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Human cytomegalovirus UL94 is a nucleocytoplasmic shuttling protein containing two NLSs and one NES

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ABSTRACT

The tegument protein UL94 is a human cytomegalovirus (HCMV) late protein and its function has yet to be determined. Using live cell fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) imaging, we found that UL94 is able to shuttle between the nucleus and cytoplasm. Analysis of UL94 mutants fused to EGFP showed that two newly characterized nuclear localization sequences (NLSs) and amino acid 343 play key roles in UL94 nuclear localization. Mutation of these sequences can alter the intracellular distribution of UL94 and disrupt its nucleocytoplasmic shuttling. Amino acid 343 of UL94 was also found to be crucial for its interaction with another HCMV tegument protein pp28. Furthermore, one nuclear export sequence (NES) was identified within UL94. Mutation of the key amino acids in the NES can also alter the intracellular distribution of UL94 and disrupt its shuttling function. Like other proteins containing a leucine-rich export signal, nuclear export of the UL94 was affected by leptomycin B, indicating that it is exported via the Crm1-dependent pathway. Our data provide a basis for further understanding the character and function of HCMV UL94.

1. Introduction

Human cytomegalovirus (HCMV) is a member of the betaherpesvirus family and can cause severe diseases, particularly in newborns and immunocompromised individuals (Mocarski and Courcelle, 2001; Pass, 2001). An infectious HCMV particle comprises three structural elements: an icosahedral capsid containing the double-stranded DNA genome, a tegument, and an envelope. The tegument, consisting of over 30 proteins, is unique to the herpesvirus family (Baldick and Shenk, 1996; Gibson, 1996; Mocarski and Courcelle, 2001). However, the characteristics and functions of many tegument proteins have not yet been determined.

The HCMV tegument protein UL94 is a 345-amino acid protein encoded by the ul94 open reading frame (ORF). This protein is a true late protein that is detected only during the late stages of a productive HCMV infection and is not synthesized in the presence of the viral DNA replication inhibitor ganciclovir (Wing and Huang, 1995; Wing et al., 1996). It is thought that HCMV UL94 is a homolog of the HSV-1 tegument protein UL16 (Chee, 1991; Chee et al., 1990; Higgins and Sharp, 1989). However, little is known about the characteristics and functions of HCMV UL94. We have recently shown that UL94 is a specific binding partner for pp28 (Liu et al., 2009). UL94, predominantly localized to the nucleus, could be directed into the cell cytoplasm by pp28 to colocalize with pp28 in a punctuate, juxtanuclear compartment, designated as the virus assembly compartment. Pp28 is an essential and abundant tegument protein required for HCMV assembly (final envelopment) and is related to the cell secretory pathway in the cytoplasm (Britt et al., 2004; Jones and Lee, 2004; Silva et al., 2003). In addition, intracellular localization of UL94 and the interaction between pp28 and UL94 may serve as a link in the sequential processes of HCMV capsidation. UL94 has also been revealed to interact with other tegument proteins UL82, UL25, and US22 by using Yeast Two Hybrid Analysis (To et al., 2011). Recently, using a murine cytomegalovirus (MCMV) model, Manninger et al.'s study revealed that the UL94 homologue gene is essential for viral trafficking at the secondary envelopment stage of MCMV (Maninger et al., 2011). Subsequently, Phillips and Bresnahan (2011) constructed a HCMV UL94-null mutant and revealed that UL94 functions late in infection to direct UL99 to the assembly complex, thereby facilitating secondary envelopment of virions. Therefore, it is important to investigate the localization and trafficking of HCMV UL94 in order to understand its function in viral infection and morphogenesis.

In the current study, we first studied the intracellular dynamic behavior of UL94 using fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) technologies. FRAP has been used extensively to examine the dynamics and

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mobility of proteins (Lippincott-Schwartz et al., 2001) and FLIP is ideal for studying the exchange of molecules between two compartments (e.g. compartments separated by lipid bilayers) (Cole et al., 1996; Nehls et al., 2000; Phair and Misteli, 2000). FRAP and FLIP imaging showed that UL94 is a nucleocytoplasmic shuttling protein. We then studied the mechanism that regulates the localization and trafficking of UL94. Two nuclear localization signals (NLSs) and one nuclear export sequence (NES) were discovered within UL94, neither of which has been reported previously. Amino acid 343 of UL94 was also found to be important for its nuclear localization and to play a key role in its interaction with pp28. Mutation of these localization-related sequences altered the intracellular distribution of UL94 and disrupted its nucleocytoplasmic shuttling. Furthermore, drug inhibition experiments indicated that the trafficking of UL94 may be linked to the classical Crm1-dependent nuclear export pathway.

2. Materials and methods

2.1. Plasmid construction

For FRAP and FLIP analysis, the UL94 gene (GenBank ID: 3077496) and its mutants were inserted into pEGFP-C1 via Bgl II and EcoRI sites to construct the plasmid pEGFPUL94 and a panel of UL94 mutants (Fig. 3A and Table S1). The construction, cloning, and propagation of plasmids were carried out using standard techniques (J. Sambrook). All constructs were verified by DNA sequencing (Invitrogen Biotechnology Co., Ltd., Shanghai, China).

2.2. Cell culture, transfection and FRET assay

Vero (African green monkey kidney) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml), at 37 °C in 5% CO_2. The day before transfection, Vero cells were seeded onto 35 mm glass bottom culture dishes at 3×10^5 cells per dish. Cells were transfected with plasmids using lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. Transfected cells were incubated at 37 °C (5% CO_2) overnight (18–20 h) and the medium was replaced with fresh medium before examination by microscopy. FRET assay was carried out as described previously (Liu et al., 2009).

2.3. Live cell imaging and fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) analysis

Fluorescence localization was imaged using an inverted wide-field fluorescence microscope (Axiovert 200, Carl Zeiss, Germany). For live cell imaging, the prepared cell culture dishes were placed in a temperature-controlled incubator at 37 °C and detection was

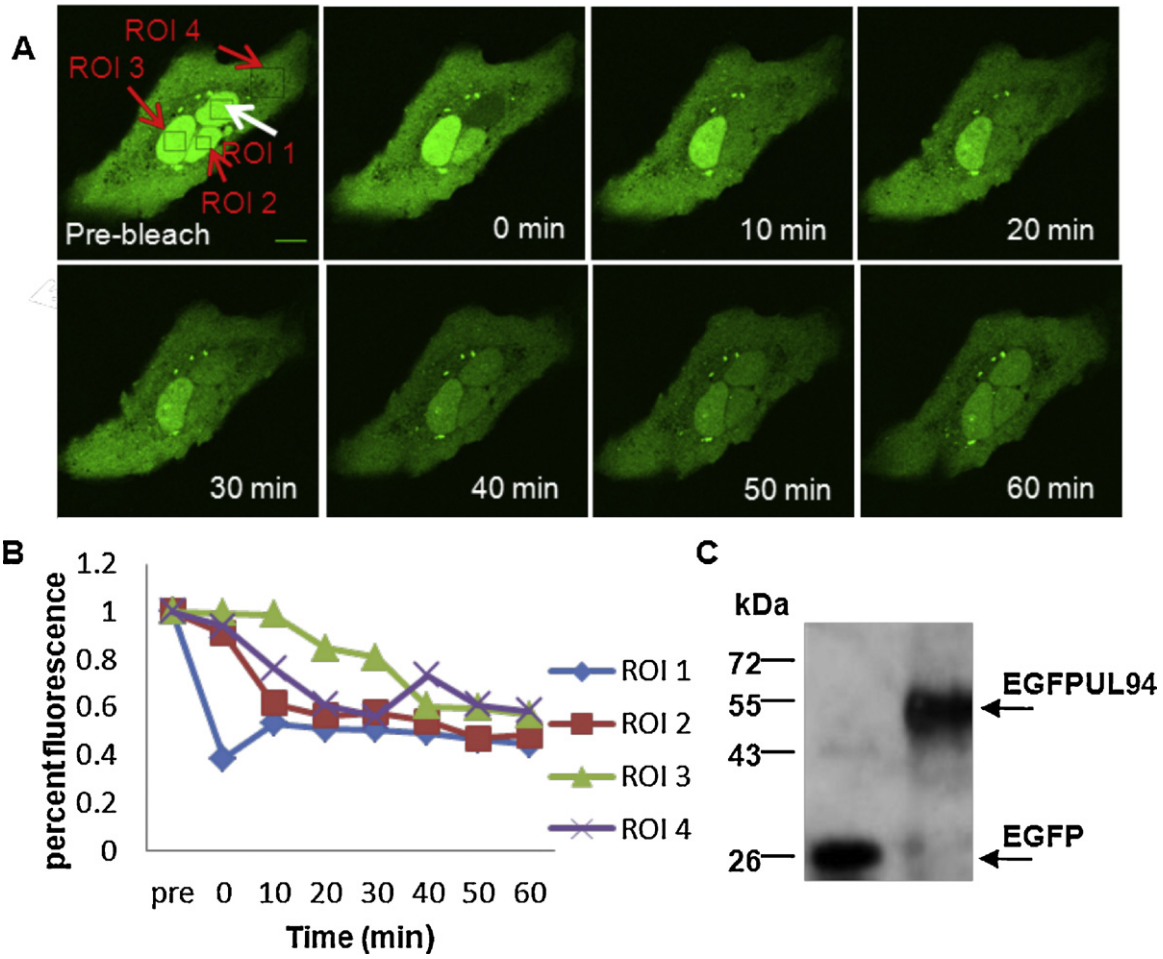

Fig. 1. Selective FRAP was used to demonstrate repeated nucleocytoplasmic shuttling of UL94 in multinuclear cells. (A) Serial images of EGFPUL94 in a selective FRAP experiment. The bleached nucleus is indicated by a white arrowhead. (B) Curves showing the fluorescence intensity of EGFPUL94 in different regions in a selective FRAP experiment (one region (ROI 1) in bleached nucleus; two regions in the unbleached nucleus (ROI 2 and ROI 3) and one region (ROI 4) in the cytoplasm). Bar = 10 μm. (C) Western blot of EGFP and EGFPUL94.