorescence, all three UL35 constructs displayed similar localization patterns and were primarily nuclear localized (~70%) with about 30% of cells showing both nuclear and cytoplasmic localization (Fig. 1B). Consistent with our previous results, in many of the UL35-S transfected cells, UL35 also formed several subnuclear dots and/or donut shaped structures that were clearly detected using the FLAG antibody (Fig. 1B). UL35-F and UL35-wt also formed subnuclear bodies in transfected cells in addition to nucleoplasmic staining (Fig. 1B). We quantified the percentage of UL35-transfected cells that contained NBs and found that UL35-wt and UL35-F formed NBs in ~50% of transfected cells, while UL35-S formed NBs in ~70% of transfected cells (Fig. 1C). In addition, although there is a big range in the number of NBs per cell for all UL35 construct, the average number of NBs per cell was similar for all UL35 constructs; namely 7.4 for UL35-S, 6.4 for UL35-F and 6.7 for UL35-wt (see Figs. 2D and 7E). However, we noticed that for both UL35-S and UL35-F, the UL35 NBs were more clearly detected using the FLAG antibody than with the UL35 antiserum (Fig. 1D), suggesting that the UL35 epitope recognized by this antiserum is not as accessible as the C-terminal tag in the context of UL35 NBs.

In addition to forming symmetrical dot or donut structures, UL35 was also found associated with irregularly shaped nucleolar-like structures (Fig. 1D, asterisks). The association of a proportion of UL35 with the nucleolus was confirmed by co-staining for UL35 and the nucleolar protein EBP2 (EBNA1 binding protein 2; Nayyar et al 2009) (Supplemental Figure 1). Notably, the dot/donut shaped UL35 NBs were not associated with EBP2 (Supplemental Figure 1, arrows) indicating that these structures are distinct from the nucleolar localization. Only these non-nucleolar structures are referred to here as UL35 NBs.

The localization of UL35a was also examined and found to be nuclear in ~70% of transfected cells. Like UL35, a small proportion of UL35a showed nucleolar localization (Fig. 1E asterisks and Supplemental Figure 1). However, unlike UL35, NBs were not observed with UL35a for any of the three constructs with the FLAG or UL35 antibodies (Fig. 1B and E). These results indicate that, although UL35 and UL35a are both primarily nuclear, UL35, but not UL35a, can form nuclear bodies in the absence of other viral proteins.

UL35 nuclear bodies associate with and remodel PML bodies

We next explored the relationship between the UL35 NBs and PML, first by expressing UL35 with and without epitope tags in U2OS cells. In the absence of UL35, PML NBs are seen as several small punctate foci within the nucleus (Fig. 2A, top row). In cells expressing UL35, PML strongly colocalized with UL35 NBs regardless of the presence or absence of an epitope tag (Fig. 2A). In addition, this colocalization with UL35 NBs resulted in a reorganization of PML NBs from several punctate bodies to crescent- and ring-shaped bodies that co-localize with the surface of the UL35 NB. In most cells, all of the visible PML was associated with UL35 NBs, however, occasionally UL35 NBs were observed that were not associated with PML (Fig. 2A, arrow). In contrast, UL35a, which does not form NBs, did not associate with cellular PML and did not appear to alter the number or morphology of PML NBs (Fig. 2B).

Herpesvirus proteins can disrupt PML NBs by various mechanisms including promoting the degradation of PML proteins, as seen for ICP0 of HSV and EBNA1 of Epstein–Barr virus (Everett et al., 1998; Sivachandran

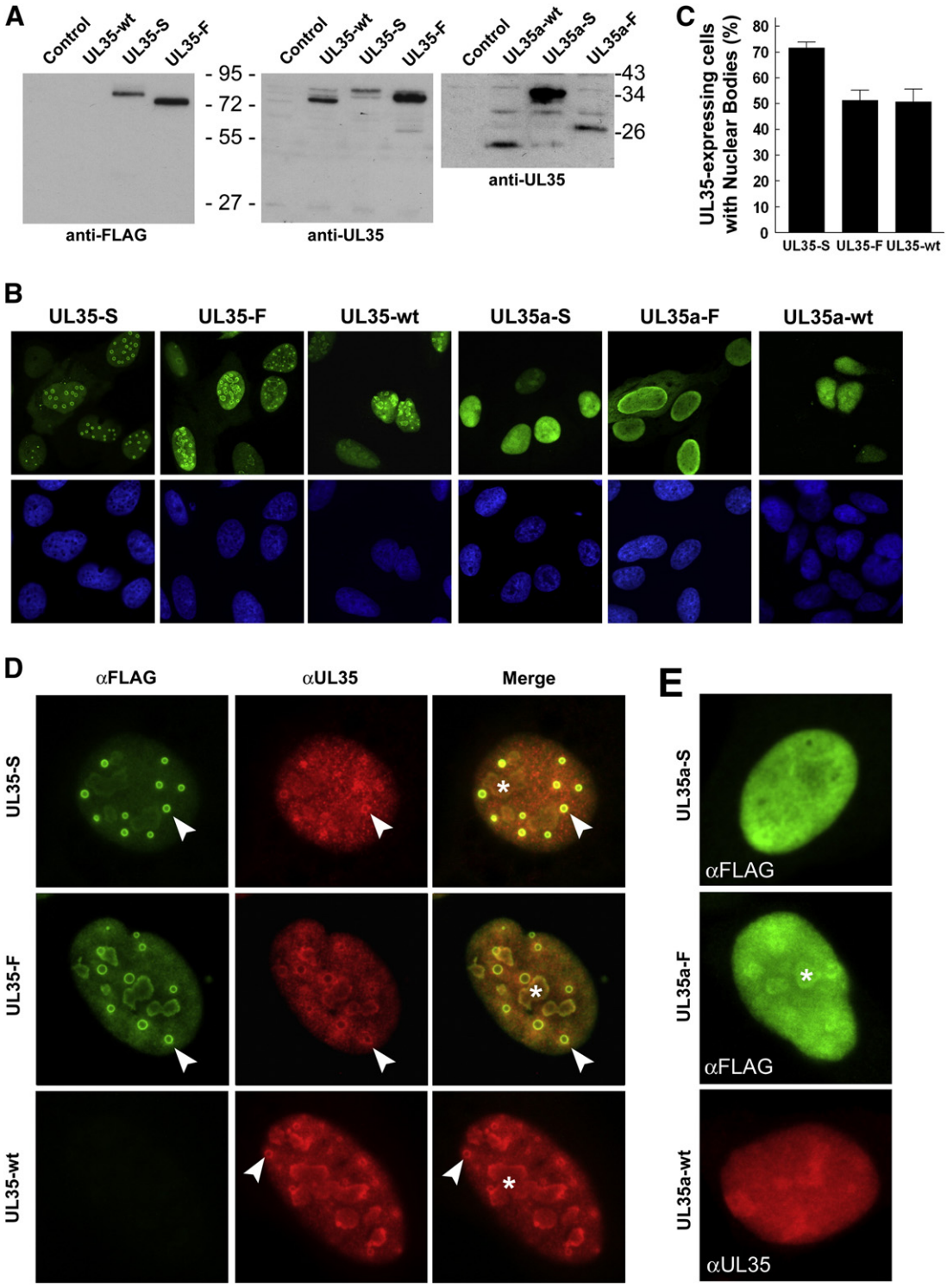

Fig. 1. UL35 forms nuclear bodies. (A) U2OS cells were transfected with different UL35 and UL35a expression plasmids as indicated and 48 h post-transfection cell lysates were analyzed by Western blotting. UL35 and UL35a were detected with anti-FLAG antibody or UL35 antiserum as indicated. (B) U2OS cells were transfected with the indicated UL35 and UL35a expression plasmids and, 48 h later, the localization of these proteins (green) was determined by immunofluorescence microscopy using anti-FLAG (UL35-F and UL35-S) or anti-UL35 (UL35-wt and UL35a-wt) antibodies. DAPI stains (blue) are also shown. (C) U2OS cells were transfected with UL35-S, UL35-F or UL35-wt and the percentage of UL35-expressing cells that contained nuclear bodies at 48 h post transfection was determined by immunofluorescence microscopy as in (D). Values represent the mean ± s.e., $n = 4–6$. (D) Examples of cells quantified in (C) in which UL35-F and UL35-S were detected with anti-FLAG antibody (green) and UL35-wt was detected with anti-UL35 antibody (red). Arrows indicate an example of UL35 NBs for each image. Asterisks in merged images indicate an example of nucleolar staining. (E) U2OS cells expressing UL35a with and without the indicated tags were imaged using anti-FLAG (green) or anti-UL35 (red) primary antibodies. Asterisks indicate an example of nucleolar staining.

et al., 2010; Sivachandran et al., 2008). To test if UL35 could affect the levels of PML proteins, UL35- and UL35a-transfected U2OS cell lysates were analyzed by PML immunoblot. Neither UL35 nor UL35a affected the overall levels of PML, despite the fact that they were expressed at high levels in over 80% of the cells (Fig. 2C). We next determined if UL35 or UL35a affected the ability of PML to form bodies by counting the PML bodies in control and UL35- and UL35a-transfected cells with and without an epitope tag (Fig. 2D and E). Control cells had an average of 11.2 ± 0.6

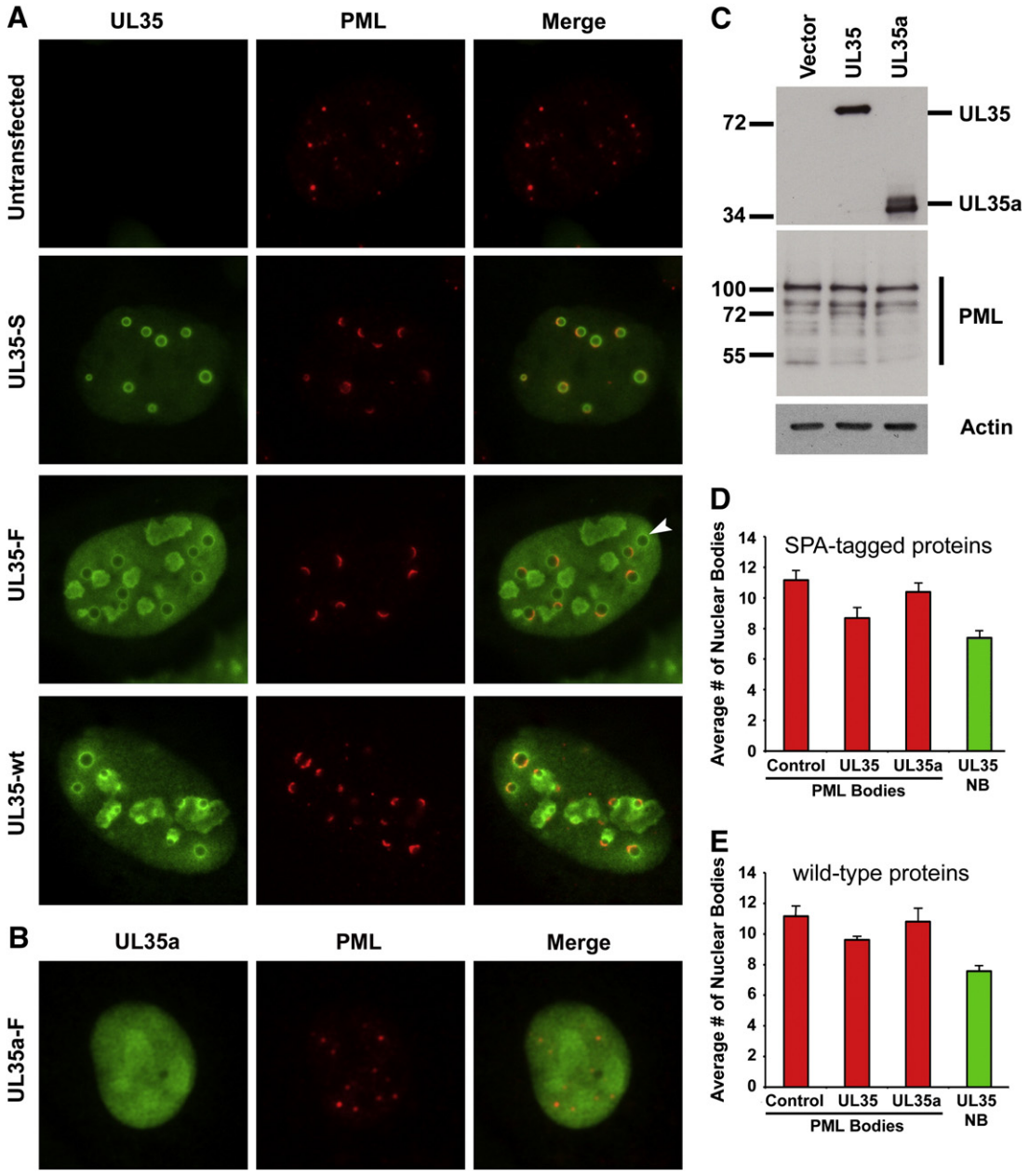

Fig. 2. UL35 associates with and alters PML bodies. (A) Representative images of single nuclei from untransfected (top row) and UL35-S, UL35-F or UL35-wt transfected cells immunostained for PML (red) and UL35 (green) using anti-FLAG (UL35-S and UL35-F) or UL35 antiserum (UL35-wt). Arrow indicates a UL35 NB not associated with PML. (B) A representative image of a UL35a-transfected U2OS cell immunostained for UL35a and PML as indicated. (C) Western blot analysis for PML, actin and FLAG of cell lysates from U2OS cells expressing UL35-S or UL35a-S. The multiple bands in the PML blot represent the different isoforms of PML. (D) The average number of PML bodies (red bars) or UL35 NBs (green bar) was determined by immunofluorescence microscopy for U2OS cells expressing UL35-S or UL35a-S as indicated. The values represent the mean ± s.e. ($n = 3–5$). (E) The same experiment as D, except that cells were transfected with untagged UL35 and UL35a. Values represent the mean ± s.e. ($n = 3–4$).

PML bodies per cell while UL35a-transfected cells had 10.0 ± 0.6 (UL35a-S, Fig. 2D) and 10.8 ± 0.9 (UL35a-wt, Fig. 2E) PML bodies per cell. In contrast, UL35-S and UL35-wt-transfected cells had somewhat fewer PML bodies (8.7 ± 0.7, and 9.6 ± 0.2, respectively) than control cells (Fig. 2D and E). Interestingly, the average number of UL35 NBs per transfected cell was determined to be 7.4 ± 0.4 (UL35-S, Fig. 2E) and 7.6 ± 0.3 (UL35-wt, Fig. 2E). Given that most of the visible PML is associated with UL35 NBs (and vice versa) (Fig. 2A) it stands to reason that the average number of PML bodies would approach the average number of UL35 NBs. Thus, although UL35 does not appear to significantly inhibit the ability of PML to form bodies, the association of PML with UL35 can cause a slight reduction in average PML body number.

Since we have shown that UL35 can alter PML localization, we investigated whether other components of PML NBs were also relocalized. In particular, we examined Sp100 and Daxx because they are common PML body components that are involved in the repression of viral gene expression and are targets of other herpesvirus proteins (Everett et al., 2006; Negorev et al., 2006; Saffert and Kalejta, 2006). We found that Sp100 and Daxx both colocalized with UL35 NBs and displayed the same type of reorganization as PML (Fig. 3). These results indicate that UL35 not only affects PML proteins, but induces remodeling of PML NBs.

UL35 NBs form independently of PML

Next, we wanted to distinguish between two possible explanations for the association between UL35 and PML. In one model, UL35 NBs represent the accumulation of UL35 at existing PML bodies. Alternatively, UL35 might form NBs independently of PML and, once assembled, these

UL35 NBs might recruit PML through interactions with PML or another PML body component. The later possibility is supported by the above observations that not all UL35 NBs contain detectable PML. Two approaches were employed in order to distinguish between these possibilities. First, UL35-F transfected cells were fixed at early times post-transfection and analyzed for UL35 NB formation and the association of those NBs with PML bodies. In U2OS cells expressing UL35-F, the distribution of UL35 was largely pan-cellular at early times post transfection, however, UL35 NB formation occurred as early as 4–6 h post transfection and the UL35 bodies appeared as multiple dot-like structures (Fig. 4A, arrows). At this early time, cellular PML bodies appeared normal and were not strongly associated with UL35 NBs (Fig. 4A). The presence of UL35 NBs that were not associated with PML bodies indicates that UL35 NBs do not form at preexisting PML bodies.

In order to further test if UL35 NB formation occurs independently of PML, UL35-F or UL35-wt were expressed in U2OS cells in which all PML isoforms had been silenced with shRNA expressed from a lentivirus, as previously described (Everett et al., 2006). Both UL35-F and UL35-wt were able to form NBs in U2OS-shPML cells despite the fact that they lacked any detectable PML NBs (Fig. 4B). The percentage of transfected cells with UL35 NBs was determined to be ~50% for both UL35-F and UL35-wt (Fig. 4C), which is nearly identical to the percentage of cells with UL35 NBs in the parental cells containing PML (Fig. 1C). These results demonstrate that UL35 NBs form independently of PML bodies or PML protein and indicate that PML protein becomes associated with UL35 after UL35 NBs are formed.

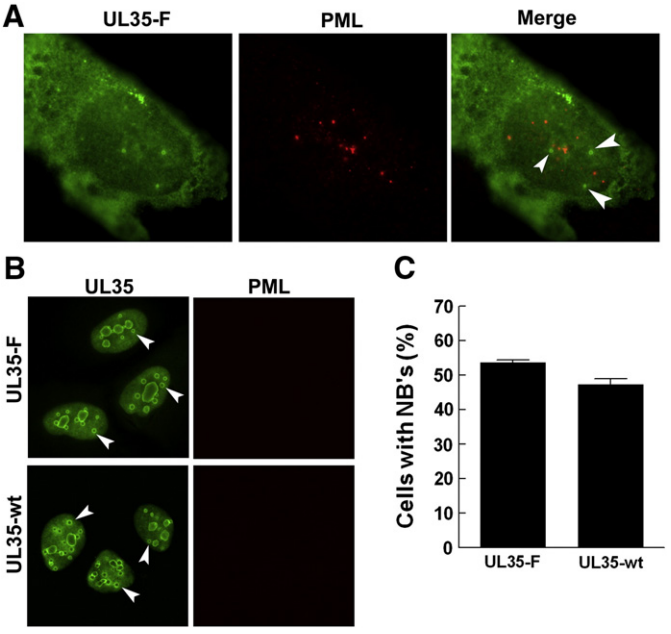

Fig. 4. UL35 NBs form independently of PML. (A) U2OS cells were transfected with UL35-F and immunostained for PML (red) and UL35 (green) at 5 h post-transfection. Examples of UL35 NBs that are not associated with PML are indicated with arrows. (B) Immunofluorescent images of U2OS cells with shRNA-silenced PML, 40 h after transfection with plasmids expressing UL35-F and UL35-wt. Arrows point to examples of UL35 NBs. (C) Quantification of UL35 NBs in shPML-U2OS cells. Cells were transfected with UL35-F or UL35-wt, fixed and immunostained at 40 h post-transfection and the percentage of transfected cells containing UL35 NBs was determined. Values represent the mean ± s.e. ($n = 3$–4).

UL35 and UL35a interact with themselves and with each other

UL35 and UL35a appear to have opposing activities with respect to the major immediate early promoter (MIEP) and UL35a is present in the cell when UL35 is expressed late in infection. Therefore, we wanted to understand more about the interplay between these two proteins. We began by investigating whether UL35 and UL35a can interact with themselves or with each other. For these experiments, we generated plasmids expressing C-terminally myc-tagged UL35 and UL35a (UL35-myc and UL35a-myc, respectively) and used them to co-transfect 293A cells along with the expression plasmids for either UL35-S or UL35a-S. FLAG immunoprecipitation of cell lysates containing UL35-myc and UL35-S recovered both proteins (Fig. 5A), indicating that UL35 can interact with itself. As expected, in the absence of UL35-S, no UL35-myc could be detected in the elution (Fig. 5A). Similarly, when UL35a-myc was co-expressed with either UL35a-S or UL35-S, FLAG immunoprecipitations recovered UL35a-myc along with both UL35-S and UL35a-S (Fig. 5B). In contrast, UL35a-myc was not recovered in the absence of UL35-S or UL35a-S (Fig. 5B). These results demonstrate that UL35 and UL35a have the ability to associate with themselves and with each other in cells. In addition, UL35a-myc was co-expressed with the FLAG-tagged N-terminal region of UL35 (UL35N-F, amino acids 1–447), which does not share sequence with UL35a (amino acids 448–640). UL35a-myc was recovered with UL35N-F by FLAG immunoprecipitation, indicating that UL35 contains at least two UL35a-interacting motifs; one in the N-terminal region and one in the C-terminal region.

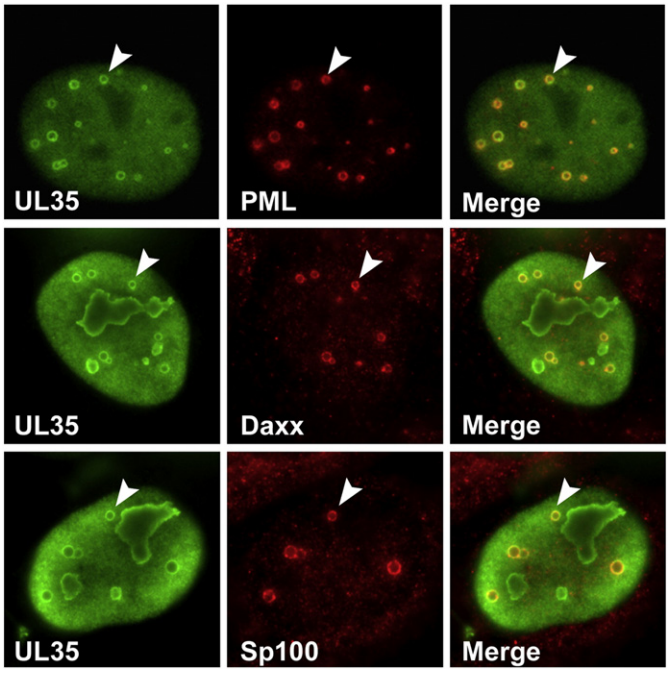

Fig. 3. Daxx and Sp100 associate with UL35 nuclear bodies. Representative immunofluorescence images of CNE-2Z cells transfected with UL35-F (green) and immunostained for PML, Daxx and Sp100 (red) as indicated. Arrows indicate an example of UL35 NBs co-localizing with PML, Daxx or Sp100.

UL35a inhibits UL35 NB formation

Since UL35 and UL35a can interact and can be expressed at the same time during infection, we examined the effect of UL35a on UL35 NB formation. To this end, U2OS cells were co-transfected with UL35-F and UL35a-myc, and the extent of UL35-NB formation was determined by immunofluorescence microscopy. When UL35-F was expressed on its own UL35 was able to form nuclear bodies in ~60% of cells at 24 h post-transfection (Fig. 6A). When UL35a-myc was co-transfected with UL35-F at an input DNA ratio of 1:9 (UL35:UL35a), many cells expressing both proteins showed reduced UL35 NB formation (but no obvious reduction in nucleolar staining, Fig. 6A). In order to quantify this effect, U2OS cells were transfected with a constant amount of UL35-F DNA and increasing amounts of UL35a-myc DNA at a 1:0, 1:3 and 1:9 ratio. We then determined the number of cells with one or more UL35 NBs, for 3

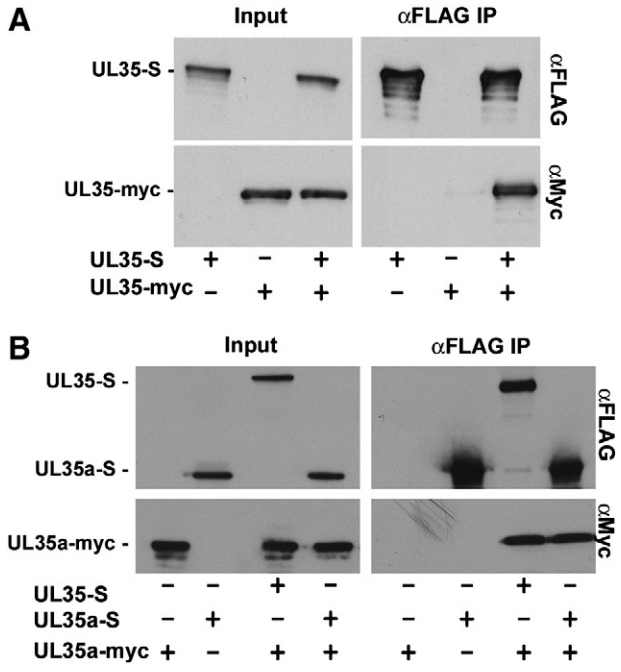

Fig. 5. UL35 and UL35a interact with themselves and with each other. 293A cells were transfected with UL35-S and/or UL35-myc (A) or with UL35-S, UL35a-S, UL35N-F and/or UL35a-myc (B) alone or in combination as indicated. FLAG-tagged proteins were immunoprecipitated from cell lysates (input) and recovered immunoprecipitants (αFLAG IP) were probed for FLAG- and myc-tagged proteins as indicated.

independent experiments, which showed a dose-dependent disruption of UL35 NBs by UL35a (Fig. 6B). A 9-fold excess of UL35a over UL35 resulted in a significant ($p = 0.001$) inhibition of UL35 NB formation such that about 70% of cells that expressed UL35a had no UL35 NBs (Fig. 6B). This decrease in UL35 NB formation was not due to a decrease in UL35 expression, since UL35 levels were the same regardless of the amount of UL35a present (Fig. 6C). These results indicate that UL35a has the ability to inhibit UL35 NB formation.

pp71 enhances UL35 NB formation

In addition to UL35a, the tegument protein pp71 also interacts with UL35, is expressed with early–late kinetics (Liu and Biegalke, 2002) and would therefore be expressed late in infection when UL35 is also present. In order to explore the effects of pp71 on UL35 NB formation, UL35-wt was co-expressed with HA-tagged pp71. In the presence of pp71, the formation of UL35 NBs was obviously increased as compared to UL35 expressed on its own and the nucleoplasmic and nucleolar-like staining of UL35 was reduced (Fig. 7A). These effects were not seen with a negative control HA-tagged protein (BZLF1 from Epstein–Barr virus; Supplemental Figure 2). When expressed on its own, UL35-wt formed NBs in ~50% of cells (Fig. 1D) and, of those cells with UL35 NBs, there was an average of 6.7 UL35 NBs per cell (Fig. 7E). In the presence of pp71, over 99% of UL35-expressing cells contained UL35 NBs (Fig. 7D) and there was an average of 12.7 UL35 NBs per co-transfected cell (Fig. 7E). Although partial pp71 localization to UL35 NBs was observed in some cells (Fig. 7A, arrow), most cells did not show a high degree of accumulation of pp71 at UL35 NBs. We also found that UL35 retains the ability to remodel PML in the presence of pp71 with PML strongly associated with UL35 NBs (Fig. 7C). In light of our observations that UL35 NB formation does not require PML, we tested whether pp71-mediated UL35 NB formation was PML-dependent. U2OS cells with silenced PML were transfected with either UL35-wt alone or co-transfected with UL35-wt and HA-pp71 (Fig. 7B) and the UL35 NBs were quantified as above (Fig. 7D and E). The results in shPML-U2OS cells were indistinguishable from those in parental U2OS cells, indicating that pp71 can increase UL35 NB formation independently of PML.

UL35a promotes cytoplasmic accumulation of pp71

Since pp71 can also interact with UL35a and both proteins are expressed with UL35 late in infection, we examined what effects UL35a and pp71 had on each other. In U2OS cells, both pp71-HA and UL35a-wt display primarily nuclear localization (Fig. 8A and B). When pp71 and UL35a were expressed together the localization of both UL35a and pp71 was obviously different with pp71 becoming predominantly cytoplasmic and UL35a showing increased cytoplasmic localization in addition to its nuclear localization (Fig. 8C and D). In addition, many co-transfected cells contained several dot-like structures in the cytoplasm that contained both UL35a and pp71 (Fig. 8C). We quantified the localization of pp71 and UL35a alone or together and determined that when expressed alone, UL35a and pp71 showed nuclear localization in about 75% of all transfected cells (Fig. 8B). About 20%–25% of pp71 or UL35a expressing cells had both nuclear and cytoplasmic (i.e. pancellular) localization, while 0%–5% of cells were predominantly cytoplasmic (Fig. 8B). When expressed together UL35a caused a dramatic shift in the distribution of pp71 from nuclear to cytoplasmic with ~75% of transfected cells containing predominantly cytoplasmic pp71 and ~25% of cells showing pancellular distribution of pp71 (Fig. 8D). Virtually no cells contained strictly nuclear-localized pp71 in the presence of UL35a. The localization of UL35a was also affected by pp71 with a pancellular distribution becoming predominant (~60%) and nuclear or cytoplasmic localizations each comprising about 20% of co-transfected cells (Fig. 8D). These results demonstrate that UL35a and pp71 can influence each other's subcellular localization.

Discussion

We have examined the subcellular localization of UL35, UL35a and pp71 and how these proteins affect the localization of each other. The reported localization pattern of UL35 is varied with cytoplasmic (Liu and Biegalke, 2002), nuclear (Schierling et al., 2004) and sub-nuclear (Salsman et al., 2008) patterns having been described. Based on these studies and our current results, two factors may contribute to the variable results that have been reported. First, the presence of a C-terminal tag (i.e. GFP, SPA, FLAG) appears to influence the subcellular localization of UL35, shifting the balance between NBs and nucleoplasmic localization and, in the case of the EGFP tag used by Liu and Biegalke (2002), may have induced the cytoplasmic localization of UL35 that they observed. The second factor that likely contributed to the reported differences in UL35 localization is the specificity of the polyclonal antibody directed against UL35 (Liu and Biegalke, 2002). Our results show that this antibody is more sensitive for nucleoplasmic UL35 than for UL35 NBs, most likely due to the masking of epitopes when UL35 is assembled in NBs.

Despite the differences in UL35 NB detection, it is clear that both tagged and untagged UL35 have the ability to frequently form NBs in transfected cells. In the context of CMV infection, UL35 NBs have not been detected (Liu and Biegalke, 2002), but this is not surprising given the limited sensitivity of the only available UL35 antibody for NBs and the interplay between UL35 and UL35a. UL35 is only present in the absence of UL35a upon initial infection when it is delivered to the cell as a minor tegument component (Varnum et al., 2004). At that time it might form NBs, which would be expected to be small due to limited amounts of UL35, and these might not be detected by the UL35 antiserum due to its limited ability to stain NBs. Later in infection when UL35 is expressed along with UL35a, detection of any potential UL35 NBs is further complicated by the cross-reactivity of the UL35 antiserum with the pan-nuclear UL35a. Therefore, whether or not UL35 forms NB in the context of CMV infection remains an open question that requires new UL35/UL35a antibodies or recombinant CMV with differently tagged UL35/UL35a to resolve. Nonetheless, UL35 NB formation in transfected

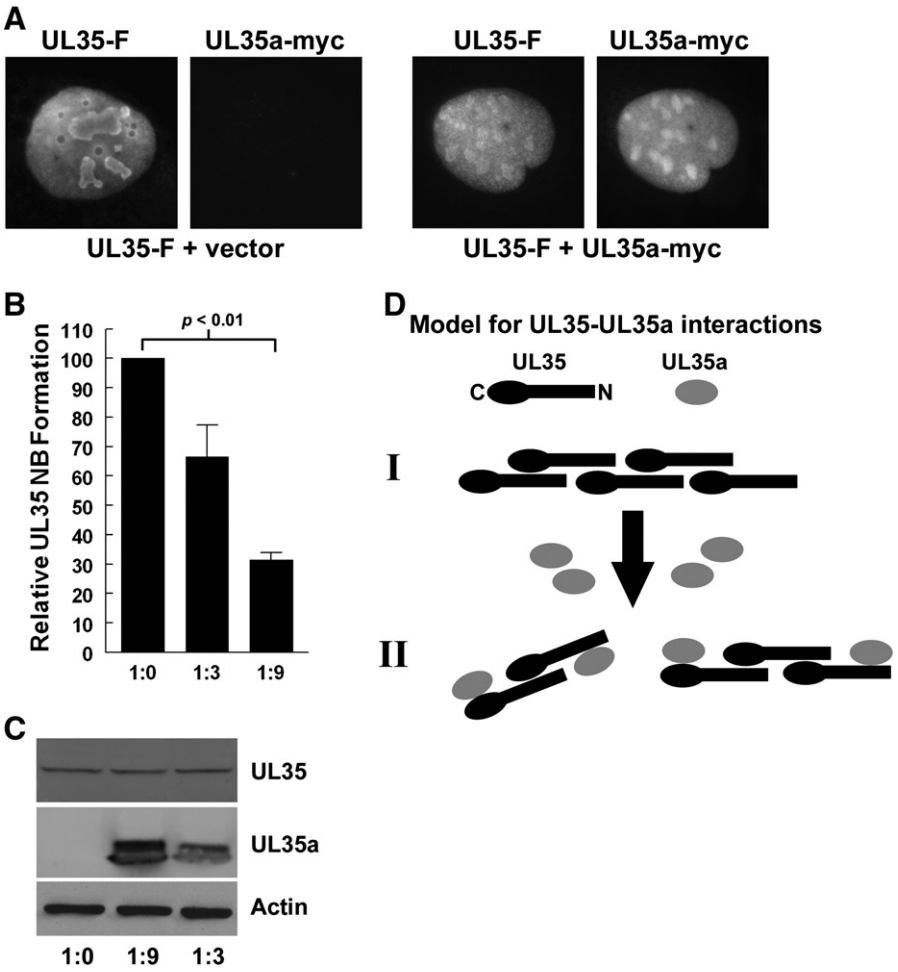

Fig. 6. UL35a inhibits UL35 NB formation. (A) U2OS cells were transfected with UL35-F ± control vector or UL35-F at a 1:9 ratio with UL35a-myc and, 24 h later, the effects on UL35 NB formation were detected by immunofluorescence using anti-FLAG and myc antibodies as indicated. (B) U2OS cells were transfected with UL35-F plasmid and an empty plasmid or with UL35-F and UL35a-myc plasmids at 1:3 and 1:9 ratios as indicated. Cells were fixed at 24 h post transfection and UL35 NBs were visualized by immunofluorescence microscopy and counted. The relative number of cells with UL35 NBs in the presence of increasing UL35a was determined for each individual experiment. Values represent the mean ± s.e. ($n = 3$). (C) Cell lysates for U2OS cells transfected as described for (B) were analyzed by Western blot for FLAG (UL35-F), myc (UL35a-myc) and actin levels as indicated. (D) A model for UL35 oligomerization and disruption by UL35a. When expressed individually, UL35 can form homotypic oligomers leading to NB formation (I). When co-expressed, UL35a can interact with both the N- and C-terminal regions of UL35, inhibiting formation of large UL35 complexes (II).

cells provides a readout for assessing interactions between UL35, UL35a and pp71 and for demonstrating interactions of PML and associated proteins with UL35.

We and others observed that UL35 has the ability to associate with PML NBs (Salsman et al., 2008; Schierling et al., 2004). Previous reports noted that this PML-association was increased in the presence of pp71, a viral tegument protein that interacts with UL35 (Schierling et al., 2004). The results of this study, however, demonstrate that UL35 can form NBs independently of PML and it is these NBs that ultimately recruit and remodel PML bodies. In addition, we have shown that pp71 is not required for UL35 NB formation and PML association. However, pp71 enhances the frequency and number of UL35 NBs in transfected cells, also in a PML independent manner. Exactly how pp71 contributes to increased UL35 NB formation is unclear. Despite the ability to interact, we did not notice strong colocalization by immunofluorescence between UL35 NBs and pp71 suggesting that pp71 might exert its effects on UL35 indirectly and independently of its ability to interact with UL35. In light of our recent findings, it is apparent that UL35 does not localize to preexisting PML bodies but rather causes the recruitment and reorganization of PML. These UL35 NB-associated PML bodies contain Sp100 and Daxx, two common PML body components, further suggesting that UL35 does not only redirect PML proteins but also PML-associated proteins.

We showed that UL35 and UL35a interact with each other and that, when present in excess, UL35a can disrupt the UL35 interactions that lead to NB formation. This effect is likely significant late in infection when UL35a levels can be substantially higher than UL35 levels (Schierling et al., 2005). However, the ratio of UL35 to UL35a appears to be affected by the MOI as another study, using a much higher MOI, found similar levels of the two proteins (Liu and Biegalke, 2002), suggesting that differences in infection conditions can alter the expression profile of the UL35 gene. In addition, the inhibitory effect of UL35a on UL35 NBs may be relevant late in infection when both proteins are expressed with pp71. Since pp71 promotes UL35 NB formation (Fig. 7, discussed below), UL35a may be required to antagonize this activity and coordinate proper virion assembly. In addition, the ability of UL35 to relocalize pp71 to cytoplasm would also limit its ability to promote NB formation late in infection.

Our data on the formation and inhibition of UL35 NBs point to a model in which UL35 NBs are the result of UL35 oligomerization which can be prevented or disrupted by UL35a binding to UL35. Since UL35a is comprised of the C-terminal 193 amino acids of UL35, this sequence must be sufficient for UL35 interactions. However, since UL35a fails to form NBs and disrupts NB formation by UL35, UL35 sequences N-terminal to this shared region must also be important for the interactions leading to UL35 NBs. This hypothesis is supported by the observation that the UL35 N-terminal portion (amino acids 1–447) interacted with the UL35 C-terminal (amino acids 448–640) region in co-immunoprecipitation experiments. Therefore, NB formation by UL35 likely requires at least two different protein interaction domains, only one of which is present in

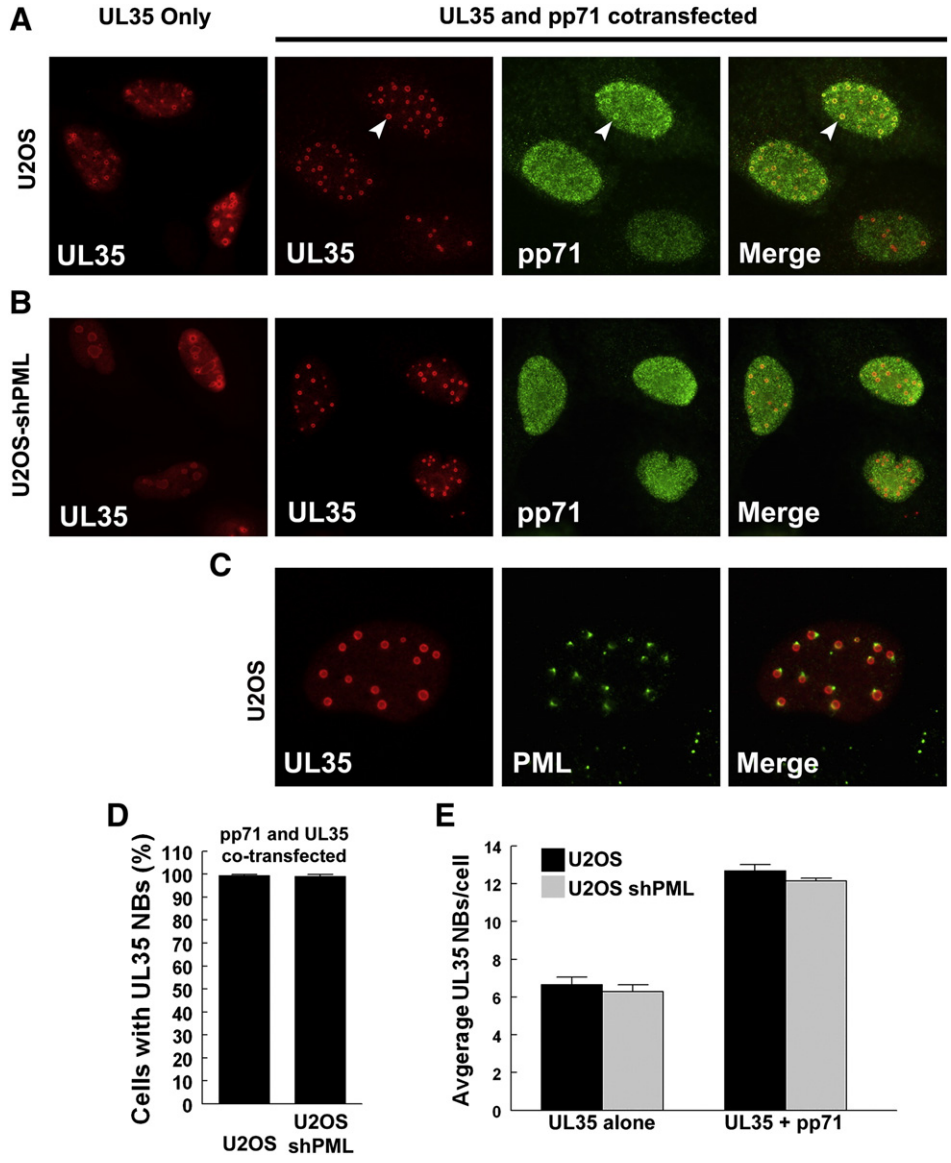

Fig. 7. pp71 enhances UL35 NB formation independently of PML. (A and B) Immunofluorescence images of U2OS (A) or U2OS-shPML cells (B) transfected with UL35-wt alone (left panel) or UL35-wt and pp71-HA as indicated. Cells were immunostained with UL35 antiserum (UL35) or HA antibody (pp71) as indicated. Arrow in (A) indicates an example of UL35 and pp71 co-localization. (C) Association of UL35 (red) and PML (green) in U2OS cells co-transfected with UL35-wt and pp71-HA. (D and E) Quantification of the experiments in A and B showing the average percentage of cells with UL35 NBs (D) and average number of UL35 NBs per cell (E). Values represent the mean ± s.e. ($n = 3–5$).

UL35a. One possible model of these interactions is shown in Fig. 6D, where the N-terminal domain of one UL35 protein interacts with the C-terminal domain of another UL35 protein, resulting in the formation of large UL35 oligomers (Fig. 6D, part I). The interaction of UL35a with either the N- or C-terminal portions of UL35 could inhibit this oligomerization by acting as chain terminators (Fig. 6D, part II).

Experiments in which UL35 was co-expressed with pp71 showed that pp71 enhances the efficiency of UL35 NB formation (Fig. 7), which can recruit PML, but that this stimulation of UL35 NB formation is independent of the presence of PML. Previous studies exploring the connection between pp71, UL35 and PML also reported that pp71 could increase the efficiency of UL35 co-localization with PML bodies (Schierling et al., 2005) and that this effect was consistent with the ability of UL35 to cooperatively activate the MIEP with pp71. Our data also show that pp71 increases the association of UL35 with PML, but demonstrate that this is due to enhanced formation of UL35 NBs, rather than enhanced association of UL35 with pre-existing PML bodies. Nonetheless, it is possible that the same UL35-host protein interaction(s) that leads to PML alteration might also contribute to relief of PML-mediated MIEP repression, even in the absence of UL35 NB formation.

Our studies on the interplay between UL35, UL35a and pp71 showed that UL35a (but not UL35) caused pp71 to relocalize from the nucleus to the cytoplasm (Fig. 8). This is interesting because, as CMV infection proceeds, the localization of pp71 is known to change from nuclear to cytoplasmic. In addition, deletion of the UL35 gene was previously shown to cause pp71 to be retained in the nucleus throughout infection (Schierling et al., 2005). Our results suggest that it is UL35a, and not UL35, that is responsible for promoting the cytoplasmic localization of pp71 during infection. In the same study using the UL35 deletion virus, the tegument protein pp65 also failed to localize to the cytoplasm late in infection, and the infection produced fewer mature virions and no dense bodies (Schierling et al., 2005). Thus, UL35 and/or UL35a are important for proper virus assembly. It is unknown if the interaction between UL35a and pp71 is required for the effects on localization, however, the presence of cytoplasmic bodies that contain both UL35a and pp71 indicate that these two proteins can associate in the cytoplasm (Fig. 8). There is also some indication that phosphorylation of pp71 contributes

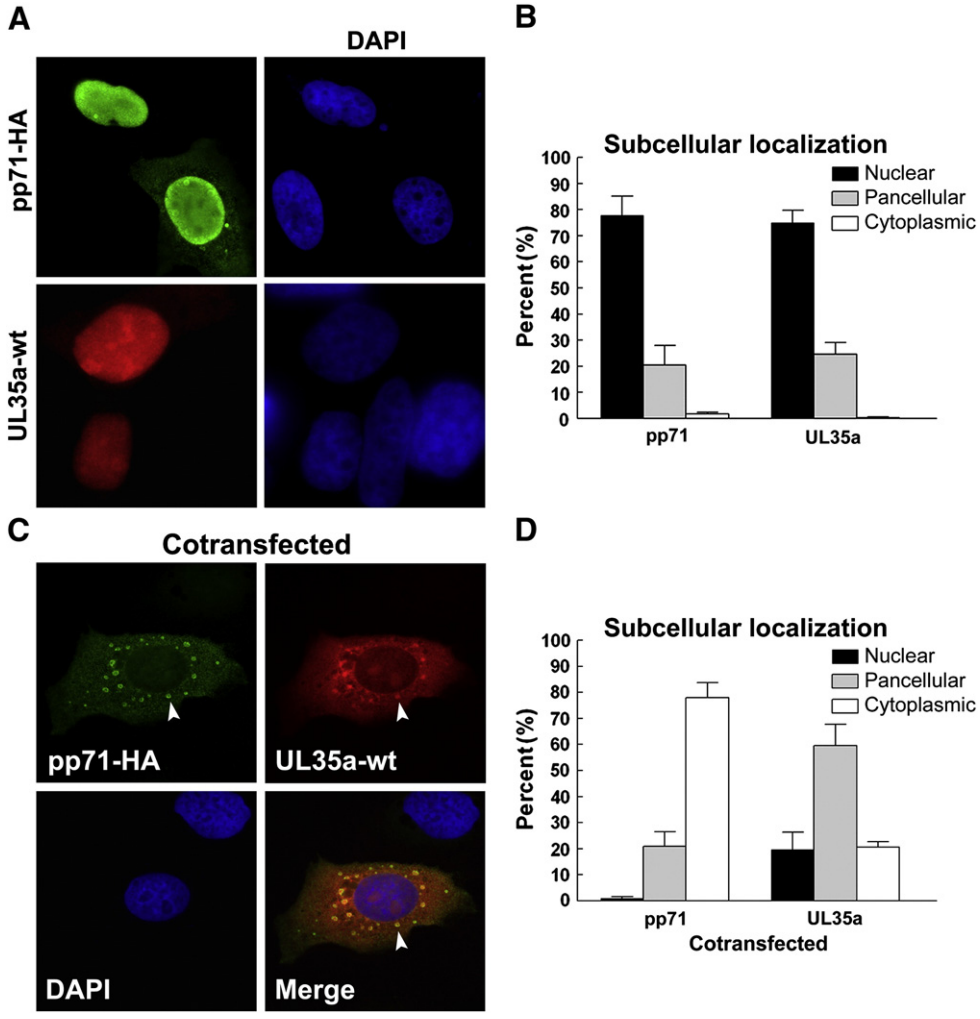

Fig. 8. UL35a promotes the cytoplasmic accumulation of pp71. (A) Immunofluorescence images of U2OS cells transfected with pp71-HA or UL35-wt as indicated and immunostained with anti-HA (pp71) or UL35 antiserum (UL35a). DNA was stained with DAPI (blue). (B) Quantification of the subcellular localization of pp71-HA and UL35a from (A). The percentage of cells with nuclear, pancellular or cytoplasmic localization is shown. Values represent the mean ± s.e. ($n = 4–5$). (C) Immunofluorescence images of U2OS cells co-transfected with pp71-HA and UL35-wt and immunostained with anti-HA (pp71) or UL35 antiserum (UL35a). DNA was stained with DAPI. Arrow indicates a cytoplasmic body. (D) Quantification of the subcellular localization of pp71-HA and UL35a when co-expressed as in (C). Values represent the mean ± s.e. ($n = 3$).

to its localization, and it is possible that UL35a could affect the efficiency of this modification (Shen et al., 2008).

In summary, we have demonstrated that UL35 forms NBs that recruit PML, and that this effect is enhanced by pp71 and inhibited by UL35a. The ability of UL35 NBs to recruit PML and associated proteins likely reflects UL35-host protein interactions that can occur at various times in infection and for various reasons. For example, the ability of UL35 from the tegument, delivered to the cell upon infection, to associate with PML may be important in order for UL35 to be in close proximity to the viral genomes, which are associated with PML NBs. In addition, the ability of UL35 to remodel PML may be an indication of UL35's involvement in cellular pathways that intersect with PML, such as transcriptional regulation, apoptosis control and DNA repair, all of which are known to be manipulated by herpesviruses to promote viral infection.

Materials and methods

Cells

U2OS human osteosarcoma and human embryonic kidney 293A cells were maintained in DMEM supplemented with 10% FBS. Human nasopharyngeal carcinoma cells CNE-2Z were maintained in α-MEM supplemented with 10% FBS. U2OS cells with stable silencing of PML (shPML-U2OS) were generated as follows. 293T cells were co-transfected with the lentiviral plasmids pLKO.shPML1, pCMV.DR8.91 and pVSV-g (kindly provided by Roger Everett; (Everett et al., 2006)) using lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. A total of 4 ml of culture supernatant containing lentiviral vector was collected at 3 days post transfection followed by 0.45 μm filtration to avoid the carryover of 293T cells. One milliliter of lentiviral supernatant was then added to 1×10^5 U2OS cells with polybrene (Sigma) at a final concentration of 8 μg/μl. The lentiviral supernatant was removed after 24 h and replaced with medium containing 0.4 μg/ml puromycin (Bioshop) for 72 h. Puromycin-resistant U2OS cells with reduced PML expression were then serial diluted in 96-well plates to obtain cultures derived from individual cell colonies. Several colonies were selected and one with undetectable PML expression, as determined by immunofluorescence and western blot, was maintained as described above for U2OS cells.

Plasmids

The plasmid encoding UL35 (HCMV strain AD169) with a C-terminal sequential peptide affinity (SPA) tag (pUL35-S), composed of a calmodulin binding peptide and a triple FLAG epitope, in the pMZS3F vector (Zeghouf et al., 2004) was described previously (Salsman et al.,

2008). A plasmid encoding UL35 with a C-terminal triple FLAG epitope tag (pUL35-F) was generated by subcloning the UL35 sequence from the pMZS3F vector between the Xho1 and Xba1 sites of the pCMV-3FC vector. To generate the pCMV-3FC vector, the triple FLAG portion of the SPA tag was PCR amplified and used to replace the YFP gene in pEYFP N1 (Invitrogen) by cloning between the Not1 and BamH1 restriction sites. Untagged wild-type UL35 (pUL35-wt) was generated by inserting the UL35 ORF with two stop codons before the C-terminal tag in pCMV-3FC using the Xho1 and Xba1 restriction sites. UL35a with a SPA tag (pUL35a-S), triple FLAG tag (pUL35a-F) and no tag (pUL35a-wt) were generated using the respective UL35 constructs as templates to amplify the UL35a sequence (amino acids 448–640). Myc-tagged UL35 (pUL35-myc) and UL35a (pUL35a-myc) in the pCMV-3FC vector were generated by PCR amplification with primers designed to add a myc tag (MEQKLISEEDL) and a stop codon to the C-terminus of the UL35 or UL35a open reading frame and inserted between the Xho1 and Xba1 sites. pUL35N-F, consisting of the EBNA1 nuclear localization signal (LKRPRSPSS, (Ambinder et al., 1991)) followed immediately by the N-terminal portion of UL35 (amino acids 1–447), was amplified by PCR and inserted between the Xho1 and Xba1 sites of pCMV-3FC. An HA-tagged pp71 expression plasmid (pCGN71) was a gift from Tom Shenk and has been previously described (Kalejta et al., 2003).

Primary antibodies

Rabbit serum raised against UL35 was a gift from Bonita Biegalke (Liu and Biegalke, 2002). Mouse anti-FLAG (M2 clone), mouse anti-PML and rabbit anti-Daxx were from Sigma-Aldrich. Rabbit anti-FLAG and rabbit anti-PML were from Bethyl Laboratories. Rabbit anti-myc was from AbCam. Rabbit anti-Sp100, anti-HA (rabbit and mouse) and mouse anti-PML were from Santa Cruz Biotechnology. Rabbit antisera against EBP2 is described in Wu et al. (2000).

Immunofluorescence

Cells were seeded into 6-well plates on glass coverslips (~700,000 cells per well) and transfected with expression plasmids using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions, using a DNA to Lipofectamine 2000 ratio of 2 μg:2 μl for 293A cells and 2 μg:4 μl for U2OS and CNE2Z cells. Transfected cells were fixed ~40 h post transfection (or at the indicated times) with 3.7% formaldehyde in PBS (20 min), permeabilized with 0.5% Triton X-100 in PBS (10 min), and blocked with 4% BSA in PBS (20 min) prior to incubation with primary (1 h) and secondary (45 min) antibodies in 4% BSA in PBS. Primary antibodies were detected using either goat anti-mouse or anti-rabbit FAb fragments conjugated with Alexafluor 488 or Alexafluor 555 (Invitrogen). Coverslips were mounted onto slides using ProLong Gold antifade fluorescent mounting medium (Invitrogen) containing DAPI for visualization of nuclear DNA. Images were acquired using the 63× oil objective (NA 1.4) on a Leica DM IRE2 inverted fluorescent microscope. Images were processed using OpenLAB (ver.4.0.2) and Adobe Photoshop version 6.0 using only linear adjustments.

Western blot

Cells were lysed in cell lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40) or in urea (50 mM Tris pH 8.0, 9M urea) for detection of PML. Cell lysates were sonicated if lysed in urea, cleared by centrifugation at 13,000*g* for 15 min at 4 °C and subjected to SDS–PAGE. Proteins were transferred to nitrocellulose membranes, blocked with 4% milk and incubated with the indicated primary antibodies (1 h) and goat anti-mouse or goat anti-rabbit HRP-conjugated secondary antibodies (45 min, Santa Cruz). Antibodies were detected by chemiluminescence using Western Lighting chemiluminescent reagent (PerkinElmer) and exposure to photographic film (Amersham).

Co-immunoprecipitation

U2OS cells in 10 cm plates were co-transfected with the indicated FLAG and/or myc tagged proteins. At 40 h post transfection, cells were washed twice with PBS and harvested. Cell pellets were lysed with cell lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40) plus protease inhibitor cocktail (P8340, Sigma). Cell lysates were clarified by centrifugation at 13,000*g* for 15 min at 4 °C. The FLAG-tagged protein was immunoprecipitated (1 mg of lysate at ~4 mg/ml) in cell lysis buffer and incubated with anti-FLAG resin (1:20,000 dilution, Sigma) for 2 h at 4 °C, while rotating. Beads were washed four times for 10 min in lysis buffer and protein was eluted with 50 μl of protein sample buffer (5% SDS, 20 mM Tris pH 8, 10% DTT, 20% glycerol). Western blots were then performed as described above to detect recovery of FLAG tagged proteins and associated myc-tagged proteins (anti-myc 1:4000 dilution, Abcam).

Quantification of nuclear bodies and protein localization

Quantification of UL35 nuclear bodies or PML bodies was conducted in U2OS, U2OS-shPML and CNE-2Z cells prepared as described above for immunofluorescence microscopy. The percent of UL35-expressing cells containing UL35 nuclear bodies was determined by observing 100 random UL35-expressing cells and scoring each for the presence of dot and/or donut shaped UL35 nuclear bodies. For co-transfections only cells expressing both transfected proteins were recorded. Quantification of the average number of PML bodies per transfected cell was determined by recording the number of PML bodies in each of 50–100 transfected cells. For localization data, the localization was categorized as nuclear or cytoplasmic if 70% or more of the signal intensity localized to either compartment. Cells were categorized as pancellular if the intensity of the cytoplasmic and nuclear was about equal. For each condition, the localization in 100 cells was recorded and for co-transfections, data was collected only for cells that expressed both of the transfected proteins. For all quantification assays, experiments were reproduced 3–5 times as indicated in the figure legends. Statistical analyses (Student's *t*-test) were conducted using Microsoft Excel 2007 software.

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