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Interactions of the HSV-1 UL25 Capsid Protein with Cellular Microtubule-associated Protein*

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Abstract: An interaction between the HSV-1 UL25 capsid protein and cellular microtubule-associated protein was found using a yeast two-hybrid screen and β–D-galactosidase activity assays. Immunofluorescence microscopy of the UL25 protein demonstrated its co-localization with cellular microtubule-associated protein in the plasma membrane. Further investigations with deletion mutants suggest that UL25 is likely to have a function in the nucleus.

Key words: HSV-1; Capsid; UL25; Microtubule-associated protein

Many of the biological studies on the Herpes Simplex Virus Type 1 (HSV-1) infectious process have elucidated the events of HSV-1 entry into host cells: HSV-1 releases its capsid and the tegument proteins into the cytosol of a host cell by fusing with the plasma membrane; the capsid is then transported by a specifically mediated mechanism to the nucleus where it binds to the cytosolic side of nuclear pore complexes, and the viral genome is rapidly released into the nucleus(6,11). Although this process is very short, it is involved in complicated infectious dynamic actions generated between associated capsid protein complexes and the cytoskeleton (3). It has been reported that the HSV-1 UL31, UL34 etc. capsid proteins play an important role in the egress of virions from the nucleus into the cytoplasm (5,12). Meanwhile, the functions and structures of several capsid proteins are most likely to offer necessary support for viral capsids and teguments to eventually complete the infection process (2). However, investigations regarding this aspect are insufficient, and the mechanistic details of several associated capsid proteins in this process remain unclear. The UL25 gene product encoding 1740 bp with a molecular weight of 62 KD is an essential capsid protein that is required for many crucial biological events that take place during viral infection. These include the facilitation of the route of transit, and proliferation and assembling etc. of virions in cells(1,7,8,9,10,13,15). These basic functions of the UL25 gene product suggest that this protein is most likely to interact with various molecules in cells. Based on this implication, we utilized biochemical

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assays for protein analysis to investigate the interaction of UL25 with other proteins, and its localization through fluorescence microscopy. Our results revealed that the UL25 protein plays an essential role in the transport of viral capsids to the nucleus.

MATERIALS AND METHODS

Plasmids, bacteria, virus, cells and reagents

Eukaryotic expression plasmid pEGFP-N2, which expresses the enhanced green fluorescent protein, and the matchmaker gail yeast two-hybrid system 3 were purchased from Clontech; competent *E.Coli* DH5α cells were purchased from Promega; the HSV-1 virus (8F strain) was isolated and maintained in this lab; African green monkey kidney cells (Vero cell) and human embryo lung diploid cells (KMB-17) were maintained by this lab; PCR Taq DNA polymerase, T4 DNA ligase and restriction enzymes *Bam*H I, *Eco*R I, RNase A were purchased from Takara; The DNA marker DL2000 was purchased from Beijing Biotech; endotoxin-free plasmid preparation kits were also purchased from Beijing Biotech; DNA fragment purification kit was from BioDev-Tech. Co.; Lipofectamine2000 was from Invitrogen; the secondary antibodies sheep anti-mouse IgG marked by BSA and TRITC were from Beijing Dingguo Biotech; the anti-tubulin mice monoclonal antibody was purchased from Biyuntian Biotech; DMEM medium was produced by the Institute; colchicine was purchased from Sigma.

Primer design and PCR amplification

UL25 fragment and other deletion mutant fragments were amplified according to GenBank HSV-1 UL25 gene sequences (AB252709) (Fig. 1).

The primers with an *Eco*R I enzyme site in the upstream primer (*up*) and a *Bam*H I enzyme site in the downstream primer (*dp*) were synthesized by Shanghai Shenggong Company. The sequences of primers are: UL25 *up*：5’-AGCGAATTCATGGACCCGTATTGCCCATTTG-3’; UL25 *dp*：5’-AATGGATCCTAACCGCCGACAGGTACTGTG-3’; UL25-N *up*: 5’-AGCGAATTCATGGACCCGTATTGCCCATTTG-3’; UL25-N *dp*: 5’-ATAGGATCCAGATCTGGACCTCCATCTCCG-3’; UL25-M *up*: 5’- GAGAATTCATGGAGATGGAGGTCCAGATCG-3’; UL25-M *dp*: 5’-ATAGGATCCGGTTTATGTCGTCGTGTG-3’; UL25-C *up*: 5’-AGCGAATTCATGCACCACGACGACATAAACC-3’; UL25-C *dp*: 5’-AATGGATCCTAACCGCCGACAGGTACTGTG-3’.

The UL25 fragment was amplified using DNA extracts from viral solutions reserved by this lab as the template, and the UL25-N, UL25-M and UL25-C deletion mutant fragments were amplified using the UL25 fragment as the template. The PCR program was set as follows: 94℃ denaturation 45s; 51℃ annealing 45s; 72℃ elongation 1 min; 30 cycles.

Recombinant plasmid construction and identification

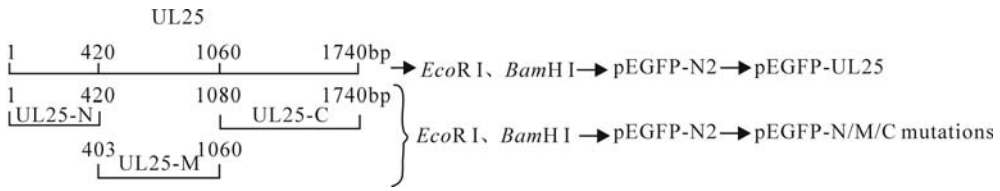

Fig.1. Design of UL25 and its mutations

The pEGFP-N2 plasmid and amplified UL25, UL25-N, UL25-M and UL25-C fragments were digested by *Eco*R I and *Bam*H I enzymes, and then ligated with T4 ligase into pEGFP-N2 followed by transformation into competent *E.Coli* DH5α cells. Clones were picked randomly and plasmids were extracted by the alkaline lysis approach or using DNA purification kits followed by identification by *Eco*R I and *Bam*H I enzyme digestion. Positive samples were sent for sequencing to Beijing Sanbo Biotech. The pGBKT7-UL25 plasmid was constructed and reserved by this lab.

Yeast two-hybrid system screening

AH109 yeast cells were transformed with the recombinant plasmid pGBKT7-UL25 and was fused with a cDNA (Clontech) library from prepared human embryonic kidney cells according to standard protocol. The resulting clones were inoculated at the same time into 2 volumes of YPDA medium for incubation at 30℃ for 20-24 h. The fused culture was inoculated onto SD/-Lir-Trp plates for incubation at 30℃ for 1 week. Clones were inoculated onto SD/-His-Leu-Trp and SD/-Ade-His-Leu-Trp plates, respectively for incubation at 30℃ for 1 week, and positive clones were screened and transformed into *E.Coli* for sequencing.

D-galactosidase activity assay

The ONPG liquid assay was employed. Clones were picked and inoculated into 5mL SD/-His-Leu-Trp liquid medium with shaking overnight at 30℃ and 200r/min, and then trans-inoculated into YPD medium with continued shaking at 30℃ for 3-5 hrs. The *OD* values were recorded. Yeast bacteria were collected when the OD_{600} value reached 0.5 ~0.8. Samples were mixed with 4mg/mL ONPG and incubated at 30℃ until the solution turned a yellow color, and the reaction time was recorded. The values for 420nm light absorption as measured by a spectrometer were recorded after removing cell debris. β–D-galactosidase unit was calculated by the following formula: β–D-galactosidase units=$1000\times OD_{420}/t\times 5\times OD_{600}$ (where OD_{600} is equal to the A_{600} absorption value of 1mL culture; t is the reaction time). The experiment was repeated three times with positive (pGBK-p53+pACT-LT) and negative (pGBK-Lam+ pACT-LT) controls.

Cell culture and tranfection

Vero cells and KMB-17 cells grown in DMEM medium with 8% bovine serum, 100U/mL penicillin, 20μg/mL streptomycin were incubated in 5% CO_2 at 37℃. Transfection of the plasmids pEGFP-N2 (negative control), pEGFP-UL25, pEGFP-UL25-N, pEGFP-UL25-M and pEGFP-UL25-C into Vero and KMB17 cells were performed accor- ding to the manufacturer's instructions. Expression was examined under an immunofluorescence microscope 24-48 h posttransfection.

Cell immunoflurescence and drug inhibition

Immunofluorescence staining was performed on cells and treated with 0.2% Triton X-100 PBS containing 2% formaldehyde at 4℃ for 15 min, and blocked with 2% BSA in 0.5% Tween-20 PBS (pH7.4) at 37℃ for 30 min. The cells were incubated with primary antibody at 37℃ for 90 min and with secondary antibody at 37℃ for 30 min, each with extensive washings with PBS between steps. The cells were examined with an immunofluorescence microscope.

Drug inhibition: normal cell culture medium was replaced with 50 μmol/L cell culture medium con-

Table 1. The genes coding proteins interacting with UL25 specifically from yeast trap

No.	Gene	GenBank No.
5	Homo sapiens ankyrin 2, neuronal (ANK2), transcript variant 2, mRNA	NM_020977.2
8	Homo sapiens eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), mRNA	NM_001402.5
25	Homo sapiens cDNA FLJ20612 fis, clone KAT05509	AK000619.1
82	tubulin, alpha, ubiquitous [Homo sapiens]	NP_006073

taining colchicine and maintained at 37℃ for 1h or 2 h before observing immunoflurescence.

RESULTS

Identification of recombinant plasmids

All of the plasmids used for co-localization in this study were constructed by PCR amplification, recombinant cloning, and verified by enzyme digestion and sequencing etc.

Interaction of the UL25 protein with cellular microtubule-associated protein

By using a yeast two-hybrid assay, UL25 protein was found to interact with cellular microtubule-associated protein. Interestingly, we also found interactions between UL25 and several cellular proteins with transcriptional and regulatory significance (Table 1). The interaction of UL25 with cellular microtubule-associated protein was analyzed in detail using a β–D-galactosidase binding assay, and the results showed a specific interaction between them (Fig. 2) .

Expression and localization of UL25 protein in different cells

In order to observe the localization of UL25 in cells, the UL25-EGFP fusion protein expression plasmid was transformed into Vero and KMB17 cells, respectively. The fluorescence examined at 24-48 posttransfection indicated that UL25 was scattered throughout the cell (Fig. 3).

UL25 protein co-localizes with microtubule-associated protein at the cytoskeleton

Based upon the yeast two-hybrid assay results and the observed localization of UL25 protein in cells, we further investigated the interaction of UL25 with microtubule-associated protein in cells using the UL25-EGFP fusion protein and an anti-cellular microtubule-associated immunofluorescence antibody. The results demonstrated that both the UL25-EFFP fusion protein and the microtubule-associated protein localized to the microtubule-organizing center (MTOC), implying that UL25 is localized to the cytoskeleton via binding to cellular microtubule-associated protein (Fig. 4).

To further testify whether UL25 interacts with microtubule-associated protein *in vivo*, we inhibited the posttransfection cell with Colchicine as methods mentioned above. Colchicine can dissociate microtubules and inhibit cell growth. At this time, location of the UL25-EGFP fusion protein in microtubule dissociated Vero or KMB -17 cell changed, most of which accumulated at MTOC, but some were scattered in a manner consistent with tubulin (Fig. 5).

Interaction of different domains of UL25 protein with cellular microtubule-associated protein

In order to further biochemically characterize the interaction of UL25 with cellular microtubule-

associated protein, different deletion mutants of the UL25 gene were constructed (Fig.1), recombined into the pEGFP-N2 vector and transformed into different cells, respectively. Interestingly, fluores-

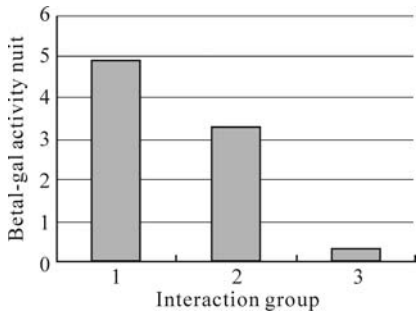

Fig.2. Binding assay of UL25 protein and tubulin. 1, Positive control (pGBK-p53+pACT-LT); 2, pGBKT7-UL25+pGADT 7-tubulin; 3, Negative control (pGBK-Lam+pACT-LT))

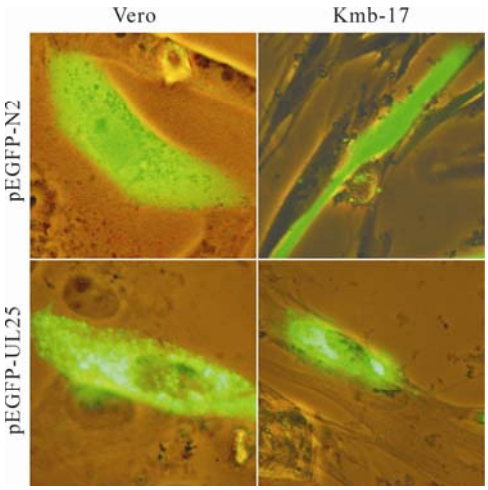

Fig.3. Location of UL25 in Vero and Kmb-17

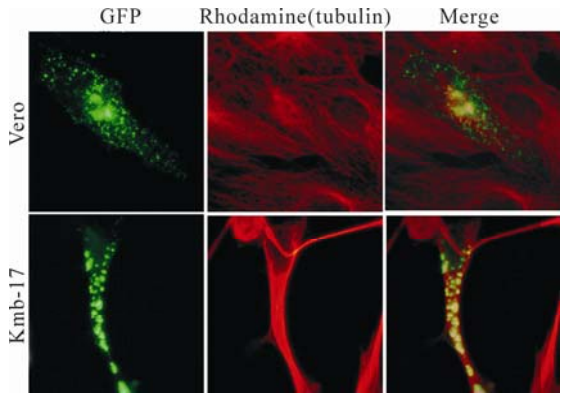

Fig.4. Fluorescence and colocalization of UL25 and tubulin

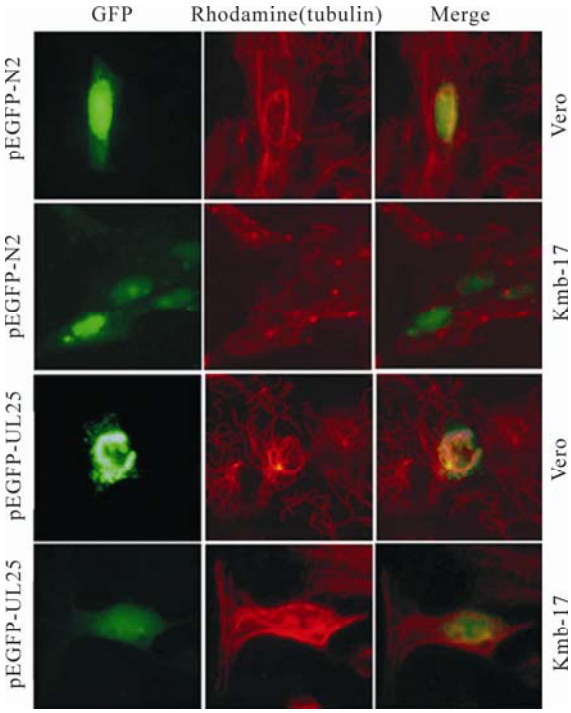

Fig.5. Colocalization fluorescence of UL25 and tubulin after colchicine treatment

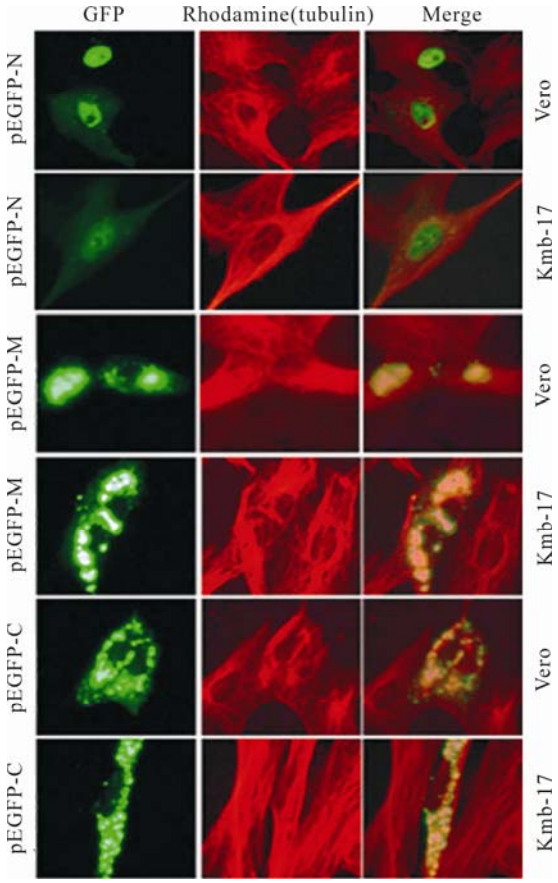

Fig.6. Mutations fluorescence and colocalize with tubulin

cence microscopy showed that the C terminus (UL-C) of UL-25 was similar to its middle fragment (UL25-M), and the N-terminus domain (1-140AA) of UL25 localizes to the plasma membrane, as well as to nuclear pore complexes (Fig. 6). Along with our yeast two-hybrid data (Table 1), this suggests that UL25 also is involved in transcriptional and regu- latory functions in nucleus. From these results, we conclude that UL25 protein is involved not only in its directed transfer in cells via interaction with cellular microtubule-associated protein, but also has unknown functions in the nucleus.

DISCUSSION

Biological studies on HSV-1 infection show evidence that incoming HSV-1 capsids are transported to the nucleus via the cytoskeleton after entry into cells (14), during which time the UL34, UL25, UL36 and US3 etc. proteins bind to the cytoskeleton and the cellular microtubule-associated dynamic protein and dynamic activating protein drive the directed transfer of incoming capsids in an ATP-dependent manner(2, 4). Nevertheless, the molecular biological functions of each viral protein in this process remain unclear. To understand the systematic and functional significance corresponding to the interactions of these proteins with cellular proteins it is critical to study the functions of each individual protein. In this work, the observation of the physical interaction of UL25 protein with cellular microtubule-associated protein and its localization in the cytoskeleton in yeast two-hybrid assays provide evidence that UL25 is involved in the directed transfer of incoming capsids in cells. Indeed, we found that UL25 binds to other proteins, such as ankyrin 2 and neuronal (ANK2) (Table 1), in our yeast two-hybrid assays and we hypothesize that such interactions facilitate the transfer of virions into cells.

The analysis of the UL25 protein using approaches for deletion mutants revealed that the N terminus domain of UL25 binds to cellular microtubule-associated proteins, while binding to other molecules in cells, particularly to some transcriptional and regulatory proteins such as eukaryotic translation elongation factor (Table 1) in the nucleus. The findings in our study are similar to previous reports, in which UL25 was deemed to correspond to the VP-19C capsid for entry into the nucleus (10). If such a finding is confirmed in subsequent studies, it might allow us to conclude that UL25 is involved in transport of virions in cells in addition to regulating mechanisms of transcription. Nevertheless, if a potential association is substantiated for these two distinct biological events, a new breakthrough into understanding the biology of HSV-1 infections will be achieved.

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