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Molecular anatomy of subcellular localization of HSV-1 tegument protein US11 in living cells

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ABSTRACT

The herpes simplex virus type I (HSV-1) US11 protein is an RNA-binding multifunctional regulator that specifically and stably associates with nucleoli. Although the C-terminal part of US11 was responsible for its nucleolar localization, the precise nucleolar localization signal (NoLS) and nuclear export signal (NES) of US11 and its nuclear import and export mechanisms are still elusive. In this study, fluorescence microscopy was employed to investigate the subcellular localization of US11 and characterize its transport mechanism in living cells. By constructing a series of deletion mutants fused with enhanced yellow fluorescent protein (EYFP), three novel NoLSs of US11 were for the first time mapped to amino acids 84–125, 126–152, and 89–146, respectively. Additionally, the NES was identified to locate between amino acids 89 and 119. Furthermore, the US11 protein was demonstrated to target to the cytoplasm through the NES by chromosomal region maintenance 1 (CRM1)-independent pathway, and to the nucleolus through Ran and importin β-dependent mechanism that does not require importin α5.

1. Introduction

Herpes simplex virus type I (HSV-1), the principal alpha herpesvirus of humans, establishes latent infections and reactivates to cause recurrently lytic infections resulting in a wide variety of clinical syndromes in newborns, children, and adults. During productive infection, the 152-kb double-stranded HSV-1 genome is rapidly translocated to the nucleus and encodes approximately 80 genes in a coordinately activated cascade that consists of the sequential expression of immediate-early (IE), early (E), and late (L) genes. It has been shown that approximately half of these genes are not essential for replication of the virus in cell culture (Roizman et al., 2007). These dispensable gene products are, however, thought to be important for virus growth and spread in the natural host.

One such nonessential protein, US11, is not necessary for viral replication in cell culture or in a mouse model (Nishiyama et al., 1993) and is a relatively small (23 kDa), highly basic phosphoprotein that is expressed late in infection but is present at early times post-infection as it enters the cell as a tegument component of the virion (Roller and Roizman, 1992). Additionally, US11 is a RNA-binding protein, which can bind a limited number of cellular and viral RNAs in a sequence- and conformation-specific manner (Diaz et al., 1996; Roller and Roizman, 1990, 1991). Structure-function analysis has demonstrated that the carboxy-terminal half of US11 is required to bind RNA, localize to nucleolus, associate with ribosomes (Roller et al., 1996) and interact with protein kinase R (PKR) and the PKR activator PACT (Cassady and Gross, 2002). Furthermore, several cellular factors have been identified by a yeast two-hybrid screen to interact with the US11 protein, such as kinesin light-chain-related protein PAT1 (Benboudjema et al., 2003) and homeodomain-interacting protein kinase HIPK2 (Giraud et al., 2004). It was shown recently that US11expression enhanced the recovery of cellular protein synthesis, increased cell survival in response to thermal stress and also protected against staurosporine induced apoptosis (Javouhey et al., 2008). Therefore, it is clear that US11 is a multifunctional protein involved in posttranscriptional regulation of gene expression and in biological processes related to the survival of cells following environmental stress.

The nucleolus is the center of ribosomal biogenesis, which is a highly complex process leading to the production of pre-ribosomal particles that are then released to the nucleoplasm and exported to the cytoplasm as mature ribosomal subunits (Carmo-Fonseca et al., 2000). Interestingly, an increasing number of key proteins from both RNA and DNA viruses have been shown to localize to the nucleolus (Hiscox, 2002). These proteins include the ORF57 protein of herpesvirus saimiri (HVS) (Boyne and Whitehouse, 2006), the Rev (Cochrane et al., 1990) and Tat proteins (Kuppuswamy et al., 1989) of human immunodeficiency virus (HIV), the nuceocapsid (N) proteins from many coronavirus and the closely related arterivirus (Tijms et al., 2002; Wurm et al., 2001), the nucleoprotein and non-structural protein 1 (NS1) protein of influenza virus (Melen et al., 2007; Ozawa et al., 2007).

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How viral and cellular proteins traffic to the nucleolus and what determines their sub-nucleolar localization is not clearly understood, but nucleolar localization signals (NoLSs) are thought to be involved (Carmo-Fonseca et al., 2000). A cluster of basic amino acids is responsible for targeting proteins to the nucleus or nucleolus (Kaffman and O'Shea, 1999). Active nuclear import of proteins is mediated by NLSs, which are then recognized by proteins of the importin super-family (importin α and β) that mediate the transport across the nuclear envelope using Ran-GTP (Bednenko et al., 2003). Similar to nuclear import, export of a protein from nucleus depends on the presence of a specific nuclear export signal (Gorlich and Mattaj, 1996). Nucleocytoplasmic trafficking of protein and RNA molecules plays an important role in eukaryotic cell function (Ullman et al., 1997). A related family of shuttling transport factors, importins and exportins, recognizes NLS or NES containing proteins and coordinates trafficking between the nucleus and the cytoplasm. Chromosomal region maintenance 1 (CRM1; exportin 1) has been identified as an export receptor that recognizes NES sequences directly and is responsible for the export of NES containing proteins (Ullman et al., 1997). The pharmacological compound leptomycin B (LMB) directly interacts with CRM1 and blocks NES-mediated protein export (Fornerod et al., 1997). Therefore, the proteins can shuttle between the nucleus and the cytoplasm with their NLSs and NESs.

It was reported previously that the unique XPR repeats of US11 were responsible for its nucleolar retention and nuclear export, which were identified by microinjection and cell fixation (Catez et al., 2002). However, such *in vitro* techniques may alter the localization of proteins, resulting in misleading conclusions in the analysis of the intracellular distribution of a specific protein. Additionally, the precise NoLS and NES of US11 and its nuclear import and export mechanism are still elusive. Therefore, we have attempted to charaterize these signals, and the molecular mechanism responsible for its nucleolar targeting. In this report, living cells fluorescence microscopy, which is widely applied (Day and Schaufele, 2008) and developed in our group (Guo et al., 2009; Zheng et al., 2004, 2005), was employed to characterize the exact nucleolar transport mechanism of US11. By sequence analysis and constructing mutants, the precise nucleolar retentionsequences and nuclear export signal (NES) of US11 were identified, and the molecular mechanism of nucleolar transport was also elucidated.

2. Materials and methods

2.1. Cells and viruses

Vero cells and COS-7 cells were grown in Dulbecco's modified MEM (DMEM, Gibco-BRL) supplemented with 10% FBS. HSV-1 (F strain) recombinant virus, pYEbac102, was a generous gift from Dr. Yasushi Kawaguchi and propagated in Vero cells for HSV-BAC DNA purification and viral infections (Tanaka et al., 2003).

2.2. Plasmids construction

All enzymes used for cloning procedures were purchased from Takara (Dalian, China) except T4 DNA ligase from New England Biolabs (MA, USA). The US11 open reading frame (ORF), including the start methionine, was amplified by PCR from the HSV-BAC DNA (pYEbac102) using primers which contained Bgl II and EcoR I sites in Table 1. The product was digested with Bgl II and EcoR I and inserted into pEYFP-N1 (Clontech), digested with Bgl II and EcoR I, to create US11-EYFP for sequencing. The cloning procedures for constructing most of recombinant plasmids were similar and all primers are listed in Table 1. Additionally, the recombinant plasmid US11(110–118)-EYFP was generated by ligating the annealed oligonucleotides as shown in Table 1 into pEYFP-N1 (Clontech) cut with Bgl II and EcoR I. pECFP-L23 was a generous gift from Dr. Johannes A. Schmid (Birbach et al., 2004) and used as nucleolar marker. pGEX-6p-1 Q69L Ran was a generous gift from Dr. Yoshinari Yasuda (Isegawa et al., 2008) and Ran Q69 L dominant negative mutant was subcloned into pECFP-N1 (Clontech) to yield Ran-Q69L-ECFP. Importin α5 dominant negative mutant (DN-importinα5) was a generous gift from Dr. Christopher F. Basler (Reid et al., 2007) and was subcloned into pECFP-N1 (Clontech) to yield DN-importin α5-ECFP. Furthermore, importin β dominant negative mutant (DN-importin β) was a generous gift from Dr. Haitao Guo (Guo et al., 2010) and was subcloned into pECFP-N1 (Clontech) to yield DN-importin β-ECFP. In addition, the plasmid pRev-NES-EGFP was a generous gift from Dr. Gillian Elliott (Williams et al., 2008) and used as positive control for LMB treatment.

2.3. Transfection and fluorescence microscopy

To express the proteins *in vitro*, COS-7 cells or Vero cells were plated onto six-well plates (Corning, USA) in DMEM (Gibco-BRL) with 10% FBS at a density of 2.5×10^5 cells per well overnight before transfection. Transfection mixtures, consisting of 1.0–1.5 μg of plasmid and Lipofectamine Plus reagent (Gibco-BRL), were prepared according to the manufacturer's instructions. Briefly, the plasmids were diluted in Optimem (Gibco-BRL) containing Lipofectamine Plus and incubated for 15 min at room temperature. Optimem containing Lipofectamine was added to the mixture and further incubated for 15 min at room temperature. COS-7 cells were washed with Optimem, and the transfection mixture, made up to 1 ml with Optimem, was added to the cells and incubated at 37 °C in a humidified 5% CO_2 incubator for 5 h, after which an equal volume of DMEM containing 10% FBS was added. LMB (Sigma) was applied in some experiments at 20 ng ml^{-1} after transfection. At 24 h after transfection, the live cells were analyzed using fluorescence microscopy (Zeiss, Germany). All the photomicrographs were taken under a magnification of 400×. Each photomicrograph represents a vast majority of the cells with similar subcellular localization. Both fluorescent images of EYFP and ECFP fusion proteins were presented in pseudocolor, green and red, respectively, and the merged images were shown to confirm the co-localization. Images were processed with Adobe Photoshop.

2.4. Immunofluorescence microscopy

Cells were either observed live or fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.137 M NaCl, 0.003 M KCl, 0.008 M Na_2HPO4, 0.001 M NaH_2PO4, pH 7.4) for 20 min, washed three times with PBS, and permeabilized with 0.5% Triton X-100 in PBS for 10 min. The cells were rinsed with PBS and then incubated with PBS containing 10% FBS for 20 min at room temperature. Subsequently, US11-specific polyclonal rabbit antibody (Roller and Roizman, 1992) diluted in PBS containing 10% FBS was added to the cells, which were again incubated for 20 min at room temperature. Finally, FITC-conjugated goat anti-rabbit IgG (Sigma, USA) in PBS containing 10% FBS was added, followed by a 20-min incubation at room temperature. After each incubation step, cells were washed extensively with PBS. Samples were analyzed using fluorescence microscopy (Zeiss, Germany). Images were processed using Adobe Photoshop.

3. Results

3.1. Molecular characterization of HSV-1 strain F US11 protein

The open reading frame of the HSV-1 strain F US11 gene located at the 3′ end of unique short (US) regions in HSV-1 genome (Fig. 1A). The sequence of the HSV-1 strain F US11 gene was deposited in

Table 1
Primers for constructing the recombinant plasmids.

Plasmids' name	Forward primer	Reverse primer
US11-EYFP	GAAGATCTATGAGCCAGACCCAACCCCCG	CGGAATTCGTACAGACCCGCGAGCCGTACGTG
EYFP-US11	GAAGATCTATGAGCCAGACCCAACCCCCG	CGGAATTCGTTATACAGACCCGCGAGCCGTAC
US11-dEYFP	CGGGATCCATGGTGAGCAAGGGCGAGGA	CGGGATCCCTTGTACAGCTCGTCCATGC
US11-tEYFP	CGGGATCCATGGTGAGCAAGGGCGAGGA	CGGGATCCCTTGTACAGCTCGTCCATGC
US11(1–83)-EYFP	GAAGATCTATGAGCCAGACCCAACCCCCG	CGGAATTCGTGTTGGCGGTCGGACGGCTTCAG
US11(84–152)-EYFP	GAAGATCTATGATCCCCAGGACACCGCGTGTTC	CGGAATTCGTACAGACCCGCGAGCCGTACGTG
US11(84–125)-EYFP	GAAGATCTATGATCCCCAGGACACCGCGTGTTC	CGGAATTCGTCGTGGATCCCTGGGGTCACGC
US11(126–152)-EYFP	GAAGATCTATGCAACCCCGGGAGCCCCGGCCT	CGGAATTCGTACAGACCCGCGAGCCGTACGTG
US11(88–125)-EYFP	GAAGATCTATGCCGCGTGTTCCCCGGGAG	CGGAATTCGTCGTGGATCCCTGGGGTCACGC
US11(89–106)-EYFP	GAAGATCTATGCGTGTTCCCCGGGAG	CGGAATTCGGGGTTCCCTAGGTTCTCG
US11(110–118)-EYFP	GATCTATGCGAGCTCCCAGAGACCCCAGGGTACCGCG	AATTCGCGGTACCCTGGGGTCTCTGGGAGCTCGCATA
US11(89–119)-EYFP	GAAGATCTATGCGTGTTCCCCGGGAG	CGGAATTCGACGCGGTACCCTGGGGTCTC
US11(84–119)-EYFP	GAAGATCTATGATCCCCAGGACACCGCGTGTTC	CGGAATTCGACGCGGTACCCTGGGGTCTC
US11(89–125)-EYFP	GAAGATCTATGCGTGTTCCCCGGGAG	CGGAATTCGTCGTGGATCCCTGGGGTCACGC
US11(126–146)-EYFP	GAAGATCTATGCAACCCCGGGAGCCCCGGCCT	CGGAATTCGACGTGGTTCGCGGGGGGTGC
US11(135–152)-EYFP	GAAGATCTATGGAGCCCCGGACCCCACG	CGGAATTCGTACAGACCCGCGAGCCGTACGTG
US11(84–146)-EYFP	GAAGATCTATGATCCCCAGGACACCGCGTGTTC	CGGAATTCGACGTGGTTCGCGGGGGGTGC
US11(89–152)-EYFP	GAAGATCTATGCGTGTTCCCCGGGAG	CGGAATTCGTACAGACCCGCGAGCCGTACGTG
US11(89–146)-EYFP	GAAGATCTATGCGTGTTCCCCGGGAG	CGGAATTCGACGTGGTTCGCGGGGGGTGC
Ran-Q69L-ECFP	TTTGAATTCATGGCTGCGCAGGGAGAG	CGCGGATCCCACAGGTCATCATCCTCAT
DN importin α5-ECFP	CGGAATTCATGACCACCCCAGGAAAAGAG	GCGGATCCCGAAGCTGGAAACCTTCCATAG
DN importin β-ECFP	CGGAATTCATGGAGCTGATCACCATTCTCG	GCGGATCCCGAGCTTGGTTCTTCAGTTTC

the GenBank with the following accession number: GQ999614. The US11 protein of HSV-1 strain F contained 152 amino acids with carboxy-terminal 21 XPR repeats followed by the TARGSV terminal hexapeptide (Fig. 1B), where X is most commonly an acidic or uncharged polar amino acid (Roller et al., 1996). Multiple alignment of the US11 protein from three HSV-1 strains (F, 17 and KOS strain) revealed that their amino acids showed more than 97% homology with different copies of XPR repeats (F strain: 21; 17 strain: 24; KOS strain: 20) (Fig. 1C). Interestingly, the potential phosphorylation site Ser was mutated into Pro (P) 132 in US11 protein of HSV-1 strain F (Fig. 1C).

3.2. Subcellular localization of US11 in infected and transfected cells

To determine the exact subcellular localization during infection, the localization of US11 in Vero cells infected with HSV-1 was investigated. The ECFP-tagged ribosomal protein L23 (ECFP-L23) encoded by plasmid pECFP-L23, was used as a nucleolar marker. The ECFP-L23 has been demonstrated to localize to the nucleoli upon over expression (Birbach et al., 2004). Immediately after transfection with pECFP-L23, Vero cells were infected with HSV-1 at a multiplicity of infection (MOI) of 0.1. The cells were fixed 16 h

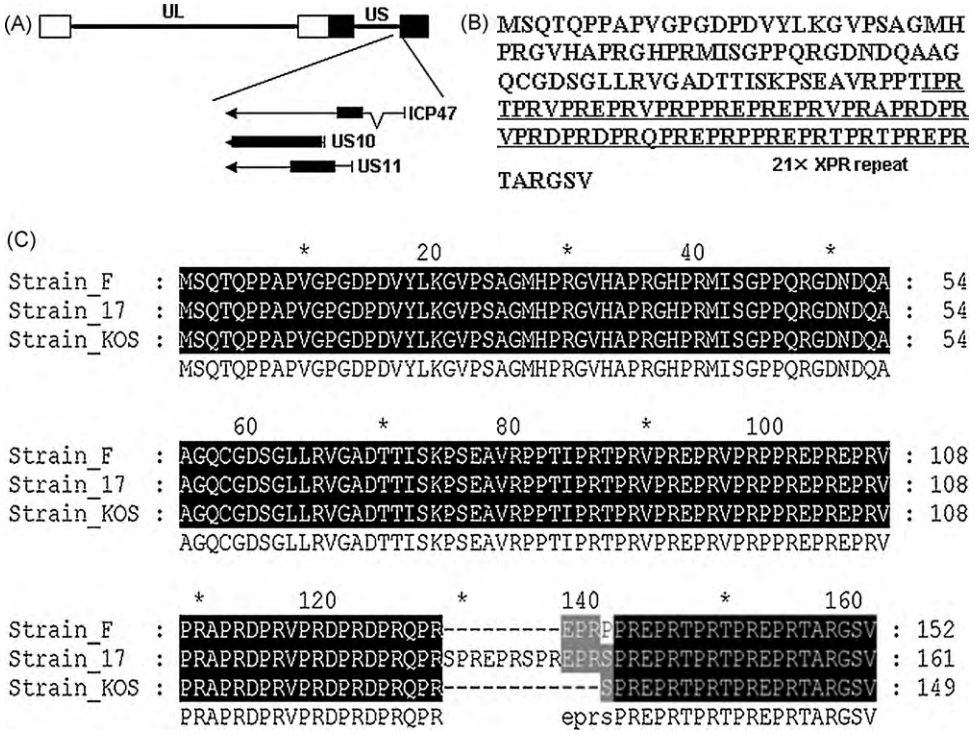

Fig. 1. The molecular characterization of the US11 protein of HSV-1 strain F. (A) Schematic representation of the HSV-1 genome showing the US11 gene. The HSV-1 genome is shown with its unique long (UL) and unique short (US) regions. Boxes and arrows indicate ORFs and mRNAs, respectively. (B) Coding sequence of the HSV-1 strain F US11 gene. The C-terminal XPR repeats of US11 are underlined. (C) Alignments of amino acids of US11 protein from three different HSV-1 strains, including strain F, strain 17 and strain KOS.

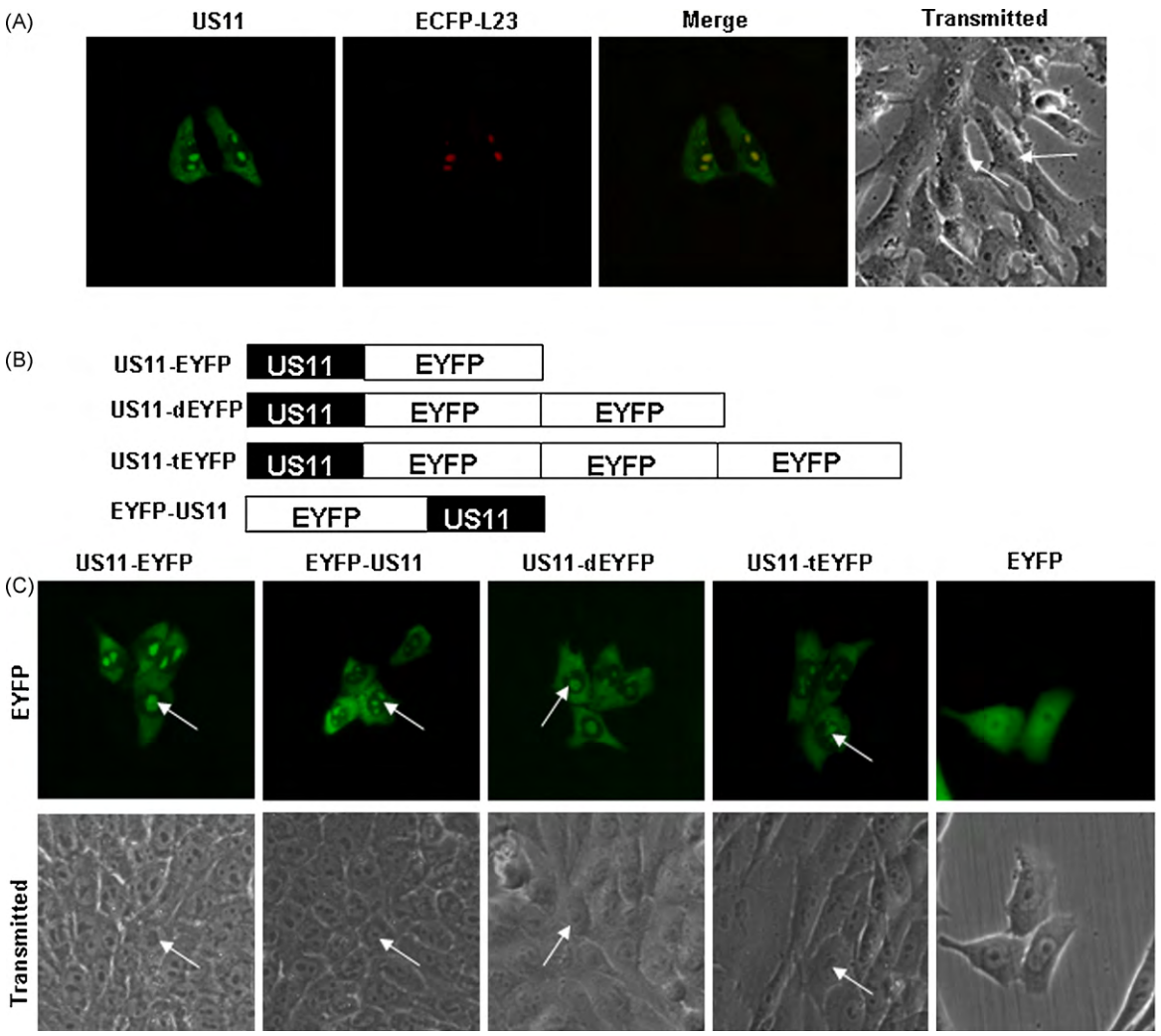

Fig. 2. Subcellular localization of US11 in HSV-1 infected cells and transiently transfected cells. (A) Vero cells were infected with HSV-1 at MOI of 0.1 immediately after transfection with pECFP-L23, 16 h after infection, immunofluorescent staining of US11 was performed using US11 specific polyclonal antibody. Immunofluorescence photomicrograph of US11, the corresponding phase-contrast image and ECFP-L23 location photomicrographs are shown. Arrows indicated the nucleoli. (B) Schematic diagram of the US11 fusion with EYFP monomer, dimmer and trimer in its C-terminus and US11 fusion with EYFP in its N-terminus. (C) Fluorescence microscopy analysis of the COS-7 cells expressing US11-EYFP, US11-dEYFP, US11-tEYFP and EYFP-US11 in comparison with phase-contrast photomicrographs of the same cells. Arrows indicate the nucleoli. Each image is representative of the vast majority of the cells observed.

post-infection and analyzed by an immunofluorescence assay using rabbit polyclonal antibody against US11. US11 staining exhibited predominant nucleolus and cytoplasm localization (Fig. 2A), which is consistent with the previous report (Roller et al., 1996). Fluorescence microscopy clearly showed the co-localization of US11 and ECFP-L23 in the nucleolus (Fig. 2A). This also demonstrated that the dense, dark-staining, irregular shaped subcellular organelles in the nucleus are nucleoli (Fig. 2A, transmitted and ECFP-L23). Thus phase-contrast images were taken to indicate the nucleolus in the following experiments.

It is well known that some fixation protocols may alter the localization of proteins, resulting in misleading conclusions in the analysis of the intracellular distribution of a specific protein. To avoid this flaw, the living cells fluorescence microscopy technique was applied in our study. To investigate whether US11 plays a role in transporting a heterogenous protein to the nucleolus, ORF of US11 was inserted into pEYFP-N1 to generate US11-EYFP. US11 fusions with EYFP dimer (dEYFP) or trimer (tEYFP) were also constructed as shown in Fig. 2B. The subcellular localization of US11-EYFP fusion protein was imaged in live cells. Low level expression of US11-EYFP was observed in the nucleolus and cytoplasm right after removing the transfection mixtures (about 4 h after transfection), and the localization of US11-EYFP does not change after 48 h transfection (data not shown). This also confirmed that the nucleolar localization was not an artifact of high expression levels. Fig. 2C showed micrographs of COS-7 cells 24 h after transfection with plasmids encoding US11 and EYFP fusion proteins. US11 fused with one, two or three copies of EYFP showed similar patterns of localization. The fluorescence was predominantly restricted to the nucleolus and cytoplasm by US11 (Fig. 2C). On the contrary, the fluorescence was evenly distributed throughout the cytoplasm and the nucleoplasm but not the nucleolar structures in cells transfected with plasmids encoding monomer EYFP (Fig. 2C). In order to investigate whether the orientation of US11 affects its localization in cells, DNA construct was also made to express US11 fused to the C-terminus of EYFP (EYFP-US11) (Fig. 2C). The fluorescence microscopy demonstrated identical subcellular distribution patterns of US11-EYFP and EYFP-US11, thus the following experiments were performed only for EYFP fusion to C-terminus of US11. Taken together, these results indicated that US11 was responsible for driving EYFP into the nucleolus.

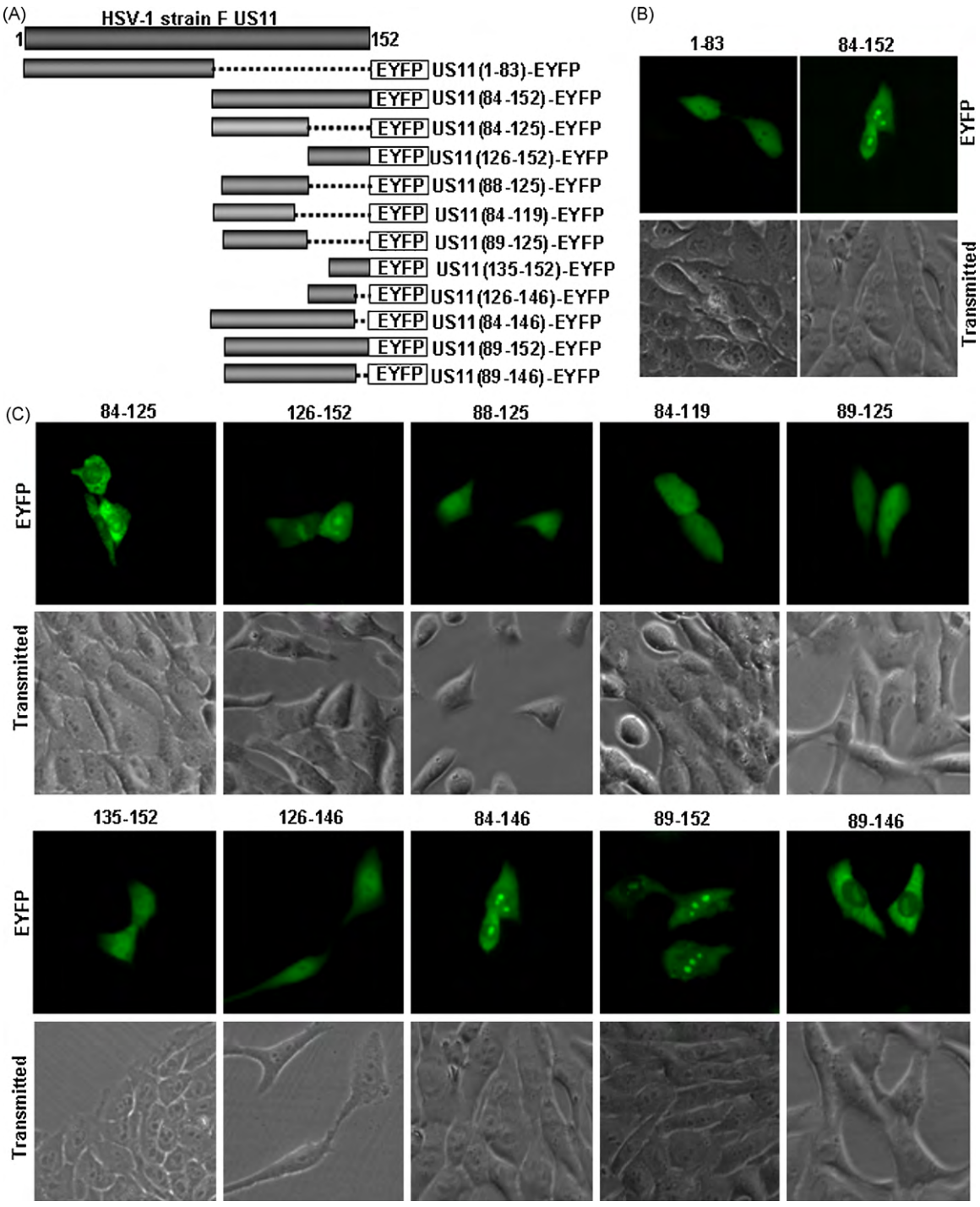

Fig. 3. Mapping and identification of the nucleolar localization signals in US11 protein. (A) Schematic representation of wild-type US11 protein and its N- and C-terminal deletion mutants fused with EYFP. (B) Subcellular localization of deletion mutants US11(1–83)-EYFP and US11(84–152)-EYFP. Each image is representative of the vast majority of the cells observed. (C) Subcellular localization of the other US11 mutants fused with EYFP. Representative fluorescence images of the vast majority of living cells for indicated EYFP fusion proteins and EYFP fluorescence were analyzed in living cells 24 h after transfection. Each image is representative of the vast majority of the cells observed. Light-translucent pictures to show cellular morphology.

3.3. Mapping and identification of the nucleolar localization signals in the US11 protein

From the above results we hypothesize that there is a nucleolar localization signal in US11 which can guide exogenous protein to the nucleolus. Since US11 fusions with EYFP monomer, dimer or trimer showed similar nucleolus localization patterns, the following experiments were performed with monomer EYFP fusion. It was reported that the XPR repeats of US11 were important for its nucleolar association (Roller et al., 1996), and the XPR motif of US11 herein started from the 84th amino acid (Fig. 1B). So, two deletion mutants encompassing amino acids 1–83 and 84–152 in frame with EYFP (Fig. 3A) were firstly constructed to investigate their subcellular localization. US11(1–83)-EYFP showed similar subcellular distribution as EYFP, with fluorescence diffuse in both the nucleus and cytoplasm, but excluded from the nucleolus (Fig. 3B). The subcellular distribution of US11(84–152)-EYFP was identical to that of US11-EYFP (Fig. 2C), with the fluorescence enriched to both

nucleoli and cytoplasm (Fig. 3B), indicating that the amino acids 84–152 were responsible for the nucleolar localization of US11, which is consistent with the previous report (Roller et al., 1996).

It was previously reported that the amino acids 88–125 of US11 was required for concentration in nucleoli (Catez et al., 2002). Therefore, the amino acids 84–152 was then sub-divided into two parts, US11(84–125) and US11(126–152), which were tagged with EYFP (Fig. 3A), respectively. Interestingly, the fluorescent patterns of US11(84–125)-EYFP and US11(126–152)-EYFP were identical to that of US11(84–152)-EYFP and US11-EYFP (Fig. 2C and 3C). Then, the plasmid US11(88–125)-EYFP (Fig. 3A) was also constructed to verify the results from Catez et al. (2002). However, our result revealed that the fluorescence of US11(88–125)-EYFP distributed evenly throughout the cytoplasm and nuclear, without nucleolar enrichment (Fig. 3C), which is not consistent with their result. These evidence demonstrated that either US11(84–125) or US11(126–152) targeted to the nucleolus with weaker enrichment, while full length XPR repeat of US11(84–152) localized to the nucleolus with stronger enrichment. So we speculate that US11(84–125) and US11(126–152) have an additive effect on the nucleolar localization of US11.

To further identify the sequence for nucleolar localization in detail, a series of deletion mutants, including US11(84–119)-EYFP, US11(89–125)-EYFP, US11(135–152)-EYFP, US11(126–146)-EYFP, US11(89–152)-EYFP and US11(84–146)-EYFP, were then constructed (Fig. 3A). The results showed that US11(84–119)-EYFP, US11(89–125)-EYFP, US11(135–152)-EYFP and US11(126–146)-EYFP all exhibited similar subcellular distribution patterns as EYFP, with diffuse fluorescence in both the nucleus and cytoplasm, but excluded from the nucleolus (Fig. 3C). The results indicates that N-terminal 84–88aa or C-terminal 120–125aa is important for nucleolar localization of US11(84–125)-EYFP, while 126–134aa or 147–152aa is critical for the nucleolar targeting of US11(126–152)-EYFP.

However, the fluorescent patterns of US11(89–152)-EYFP and US11(84–146)-EYFP were identical to that of US11-EYFP (Fig. 3C), indicating that 84–88aa and 147–152aa were dispensable for nucleolar localization of US11(84–152)-EYFP. These results indicates that amino acids 89–146 may play an important role for the nucleolar localization of US11. Interestingly, US11(89–146)-EYFP exhibited similar fluorescent pattern as that of wild-type US11 (Fig. 3C). Also, US11(126–146)-EYFP could not target to the nucleoli (Fig. 3C). Therefore, US11(89–146) was deduced to be another smallest peptide of US11 capable of retention in the nucleoli.

3.4. Identification of the nuclear export signal in the US11 protein

It was previously reported that the amino acids 88–125 of US11 may have nuclear export activity (Catez et al., 2002), however, the precise nuclear export signal (NES) of US11 was still not known. To map the amino acid sequence of US11 responsible for its nuclear export, a series of deletion mutants encompassing amino acids 89–106, 89–119 and 110–118 fused with EYFP were constructed (Fig. 4A). As a result, US11(89–106)-EYFP and US11(110–118)-EYFP showed identical subcellular localization as EYFP, with fluorescence diffuse in both the nucleus and cytoplasm (Fig. 4B), which

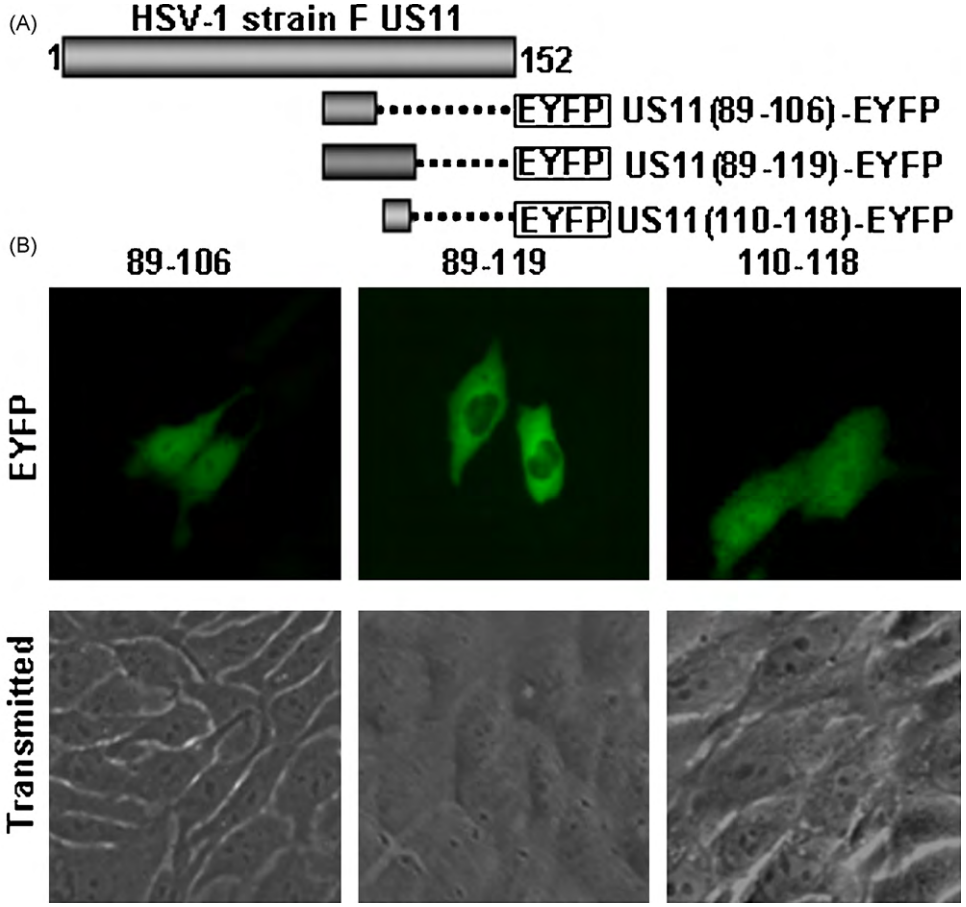

Fig. 4. Identification of the nuclear export signal in the US11 protein. (A) Schematic diagram of wild-type US11 and its deletion mutants fused with EYFP. (B) Subcellular localization of these US11 mutants fused with EYFP. Representative fluorescence images of the vast majority of living cells for indicated EYFP fusion proteins and EYFP fluorescence were analyzed in living cells 24 h after transfection. Each image is representative of the vast majority of the cells observed in several fields. Light-translucent pictures to show cellular morphology.

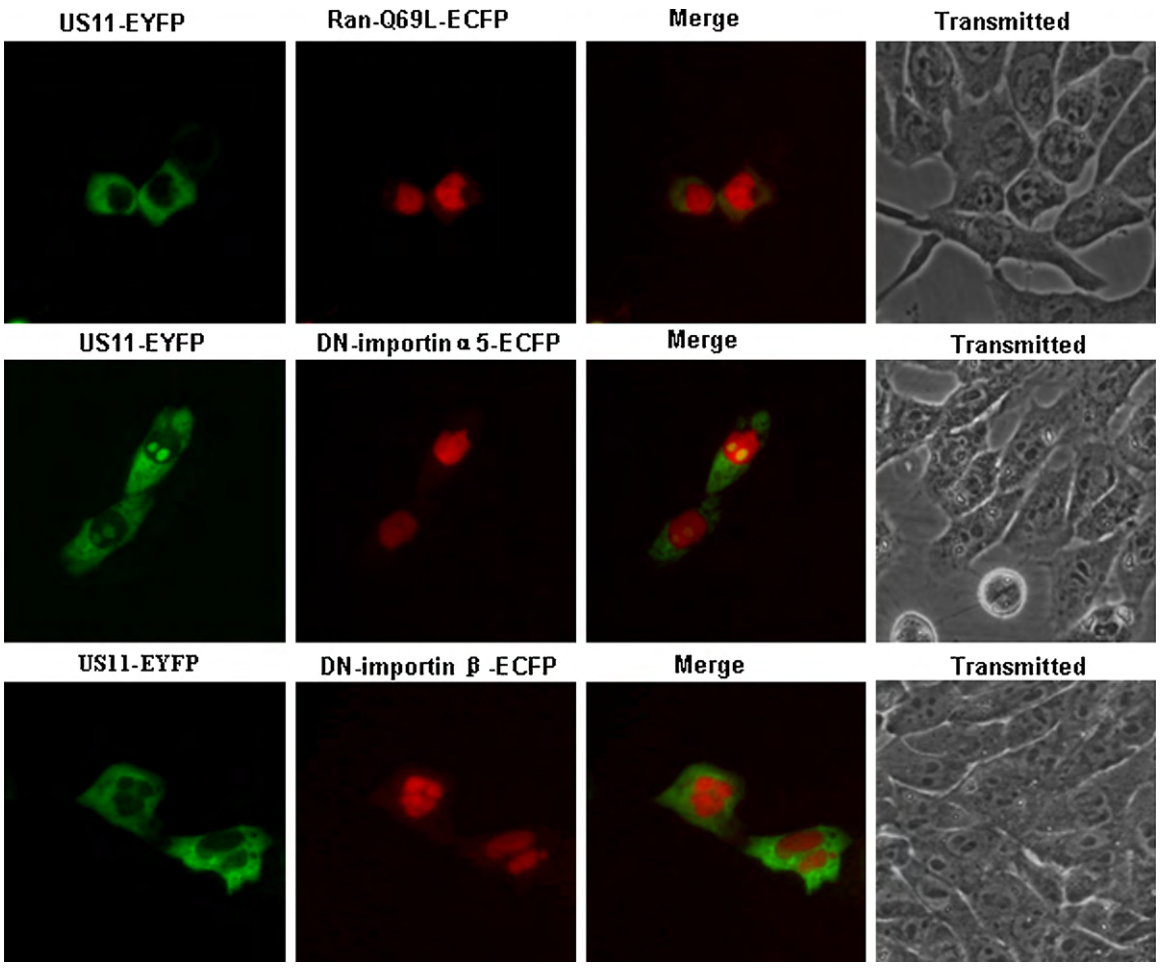

Fig. 5. The nuclear import mechanism of US11. COS-7 cells were co-transfected with plasmid US11-EYFP and plasmids encoding dominant negative Ran Q69L, importin α5, importin β, respectively. EYFP and ECFP fluorescence were analyzed in living cells 24 h after transfection. Representative fluorescence images of the vast majority living cells expressing indicated fusion proteins were shown. Light-translucent pictures to show cellular morphology.

were not consistent with previous report that US11(89–106) and US(110–118) were critical for NoLS function of 88–125 peptide (Catez et al., 2002). Interestingly, US11(89–119)-EYFP was distributed exclusively in the cytoplasm, with no fluorescence in both the nucleus and nucleolus (Fig. 4B), indicating that the US11(89–119) was responsible for the nuclear export of US11. Taken together, these evidences indicate that the NES of US11 is located in its amino acids 89–119.

3.5. Characterization of the nuclear import mechanism of US11

The Ran protein has been shown to be required for classic NLS dependent nuclear transport (Moore and Blobel, 1993). To further explore the nuclear import mechanism of US11, a dominant negative Ran protein, Ran-GTP Q69L, which is deficient in GTP hydrolysis (Palacios et al., 1996) was introduced to determine whether Ran is required for the nuclear transport of US11. COS-7 cells were co-transfected with Ran-Q69L-ECFP and US11-EYFP and the subcellular localization of US11 and Ran Q69L was monitored. As shown in Fig. 5, co-transfection of Ran-GTP Q69L significantly blocked the nuclear import and nucleolar targeting of US11, whereas US11 alone could target to the nucleolus from cytoplasm. This result demonstrated that the nucleolar translocation of US11 was Ran dependent and required GTP hydrolysis.

A well-characterized class of NLS is recognized by the importin α/β heterodimer, where importin α recognizes the NLS, and importin β facilitates the importin α-NLS interaction by mediating a conformational change in importin α (Kobe, 1999). To identify the cellular receptor responsible for US11 nucleolar targeting and further characterize the nuclear import pathway of US11, a dominant negative importin α5 (Reid et al., 2007) and importin β (Guo et al., 2010), which lack the ability to bind importin β and Ran, respectively (Chi et al., 1997; Kutay et al., 1997), was introduced to determine whether they are required for the nuclear transport of US11. COS-7 cells were co-transfected with US11-EYFP and DN importin α5-ECFP or DN importin β-ECFP, and their subcellular localization was monitored. As shown in Fig. 5, co-transfection of DN importin β significantly blocked the nuclear import and nucleolar targeting of US11. However, dominant negative importin α5 did not inhibit the nuclear import and nucleolar localization of US11 protein. These results demonstrated that US11 nuclear import is mediated by a classic importin β-dependent mechanism, but does not require importin α5.

3.6. Characterization of the nuclear export mechanism of US11

Leucine-rich NES has been identified in an increasing number of cellular and viral proteins executing quite heterologous biological functions. Most studies pertaining to nuclear export have implicated the CRM-1 protein in facilitating the export of NES-containing proteins. An antibiotic compound, LMB, has been found to specifically inhibit CRM-1-mediated nuclear export (Fornerod et al., 1997; Ullman et al., 1997). To investigate the nuclear export mechanism of US11, LMB treatment was performed after transfection of US11-

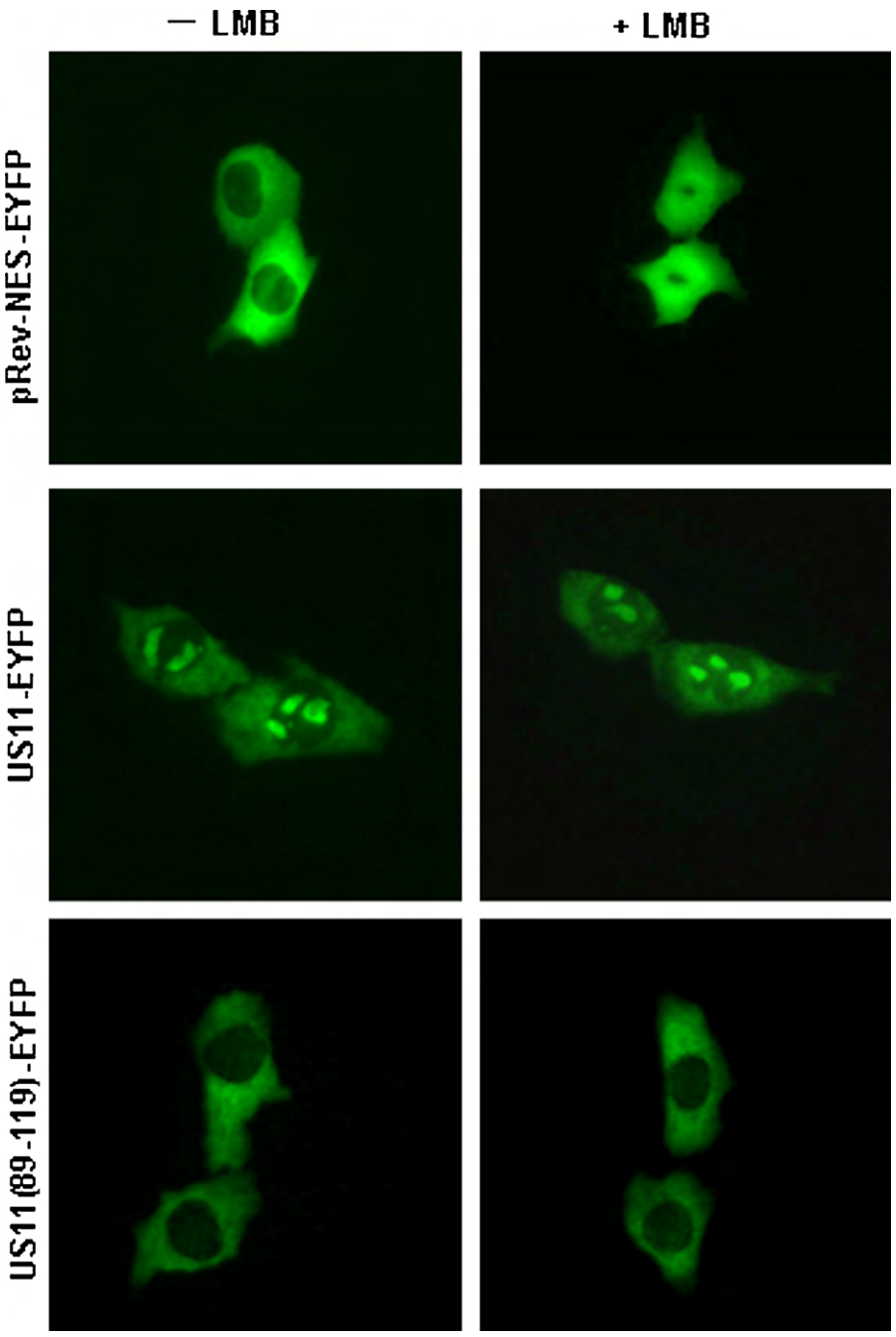

Fig. 6. The nuclear export mechanism of US11. COS-7 cells were transiently transfected with plasmids encoding US11-EYFP, US11(89–119)-EYFP and positive control plasmid pRev-NES-EGFP, with or without treatment with LMB, and examined live 24 h after transfection by fluorescence microscopy. Each image is representative of the majority of the cells observed in the same cells.

EYFP, US11(89–119)-EYFP and positive control pRev-NES-EGFP. The results showed that the nuclear export activities of US11 and its NES were not affected by a treatment with LMB at 20 ng ml^{-1} after transfection (Fig. 6). In contrast, the LMB treatment could completely abolished the nuclear export of pRev-NES-EGFP containing Leucine-rich NES (Williams et al., 2008). The evidence indicated that the export of US11 from the nucleolous to the cytoplasm is CRM-1 independent.

4. Discussion

As a multifunctional viral protein, the US11 protein is well conserved between HSV-1 and -2 (63% homology), with the greater variation at the N terminus. There are no US11 homologues in other neurotropic herpesviruses, such as varicella-zoster virus (VZV), bovine herpesvirus (BoHV) and pseudorabies virus (PRV). The US11 protein is among the most-abundant viral proteins present in cells late in infection and is packaged in the tegument of the native virions to be delivered into cells after infection (Roller et al., 1996). Soon after infection, the US11 protein is found in the cytoplasm, either as heterogeneous oligomers or associated with ribosomes or both (Diaz et al., 1993; Roller and Roizman, 1992). Later during infection, the US11 protein accumulates into nucleoli and is also found in ribonucleoprotein (RNP) fibrils as well as in clusters of interchromatin granules (Giraud et al., 2004). It was reported that HSV-1 infection affected the nucleolus, a prominent nuclear sub-structure implicated in the synthesis of ribosomal RNA (rRNA), cell cycle regulation and nucleo-cytoplasmic shuttling (Besse and Puvion-

Dutilleul, 1996; Boisvert et al., 2007). Nucleoli become elongated following infection, and the synthesis of mature rRNA is reduced (Besse and Puvion-Dutilleul, 1996). Apart from US11, several other HSV-1 proteins have also been shown to localize to, or associate with, the nucleolus (Emmott and Hiscox, 2009). The viral protein VP22 associates with the nucleolus and with dispersed nucleolin in HSV-1 infected cells (Lopez et al., 2008), and RL1 (ICP34.5), ICP0, ICP27 and UL24 have also been shown to localize to nucleoli (Bertrand et al., 2009; Mears et al., 1995; Morency et al., 2005; Salsman et al., 2008). Additionally, bovine infected cell protein 27 (BICP27) was, to date, the first viral protein of bovine herpesvirus type I identified to localize in the nucleoli (Guo et al., 2009).

It was reported that retention of US11 within the nucleoli and cytoplasm is mediated by a unique motif embedded into the C-terminal part of the protein (Catez et al., 2002), but the study was performed by *in vitro* microinjection of purified glutathione *S*-transferase linked to full length or deletion mutants of HSV-1 US11 protein into either the nucleus or the cytoplasm. Such *in vitro* techniques relying on analysis of exogenously expressed/purified protein and cytoplasmic factors have been previously used to characterize the different NLS-dependent nuclear import pathways, and determine basic parameters of nuclear import kinetics (Hu et al., 2005; Jans and Jans, 1994; Seydel and Jans, 1996). However, these methods are restricted to semi-intact or damaged cell systems that use high concentrations of nuclear localizing substrates and importins. In addition, the MT network, which has been implicated in facilitating the nuclear import of several proteins (Giannakakou et al., 2000; Lam et al., 2002; Roth et al., 2007), is either lacking or damaged in semi-intact cells in such an approach. In this study, however, the living cells fluorescence microscopy was employed for investigating the subcellular localization and nuclear import and export mechanisms of US11 protein. It is well known that living cells fluorescence microscopy has an advantage over conventional *in vitro* nuclear transport assays in that cells are not physically damaged by microinjection, detergent, or mechanical perforation, this means that cellular components important for trafficking, such as nuclear import and export receptors, the MT network, are intact (Dohner et al., 2002; Giannakakou et al., 2000).

In this study, the US11 protein of HSV-1 strain F showed highly homologous to that of other HSV-1 strains, with only one to three variations in XPR repeats (Fig. 1). It localized to both the nucleoli and cytoplasm in infected and transfected cells, and its C-terminal 84–152 peptide containing the XPR repeats showed identical subcellular localization to the wild-type US11. These results are in accordance with the previous reports (Diaz-Latoud et al., 1997; Diaz et al., 1996; MacLean et al., 1987). Catez et al. reported that the nucleolar retention signal (NoLS) of US11 located between amino acids 88 and 125, which was described as a new type of bipartite, while 89–97aa and 110–118aa were deduced to be the critical parts of the NoLS (Catez et al., 2002). However, our results showed that US11(88–125)-EYFP, US11(89–106)-EYFP and US11(110–118)-EYFP did not display any nucleolar enrichment, which are all inconsistent with their results (Catez et al., 2002). On the contrary, US11(84–125)-EYFP and US11(126–152)-EYFP showed identical subcellular localization to US11, while US11(84–88) or US11(120–125) deletion abolished the nucleolar retention of US11(84–125), and US11(126–134) or US11(147–152) deletion destroy completely the nucleolar retention of US11(126–152), these results indicate that these short stretch aa were critical for their nucleolar localization of US11(84–125) or US11(126–152), respectively, the discrepancy between our and previous results may result from the different experimental assay as mentioned above.

Interestingly, by further deleting the N- and C-terminal parts of US11(84–152), US11(89–146) also showed identical subcellular localization to US11 and targeted to the nucleoli. Roller et al. reported previously that the US11 protein of its deletion mutant viruse (deletion of amino acids 91–121) could located partially in the nucleoli by immunofluorescence assay (Roller et al., 1996), which further supported our results. In addition, it was reported that phosphorylation of Ser 129 stimulated the NES activity of GST-US11 (Catez et al., 2002). Interestingly, there is a site mutation in position 132 of HSV-1 strain F US11, from Ser (S) to Pro (P) (Fig. 1C). So, the mutation of Ser into Pro may be responsible for the nucleolar localization of US11(126–152) and US11(89–146). Taken together, US11(84–125), US11(126–152) or US11(89–146), alone or in combination, contribute to the nucleolar localization of US11. These results suggested that the XPR repeat number might determine its ability to target to the nucleolus.

It was reported that the 88–125 domain of US11 protein may contain an active NES, however, they did not identified the precise NES. Here, we mapped the NES of US11 to amino acids 89–119. In addition, the NES identified here did not display similarities with any classical leucine-rich NES. To our knowledge, the proline-rich NoLS and NES identified herein are to date novel and unique to US11 protein of HSV-1, and they may carry out their function with the conformation exhibited by unique XPR repeats. As reported previously (Diaz et al., 1996; Schaerer-Uthurralt et al., 1998), this region may form a poly-L-proline type II helix due to the proline residue present at every third position. This putative secondary structure, a right-handed single helix characterized by three residues per turn (Williamson, 1994), has a strong electrostatic polarity. Based on this, it is speculated that the formed helix may exhibited a special conformation for nucleolar retention and nuclear export.

Recently, it has become appreciated that some nuclear proteins are not confined to the nucleus once they are transported there. Rather, these proteins continuously shuttle between the nuclear and the cytoplasmic compartments. To date, the receptors for nuclear import and export of US11 protein are still not understood. Our results showed that the nuclear transport of the US11-EYFP fusion protein was inhibited by the Ran-GTP Q69L mutant, indicating that the US11 protein is a Ran dependent protein and is transported into the nucleus from the cytoplasm through classic nuclear transport machinery. Furthermore, cotransfection of dominant negative importins demonstrated that US11 import is mediated by an importin β-dependent mechanism that does not require importin α5. Additionally, chemical LMB did not inhibit the exports of US 11 and its NES (89–119aa) from the nucleolus to the cytoplasm, indicating the export of US11 protein do not achieved through its direct interaction with the nuclear export receptor CRM1.

In conclusion, we identified three nucleolar retention sequences and an NES of US11. Additionally, the US11 protein of HSV-1 was demonstrated to transport between the nucleolus and the cytoplasm through the non-classical NES by CRM1-independent pathway and through these nucleolar retention sequences by Ran-dependent and importin β-dependent mechanism that does not require importin α5.

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