Involvement of UL24 in herpes-simplex-virus-1-induced dispersal of nucleolin

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Abstract

UL24 of herpes simplex virus 1 is important for efficient viral replication, but its function is unknown. We generated a recombinant virus, vHA-UL24, encoding UL24 with an N-terminal hemagglutinin tag. By indirect immunofluorescence at 9 h post-infection (hpi), we detected HA-UL24 in nuclear foci and in cytoplasmic speckles. HA-UL24 partially co-localized with nucleolin, but not with ICP8 or coilin, markers for nucleoli, viral replication compartments, and Cajal bodies respectively. HA-UL24 staining was often juxtaposed to that of another nucleolar protein, fibrillarin. Analysis of HSV-1-induced nucleolar modifications revealed that by 18 hpi, nucleolin staining had dispersed, and fibrillarin staining went from clusters of small spots to a few separate but prominent spots. Fibrillarin redistribution appeared to be independent of UL24. In contrast, cells infected with a UL24-deficient virus retained foci of nucleolin staining. Our results demonstrate involvement of UL24 in dispersal of nucleolin during infection.

Keywords: UL24; Nucleolus; Nucleolin; Fibrillarin; Herpes simplex virus 1

Introduction

UL24 is considered a core herpesvirus gene (Davison, 2002) and it has been found in all mammalian and avian herpesviruses sequenced to date. UL24 is important for efficient viral replication, especially in neurons, and for efficient reactivation from latently infected trigeminal ganglia in a mouse model of herpes simplex virus 1 (HSV-1) eye infection (Jacobson et al., 1998). In cell culture, UL24-null strains exhibit a small plaque and a syncytial plaque phenotype (syn), the latter of which is especially prominent at elevated temperatures (Jacobson et al., 1989; Sanders et al., 1982; Tognon et al., 1991). The UL24 and tk (UL23) genes of HSV-1 partially overlap at their 5′ ends and are transcribed divergently (Fig. 1). UL24 encodes a 29.5 kDa protein expressed with leaky-late kinetics (Pearson and Coen, 2002). Recently, UL24 was shown to contain a putative PD-(D-E)XK endonuclease motif that is present in certain restriction enzymes and recombinases (Knizewski et al., 2006). UL24 is one of four HSV-1 genes that when mutated can confer a syn phenotype (reviewed in Spear, 1993). The others, UL20, gK and gB, each encode membrane proteins. gB is required for virus entry (Cai et al., 1988) and is necessary for membrane fusion (Cai et al., 1988; Turner et al., 1998). Both UL20 and gK encode proteins that exhibit perinuclear localization and function during viral egress at the stages of de-envelopment and secondary envelopment respectively (Avitabile et al., 1994; Foster and Kousoulas, 1999; Hutchinson and Johnson, 1995). The function of UL24 is unknown.

The biology of HSV-1 is closely tied to the nucleus as viral DNA replication, transcription, capsid assembly, and DNA packaging all occur within this organelle (reviewed in Roizman and Knipe, 2001). The nucleus contains multiple sub-domains (reviewed in Lamond and Sleeman, 2003), the most well-characterized of these being nucleoli, which are the sites of rRNA synthesis and the initial assembly of ribonuclear particles (reviewed in Carmo-Fonseca et al., 2000). The nucleolus forms around the nucleolar organizer region, which consists of the sites on chromosomes that contain rDNA. Nucleoli are comprised of three functionally distinct sub-compartments, namely the fibrillar center (FC) containing the rDNA, which is...
As infection proceeds, viral replication compartments (Maul et al., 1993; Maul et al., 1996; Uprichard and Knipe, 1997) and the GC are disrupted soon after HSV infection (Taylor et al., 2003), chromatin is marginalized (Monier et al., 2000), the nuclear lamina are disrupted (Simpson-Holley et al., 2004; Scott and O’Hare, 2001) and several can be isolated biochemically and are amenable to proteomic analysis (Andersen et al., 2002; Lam et al., 2002). Such studies on nucleoli have revealed the presence of multiple proteins other than those involved in ribosome biogenesis. In accordance with these findings, new roles have recently been discovered for nucleoli. For example, they appear to be involved in the regulation of protein activity by acting as sites of sequestration (Kashuba et al., 2003 and reviewed in Carmo-Fonseca et al., 2000; Olson et al., 2000). Even though nucleoli and other nuclear sub-structures are not membrane-bound, it has been proposed that they associate with chromatin and thereby affect gene expression (Ching et al., 2005). In previous fractionation experiments we found that UL24 of HSV-1 was between 50 and 70% nuclear-associated (Pearson and Coen, 2002), however the precise spatial and temporal distribution of UL24 during infection remains to be elucidated. We generated a virus that expresses the UL24 protein with a hemagglutinin (HA) affinity tag allowing for easy detection by indirect immunofluorescence (IF) microscopy. With this virus we investigated the subcellular localization of UL24 over time in cells infected in culture and identified nucleoli as an organelle targeted by UL24. We also investigated the impact of HSV-1 infection on the subnuclear distribution of nucleolar proteins and tested the impact of UL24 on this phenomenon. Our results have uncovered a role for UL24 in the redistribution of nucleolin during infection thus suggesting a novel function for UL24 in the host cell.

Results

Construction and characterization of vHA-UL24

In order to investigate the function of UL24 we wished to determine with which subcellular compartment(s) UL24 associated during infection. Original attempts to use our UL24 anti-serum (Pearson and Coen, 2002) in indirect IF experiments were unsuccessful due to insufficient sensitivity. Thus, we engineered a recombinant virus in the KOS background such that it encoded the UL24 protein as a C-terminal fusion to a nine amino acid HA tag (Fig. 1). Because the 5’ ends of the UL24 and tk open reading frames overlap, we designed the sequence of the tag such that none of the nine codons inserted within tk was a stop codon. Infectious viral DNA from tkLTRZ-1 was co-transfected with the BamHI fragment of pKOSHA-UL24 into Vero cells. Screening for recombinant viruses was based on the formation of white plaques, which differentiated them from the starting virus, tkLTRZ-1, that contains the lacZ gene inserted within tk and thus forms blue plaques upon X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) staining. To test whether HA-UL24 was stably expressed, we infected Vero cells in culture with either vHA-UL24 or KOS and harvested cell lysates 16 hpi. Proteins were resolved by SDS–polyacrylamide gel electrophoresis, electro-transferred to PVDF membrane, and analyzed by Western blot with a monoclonal antibody directed against the HA affinity tag (Fig. 2A, top panel). A band corresponding to the expected molecular mass of HA-UL24 was detected in the lysates of cells infected with vHA-UL24 (lane 3) but not of cells infected with KOS (lane 2) or mock-infected cells (lane 1). The membrane was then stripped and incubated with antisemur raised against recombinant UL24 (Pearson and Coen, 2002) (Fig. 2A, middle panel). A protein of the same molecular mass as that detected by the HA antibody was present on the anti-UL24 blot (lane 3), however the precise spatial and temporal distribution of UL24 during infection remains to be elucidated.
Fig. 2. Characterization of vHA-UL24. (A) Western blot analysis of vHA-UL24-infected cells. Lysates from mock-infected cells or cells infected with either KOS or vHA-UL24 were subjected to Western blotting with an antibody directed against the HA epitope (top panel). The blot was subsequently stripped and incubated with antiserum raised against recombinant UL24 (middle panel). As a loading control, the blot was stripped and incubated with an antibody against gB (bottom panel). The positions of molecular mass makers are indicated to the left of the panels. Arrows indicate the positions of HA-UL24, UL24, or gB. (B) Viral yield assay. Vero cells were infected in duplicate with either KOS, vHA-UL24, or UL24XB at an MOI of 5. At the indicated hpi, total virus (supernatant and cell-associated) was harvested. Titers of harvested virus are presented in pfu per ml. Error bars represent standard error of the means. (C) ACV sensitivity plaque reduction assay. Monolayers of Vero cells were infected in triplicate with either KOS, vHA-UL24, or UL24XB at an MOI of 5. At the indicated hpi, total virus (supernatant and cell-associated) was harvested. Titers of harvested virus are presented in pfu per ml. Error bars represent standard error of the means. (D) Plaque morphology of vHA-UL24. Shown are photographs of Vero cells infected with KOS, vHA-UL24, or UL24XB, grown at 34 °C (top panels), 37 °C (middle panels), or 39 °C (bottom panels).
expressed the full-length tagged UL24. As a loading control, the membrane was stripped and incubated with an antibody against the viral protein gB (Fig. 2A, bottom panel). Expression levels of HA-UL24 and UL24 were similar despite the presence of a nucleotide substitution in the Kozak consensus sequence resulting from insertion of the HA tag sequence.

We characterized the replication of vHA-UL24 in cell culture. The viral yield of vHA-UL24 was compared to that of the wild-type KOS virus and the UL24-null virus UL24XB (Jacobson et al., 1998) in a one-step growth curve. Vero cells were infected at an MOI of 5 and total virus (supernatant and cell-associated) was harvested and titrated. The viral yield obtained for vHA-UL24 was similar to that obtained for KOS (Fig. 2B). In contrast, the viral yield of UL24XB was decreased between 5 and 10-fold as described previously (Jacobson et al., 1998).

We tested whether the nine amino acid insertion within TK, caused by the addition of the HA tag to UL24, resulted in the inactivation of the enzyme. Because TK is required to activate the drug acyclovir (ACV), viruses that do not express TK are not sensitive to the drug (Coen and Schaffer, 1980). To determine if vHA-UL24 expressed functional TK, sensitivity to ACV was tested in a plaque reduction assay comparing vHA-UL24 to KOS, using tkLTRZ1 as a negative control. We found that vHA-UL24 exhibited sensitivity to ACV similar to that of KOS, while tkLTRZ1 was resistant (Fig. 2C). Thus, we concluded that vHA-UL24 retained TK activity.

Viruses deficient in UL24 form small plaques at 37 °C, and syncytial plaques especially at 39 °C, while at 34 °C the plaques are non-syncytial (Jacobson et al., 1989). We compared the plaque phenotype of vHA-UL24 to that of KOS and UL24XB at 34, 37, and 39 °C. At 34 °C no syncytial plaques were observed with any of the viruses tested (Fig. 2D, top panels). At 37 °C the plaques formed by vHA-UL24 and by KOS were similar and were clearly larger than those formed by UL24X (Fig. 2D, middle panels). At 39 °C (Fig. 2D, bottom panels) the UL24-deficient phenotype is fully penetrant and, accordingly, we observed that all plaques formed by UL24XB at this elevated temperature were syncytial. In contrast, even at 39 °C the plaques formed by vHA-UL24, while appearing slightly smaller than those formed by KOS, were non-syncytial, suggesting that the HA tag did not impair whatever UL24 activity is responsible for inhibiting the formation of syncytial plaques. We concluded from our characterization of vHA-UL24 that HA-tagged UL24 expressed upon infection was at least partially functional and appeared to retain all activities of wild-type UL24 at 37 °C in cell culture, the temperature at which all of our subsequent experiments were conducted.

Time course of HA-UL24 expression

We investigated the kinetics of HA-UL24 expression and localization. Vero cells grown on coverslips were infected in parallel with either vHA-UL24 (Fig. 3, A panels) or, as a negative control, KOS (Fig. 3, B panels), at an MOI of 10. At 3, 6, 9, 12, 15, 18, 21, and 24 hpi cells were fixed and stored at 4 °C until the end of the time course. A negative control was included for each time point because we expected that non-specific staining, much of which was likely due to the expression of Fc receptors encoded by the late genes gI and gE (Johnson et al., 1988), would vary with time. Once all cells were fixed, the coverslips were processed in parallel to minimize variability in staining. Cells were visualized by confocal microscopy with the same laser intensity and other parameters used for each time point. At 3 hpi we did not observe any difference in staining between cells infected with KOS and vHA-UL24 (data not shown). At 6 hpi we observed slightly stronger staining in vHA-UL24-infected than in KOS-infected cells. At 9 hpi there was some cytoplasmic speckled staining. There was also diffuse nuclear staining, and foci of nuclear staining. This represented the major staining pattern observed. By 12 hpi the foci of nuclear staining were not as evident but the diffuse nuclear staining and the speckled cytoplasmic staining appeared to increase. We did not observe a large difference between 12 and 15 hpi, however, by 18 hpi the diffuse nuclear staining had cleared from many of the cells and a more prominent perinuclear staining was observed. This staining pattern intensified at 21 hpi. By 24 hpi this pattern was lost and there was intense staining throughout the cells (data not shown).

Subnuclear localization of HA-UL24

The foci of nuclear staining at 9 hpi were striking in that none of the other three proteins encoded by genes associated with the syn phenotype exhibits this localization, and thus we examined it further. Through a series of co-staining confocal microscopy experiments we tested whether the staining we observed corresponded to a known nuclear sub-structure, which might provide clues as to the function of UL24. The nucleus comprises multiple sub-domains that are structurally and functionally distinct. Viral replication compartments are one of the major nuclear sub-structures in HSV-1-infected cells. We tested whether nuclear HA-UL24 localized to replication compartments by co-staining for HA and the viral single-stranded binding protein ICP8 at 9 hpi (Fig. 4A, panels 1–3). At this time in infection the replication compartments occupied most, but not all, of the nucleus. Despite this distribution, we noted that the foci of HA-staining in nuclei did not co-localize with ICP8, implying that UL24 does not localize to viral replication compartments. We next co-stained infected cells for HA and nucleolin, a marker for nucleoli (Fig. 4B, panels 4–6). We found that the foci of HA staining did co-localize with nucleolin suggesting that HA-UL24 was present in nucleoli. To confirm the nucleolar localization of HA-UL24, we co-stained infected cells for HA and fibrillarin, a marker for the DFCs of nucleoli (Fig. 4C, panels 7–9). We found that foci of HA nuclear staining were generally located in the same regions that were stained for fibrillarin. However, upon closer examination it became evident that this was not strict co-localization, but that the...
staining for HA and fibrillarin was closely juxtaposed. This result was consistent with nucleolar localization of HA-UL24, but implied that HA-UL24 did not specifically localize to DFCs. At this time post-infection, both chromatin and nucleoli are marginalized to the edges of nuclei due to the growing replication compartments. To verify that the foci of nuclear staining we observed for HA-UL24 did not simply correspond to the subnuclear space left unoccupied by viral replication compartments, we tested whether HA-UL24 co-localized with another nuclear sub-structure by co-staining for HA and fibrillarin.

Fig. 3. Time course of HA-UL24 localization in infected cells. Vero cells were infected with either vHA-UL24 (A panels) or KOS (B panels) at an MOI of 10. Cells were fixed at the indicated times post-infection and processed for IF using a monoclonal antibody directed against HA and a secondary antibody conjugated to Alexa 488. A closeup of the staining at 9 hpi is included below the corresponding panel.
coilin, a marker for Cajal bodies. We did not observe co-localization using these two antibodies (Fig. 4D, panels 9–12). We also co-stained for HA and histone H1, which is present diffusely throughout the nucleoplasm. Such as has been seen previously (Simpson-Holley et al., 2004), we observed similar intensity staining for histone H1 both along the inner periphery of nuclei and extending to protrusions within nuclei (Fig. 4E, panel 14). As expected, we observed some co-staining between...
HA and histone H1 due to the diffuse nature of histone H1 localization and the partial diffuse nuclear distribution of HA-UL24. However, the pattern of histone H1 staining differed from the general pattern observed for HA-UL24 in that for the latter, little peripheral nuclear staining was observed, and the foci of staining were typically the most intense staining seen within nuclei (panels 13–15). This result shows that the pattern of nuclear staining for HA-UL24 did not simply represent all spaces left unoccupied by viral replication compartments and is consistent with our conclusion that the foci of nuclear staining for HA-UL24 we observed correspond to a distinct subnuclear compartment—the nucleolus.

The role of UL24 in virus-induced nucleolar modifications

It has been observed that nucleoli are altered during the course of HSV-1 infection (Besse and Puvion-Dutilleul, 1996) but little is known about the details of this alteration. In our staining for nucleolar markers, we found that at 9 hpi, the staining for nucleolin was more diffuse than expected. We therefore went on to test the effect of HSV-1 infection on the staining patterns of nucleolin and also of fibrillarin later in infection (Figs. 5 and 6). Mock-infected cells, or cells infected at an MOI of 10 with KOS, were fixed and stained at 9 and 18 hpi. Inocula were back-titrated to confirm the MOI used in

![Fig. 5. Effect of HSV-1 infection on the subnuclear distribution of nucleolin. Vero cells were mock-infected (panels 1 and 2) or infected at an MOI of 10 with either KOS (panels 3–6), UL24XB (panels 7–10), or UL24XG (panels 11–14). Cells were processed for IF at 9 and 18 hpi and were visualized by confocal microscopy. Cells were stained with a monoclonal antibody directed against nucleolin and an Alexa 488-coupled secondary antibody. Nuclei were stained with Draq5. The right-hand image of each pair of panels is a closeup of nuclei from the corresponding panel to the left.](image-url)
the experiments. As expected, in mock-infected cells, nucleolin staining was present in one or two relatively large, prominent spots within each nucleus. Very little staining in the nucleoplasm was visible (Fig. 5, panels 1 and 2). As we had seen before, at 9 hpi with KOS the foci of staining were still often visible though less prominent, and the diffuse nuclear staining seemed to increase somewhat (Fig. 5, panels 3 and 4). Strikingly, in KOS-infected cells at 18 hpi, foci of nucleolin staining were rarely visible and only some diffuse staining remained (Fig. 5, panels 5 and 6). We also looked at the effect of HSV-1 infection on the staining pattern of fibrillarin. In a previous report, fibrillarin staining was found to increase in intensity over the course of HSV-1 infection of rabbit fibroblasts (Lopez-Iglesias et al., 1988). Interestingly, in our experiments we did not observe an increase in fibrillarin staining in infected cells. In mock-infected Vero cells, fibrillarin was present in a characteristic collection of small spots clustered together at one or two sites within the nucleus (Fig. 6, panels 1 and 2). At 9 hpi with KOS, the clusters of spots appeared to disperse (Fig. 6, panels 3 and 4), and by 18 hpi, instead of clusters of smaller spots, only a few distinct, sometimes slightly larger spots were detected within nuclei (Fig. 6, panels 5 and 6).

Fig. 6. Effect of HSV-1 infection on the subnuclear distribution of fibrillarin. Vero cells were mock-infected (panels 1 and 2) or infected at an MOI of 10 with either KOS (panels 3–6), UL24XB (panels 7–10), or UL24XG (panels 11–14). Cells were processed for IF at 9 and 18 hpi and were visualized by confocal microscopy. Cells were stained with a monoclonal antibody directed against fibrillarin and an Alexa 488-coupled secondary antibody. Nuclei were stained with Draq5. The right-hand image of each pair of panels is a closeup of nuclei from the corresponding panel to the left.
Because of the co-staining between HA-UL24 and nucleolar markers at 9 hpi, when we began to observe more diffuse staining for nucleolin, we hypothesized that UL24 had a role in virus-induced nucleolar modifications. To test this hypothesis, we compared the staining patterns for nucleolin and fibrillarin of cells infected with two independent isolates of a UL24-deficient virus, UL24XB and UL24XG (Jacobson et al., 1998), to that which we saw with KOS-infected cells. In cells infected with either UL24XB or UL24XG, changes in the pattern of fibrillarin staining were similar to that which we observed in wild-type-infected cells, both at 9 and 18 hpi (Fig. 6, panels 7–14). However, we obtained a different result for nucleolin. In the absence of a functional UL24 gene, we found that prominent foci of nucleolin staining persisted, even at 18 hpi (Fig. 5, panels 7–14). On average, at 18 hpi, 83% of cells infected with a UL24-deficient virus (113 out of 139 cells total for UL24XB, and 119 out of 142 cells total for UL24XG) retained foci of nucleolin staining, as compared to 2% of KOS-infected cells (3 out of 165 cells total). The staining pattern was not perfectly identical to that of uninfected cells, which would be expected due to the marginalization of chromatin and nucleoli during infection.

We wanted to determine if the pattern of nucleolin staining that we observed in wild-type-infected cells late in infection was indeed due to the physical dispersal of the protein from the original nucleolar foci as opposed to a decrease in steady-state levels of the protein. To test this latter possibility, mock-infected Vero cells, or cells infected at an MOI of 10 with either KOS or UL24XB, were harvested at 18 hpi. Total cell lysates were resolved by SDS–PAGE, and nucleolin protein detected by Western blot (Fig. 7). The intensity of the signal for nucleolin in the three lysates was similar, indicating that there was no major difference in the steady-state levels of nucleolin. These results led us to conclude that UL24 is involved in HSV-1-induced nucleolar modifications at the level of nucleolin spatial distribution.

Discussion

We have taken a cell biological approach to investigate the role of UL24 in the infected cell. We generated a recombinant virus that expresses UL24 as a fusion protein with an HA tag and determined the localization of this protein in cells infected in culture through indirect IF. We observed staining for HA-UL24 in both the cytoplasm and the nucleus and identified the nucleolus as a nuclear sub-compartment targeted by UL24. We investigated virus-induced nucleolar modifications and found that, in addition to fibrillarin, the distribution of nucleolin was markedly altered in HSV-1-infected cells. Interestingly, this alteration of the host cell architecture was partially dependent on UL24, identifying a novel function for this widely conserved, but poorly understood gene.

Kinetics of UL24 localization

We did not detect any specific staining for HA-UL24 until 6 hpi. This is earlier than we had detected previously by Western blotting with UL24 antiserum (Pearson and Coen, 2002) and likely reflects increased sensitivity of the assay used here. The staining at this early time was very weak. At 9 hpi we observed accumulation of HA-UL24 in nucleolar structures as well as diffuse nuclear staining and speckled staining in the cytoplasm. From 9 to 15 hpi, the overall intensity of staining increased and we saw staining for HA-UL24 distributed between the cytoplasmic and nuclear compartments, which is similar to our previous fractionation results, where over the time period of 9 to 15 hpi, 50–70% of UL24 was nuclear-associated (Pearson and Coen, 2002). In fractionation experiments, HA-UL24 was also detected in both the nuclear-associated and cytoplasmic fractions (data not shown). The nucleolar localization of HA-UL24 was transient and by 12 hpi few foci of nuclear staining remained. This result may suggest that the function of nucleolar-localized UL24 is important prior to the very late stages of the HSV-1 life cycle. An association between both UL24 of HSV-2 (Hong-Yan et al., 2001) and the HCMV homolog UL76 (Wang et al., 2000) with nucleoli in the context of transient transfections has also been seen. This similarity suggests a conserved function for this pool of UL24. However, it is worth noting that, in HCMV-infected cell lysates, UL76 was detected as early as 1 hpi, even in the presence of the protein synthesis inhibitor cycloheximide. It is possible that differences in detection sensitivities are responsible for the discrepancy between the kinetics of appearance of UL76 and the UL24 proteins of HSV-1 and HSV-2 in infected cells, however it may also represent a fundamental difference in the role of UL24 between alpha and beta herpesviruses. At 18 and 21 hpi, we observed what appeared to be clearing of the diffuse nuclear staining concomitant with prominent perinuclear staining. The perinuclear staining pattern was similar to what is observed for other viral proteins encoded by genes that when deleted lead to a syncytial plaque phenotype, namely UL20 (Ward et al., 1994) and gK (Hutchinson and Johnson, 1995). This result could indicate that, like UL20 and gK, UL24 functions during viral egress, as proposed previously (Pearson and Coen, 2002). UL24 of HSV-2 and UL76 of HCMV have been detected in gradient purified virions (Hong-Yan et al., 2001; Wang et al.,...
2004), thus the cytoplasmic staining that we have observed could also represent staining of virions. The exact nature of the perinuclear localization at late times, and whether loss of UL24 activity affects assembly and egress of newly synthesized viral particles, remains to be established.

Nucleolar localization of UL24

We found that UL24 localized to nucleoli during infection, although a virally modified form of nucleoli. Nucleoli form around rDNA. Although we have no direct evidence that UL24 interacts with RNA or DNA, it is a highly basic protein with a predicted pi of 10.87, which would be consistent with nucleic acid binding activity. Furthermore, the putative endonuclease motif recently identified in UL24, if functional, would also suggest nucleic acid binding activity. It is known that during the course of HSV infection rRNA is retained in nucleoli (Besse and Puvion-Dutilleul, 1996), and thus UL24 might somehow contribute to this retention. Interestingly, UL24 appeared to be excluded from viral replication compartments, which are involved in viral DNA replication (de Bruyn Kops and Knipe, 1988), viral gene expression (Leopardi et al., 1997; Phelan et al., 1997; Rice et al., 1994), and encasement of viral DNA (de Bruyn Kops et al., 1998; Lamberti and Weller, 1998; Taus et al., 1998); however, we do not believe that its absence necessarily precludes a role for UL24 in these processes. In addition to UL24, only a limited number of HSV-1-encoded proteins have been detected in nucleoli during the course of infection, namely US11 (MacLean et al., 1987; Puvion-Dutilleul, 1987), ICP27 (Rice and Knipe, 1990), and ICP0 (Morency et al., 2005). The relationship, if any, between UL24 and these other nucleolar-associated viral proteins, remains to be determined. All of these proteins are known to play a role in the regulation of gene expression, which by association suggests that the nucleolar pool of UL24 may also have a regulatory function. Consistent with this hypothesis is the observation that in transient transfection assays HCMV UL76 modulates expression of certain reporter constructs (Wang et al., 2000).

A role for UL24 in HSV-1-induced nucleolar modification

Although it has been well established that HSV-1 infection results in dramatic changes to nuclear architecture, knowledge regarding the impact on nucleoli is limited. Electron microscopic analyses have shown that nucleoli become elongated and are marginalized to the inner edges of the nucleus, and rRNA is retained within nucleoli (Besse and Puvion-Dutilleul, 1996). By immunogold staining of infected rabbit fibroblasts, fibrillarin was found to increase in intensity and was localized not only to nucleoli, but also to viral dense bodies (Lopez-Iglesias et al., 1988). Fibrillarin is involved in multiple aspects of ribosome biogenesis. Temperature sensitive mutations in yeast fibrillarin have revealed roles in rRNA processing, rRNA methylation, and assembly of ribosomes (Tollervey et al., 1993). Interestingly, in KOS-infected Vero cells, although we did observe a disaggregation of fibrillarin spots, we did not detect an overall increase in staining for this marker as was seen in the previous report (Lopez-Iglesias et al., 1988). This difference cannot be attributed to strain variation because KOS was used in both studies, and thus perhaps reflects differences due to cell type.

During the course of infection, we found that staining for nucleolin dispersed becoming diffuse throughout the nucleus, despite there being little, if any change in steady-state levels of nucleolin in mock, KOS-, or UL24X-infected cells. Nucleolin is a multifunctional nuclear phosphoprotein involved in rDNA transcription, rRNA processing, and ribosome assembly, and it has been attributed with both positive and negative regulatory functions (reviewed in Srivastava and Pollard, 1999; Tuteja and Tuteja, 1998). Because the formation of nucleoli depends on active rDNA transcription, the dispersal of nucleolin and fibrillarin may be related to the approximate 85% decrease in rRNA synthesis that is associated with HSV-1 infection (Stenberg and Pizer, 1982; Wagner and Roizman, 1969). In addition, because the phosphorylation state of nucleolin regulates its function, it is possible that the major change in its subnuclear localization is mediated by post-translational modification. However, further experimentation will be required to test this hypothesis.

We discovered a role for UL24 in HSV-1-induced nucleolar modification. We made the intriguing observation that the disappearance of foci of nucleolin staining in KOS-infected cells was not evident in cells infected with either of two independent isolates tested of a UL24-deficient virus. The UL24XB and UL24XG strains were generated by insertion of stop codons in all three reading frames within sequence between the first and second UL24 homology domain (Jacobson et al., 1998). These viruses were shown to retain TK activity, thus the observed effect on nucleolin distribution we have seen with UL24XB and UL24XG can be attributed to the mutation in UL24. It remains to be determined whether the dispersal of nucleolin is a secondary consequence due to an effect of UL24 on rDNA transcription, or alternatively, via a direct or indirect effect of UL24 on nucleolin. Finally, we did not observe a dramatic effect of UL24 on fibrillarin redistribution, implying a dissociation between the spatial regulation of fibrillarin and nucleolin during infection.

It is not evident how nucleolar-localized UL24 would have an impact on fusion at the plasma membrane. One possible explanation is that different subcellular pools of UL24 may have different functions, and that only the cytoplasmic pool of UL24 is involved in membrane fusion events, while nucleolar UL24 would affect ribosome biogenesis for example. Consistent with this hypothesis would be that these multiple functions segregate with different domains of the protein. A detailed structure–function analysis of UL24 will be required to elucidate how this apparently multifunctional protein affects both subnucleolar and plasma membrane events. Further questions to be addressed concern the molecular mechanisms underlying the role of UL24 in nucleolar modifications during infection, in addition to the functional consequences of these viral induced changes.
Materials and methods

Viruses and cells

The HSV-1 strains KOS, tkLTRZ-1 (Davar et al., 1994), UL24XB, and UL24XG (Jacobson et al., 1998) were obtained from Donald M. Coen (Harvard Medical School). All viruses were propagated on Vero cells (African green monkey kidney cells) grown in Dulbecco’s Modified Eagle’s Media (DMEM) containing 4500 mg/l of glucose and supplemented with 5% newborn calf serum and the antibiotics penicillin and streptomycin. Cells were maintained in a humidified incubator with 5% CO₂ at 37 °C unless indicated otherwise.

Recombinant virus construction

To construct the transfer vector pKOSHA-UL24 two PCR products were generated such that their ligation resulted in an in-frame insertion of sequence encoding an HA tag at the 5’ end of UL24. The 5’ HA-UL24 fragment was generated using the primers 5’ phos-gtt cca gat tac get ggc geg aga acg cgc a and 5’ tgc tgg cgg tgt ccc cg (Integrated DNA Technologies) and was then digested with SacI. The 3’ HA-UL24 fragment was generated using the primers 5’ phos-atc gta tgg gta cat aac cga cgt acg g and 5’ gag ggc ggc gat ggg atg gc and was then digested with Bgl II. In each case the template used was pKOS17B2, which consists of the KOS BamHI “P” fragment subcloned into pBR325 (Weller et al., 1983), and the thermophilic polymerase used was Pfu turbo (Stratagene). To construct pKOSHA-UL24, a triple ligation was carried out between (1) pKOS17B2 digested with SacI and BglII; (2) the SacI-digested 5’HA-UL24 fragment; and (3) the BglII-digested 3’HA-UL24 fragment. After sequencing confirmed the correct product, the transfer vector was co-transfected with infectious tkLTRZ-1 DNA (Coen et al., 1989; Leib et al., 1989) into Vero cells using Effectene (Qiagen) transfection reagent following the manufacturer’s instructions. Screening for recombinant virus was done based on the formation of white plaques and confirmed by sequencing. Recombinant virus was purified by limiting dilution three times as described previously (Griffiths and Coen, 2003).

Acyclovir sensitivity assays

Sensitivity of viruses to acyclovir was assessed by plaque reduction assay, essentially as described previously (Coen et al., 1985).

Antibodies

The rat antiserum raised against UL24 has been described previously (Coen et al., 2002). For Western blotting of HA-tagged protein, anti-HA monoclonal antibodies from Covance were used. The remaining primary antibodies were as follows: rat monoclonal anti-HA high specificity (Roche), mouse monoclonal anti-gB (Fitzgerald Intl.), mouse monoclonal anti-ICP8 (Chemicon), rabbit monoclonal anti-coilin (Sigma), mouse monoclonal anti-C23 (nucleolin) (Santa Cruz Biotechnology), mouse monoclonal anti-fibrillarin (Covance), mouse monoclonal anti-histone H1 (Upstate), rabbit polyclonal anti-β actin (Biologend). Secondary antibodies used were as follows: ECL anti-rat IgG horseradish peroxidase conjugate (HRP) (GE), anti-mouse IgG HRP (Calbiochem), anti-rabbit IgG HRP (Jackson ImmunoResearch), Alexa Fluor 488 goat anti-rat IgG, Alexa Fluor 568 goat anti-mouse IgG, Alexa Fluor 568 goat anti-rabbit IgG, and Alexa Fluor 488 goat anti-mouse IgG (Invitrogen).

Immunostaining and fluorescence microscopy

Vero cells were grown on coverslips and infected at an MOI of 10 with KOS, vHA-UL24, UL24XB, or UL24XG. At the indicated hpi, cells were fixed and stained essentially as described previously (Chi and Wilson, 2000). Cells were washed with phosphate buffered saline (PBS) and fixed in 2% paraformaldehyde for 10 min. Fixed cells were stored at 4 °C overnight in PBS. Cells were permeabilized by incubation in 0.1% Triton X-100 diluted in PBS for 10 min, washed twice with PBS, and then blocked with NATS (20% NCS and 0.5% Tween 20 in PBS) for 30 min. Incubation with the appropriate primary antibody was carried out in a humidified chamber at 37 °C for 1 h. Cells were washed 4×5 min in PBS and incubated with the secondary antibody for 1 h. Coverslips were washed as described above, and, where indicated, nuclei were then stained with 2 μM Draq5 (Biostatus limited) for 3 min at 37 °C. After rinsing 1× in PBS, coverslips were mounted on microscope slides using Prolong or ProlongGold antifade reagent (Invitrogen). For the confocal images, cells were visualized using the BioRad Radiance 2000 confocal with an argon–krypton laser at 488 and 568 nm (diode 638) mounted onto the Nikon E800 microscope (100× objective, N.A. 1.4). Figures based on the TIF files of images were assembled using Adobe Photoshop.

Western blotting

Western blot analyses were carried out essentially as described previously (Pearson and Coen, 2002). Proteins were transferred to PVDF membrane. Detection was by Enhanced chemiluminescence plus (GE) according to the manufacturer’s instructions.

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References


Scott, E.S., O’Hare, P., 2001. Fate of the inner nuclear membrane protein lamin B receptor and nuclear lamins in herpes simplex virus type 1 infection. J. Virol. 75 (18), 8818–8830.


Taus, N.S., Salmon, B., Baines, J.D., 1998. The herpes simplex virus 1 UL 17 gene is required for localization of capsids and major and minor capsid proteins to intranuclear sites where viral DNA is cleaved and packaged. Virology 252 (1), 115–125.


Turner, A., Brun, B., Minson, T., Browne, H., 1998. Glycoproteins gB, gD, and gHgL of herpes simplex virus type 1 are necessary and sufficient to mediate membrane fusion in a Cos cell transfection system. J. Virol. 72 (1), 873–875.


