Nucleolin interacts with Us11 protein of herpes simplex virus type 1 and is involved in its trafficking.
ABSTRACT

Herpes simplex virus type 1 infection induces profound nucleolar modifications at the functional and organizational levels, including nucleolar invasion by several viral proteins. US11 is one of these proteins that exhibits several different functions and displays both cytoplasmic localization and clear nucleolar localization that is very similar to that of the major multifunctional nucleolar protein nucleolin. To determine whether US11 interacts with nucleolin, we purified US11 protein partners by co-immunoprecipitations using a tagged protein, Flag-US11. From extracts of cells expressing Flag-US11 protein, we co-purified a protein of about 100 kDa that was further identified as nucleolin. *In vitro* studies have demonstrated that nucleolin interacts with US11 and that the C-terminal domain of US11, which is required for US11 nucleolar accumulation, is sufficient for interaction with nucleolin. This association was confirmed in HSV-1-infected cells. We found an increase in the nucleolar accumulation of US11 in nucleolin-depleted cells, thereby revealing that nucleolin could play a role in US11 nucleo-cytoplasmic trafficking through a one-way directional transport out of the nucleolus. Since nucleolin is required for HSV-1 nuclear egress, the interaction of US11 with nucleolin may participate in the outcome of infection.
INTRODUCTION

The genome of herpes simplex virus type 1 (HSV-1) contains more than 80 genes that have been grouped into two different categories related to the function of their products. *In vivo*, most are “essential genes” that code for proteins that are essential for viral gene expression, DNA synthesis, and the assembly of viral particles; the remaining are “non-essential genes” that encode products that allow viral production in various cellular conditions (29). Because all of these non-essential genes are dispensable for virus growth in cell culture, the precise functions of most of them remain to be elucidated.

HSV-1 *US11* gene was initially described as one of these non-essential genes (5, 21, 22). US11 is an abundant 21-kDa late viral protein, packaged within the viral tegument and delivered into newly infected cells prior to the expression of viral genes. Although US11 is dispensable for infection of cell cultures, it may play a role in the replication of HSV-1 in the adrenal gland, an organ important for viral penetration into the spinal cord and the brain (18, 26), and in cells subjected to thermal stress (5, 13). Also, US11 is involved in the anti-viral response (20) and displays anti-apoptotic activity, notably against heat-induced apoptosis, which appears to be located at the level of mitochondria or upstream signaling (19).

Since the first identification of US11 as a DNA-binding protein (9), US11 has been demonstrated to exhibit several different functions. Strong evidence shows that US11 is an RNA-binding protein that binds RNA in a sequence- and conformation-specific fashion and also displays a role in post-transcriptional regulation of gene expression (30, 31). Moreover, by its ability to bind certain mRNAs, we have demonstrated that US11 displays striking functional similarities to HIV-1 Rev and human T-cell leukemia/lymphoma virus type I (HTLV-I) Rex proteins. US11 can substitute for Rev and Rex and intervenes post-transcriptionally in the life cycle of these retroviruses by transactivating expression of the genes that encode HIV-1 and HTLV-I envelope (env) glycoproteins (10).

In addition to RNAs, US11 interacts with cellular proteins. Until now, the only proteins known to interact with US11 were human ubiquitous kinesin heavy chain (14), PAT1,
which is a homolog of kinesin light chain (2), PKR a double-stranded RNA-dependent
protein kinase (27), PACT, which is a dsRNA-independent protein activator of PKR (20,
27), and 2'-5'-oligoadenylate synthetase (33).

During HSV-1 infection, the incoming US11 protein is delivered early into the cells. Soon
after infection, US11 is found in the cytoplasm, either as a heterogeneous oligomer or
associated with the ribosomes or both. Later during infection, US11 accumulates in
nucleoli but is also found in ribonucleoprotein fibrils and in clusters of interchromatin
granules (12, 25, 28).

The nucleolus consists of fibrillar centers (FC), dense fibrillar components (DFC), and
granular components (GC). Ribosomal RNA genes are localized in the FC, pre-rRNA
resides in the DFC, and the late processing steps of ribosome biogenesis occur in the
GC. When US11 concentrates in nucleoli, it is abundant in the DFC and GC but absent
from the FC (3), an intra-nucleolar distribution that is similar to that of nucleolin.
Indeed, nucleolin, one of the most abundant nucleolar components, is usually found in
the DFC and GC of nucleoli. Its interaction with ribosomal proteins and with specific pre-
rRNA sequences and its implication in pre-rRNA maturation suggest that nucleolin could
be an important ribosome assembly factor (4). Several of our observations suggest that
nucleolin is also a major factor in promoting cell proliferation (39, 40).

During HSV-1 infection, a fraction of nucleolin is depleted from the nucleolus and is
found in the viral replication compartments (6, 23, 24). Nucleolin is one of the few
cellular proteins that are required for HSV-1 infection (6); recent data indicate that
nucleolin is needed for efficient nuclear egress of HSV-1 nucleocapsids (32).

Since US11 and nucleolin share a similar localization and because we have shown that
HSV-1 infection is prevented in nucleolin-depleted cells, we designed a strategy to
analyze whether these two proteins interact. Using a co-immunoprecipitation approach
coupled with western blot and mass spectrometry analysis, we show that nucleolin and
US11 are present in the same protein complexes in cells expressing recombinant US11
proteins and, more importantly, in HSV-1-infected cells. In vitro interaction experiments
and far-western blot analysis reveal that US11 interacts with nucleolin and that the polyproline type II helix-containing domain of US11 is responsible for this interaction. The fact that this domain is also responsible for US11 nucleolar accumulation and that nucleolin is a nucleo-cytoplasmic shuttling protein suggests that the interaction between US11 and nucleolin could regulate the intracellular trafficking of US11 or the molecular processes in which these proteins are involved during infection. Indeed, we show that in nucleolin-depleted cells, US11 accumulates in nucleoli, indicating that nucleolin contributes to US11 nucleolar export and strongly supporting the notion that the interaction between nucleolin and US11 plays a role in the outcome of infection.
MATERIALS AND METHODS

Plasmids

The plasmids pHTLV-env, pcREX, and pcTax that express HTLV env, Rex, and Tax, respectively, pCMV-US11 that expresses US11, plasmids that code for the recombinant proteins GST-US11, GST-US11(Δ1-40), GST-US11-m5, GST-US11-m6, and GST-US11-m15 derived from pGEX-2T that have been described previously (7, 10, 34) were used in this study. Briefly, GST-US11 contains the 149 amino acids (aa) of full-length US11 from the KOS strain, whereas GST-US11 (Δ1–40) contains aa 41 to 149 of the US11 protein, GST-US11-m5 contains aa 36 to 149, GST-US11-m6 contains aa 62 to 149, and GST-US11-m15 contains aa 1 to 40. pSG5-Flag-US11 vector expressing flag-US11 (full-length US11 fused to Flag sequence) was obtained by inserting the HindIII/BglII fragment of plasmid pGEX-2T-US11 (7) into the pSG5-flag vector.

Cell culture, transfection, infection, and functional assay

HeLa cells were grown in Eagle’s essential minimal medium (E-MEM) containing 100 U/ml penicillin and 100 µg/ml streptomycin with or without 5% heat-inactivated fetal calf serum (FCS) at 37°C under 5% CO2. Cells were transfected for 48 h using the calcium phosphate precipitation procedure (17). When indicated, HeLa cells were infected just before confluence with HSV-1 17+ wt strain with 10 plaque-forming units (PFU) per cell. After 1 h of adsorption of the virus at 33°C under 5% CO2, the media was removed, and the cells were washed and then incubated in E-MEM at 37°C until harvesting at the indicated times post-infection. US11 can substitute for Rex in transactivating expression of the retroviral genes encoding HTLV-I env glycoproteins (10). Nucleolin silencing by siRNA

A mixture of functional siRNAs (Eurogentec) specific for human nucleolin siRNA #4 (UUCUUGACAGGCUCUCCCU) and siRNA #2 (UCCAAGGAUCUUUAUUCUU) was used as previously described (6, 40). siRNAs were reconstituted at a concentration of 100 nM and stored at −20°C. The HL5a1 clones of HeLa cells that stably expressed US11 (13, 37) were transfected in a 6-well dish using siRNA at 2 nM final concentration. siRNAs were diluted in 200 µl of Opti-MEM and plated in a well. Eighty microliters of INTERFERin (Polyplus) diluted 1:10 in RNase-free water was added. After 10 min of incubation, 2 ml of media containing 3 x10^5 cells was added.
Immunofluorescence and fluorescence quantification

Cells were either infected for 8 h with the HSV-1 17+ wt strain or transfected for 48 h with the indicated expression vectors. Then, cells were washed with phosphate-buffered saline (PBS), fixed and submitted to immunohistochemistry as described (6). For nucleolin siRNA treatment, cells were plated at \(4 \times 10^4\) cells/well in 24-well dishes onto glass coverslips 2 days after nucleolin siRNA transfection. Four days after siRNA transfection, cells were fixed and treated as previously described (40). Rabbit polyclonal antibodies for US11 (12) or nucleolin (15) and mouse monoclonal anti-nucleolin (4E2) antibodies were the primary antibodies used. Secondary antibodies were coupled to Alexa dyes (A647 or A488, Invitrogen, A546, A555, Interchim). For Fig. 1, appropriate confocal emission filters were used for double-labeling experiments. Images were merged by computer. To control for cross-reactivity, samples were stained with one primary antibody and appropriate secondary antibodies. No overlap between the channels was observed for any of the samples at the settings used. For Fig. 6 (and Fig. 1B, bottom), 12 bit-images were acquired with the same exposure times using a Cool Snap HQ CCD camera mounted on a Zeiss Axio-Imager equipped with a 63X oil-immersion objective lens (NA = 1.4) and fluorescence filters suited to visualize 4′-6-diamidino-2-phenylindole (DAPI), A488 and A647. For each field of view, Z-stacks of about 10 images with a pixel size of 102 nm were obtained by setting the Z-step at 200 nm. For each analyzed cell, the optical section in which the nucleoli were the most in focus was chosen for quantification. We ensured that the choice of the section did not significantly affect the US11 nucleo-cytoplasmic ratio (US11 ratio variations <0.1 among 5 successive optical sections). Quantification of US11 intensities was performed on the raw selected 12-bit-section using ImageJ freeware. Outlines of the cells were hand drawn on the green (US11) channel section and used to obtain the mean integrated intensity, referred to as the US11 cellular level in Fig. 6F. The US11 nucleo-cytoplasmic ratio was calculated for each cell by dividing the mean nucleolar intensity by the mean cytoplasmic intensity obtained on the green channel section as follows. For each cell, outlines of every nucleolus were hand drawn, and the corresponding integrated
intensities were summed and divided by the sum of their areas to obtain the mean nucleolar intensity. The mean cytoplasmic intensity was calculated by subtracting US11 integrated intensity of the nucleus to that of the cytoplasm divided by the difference of their areas. The outline of the nucleus was hand drawn on the blue (DAPI) channel section and was copied onto the green channel to determine the mean US11 nuclear integrated intensity. This analysis was performed on at least 16 individual cells and observed on 5 (C, MT, SC conditions) or 10 (NCL condition) different fields of view. The corresponding means and standard deviations are plotted on Fig. 6F for each condition.

Immunoprecipitation and 1-DE electrophoresis of proteins and protein identification by mass spectrometry and western blot analysis

Cells were harvested 48 h post-transfection, or 8 and 16 h after infection with the HSV-1 17+ wt strain. To immunoprecipitate cellular multiprotein complexes that contained US11, several cell lysis procedures were evaluated including different detergents such as NP-40, Triton X-100, SDS (Sodium Dodecyl Sulfate), DOC (deoxycholic acid), and CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate). The experimental conditions using an anti-Flag antibody were optimized to efficiently and reproducibly co-purify Flag-US11 and proteins that specifically bind to Flag-US11 and not to Flag alone (data not shown). The optimal lysis buffer retained contained 0.1% Triton X-100 and 0.1% CHAPS. Cells were washed with PBS and then lysed at 4°C for 30 min in 1 ml of RIPA buffer (50 mM Tris-HCl pH 7.2, 0.1% Triton X-100, 0.1% CHAPS) that contained protease inhibitor and was either supplemented or not with NaCl from 50 to 500 mM. Cell extracts were centrifuged at 75000 rpm (TLA 100.3 Beckman rotor) for 30 min at 4°C. Supernatants were incubated overnight with the M2 anti-Flag antibody or a rabbit polyclonal anti-nucleolin antibody (PB, unpublished data). Immunocomplexes were immobilized for 30 min at 4°C on protein-A sepharose beads. After extensive washing with RIPA buffer supplemented with the appropriate amount of NaCl, bound proteins were eluted by addition of Laemmli buffer and were then separated by monodimensional gel electrophoresis (1-DE) (11) and stained with either Coomassie blue R250 Biosafe or silver nitrate. Western blot analyses were performed as described (6). For mass spectrometry analyses, proteins present around 100 kDa in Coomassie blue-stained gel slices were submitted to in-gel digestion as described previously (35). Resulting
peptides were analyzed by nano-liquid chromatography coupled to tandem mass spectrometry as described in (8), and proteins were identified using Phenyx software (Genebio).

**Protein purification and protein-protein interaction assays**

Recombinant GST-US11 proteins and GST protein were produced in *Escherichia coli* DH5a and purified from bacterial lysates by affinity chromatography on glutathione-agarose beads as previously described (34). Nucleolin-p50 (also called R1234G in 36) was produced in E. coli and purified as previously described (36). Nucleolin purified from CHO cells was biotinylated as previously described (15). Biotinylated nucleolin-US11 complexes were recovered with magnetic streptavidin beads (Dynal). Beads were first extensively washed with binding buffer (BB: 15 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 150 mM NaCl, 0.1% Tween 20, 0.1% BSA). One hundred nanograms of biotinylated nucleolin was incubated with approximately 200 ng of GST or GST-US11 protein in 100 µl of BB for 60 min at 4°C. The beads were then extensively washed with BB, and proteins bound to streptavidin beads were eluted in Laemmli electrophoresis buffer and then separated by 1-DE.

**Far-western blot analysis**

Far-western blot analysis of the interaction of nucleolin with US11 deletion mutants was performed as previously described (4, 15). After 1-DE separation, proteins were transferred to nitrocellulose membranes, and immobilized proteins were stained with Ponceau in 5% TCA. Membranes were washed twice with PBS and then in 10 mM Tris-HCl pH 7.5, 150 mM KCl and 0.1% Tween 20. Membranes were incubated for 90 min at RT in buffer A (15 mM Tris-HCl pH 7.5, 150 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.1 mM PMSF, 0.1% Tween 20, and 0.1 mg/ml BSA). Nucleolin was added at a final concentration of 2.5 µg/ml for 2 h at RT. Membranes were washed four times in buffer A and then subjected to western blot.
RESULTS

Intracellular localization and functional analysis of the Flag-US11 protein
For the purification of the native multiprotein complexes containing US11, we generated an expression vector allowing the expression of Flag-US11 protein. To validate this strategy, we first verified whether Flag-US11 and wild-type (wt) US11 proteins have the same intracellular distribution. HeLa cells were either infected with HSV-1 or transfected with expression vectors coding for US11 or Flag-US11. The results shown in Fig. 1A confirm that in HSV-1-infected cells, US11 was present in the cytoplasm and accumulated in the nucleoli where it co-localized with nucleolin, a nucleolar marker. We then analyzed the intracellular distribution of wt US11 and Flag-US11 (Fig. 1B). HeLa cells were transfected with the pCMV-US11 vector allowing the expression of wt US11 (Fig. 1B, upper panels) or the pSG5-Flag-US11 vector for the expression of Flag-US11 (Fig. 1B, lower panels). This analysis showed that both proteins displayed a very similar intracellular distribution (Fig. 1B, upper and lower panels). They are concentrated within the nucleoli and distributed throughout the entire cytoplasm. Nucleolin localization is unchanged in cells expressing untagged-US11 (Fig. 1B, upper panels). Therefore, the intracellular distribution of Flag-US11 protein is very similar to that of wt US11 protein present in HSV-1 infected cells.

We then determined whether Flag-US11 protein retained the known function of the wt US11 protein. We have previously demonstrated that US11 can substitute for Rex in transactivating expression of the genes encoding HTLV-I env glycoproteins (10). We therefore determined whether Flag-US11, like US11 and Rex, exhibited the ability to transactivate the expression of an mRNA coding for HTLV-I env (10). HeLa cells were co-transfected with expression vectors coding for the envelope protein and the transcriptional activator Tax of HTLV-I combined with the expression vector coding for Rex, or wt US11, or Flag-US11. Control experiments included the co-transfection of HeLa cells with the env vector only, as well as with the env and tax vectors. After transfection, expression of functional envelope glycoproteins was evaluated by formation of multinucleated cells (Fig. 1C). As expected, expression of Rex or wt US11 together with env and tax led to the formation of syncytia indicated by arrows in Fig. 1C. No such multinucleated cells were observed in the negative controls (Fig. 1C, lower panels).
Syncytia were also observed in cells expressing Flag-US11 protein (Fig. 1C, arrow in right upper panel). Altogether, these data demonstrate that Flag-US11 displays an intracellular localization and a function similar to wild type US11.

**Nucleolin is associated with Flag-US11 protein in transfected cells**

Immunoprecipitation experiments with the Flag tag were optimized to allow the co-immunoprecipitation of US11 associated proteins. Because the ionic strength of the buffer can modify the electrostatic protein-protein interactions between Flag-US11 and its binding partners, we have evaluated various NaCl concentrations for purification of the US11-containing complexes. The proteins that co-immunoprecipitated with Flag-US11 in buffers containing NaCl concentrations ranging from 0 to 500 mM are shown in Fig. 2A. Numerous proteins co-immunoprecipitated with Flag-US11 when no NaCl was added to the extraction buffer. Most of these proteins were also detected after immunoprecipitation with Flag alone, although in smaller amounts (Fig. 2A, lane 1).

Increasing the NaCl concentration from 0 to 150 mM led to an enrichment of proteins that bind specifically to Flag-US11 (Fig. 2A, lanes 1–6). Concentrations higher than 150 mM induced a strong reduction in the number of proteins co-immunoprecipitated.

Therefore, the concentration of 150 mM NaCl was selected for the affinity proteomic analyses.

To determine whether nucleolin was a US11 binding partner, the presence of nucleolin in these purified protein complexes was evaluated by western blot with an anti-nucleolin antibody (Fig. 2A, lower panel). Results clearly showed that nucleolin was present in the pool of proteins co-immunoprecipitated with the anti-Flag antibody in cells expressing Flag-US11 (Fig. 2A, lanes 1–6) and not detected in cells expressing the Flag only (Fig. 2A, lanes 1, 3, 5, 7, 9). The amount of nucleolin was proportional to that of Flag-US11 and was maximal at 50 and 150 mM NaCl.

We then used mass spectrometry to confirm the presence of nucleolin in the US11 binding partners. Co-purified proteins were separated by 1-DE and stained with Biosafe Coomassie blue (Fig. 2B). Several gel fragments of molecular weights of approximately 100 kDa were cut out, and the proteins contained in each fragment were digested in-gel with trypsin. Peptides were then analyzed by MALDI-TOF and Q-TOF mass spectrometry. The sequences of the 5 peptides (Table 1) obtained in the portion of the
gel indicated by an arrow in Fig. 2B (upper panel) unambiguously demonstrates the presence of nucleolin. Western blot analysis with the anti-nucleolin antibody (Fig. 2B, lower panel) validates the presence of nucleolin in complexes purified from cells expressing Flag-US11. Altogether, these results demonstrate that US11 protein and nucleolin are binding partners.

Nucleolin interacts with US11 in vitro The experiments described above show that nucleolin and US11 are co-immunoprecipitated. To determine whether these two proteins interact with each other, we performed in vitro interaction experiments using purified nucleolin and purified GST-US11. Full length nucleolin was purified and biotinylated as previously described (15). GST-US11 was expressed and purified from E. coli (34). GST-US11 or GST was incubated with streptavidin beads that were either loaded or not with biotinylated nucleolin. The results in Fig. 3A clearly show that GST-US11 was specifically bound to nucleolin (lane 6) and not to streptavidin beads alone (lane 5). This finding was specific to US11 since GST alone did not interact at all with nucleolin (lane 3). To strengthen this result, and in particular to exclude the possibility that the interaction was mediated by a contaminating protein, we performed an additional pull-down experiment with a recombinant nucleolin protein (nucleolin-p50), which is produced in E. coli, and GST-US11 (Fig. 3B). GST-US11 and GST alone were bound to glutathione sepharose beads and incubated with purified nucleolin-p50 protein (lanes 3 and 7). Only GST-US11 was able to retain nucleolin-p50 on the beads (lane 3), indicating that this interaction was mediated by US11. Altogether, these experiments demonstrate an association between nucleolin and US11. To further determine which domain(s) of US11 are required for the interaction with nucleolin, several mutants of US11 protein (Fig. 4A) were produced in E. coli and used in a far-western blot analysis (Fig. 4C). Each GST-US11 protein mutant was loaded on a denaturing gel and transferred to a nitrocellulose membrane (Fig. 4B). Transferred proteins were then renatured and incubated with purified nucleolin protein. After extensive washing, the presence of nucleolin bound to US11 proteins was revealed with an anti-nucleolin antibody (Fig. 4C). As a control experiment (Fig. 4D), a western blot performed with the anti-nucleolin antibody on a membrane similar to that of Fig. 4C, but which had not been
incubated with the purified hamster nucleolin, allowed us to conclude that the signal
visualized in panel C was indeed due to the interaction of nucleolin with the US11
protein and not to a cross-reactivity of nucleolin antibody with these proteins. This far-
western blot analysis indicates that full-length US11 (lane 2) and US11 mutants ∆1-40,
m5 and m6 (lanes 3–5) bind efficiently nucleolin, whereas US11 mutant m15 (lane 6)
and GST alone (lanes 1 and 7) do not bind nucleolin. This indicates that the C-terminal
domain of US11, that is required for the nucleolar localization of US11, contains
residues also required for direct interaction with nucleolin (7, 43).

Nucleolin is associated with native wild-type US11 protein in HSV-1-infected cells

To determine whether this association occurs with the native proteins in HSV-1-infected
cells, we performed reverse co-immunoprecipitation experiments using an anti-nucleolin
antibody on protein extracts prepared from cells infected for 8 h and 16 h with wt HSV-1.
Extracts from mock-infected cells were used as the negative controls. Results in Fig. 5
demonstrate that nucleolin is specifically immunoprecipitated by the rabbit polyclonal
anti-nucleolin antibody (Fig. 5A, lanes 7–9) and not by the rabbit polyclonal anti-HA
antibody (Fig. 5A, lanes 4–6) or by rabbit pre-immune sera (Fig. 5A, lanes 1–3), which
were used as negative controls. In addition, US11 was present only in complexes
containing nucleolin that were purified from HSV-1-infected cells and not from mock-
infected cells (Fig. 5B, lanes 7–9 and lanes 10–12). Furthermore, US11 was detected in
larger amount in complexes purified from cells infected for 16 h (Fig. 5B, lane 9)
compared to cells infected for 8 h (Fig. 5B, lane 8). These results further validate the
specific association between nucleolin and US11 in HSV-1-infected cells.

Nucleolin depletion leads to US11 accumulation in nucleoli

In order to analyze the functional implications of nucleolin and US11 association, the
cellular localization of US11 in nucleolin-depleted cells was investigated (Fig. 6). To this
aim, we performed siRNA nucleolin silencing in a HeLa cell line stably expressing wt
US11 (13, 37). As previously described in HSV-1 infected cells (Fig. 1), US11 localized
to nucleoli and to the cytoplasm in this stable cell line (Fig. 6A and C). In nucleolin-
depleted cells, US11 was still present in nucleoli (Fig. 6B), thereby ruling out the
possibility that nucleolar localization of US11 is nucleolin dependent. Fluorescence quantification of US11 intensities in a total of 87 cells (Fig. 6E) revealed that the US11 ratio between nucleolar and cytoplasmic compartments is two-fold higher in nucleolin-depleted cells than in any-type of control cells (Fig. 6E). This significantly higher level of nucleolar US11 was not a consequence of overexpression since US11 level remained constant after nucleolin silencing (Fig. 6F). Therefore, our results point to a redistribution of US11 towards the nucleolar compartment in nucleolin-depleted cells, thereby indicating that the absence of nucleolin leads to US11 accumulation within nucleoli to the detriment of the US11 cytoplasmic fraction. In summary, our results reveal that nucleolin plays a role in US11 export from nucleoli to the cytoplasm.
DISCUSSION

The function of US11, an abundant HSV-1 protein, in viral infection is still not well understood. Previous studies have demonstrated that US11 is present in different cell compartments during the time course of HSV-1 infection, and in particular it seems to accumulate within nucleoli (3, 12, 25, 28). The function of US11 during HSV-1 infection probably requires the interaction with cellular proteins, but so far, only a very limited number of proteins have been shown to interact with US11 (2, 14, 20, 27, 33).

In order to better understand US11 function and trafficking within the cell, we have identified and characterized a new nucleolar US11 protein partner. Taking advantage of a Flag-US11 tagged protein that behaves as a wild type US11 protein produced during the infection (Fig. 1), we determined by proteomic analysis that nucleolin, an abundant nucleolar protein, interacts with US11 (Fig. 2).

What could be the role of the interaction of nucleolin with US11?

In addition of showing a strong nucleolar accumulation, US11 is also found associated with cytoplasmic ribosomes (12, 31). We and others have shown that upon HSV-1 infection, rRNA synthesis is altered (1, 41), whereas ribosomal protein synthesis is maintained until later in the infection (16, 38). We have also shown that nucleolin interacts with certain ribosomal proteins and might be involved in their assembly within ribosomal subunits (4). Therefore, the interaction between nucleolin and US11 could be involved in the association of US11 into the ribosomal subunits.

Another possibility is that interaction of nucleolin with US11 participates to the intracellular trafficking of US11.

US11 shuttling between the nucleus and the cytoplasm in HSV-1-infected cells or in transiently transfected cells (7) has recently been confirmed in living cells (43). This nucleo-cytoplasmic shuttling requires the C-terminal extremity of US11 (7). This C-terminal domain of US11 is composed of the 20 XPR tripeptide repetitions that are organized into a poly-prolin type II helix structure that is commonly involved in protein-protein interaction (42). In this study using several in vitro protein interaction assays...
(Figs. 3 and 4), we show that this C-terminal domain is indeed involved in the interaction with nucleolin. A previous study using saturated mutagenesis and computer modeling of this C-terminal domain of US11 that interacts with nucleolin has shown that this domain carries the capacity of US11 to be concentrated within nucleoli and also to be exported to the cytoplasm (7, 34).

Interestingly, the silencing of nucleolin prevents HSV-1 infection (6), and the expression of nucleolin is required for nuclear egress of HSV-1 nucleocapsids (32), demonstrating that nucleolin is an important protein for HSV-1 infection and that the interaction with US11 could play an important role.

We demonstrate that the silencing of nucleolin affects the trafficking of US11 between the nucleolus and the cytoplasm (Fig. 6). Indeed, we observed a significant increase in the accumulation of US11 within nucleoli when nucleolin expression is inhibited (more than four-fold higher in the nucleoli than in the cytoplasm in silenced cells compared to two-fold in control cells, Fig. 6), suggesting that nucleolin participates in US11 nucleo-cytoplasmic transport by promoting US11 nucleolar export.

The role of nucleolin in US11 nucleo-cytoplasmic trafficking seems to be a one-way directional transport out of the nucleoli since US11 is still present in the nucleoli of nucleolin-silenced cells.

This hypothesis is also supported by the fact that US11 exhibits a non-canonical nuclear export signal (7) and based on recent data that show that US11 nucleo-cytoplasmic export does not require the conventional export factor CRM1 (43). We show here that the level of cytoplasmic localization of US11 depends on the presence of nucleolin.

These results, together with the fact that nucleolin has been recently shown to be required for efficient nuclear egress of HSV-1 nucleocapsids (32), and that US11 is a structural protein that is co-transported with capsids (14), suggest that nucleolin, through its interaction with US11, could participate to the transport of the viral particles.
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**FIGURE LEGENDS**

Figure 1: Intracellular localization and functional analysis of US11 protein.

A- Intracellular localization of US11 and nucleolin (NCL) in HeLa cells infected for 8 h with HSV-1 (wt 17+ strain, 10 PFU/cell) was visualized by indirect immunofluorescence using anti-US11 polyclonal and anti-nucleolin monoclonal antibodies. The merged image is presented. B- Sub-cellular localization of wt-US11 and Flag-US11 proteins in HeLa cells transfected with vectors expressing either untagged-US11 (upper panels) or Flag-US11 fusion protein (lower panels). Forty-eight hours after transfection, cells were fixed, and the localization of the proteins was assayed with a polyclonal anti-US11 antibody, a monoclonal anti-nucleolin antibody (upper panels), a monoclonal anti-Flag antibody and with a polyclonal anti-US11 antibody (lower panels). The merged images are presented. Scale bars represent 5 µm. C- Transactivation of HTLV-I envelope glycoprotein expression by Rex, wt US11, and Flag-US11 proteins. HeLa cells were transfected with vectors expressing HTLV-env and Tax, plus vectors expressing either HTLV-Rex (upper left) or US11 (upper middle), or Flag-US11 fusion protein (upper right), or Flag only (lower left). Transactivation of the expression of HTLV-I env by either Rex or wt US11 leads to the formation of syncytia (arrows in left and middle upper panels) and as described in details previously (10). The same transactivating property is displayed by Flag-US11 as visualized by syncytia formation (upper right panel). As control experiments, HeLa cells were either not transfected (lower right) or transfected with vectors expressing HTLV-env and Tax, plus vectors expressing either Flag only (lower left) or no additional vector (lower middle). As expected, in these three lower panels, only individual cells are visible and no syncytium was detected.

Figure 2: Nucleolin is associated with Flag-US11 fusion protein in transfected cells: purification of the Flag-US11-containing complexes.

A- Optimization of the co-immunoprecipitation conditions of the Flag-US11 binding partners. HeLa cells were transfected with pSG5-Flag (lanes 1, 3, 5, 7, and 9) or with pSG5-Flag-US11 (lanes 2, 4, 6, 8, and 10) expression vectors. Upper panel: Effect of NaCl concentration on the isolation of protein complexes containing Flag-US11 protein.
by co-immunoprecipitation experiment using an anti-Flag antibody. Protein complexes were purified with an optimized buffer (see Methods) in the presence of an anti-Flag antibody and of various NaCl concentrations as indicated on the figure, and they were then analyzed by 1-DE and silver staining (upper panel). The positions of the Flag-US11 protein and that of molecular weight markers are indicated on the left. The presence of nucleolin (NCL) in the corresponding purified complexes was checked by western blot analysis using a polyclonal anti-nucleolin antibody (lower panel). B- Identification of nucleolin as a Flag-US11- interacting protein. Protein complexes purified as in A in the presence of 150mM NaCl from cells transfected with a plasmid expressing either Flag or Flag-US11 were separated by 1-DE and stained with Coomassie blue (upper panel).

The portion of the gel corresponding to proteins of about 100 kDa in size (arrow on the right) was cut out, and the corresponding nucleolin protein was identified by mass spectrometry and bio-informatics analyses (see Table 1). The identity of this protein in the purified complexes was confirmed by western blot analysis using a specific anti-nucleolin antibody (lower panel). The position of nucleolin (NCL) is indicated by an arrow on the right.

Figure 3: Nucleolin interacts in vitro with full-length US11

A- Nucleolin purified from CHO cells was biotinylated as previously described (15) and used for interaction studies with E. coli expressed GST (lanes 1-3) or GST-US11 (lanes 4-6) proteins. Streptavidin beads (Strept. Beads) were incubated with GST (lane 2, 3), or GST-US11 (lanes 5, 6) in the absence (lanes 2, 5) or presence (lanes 3, 6) of biotinylated nucleolin. After extensive washing, streptavidin beads were incubated in Laemmli loading buffer, and recovered proteins were separated by 1-DE then visualized by silver staining. Position of nucleolin and GST-US11 is indicated. B- Nucleolin-p50 protein was produced in E. coli and purified as previously described (36). Glutathione sepharose beads were incubated with binding buffer (lane 1), GST-US11 (lanes 2, 3), or GST protein (lanes 6, 7). Nucleolin-p50 protein was then added to the beads (lanes 1, 3, 7). After extensive washing, beads were incubated with Laemmli buffer and protein separated by 1-DE then visualized by silver staining.
Figure 4: Interaction of nucleolin with US11 mutants  
A- Schematic representation of the different GST-US11 mutants used in this study. B- 
The different proteins (1 µg) were separated by 1-DE, transferred on a nitrocellulose 
membrane, then stained with Ponceau dye. The nucleolin-p50 protein (lane 8) 
corresponding to the C-terminal domain of nucleolin was used as a positive control for 
the western blot with anti-nucleolin antibody. C- The membrane was incubated with the 
purified nucleolin protein as described in the Methods section, and after extensive 
washing, it was subjected to western blot with a polyclonal anti-nucleolin antibody. Then 
nucleolin detection was revealed using ECL. D- As a control, a membrane similar to the 
one shown in B was subjected directly to a western blot with the anti-nucleolin antibody. 
Only the p50 nucleolin protein could be detected showing that the nucleolin antibody 
does not cross-react with any of the GST-US11 protein.

Figure 5: US11 is present in nucleolin-containing complexes purified from extracts of 
HSV-1-infected cells. 
HeLa cells were mock-infected at 0 h (lanes 1, 4, 7, and 10) or infected with HSV-1 for 8 
h (lanes 2, 5, 8, and 11) and 16 h (lanes 3, 6, 9, and 12) at 10 PFU/cell. Co-
immunoprecipitations (lanes 1–9) were performed with the corresponding total cell 
extract proteins and used either a polyclonal anti-nucleolin (anti-NCL, lanes 7–9), or an 
anti HA (lanes 4–6) antibodies, or a pre-immune serum (lanes 1–3) as indicated at the 
top of the figure. The presence of nucleolin (panel A) and of US11 (panel B) in the 
purified complexes was checked by western blot analysis using anti-nucleolin and anti-
US11 specific antibodies, respectively. The position of nucleolin and of US11 is 
indicated. Total cell extract proteins not submitted to the co-immunoprecipitation were 
used as positive controls (lanes 10–12).

Figure 6: Nucleolin depletion leads to US11 accumulation in nucleoli  
A and B- Immunofluorescence in a HeLa cell line stably expressing untagged US11 
(clone HL5a1) transfected with scramble siRNA (SC) (A) or siRNA against nucleolin (B). 
Nucleolin (NCL) is detected in red and US11 in green, and DNA is stained with DAPI 
(blue). Scale bars represent 5 µm. C and D- Fluorescence intensity profiles (gray level
values) obtained on 16-bit images along the white lines selected on A and B, respectively. Profiles correspond to nucleolin (red), US11 (green), and DAPI (blue) fluorescence intensities. E- Quantification of the nucleolar-cytoplasmic ratio of US11. The ratio of nucleolar over cytoplasmic US11 fluorescence intensity was calculated in control untransfected cells (C, n=18), mock-transfected cells (MT, n=24), cells transfected with scramble siRNA (SC, n=16), and cells transfected with nucleolin siRNA (NCL, n=29). Five to 10 fields of view were analyzed for each condition. Error bars correspond to standard deviations. Mann-Whitney and Wilcoxon statistical tests showed that the US11 ratio in nucleolin-depleted cells is significantly higher than all the control cell populations (P < 2x10^{-7}). F- US11 cellular intensity in control untransfected cells (C), mock transfected cells (MT), cells transfected with scramble siRNA (SC), and cells transfected with nucleolin siRNA (NCL) (gray level values).

Table 1: Nucleolin is present in Flag-US11-containing complexes purified from transfected cells. Proteins co-immunoprecipitated with an anti-Flag antibody from cells expressing Flag-US11 protein were separated by 1-DE, and the proteins present in the portion of the gel corresponding to proteins, of which the molecular weights (MW) are approximately 100 kDa (apparent nucleolin MW) were identified by mass spectrometry analysis. Only identified peptides corresponding to nucleolin are presented. DB (database) entry: database entry name of nucleolin in SWISS-PROT, TrEMBL, or GenBank databases. MW and pI (isoelectric point) correspond to the theoretical MW and pI calculated using Compute pI/Mw software (www.expasy.org). Coverage: percentage of coverage of peptides in the nucleolin sequence.
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