The conserved N-terminal domain of herpes simplex virus 1 UL24 protein is sufficient to induce the spatial redistribution of nucleolin

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UL24 is widely conserved among herpesviruses but its function during infection is poorly understood. Previously, we discovered a genetic link between UL24 and the herpes simplex virus 1-induced dispersal of the nucleolar protein nucleolin. Here, we report that in the absence of viral infection, transiently expressed UL24 accumulated in both the nucleus and the Golgi apparatus. In the majority of transfected cells, nuclear staining for UL24 was diffuse, but a minor staining pattern, whereby UL24 was present in nuclear foci corresponding to nucleoli, was also observed. Expression of UL24 correlated with the dispersal of nucleolin. This dispersal did not appear to be a consequence of a general disaggregation of nucleoli, as foci of fibrillarin staining persisted in cells expressing UL24. The conserved N-terminal region of UL24 was sufficient to cause this change in subcellular distribution of nucleolin. Interestingly, a bipartite nuclear localization signal predicted within the C terminus of UL24 was dispensable for nuclear localization. None of the five individual UL24 homology domains was required for nuclear or Golgi localization, but deletion of these domains resulted in the loss of nucleolin-dispersal activity. We determined that a nucleolar-targeting signal was contained within the first 60 aa of UL24. Our results show that the conserved N-terminal domain of UL24 is sufficient to specifically induce dispersal of nucleolin in the absence of other viral proteins or virus-induced cellular modifications. These results suggest that UL24 directly targets cellular factors that affect the composition of nucleoli.

INTRODUCTION

Herpes simplex virus 1 (HSV-1) encodes over 80 proteins. The UL24 gene is conserved among all three subfamilies of Herpesviridae. UL24 is required for efficient viral replication both in cell culture and in a mouse model of ocular HSV-1 infection, as well as for efficient reactivation (Jacobson et al., 1998). UL24 homologues in HSV-2 and varicella-zoster virus (VZV) have also been shown to play a role in viral pathogenesis (Blakeney et al., 2005; Ito et al., 2005). In cell culture, UL24 is one of a limited number of HSV-1 genes that when mutated causes the formation of syncytial plaques. The molecular basis for the phenotypes associated with UL24 mutations is unknown.

UL24 of HSV-1 is a highly basic protein of 269 aa. In contrast to the C-terminal domain of UL24, the N-terminal portion of the protein is highly conserved (Jacobson et al., 1989). Within this region, five clusters of well-conserved stretches of amino acids have been identified, termed UL24 homology domains (HD) (Fig. 1). More recently, a bioinformatics analysis suggested a PD-(D/E)XK endonuclease motif within the conserved region, the specific residues of which are contained within the second and third HD (Knizewski et al., 2006). A putative bipartite nuclear localization signal has been identified in the C-terminal domain of the protein, the importance of which has yet to be determined. In both cellular fractionation studies and confocal microscopy, UL24 protein has been found associated with the nucleus and is also present in the cytoplasm of infected cells (Lymberopoulos & Pearson, 2007; Pearson & Coen, 2002).

HSV-1 DNA replication, transcription and capsid assembly occur within nuclear structures termed viral replication compartments (reviewed by Knipe et al., 2007). Infection with HSV results in dramatic changes to nuclear structure and organization. These include the marginalization of chromatin following the expansion of viral replication compartments (Monier et al., 2000) and changes to the nuclear lamina (Scott & O’Hare, 2001; Simpson-Holley et al., 2004). The centromeric proteins CENP-A, -B, -C are degraded (Everett et al., 1999; Lomonte & Morency, 2007; Lomonte et al., 2001). Promyelocytic leukaemia (PML) bodies are disrupted (Maul et al., 1993, 1996; Uprichard & Knipe, 1997); however, new subnuclear compartments are formed including VICE domains, which are positioned on...
the external periphery of viral replication compartments and are involved in the unfolded protein response (Burch & Weller, 2004). Electron microscopy has also revealed that during HSV infection, the morphology of nucleoli is altered showing a more elongated structure (Besse & Puvion-Dutilleul, 1996).

Nucleoli constitute the sites of ribosome biogenesis, and more recently have been ascribed roles in cell-cycle control as sites of sequestration of cell-cycle modulators, and in response to stress (reviewed by Boisvert et al., 2007). In previous work, we discovered that upon HSV-1 infection, UL24 partially localizes to nucleoli. Furthermore, we found that in addition to changes in the subnuclear distribution of the nucleolar protein fibrillarin, infection entrains the dispersal of the nucleolar protein nucleolin throughout the nucleus, in a manner dependent on the expression of UL24 (Lymberopoulos & Pearson, 2007).

The principal aim of this study was to test the hypothesis that UL24 is not only necessary, but is sufficient to induce the dispersal of nucleolin, as part of our larger goal of understanding the role of nucleolar-localized UL24 in the modification of nucleolar organization during infection.

**METHODS**

**Cell culture.** COS-7 cells were propagated in Dulbecco’s modified Eagle’s media (DMEM) with high glucose, supplemented with 5% newborn calf serum, 50 U penicillin ml

![Image](http://vir.sgmjournals.org/1143)

**Fig. 1.** UL24 deletions. Diagram illustrating the deletion series of HA-tagged UL24 proteins used. (a) The N-terminal HA tag is represented by a hatched box. For the wild-type protein, the HDs are shown in solid black; the remainder of the protein is in grey. Dotted lines represent the deleted regions. (b) Western blot showing the expression of UL24 HD deletions. (c) Western blot showing the expression of the C-terminal and N-terminal portions of UL24. The positions of molecular mass markers are indicated to the left of the panels, and the position of the different UL24 proteins are indicated by arrows to the right.

site was produced by annealing the synthetic oligonucleotides 5'-GATCTTAATACGACTCACTATAGGGTGATGGCGG-3' and 5'-GATCCGAATTCGTACGCCCTATAGTGAGTCGTATTAA-3' (Integrated DNA Technologies) and was ligated with T4 DNA ligase (New England Biolabs) into the BamHI site of a pCG-Zeo based vector (von Messling et al., 2003) (provided by Veronika von Messling), forming pLBpL. The UL24 gene from the vector pAG5 (Griffiths & Coen, 2003) (provided by Donald M. Coen) or a version expressing UL24 fused with a haemagglutinin (HA) epitope tag from pKOSHA-UL24 (Lymberopoulos & Pearson, 2007) were excised using Pfl23HI (Fermentas) and BamHI (New England Biolabs) and inserted into pLBpL that had been digested with the same restriction enzymes, forming the vectors pLBpL-UL24 and pLBpL-HA–UL24, respectively.

Deletions of the sequences corresponding to individual UL24 HDs were generated by PCR-based site-directed mutagenesis using pairs of primers flanking each HD, and the vector pKOSHA-UL24 as template. Primers either were obtained with 5’ phosphate groups, or were phosphorylated in vitro using T4 polynucleotide kinase (Fermentas). Deletion of the individual HDs was performed as follows: delHD1 (aa 14–28 inclusively) was constructed using the primers DelHD1-5' and DelHD1-3'; delHD2 (aa 61–81 inclusively) with DelHD2-5' and DelHD2-3'; delHD3 (aa 111–131 inclusively) with DelHD3-5' and DelHD3-3'; the clone obtained for the deletion of HD3 containing primer DelHD3-5' and DelHD3-3' contained an unexpected additional deletion of 2 aa at the 5’ end of the HD3 domain, resulting in a deletion in aa 92–107 instead of 94–107. This mutation was particularly difficult to obtain, and thus we went forward with this clone despite the deletion of the two extra residues.

A deletion corresponding to the N-terminal portion of UL24, which includes all five HDs, was obtained by restriction digest of pKOSHA-UL24 with Pfl23HI and Bst1107I (Fermentas). The plasmid was then religated by inserting a synthetic linker that restored the sequence from the Pfl23HI site to the end of the sequence corresponding to the HA tag (annealed oligonucleotides HADE2N-top and HADE2N-bot). This resulted in an 86 aa protein beginning at residue 190 of UL24. To generate the C-terminal truncated version of HA–UL24, stop codons were introduced into the UL24 open reading frame (ORF) in
all three frames by inserting the annealed oligonucleotides DelC-top and DelC-bot into the BsrI107I restriction site, causing translation to end at aa 192.

Vectors expressing fragments of UL24 as fusion proteins with the enhanced green fluorescent protein (eGFP) were constructed as follows. Segments of UL24 flanked by 5′ HinIII and 3′ KpnI restriction sites were amplified by PCR using Pfu polymerase. The primers S1-S and S2-E were used for fragment S1-S2, S1-S and S3-E for S1-S3, S2-S and S3-E for S2-S3 and S3-S and S6-E for S3-S6. Primer sequences are detailed in Supplementary Table S1 (available in IGV Online). The PCR products were cloned into the plasmid pCR-TOPO-BluntII (Invitrogen) and subsequently excised with HinIII and KpnI (New England Biolabs). The inserts obtained were each ligated to the vector pEGFP-N1 (Clontech) that had also been digested with HinIII and KpnI.

**Protein expression.** To assess protein expression levels, 1 × 10⁵ COS-7 cells were seeded per well in six-well plates. The following day, cells were transfected with 5 μg (unless indicated otherwise) pLBPI-HA–UL24 or mutant versions using Lipofectamine transfection reagent (Invitrogen) according to the manufacturer’s instructions. The luciferase expression vector pGL3 (Promega) was co-transfected to normalize loading volumes. Two days post-transfection, cells were washed with PBS, lysed in luciferase assay lysis buffer, and assayed for luciferase activity using LARII luciferase assay reagent according to the manufacturer’s instructions (Promega). Lysates were resolved by PAGE on a denaturing SDS 12.5 % gel. The proteins were then transferred to a PVDF membrane, and analysed by Western blotting using a monoclonal antibody directed against HA (Covance) and a secondary antibody coupled to horseradish peroxidase (Calbiochem). Detection was by Enhanced Chemiluminescence Plus reagents (GE).

**Confocal microscopy.** COS-7 cells (4 × 10⁵) were seeded onto glass coverslips in 24-well plates. After 24 h, cells were transfected with expression plasmids using Fugene6 transfection reagent (Roche) according to the manufacturer’s instructions. Two days post-transfection, cells were immunostained as described previously (Lymberopoulos & Pearson, 2007). The following primary antibodies were used: anti-HA high affinity (Roche), anti-nucleolin (Abcam), anti-fibillin (Covance), anti-GM130 (BD Bioscience), anti-golgin-97 (Invitrogen), anti-mannosidase II (Abcam) and anti-z-tubulin (Calbiochem). Secondary antibodies included anti-rat, anti-mouse or anti-rabbit polyclonals coupled to Alexa-488, Alexa-568 or Alexa-647 (Invitrogen), as indicated in the text. Following immunostaining, where indicated, the coverslips were washed three times in PBS and incubated for 5 min with Draq5 (Biostatus) diluted 1 : 2000 in PBS. The coverslips were washed twice more in PBS and mounted onto glass slides using Prolong Gold Anti-Fade reagent (Invitrogen). For the nocodazole assays, cells were exposed to either 1 % DMSO or 1 % DMSO with nocodazole (Sigma) at a final concentration of 1 μg ml⁻¹ at 12 h post-transfection, and then fixed a further 12 h later. The slides were visualized using the confocal BioRad Radiance 2000 with an argon–krypton laser at 488 and 568 nm (diode 638 nm) mounted onto a Nikon E800 microscope. Images were prepared using Adobe Photoshop CS2.

**RESULTS**

**Construction of UL24 mammalian expression vectors**

In order to study the activity of UL24 in the absence of other viral proteins, and as part of a structure–function analysis, we generated a series of mammalian expression vectors encoding UL24 of HSV-1 with an N-terminal HA tag. We previously demonstrated that HA–UL24 retains wild-type activity when introduced into the viral genome in replacement of the endogenous UL24 gene (Lymberopoulos & Pearson, 2007). A diagram showing the various constructs is presented in Fig. 1(a). In addition to the wild-type version (HA–UL24), plasmids encoding only the N-terminal (HA–UL24Nterm) or C-terminal domain (HA–UL24Cterm) of the protein, or full-length versions lacking individual HDs (HA–UL24delHD 1–5) were engineered. Expression of the modified proteins was confirmed by Western blot analysis of lysates prepared from transiently transfected COS-7 cells (3 μg plasmid DNA) (Fig. 1b and c). We noted that steady-state levels of the proteins were similar, except for HA–UL24Nterm and HA–UL24Cterm for which we detected lower and higher levels, respectively, than those seen for the full-length forms of the protein. In order to observe both HA–UL24Nterm and HA–UL24Cterm on the same Western blot, 20 times less vector DNA corresponding to HA–UL24Cterm (0.15 μg) was transfected than for HA–UL24 wild-type and HA–UL24Nterm (3 μg) (Fig. 1c).

**Localization of UL24 in the absence of other viral proteins**

We tested how the subcellular localization of HA–UL24 in transfected cells compared to what we had found during infection by confocal microscopy (Lymberopoulos & Pearson, 2007). We observed pronounced perinuclear staining in virtually all transfected cells, as well as nuclear staining (Fig. 2). Two patterns of nuclear staining were evident. In the majority of nuclei (95 % of 263 cells counted), staining for HA–UL24 was diffuse (Fig. 2b); however, in a minority of cells (5 %), HA–UL24 was present in nuclear foci (Fig. 2c). No immunostaining was detected when only the secondary antibody was used (Fig. 2a). We next undertook co-staining experiments to determine the nature of the cellular structures targeted by HA–UL24. The pattern of perinuclear staining was

![Fig. 2. Cellular localization of UL24 in the absence of viral infection. The localization of HA–UL24 in transiently transfected COS-7 cells was assessed by confocal microscopy. Nuclei were stained with Draq5 (blue) and HA–UL24 was visualized by indirect immunofluorescence using a primary antibody specific for HA and a secondary antibody conjugated to the fluorophor Alexa-488 (green). (a) Negative control showing staining with the secondary antibody only. (b) The major staining pattern observed for HA–UL24. (c) The minor HA–UL24 staining pattern.](image-url)
suggestive of the Golgi apparatus, which is composed of three major compartments, the cis-Golgi, medial-Golgi and trans-Golgi. Evidence points to Golgi- and possibly endosome-derived vesicles as playing roles in the secondary envelopment of viral particles during egress (Harley et al., 2001; Turcotte et al., 2005). To determine if indeed the perinuclear staining we observed for HA–UL24 corresponded to the Golgi apparatus, we co-stained for HA and markers of different Golgi compartments. The images shown represent the most common staining pattern observed for each marker (Fig. 3). In co-staining experiments for HA–UL24 and golgin-97, a trans-Golgi-associated protein, we observed direct overlap of staining in 64% of cells, and partial overlap of staining in 15% of cells (Fig. 3a). In contrast, staining for GM130, a cis-Golgi marker, appeared to surround that of HA–UL24 in many cells with no overlap detected in 48% of cells. Furthermore, only 15% of cells expressing HA–UL24 exhibited direct overlap of staining for HA and GM130, and in 37% of cells a partial overlap of staining was seen (Fig. 3b). We obtained an intermediate result when we co-stained using an antibody specific for mannosidase II (Fig. 3c), a marker for medial-Golgi. We found that 18% of cells exhibited direct overlap of staining with HA, and 59% showed a partial overlap of staining. Between 75 and 86 HA–UL24-expressing cells were counted in each co-staining experiment with the Golgi markers. The perinuclear staining we observed for HA–UL24 could also be indicative of the formation of aggresomes, which consist mainly of an accumulation of misfolded proteins. To distinguish between these two possibilities, we tested the effect of nocodazole treatment, a microtubule depolymerization agent (De Brabander et al., 1976), on the cytoplasmic staining pattern seen for HA–UL24. Aggresome formation is tightly linked to the microtubule-organizing centre, but once formed, its localization and shape is not affected by the disruption of the microtubule network (Johnston et al., 1998). In contrast, the Golgi apparatus becomes fragmented under these same conditions (Rogalski & Singer, 1984). We found that treatment of HA–UL24-expressing cells with 1 mg nocodazole ml⁻¹ 12 h post-transfection (Fig. 3e), but not with DMSO alone (Fig. 3d), resulted in the absence of perinuclear staining for HA–UL24. These results support the notion that the perinuclear staining seen for HA–UL24 represents genuine Golgi localization.

To determine if the nuclear foci of HA–UL24 labelling we observed corresponded to nucleoli, cells were co-stained for HA and fibrillarin (Fig. 4a). Where foci of HA–UL24 staining were observed, the staining co-localized with that of fibrillarin, indicating that nucleolar localization of HA–UL24 was possible in the absence of other viral proteins. Interestingly, when we co-stained for nucleolin, we observed two patterns. In cells exhibiting foci of HA–UL24 staining, these foci co-localized with foci of nucleolin staining (Fig. 4b). However, in those cells where HA–UL24 staining was diffuse throughout the nucleus, the major staining pattern, staining for nucleolin was also diffuse (Fig. 4c).

UL24-induced dispersal of nucleolin

To establish that the dispersal of nucleolin was indeed associated with UL24, we quantified the effect of UL24 expression on the percentage of cells exhibiting this
staining pattern. To identify transfected cells, the reporter construct pEGFP-N1 was co-transfected with either pLBPlf-UL24, pLBPlf-HA–UL24 or the corresponding empty vector. We assumed that the majority of cells expressing eGFP would also contain the second expression vector. When we stained cells for nucleolin and counted the number of eGFP-positive cells that retained foci of nucleolin staining, we found that there was a positive correlation between UL24 expression and nucleolin dispersal (Fig. 5a). The effect was similar for UL24 and HA–UL24, in that an average of 70% of eGFP-positive cells co-transfected with a UL24 expression plasmid exhibited diffuse staining for nucleolin, as opposed to less than 10% of cells transfected with the empty vector. Thus, UL24 was sufficient to induce the dispersal of nucleolin. This effect appeared to be specific, because in over 75% of cells expressing either UL24 or HA–UL24, foci of fibrillarin staining were detected (Fig. 5b). Furthermore, in triple labelling experiments, we found that foci of fibrillarin staining persisted in cells exhibiting diffuse nuclear staining for both HA–UL24 and nucleolin (Fig. 5c).

The N-terminal domain of UL24 is sufficient for targeting to the nucleus and dispersal of nucleolin

Analysis of the primary sequence of UL24 identified a putative bipartite nuclear localization signal in the C-terminal non-conserved region of HSV-1 UL24 (Pearson & Coen, 2002). We tested whether this domain functioned as the nuclear localization signal for UL24, and hence contributed to its ability to reach the nucleolus. We compared the localization and effect on nucleolin of the N-terminal domain, which contains the five HDs, to that observed with the C-terminal domain (Fig. 6). Because we had observed an approximately 40-fold difference in expression levels of HA–UL24Nterm and HA–UL24Cterm, we adjusted the relative amounts of plasmid DNA transfected accordingly (25 ng for HA–UL24Cterm and 1 μg for HA–UL24Nterm). We previously confirmed that the staining patterns we observed for HA–UL24 did not vary over a range of 25 ng to 1 μg of transfected plasmid DNA (Supplementary Material available in JGV Online). We found a clear segregation with regards to the subcellular localization of the two parts of UL24. Surprisingly, the C-terminal domain containing the putative nuclear localization signal was excluded from the nucleus and accumulated in the Golgi apparatus (Fig. 6a and c). In contrast, the conserved N-terminal domain was detected exclusively in the nucleus (Fig. 6b and d), primarily in a diffuse pattern similar to what we had observed for full-length HA–UL24. Furthermore, foci of nucleolin were intact in cells expressing only the C terminus of UL24 (Fig. 6a), while those expressing the N-terminal domain exhibited diffuse nucleolin staining.

Fig. 4. Targeting of UL24 to nucleoli. Confocal microscopy images showing staining patterns for HA–UL24 and nucleolar markers. (a) COS-7 cells transiently expressing HA–UL24 were co-stained for HA and the nucleolar protein fibrillarin. Shown is a cell that exhibits the minor HA–UL24 distribution pattern. (b) A cell that exhibits the minor HA–UL24 distribution pattern co-stained for HA and nucleolin. (c) A cell that exhibits the major HA–UL24 distribution pattern co-stained for HA and nucleolin. Secondary antibodies coupled to Alexa-488 (green) or Alexa-568 (red) were used for HA and nucleolar proteins, respectively. Merged images are shown in the right-hand panels. Arrows indicate sites of co-localization.
We obtained the same results when identical amounts of each plasmid (200 ng) were used (data not shown). Thus, we concluded that the putative C-terminal nuclear localization signal was not required for the nuclear localization of UL24, and that the N-terminal domain of UL24 was sufficient for nucleolin dispersal.

Having determined that the conserved N-terminal domain of UL24 accumulated efficiently in the nucleus and induced the dispersal of nucleolin, we next tested the role of the HDs on these activities. A series of expression vectors encoding versions of UL24 lacking individual HDs were tested in transient transfection experiments (Fig. 7). Co-staining of cells for GM130 and HA–UL24 revealed that the HDs were dispensable for localization of the protein to the Golgi apparatus (data not shown). We also found that HA–UL24 proteins lacking any one of the five HDs were still able to localize to nuclear foci. The means from two independent experiments, where between 93 and 266 cells were counted for each construct, were calculated. Unlike wild-type HA–UL24 that exhibited nucleolar foci of

**UL24 homology domains are dispensable for nucleolar targeting**

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Fig. 7. Cellular localization of UL24 HD deletion variants. Confocal images showing co-staining for HA and nucleolin of cells expressing HA–UL24 proteins harbouring HD deletions. (a) The major staining pattern for wild-type HA–UL24 is shown. (b–f) Localization of UL24 variants with deletions corresponding to HD 1–5, respectively. Secondary antibodies used were as described in Fig. 4. Merged images are shown in the right-hand panels. Arrows indicate sites of co-localization.
staining in only 5% of cells, the value was greater than 90% for each of the deleted-forms of HA–UL24, namely 91 ± 2.4% for HA–UL24del1, 96 ± 1.3% for HA–UL24del2, 97 ± 0.05% for HA–UL24del3, 96 ± 0.60% for HA–UL24del4 and 97.0 ± 0.74% for HA–UL24del5. Co-staining for fibrillarin in cells expressing the HD-deleted proteins revealed that these foci were nucleoli (data not shown). Interestingly, similar foci were also seen for nucleolin, indicating that, unlike wild-type UL24 (Fig. 7a), the deleted forms of UL24 had lost the ability to induce nucleolin dispersal (Fig. 7b–f). Thus, the integrity of each of the HDs was required for UL24 to induce the dispersal of nucleolin, but not for targeting to nucleoli.

Identification of a nucleolar targeting signal in UL24

To identify the sequence in UL24 that directs nucleolar targeting, we undertook a systematic analysis. Vectors expressing various segments of UL24 as fusion proteins with eGFP were transiently transfected into COS-7 cells, and the ability of the fragments to direct eGFP to nucleoli was determined by confocal microscopy. Transiently expressed eGFP was distributed between the nuclear and cytoplasmic compartments, but was excluded from nucleoli (Fig. 8c, left-hand panel), thus allowing the unambiguous determination of nucleolar-targeting in this assay. A schematic representation illustrating the various constructs tested is presented in Fig. 8(a). Full-length eGFP fusion proteins to fragments S1–S2, S2–S3, S1–S3 and S3–S6 were detected by Western blot analysis (data not shown). Through standard deletion analysis we found that the N-terminal 60 aa fragment of UL24, when fused to eGFP, was sufficient to direct the protein to nucleoli (Fig. 8b). We found that in approximately 40% of cells expressing eGFP–S1–S2, the eGFP signal was not excluded from the nucleolus as seen with unfused eGFP alone. Of those, between 15 and 20% exhibited a prominent nucleolar signal as shown in Fig. 8(c), middle panel. Thus, we concluded that fragment S1–S2 contains a nucleolar localization signal.

DISCUSSION

Localization of UL24 in the absence of other viral proteins

The staining pattern we observed for HA–UL24 when expressed out of the context of infection showed similarities with the localization of HA–UL24 in infected cells (Lymberopoulos & Pearson, 2007). In each case, co-localization with nucleolar markers was observed, as well as perinuclear staining. However, while the perinuclear staining in transfected cells co-localized with the trans-Golgi marker golgin-97, in HSV-1-infected Vero cells, the Golgi apparatus becomes fragmented (Avitabile et al., 1995; Campadelli et al., 1993). The staining pattern for golgin-97 is greatly altered in HSV-infected Vero cells and we have not observed co-localization with HA–UL24 (M. H. Lymberopoulos & A. Pearson, unpublished data). Thus, it is possible that the perinuclear accumulation of HA–UL24 upon transient transfection and in the context of infection correspond to different structures, or perhaps virus-modified Golgi-related vesicles in the case of infected cells.

Our observation that wild-type UL24 of HSV-1, while able to target nucleoli, is more often detected dispersed throughout the nucleus may explain previous reports of dispersed nuclear staining, but not nucleolar localization, of the VZV UL24 homologue, gp35 (Ito et al., 2005), and the murine gammaherpesvirus 68 homologue ORF20 (Nascimento & Parkhouse, 2007). However, foci of nuclear staining suggestive of nucleoli have been observed for the HSV-1 UL24 homologues of HSV-2 (Hong-Yan et al., 2001) and human cytomegalovirus (Wang et al., 2000). When cells were observed where both UL24 and nucleolin were dispersed but fibrillarin exhibited the typical staining pattern for cells in interphase, this strongly suggests that
UL24-induced dispersal of nucleolin is not a consequence of a general disaggregation of nucleoli. We propose that wild-type UL24 targets nucleoli leading to the dispersal of nucleolin, which is associated with the dispersal throughout the nucleus of UL24 itself.

**UL24 induces dispersal of nucleolin**

Ribosome biogenesis is downregulated during HSV infection (Stenberg & Pizer, 1982; Wagner & Roizman, 1969). Nucleolin functions in both RNA polymerase I transcription and rRNA maturation, and thus plays an important role in ribosome biogenesis (reviewed by Mongelard & Bouvet, 2007; Rickards et al., 2007). An attractive hypothesis is that, during infection, UL24 contributes to this change in cellular metabolism through an effect on nucleolin. It remains to be determined if UL24 is either necessary or sufficient to direct this down-regulation, and if so, whether the effect is direct or indirect. However, our finding that UL24 was sufficient to induce dispersal of nucleolin indicates that UL24 interacts directly with one or multiple cellular components. Nucleoli are also involved in cell-cycle regulation (reviewed by Boisvert et al., 2007). Because the spatial distribution of nucleolar proteins varies during the cell cycle, dispersing in the M phase, it is possible that the effect of UL24 on nucleolin is related to an effect on cell-cycle progression. Consistent with this hypothesis, it was recently reported that the UL24 homologue of murine gammaherpesvirus 68, ORF20, when expressed in 293T cells, is associated with a cell-cycle block in G2 (Nascimento & Parkhouse, 2007).

**Functional dissection of UL24**

We discovered that the C-terminal domain of UL24 was both necessary and sufficient for targeting the Golgi apparatus. Because Golgi-derived vesicles appear to play a role in the final envelopment of viral particles, one hypothesis is that this C-terminal domain would play a role in the modulation of membrane fusion events late in infection. Disruption of these activities would then explain the syncytial plaque phenotype associated with UL24 mutations. However, it is also possible that, while Golgi-targeting information is contained within the C terminus, functions encoded in the N-terminal portion of the protein are still required for fusion-related activities.

We found that the nucleolin-dispersal activity was contained within the conserved N-terminal domain of UL24, suggesting that this function may be shared among virtually all herpesviruses, and represents a critical function in the viral life cycle. Our observation that deletion of any one HD abrogated the ability to disperse nucleolin could reflect the loss of specific interactions with cellular components, but it is also possible that the deletions impede correct folding without changing steady-state levels of the protein or inhibiting the nucleolar localizing activity. Contained within the UL24 HDs are the sequences that make up the putative PD-(D/E)XK endonuclease motif. At present, the functionality of this motif is unknown; however, the amino acids constituting the predicted catalytic domain are highly conserved among herpesviruses of all families. The experiments presented here do not allow us to establish if the putative endonuclease motif plays a role in nucleolin dispersal. Further analyses involving mutations that specifically target the core residues of the putative catalytic site will be required to establish the importance of this motif in nucleolar modifications.

We found that a fragment corresponding to the first 60 aa of UL24 was able to target nucleoli; however, it did not appear to be sufficient to disperse nucleolin (data not shown), which suggests that functions in the remainder of the N-terminal domain of UL24 must be important for this activity. This result is consistent with our finding that each of the HD deletion variants could target nucleoli but not affect the distribution of nucleolin.

Our discovery that expression of UL24 alone is sufficient to modify the composition of nucleoli leads to further questions regarding the function of this cellular modification and the mechanisms involved. It will be of particular interest to determine the relationship between the nucleolin-dispersal activity of UL24 and UL24-mutant phenotypes observed in cell culture and in an animal model of infection.

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