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Virus Laboratory, the Affiliated Shengjing Hospital, China Medical University, Shenyang, Liaoning, China

Abstract

Interplay between the host and human cytomegalovirus (HCMV) has a pivotal role in the outcome of infection. A region (referred to as UL/b') present in the Toledo strain of HCMV and low passage clinical isolates contains 19 additional genes, which are absent in the highly passaged laboratory strain AD169. Products of the *UL/b'* genes may determine the manifestations of HCMV infection *in vivo.* However, little is known about the host factors, which interact with UL/b' proteins. This study was conducted to investigate the function of the HCMV UL136 protein. By yeast two-hybrid screening, the β1 subunit of the host Na^+/K^+-ATPase (ATP1B1) was identified to be a candidate protein, which interacts with the HCMV UL136 protein. The interaction was further evaluated both *in vitro* by pull-down assay and *in vivo* by immunofluorescent co-localization. The results showed that the UL136 protein can interact with ATP1B1 *in vitro*. Co-localization of UL136-EGFP and ATP1B1-DsRed in cell membranes suggests that ATP1B1 was a partner of the UL136 protein. It can be proposed that the HCMV UL136 protein may have important roles in processes such as cell-to-cell spread, and in maintaining cell osmotic pressure and intracellular ion homeostasis during HCMV infection.

Key words: HCMV; UL136; ATPase; Yeast two hybridization

Introduction

Human cytomegalovirus (HCMV), a member of the Betaherpesvirinae subfamily, is widely distributed in human populations. It can cause a minor or asymptomatic infection in immunocompetent individuals, and cause severe disease in neonates and immunosuppressed individuals such as allograft transplant recipients and AIDS patients (1). Although the exact mechanisms are not known, a majority of researchers believe that a substantial portion of the HCMV-encoded proteins have the potential to affect virulence through cell tropism, immune evasion, molecular mimicry, or interference with host chemokines (2,3).

The HCMV genome comprises 236 kb dsDNA, and many of its genes are nonessential for viral replication in fibroblasts (4). Since 1996, it has been recognized that a 15-kb unique region (UL/b') contains at least 19 open reading frames (ORFs). These genes are found in clinical HCMV isolates but not in the extensively passaged laboratory AD169 strains, which are dispensable for growth *in vitro* (5). The retention of these ORFs in clinical HCMV isolates, and the fact that the virus can replicate in SCID mice implanted with human tissues suggest that the UL/b' region is required for viral infection *in vivo* (6). The functions of several UL/b' region genes, such as UL132, UL141, UL142, UL144, UL146, and UL147 have been previously investigated. The gpUL132 was observed to co-localize with markers for the trans-Golgi network (7) and with other viral envelope glycoproteins such as gB and gH, serving as mediators for attachment and fusion with the target cells. The gpUL141 specifically acts to downregulate cell surface expression of the natural killer (NK) cell-activating receptor CD155 (8). Soluble tagged gpUL141 inhibits the binding of the monoclonal antibody D171 to CD155, suggesting a direct interaction between CD155 and gpUL141. The UL142 gene product is capable of modulating natural killer NK cell recognition when it is expressed in isolation or in the context of viral infection and inhibiting NK cell-mediated lysis (9). The UL144 gene encodes a structural homologue of the herpes virus entry mediator, upregulating

Correspondence: Zheng-Rong Sun or Qiang Ruan, Virus Laboratory, the Affiliated Shengjing Hospital, China Medical University, Shenyang, Liaoning 110004, China. Fax: +86-024-2389-2617. E-mail: ruanq@sj-hospital.org (Qiang Ruan), or sunzr@sj-hospital.org (Zheng-Rong Sun).

*These authors contributed equally to this study.

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NFκB-dependent transcription through a mechanism involving TNFR-activated factor 6 (TRAF6) (10). The products of the UL146 and UL147 genes have limited homology to CXC chemokines. The pUL146 protein induces neutrophil chemotaxis and calcium mobilization (11).

The UL136 ORF is 723 bp in size, and has the potential to encode a 241-amino acid protein. The UL136 protein is a weakly alkaline protein with a molecular mass of ~27.3 kDa; however, little is known about the function of the UL136 gene product. In the present study, ATPase, a Na/K transporting beta 1 polypeptide was identified as one of the binding candidates of the UL136 putative protein.

Material and Methods

Yeast two-hybrid screen

The sequence of UL136 ORF was amplified by the polymerase chain reaction (PCR) using HCMV H strain DNA (GenBank No. GQ981646) as a template with primers 5′-CCGGAATTCTTGTCGACCTCAGTCAAGGGCGTGG AGATG-3′ (forward, *Eco*RI included) and 5′-CGCGGAT CCGGTACCTTACGTAGCGGGAGATACGG-3′ (reverse, *Bam*HI included), and then cloned into the C-terminal domain of the shuttle plasmid pGBKT7 (Clontech, USA) vector via *Eco*RI and *Bam*HI restriction sites. The construct of pGBKT7-UL136 was used as the bait in yeast two-hybrid experiments.

The yeast two-hybrid experiments were performed according to manufacturer instructions (Matchmaker GAL4 Two-Hybrid System 3; Clontech). *Saccharomyces cerevisae* AH109 was co-transformed with plasmids of both pGBKT7-UL136 and the human fetus brain cDNA Library, pACT2-cDNA. Positive clones were selected on synthetic dropout medium in the absence of 4 nutrients (Leu/Trp/Ade/His) and the selected clones were then confirmed by detection of α-galactosidase activity. Colonies turning blue were kept, and the positive results were confirmed by repeat assays. The recombinant plasmids isolated from the positive clones were co-transformed into yeast with the pGBKT7-UL136 for further confirmation of the interaction. Sequencing of the selected clones was performed by the Blast network service at the National Center for Biotechnology Information (http://www.ncbi.nlm.gov/blast).

GST-pull-down assay

The sequence of one candidate of the pUL136 interacting proteins (pACT2-ATP1B1) was cloned into the glutathione S transferase (GST)-tagged pGEX-4T-2 vector in *Eco*RI and *Xho*I sites, designated as GST-ATP1B1.

The GST-pull-down experiment was performed according to manufacturer instructions (MagneGST™ Pull-Down System, Promega, USA). The c-Myc-labeled protein expressed from the pGBKT7-UL136 was used as a prey protein in a TNT T7 Quick Reaction. As a bait protein, 20 µL GST-ATP1B1 protein was incubated with 80 µL pGBKT7-UL136 expressed protein at room temperature for 1.5 h on a rotating platform. The reaction products were then incubated with MagneGST particles for 30 min. After 3 washes with buffer, the binding proteins on the MagneGST particles were eluted with elution buffer and solubilized in 2X SDS sample buffer.

Protein samples were subjected to electrophoresis on a 12% SDS-polyacrylamide gel, and the separated proteins were blotted onto a nitrocellulose membrane (Sigma, USA). Western blot analyses were performed using mouse anti-c-Myc or goat anti-GST monoclonal antibodies (Pierce, USA). Blots were treated with horseradish peroxidase-labeled anti-mouse or anti-goat secondary antibodies, respectively. Signals were revealed by the Molecular Imager ChemiDoc XRS System (Bio-Rad, Inc., USA).

Immunofluorescent co-localization

Commercial pEGFP-C1 and pDsRed-C1 (Clontech) vectors were used to express fusion proteins with fluorescent tags. For the pEGFP-UL136 construct, the UL136 coding region was amplified from the HCMV H strain using the primers 5′-CGGAATTCCTCAGTCAAGGGCGTG-3′ (forward, *Eco*RI included) and 5′-CGCGGATCCTTACGT AGCGGGAGA-3′ (reverse, *Bam*HI included). The product was then cloned into the pEGFP-C1 vector via *Eco*RI and *Bam*HI sites, yielding pEGFP-UL136. For the pDsRed-ATP1B1 construct, the fragment of ATP1B1 was amplified by PCR using pACT2-ATP1B1 as a template with primers 5′-CGGAATTCCGTCTGCAGATTCAAG-3′ (forward, *Eco*RI included) and 5′-CGCGGATCCTGTCATTTACAGTAG-3′ (reverse, *Bam*HI included). Next, the amplicon was cloned into the pDsRed-C1 vector via *Eco*RI and *Bam*HI sites, yielding pDsRed-ATP1B1. All constructs were verified by DNA sequencing (Invitrogen Biotechnology Co., Ltd., China).

293T cells were grown in DMEM containing 10% fetal calf serum. At 75% confluence, cells were co-transfected with a mixture of 3 µg pEGFP-UL136 and 5 µg pDsRed-ATP1B1 using lipofectamine 2000 (Invitrogen) according to manufacturer instructions. Cells were analyzed using a TCS SP2 Leica laser scanning confocal microscope (Nikon Eclipse Ti-E, Japan) equipped with a cooled CCD camera with a 488-nm and a 543-nm excitation beam.

Results

Results of sequence blast online showed that several positive clones contained the coding sequence of the ATPase beta 1 polypeptide (GenBank No. NM001677), showing 99% nucleotide sequence identity. Biotechnology Information indicated that the UL136 protein could possibly interact with the C-terminal of the ATP1B1 protein *in vitro*.

To confirm the interaction between ATP1B1 and UL136 proteins, pull-down experiments were employed using expressed tagged proteins. A strong and specific interaction

between the ATP1B1 and UL136 proteins was demonstrated (Figure 1).

In an immunofluorescent co-localization test, pDsRed/ATP1B1 was found in the cell membrane, and the pEGFP/UL136 fusion protein was localized in both the cell membrane and the cytoplasm. Results of confocal microscopic analysis revealed that the UL136 fusion protein showed the same location as the ATP1B1 fusion protein on the membrane of mammalian cells (Figure 2).

Discussion

Genes in the HCMV UL/b' region encode several important proteins including the cell-tropic factors (UL131A -128), virion structure (UL132) (2,7), viral latency determinant (UL138), and host cell machinery modulation factors (UL141, 142, 144, and 148) (8-10,12,13). This region is one of the most important targets to elucidate the mechanism of pathogenesis related to clinical HCMV isolates. So far, research on targets of the UL136 protein has been found in only one publication concerning HCMV UL138, but there was no further functional analysis of the UL136 protein (13).

Host factors may have important roles in viral entry, replication, budding, release, pathogenesis, and restricting cross-species transmission. Many host factors have been identified to interact with HCMV proteins and are involved in all stages of the virus life cycle (14). Similar to other viral infections, HCMV is known to perturb a number of host cell functions. The majority of these perturbations presumably optimize host cell functions and conditions to support viral persistence and productive viral replication (15,16). Earlier studies have demonstrated that both cytomegaly and viral replication strongly depend on the presence of extracellular

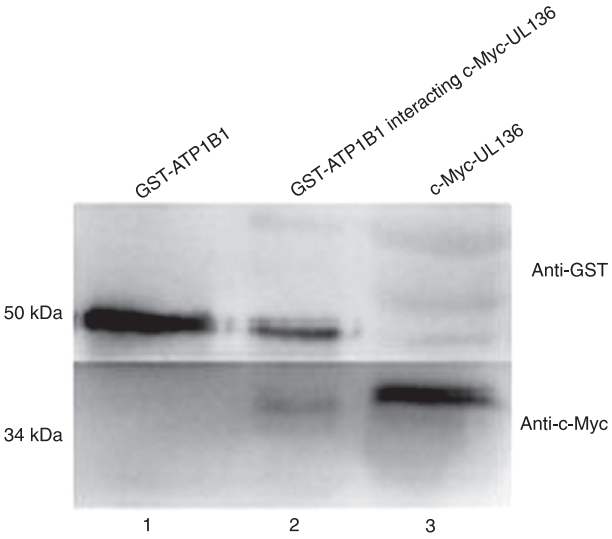

Figure 1. Result of a pull-down experiment analyzed by Western blot. The upper part of the figure was reacted with a goat anti-GST monoclonal antibody, and the lower part was reacted with a mouse anti-c-Myc monoclonal antibody, separately. *Lane 1* shows the protein lysates of transfected DH5α expressing GST-ATP1B1 bait protein. *Lane 2* shows interaction between c-Myc-tagged pUL136 protein and GST-ATP1B1. *Lane 3* shows c-Myc-tagged pUL136 protein.

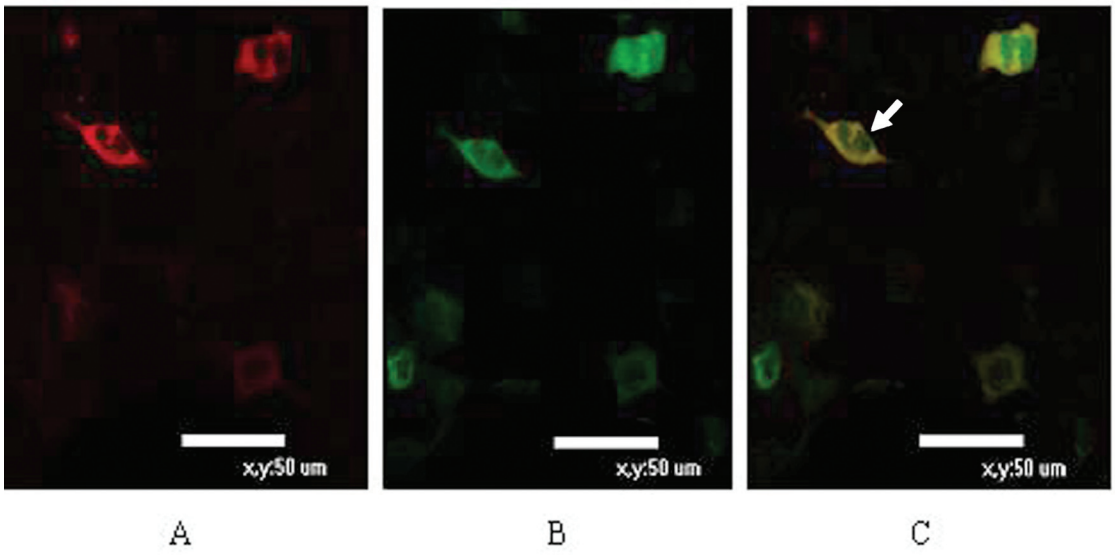

Figure 2. Immunofluorescent co-localization test. *A*, pDsRed/ATP1B1 localized in the cell membrane; *B*, the pEGFP/UL136 fusion protein localized in both the cell membrane and cytoplasm; *C*, confocal microscopic analysis revealed that UL136 fusion protein co-localized with ATP1B1 fusion protein in mammalian cells on the membrane.

Na^+ (17). Therefore, it is not surprising that HCMV infection is characterized by significant effects on a variety of membrane ion transporters that mediate Na^+ transmembrane movements. Examples of such transporters include the sodium pump and the Na^+/H^+ exchanger (NHE), as well as the Cl^-/HCO_3^- exchanger, which works in concert with NHE to mediate net uptake of inorganic osmolytes promoting cell volume increase. Previously, Fons et al. (18) reported that treatment of HCMV-infected cells with an inhibitor of NHE (amiloride) inhibited HCMV replication by almost 2 orders of magnitude.

ATP1B1 is a member of the Na, K-ATPase family of proteins, which are ubiquitous transmembrane proteins that establish and maintain an electrochemical gradient across the plasma membrane in epithelial cells (19). Na, K-ATPase consists of α and β subunits. The glycosylated β subunit has been proposed to facilitate the assembly and transport of the α subunit from the endoplasmic reticulum to the plasma membrane (20,21), and the β subunit is the key factor in polarizing distribution of Na, K-ATPase (22). The results of the present study showed that the HCMV UL136 protein has the ability to interact with ATP1B1. Biotechnology Information indicated that the UL136 protein could interact with the C-terminal of ATP1B1 *in vitro*. It is predicted that the N-terminal of ATP1B1 located inside the transmembrane region and the UL136-encoded protein also has a potential transmembrane domain. These findings suggest that the UL136 protein may interact with ATP1B1 inside the membrane. To further confirm whether the UL136-encoded protein shares the same location with ATP1B1 in cells, the UL136 and ATP1B1 expressing vectors were co-transferred in mammalian cells. Confocal microscopic analysis revealed that the UL136-encoded protein was co-localized with the ATP1B1 protein on the membranes of mammalian cells.

ATP1B1 is an auxiliary subunit of Na^+/K^+-ATPase, which is an important regulator of cellular ion homeostasis (23). Recent studies have shown that ATP1B1 can interact with proteins other than the Na^+/K^+-ATPase α subunit, such as NKIP and BKCa (24,25). Reduced expression of endogenous ATP1B1 can markedly inhibit evoked BKCa currents (25). Therefore, we hypothesize that interaction of ATP1B1 with the UL136 protein possibly facilitates the transport and correct assembly of the Na^+/K^+-ATPase α subunit in the cell membrane and affects ion channel activity.

Based on the results of this study, we propose that the UL136-encoded protein may be important for maintaining cell osmotic pressure and intracellular ion homeostasis. Details of the mechanisms involved in this process require further investigation.

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