Herpes simplex virus type 2 membrane protein UL56 associates with the kinesin motor protein KIF1A

Tetsuo Koshizuka,1 Yasushi Kawaguchi1,2 and Yukihiro Nishiyama1

1Department of Virology, Graduate School of Medicine, Nagoya University, 65 Showa-ku, Nagoya 466-8550, Japan
2PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama, Japan

The herpes simplex virus UL56 gene product is a C-terminal-anchored, type II membrane protein of unknown function. UL56 was found to interact with KIF1A, a member of the kinesin-3 family, in a yeast two-hybrid screen and a GST pull-down assay. KIF1A mediates the transport of synaptic vesicle precursors and is essential for the function and viability of neurons. When overexpressed, KIF1A co-localized with full-sized UL56, but no clear co-localization was observed when co-expressed with the UL56 mutant protein lacking its C-terminal transmembrane domain (TMD). Although the C-terminal TMD was not essential for the interaction with KIF1A in the yeast two-hybrid screen and GST pull-down assays, these results indicate that the C-terminal TMD, as well as aa 69–217, of UL56 are important for the interaction with KIF1A in vivo. The hypothesis that the UL56 protein affects vesicular trafficking in infected cells, potentially by acting as a receptor for motor proteins in neurons, is discussed.

INTRODUCTION

Herpes simplex virus (HSV) is a large, enveloped DNA virus whose genome encodes at least 74 different genes (Dolan et al., 1998; Roizman & Knipe, 2001), although approximately half of these genes are not essential for virus replication in cell culture (Whitley, 2001). However, the dispensable gene products are thought to be important for virus growth and spread in natural hosts.

The UL56 genes of HSV type 1 (HSV-1) and type 2 (HSV-2) encode proteins of 234 and 235 aa, respectively, both of which possess a C-terminal 17 aa stretch of uncharged or hydrophobic amino acids. The UL56 protein localizes to the Golgi apparatus and cytoplasmic vesicles as a C-terminal-anchored, type II membrane protein (Koshizuka et al., 2002). Members of this protein class have no N-terminal signal sequence, but instead possess a hydrophobic segment near the C terminus that orients the N terminus of the protein in the cytoplasm (Kutay et al., 1993). Although UL56 is dispensable for HSV replication in tissue culture, virus strains lacking UL56 are substantially less neuroinvasive. The apathogenic HSV-1 strain HFEM, in which the UL56 promoter is deleted, is unable to replicate in the adrenal glands or to penetrate the spinal cord and brain after intraperitoneal injection (Peles et al., 1990). Additionally, the recombinant virus HSV-1-M-lacZ, in which UL56 was eliminated by the insertion of lacZ into the genome of HSV-1 strain F, is avirulent and unable to penetrate the spinal cord (Berkowitz et al., 1994; Rosen-Wolff et al., 1991). The C-terminal hydrophobic region of UL56 is also important for HSV-1 pathogenicity (Kehm et al., 1996). However, Nash & Spivack (1994) reported that UL56 does not play a role in HSV-1 virulence following intraperitoneal infection in mice.

KIF1A is a member of the kinesin superfamily of proteins and is now classified into the kinesin-3 family in the standard kinesin nomenclature (Lawrence et al., 2004). unc-104/KIF1A kinesin was originally identified as the gene underlying the Caenorhabditis elegans unc-104 paralysed mutant (Hall & Hedgecock, 1991; Otsuka et al., 1991) and was later cloned as mouse KIF1A (mKIF1A) (Okada et al., 1995) and human ATSV (axonal transport of synaptic vesicles) (Furlong et al., 1996). The lack of Unc-104 leads to a decreased number of synaptic vesicles and the accumulation of similar vesicles in the neuron cell body (Otsuka et al., 1991). Mice deficient in KIF1A display a similar accumulation of vesicles in the cell body, as well as neuronal death (Yonekawa et al., 1998). KIF1A associates with organelles containing synaptic vesicle proteins such as synaptotagmin, synaptophysin and Rab3A (Okada et al., 1995), and it has been suggested that KIF1A plays a role in the axonal transport of synaptic vesicle precursors.

In this study, we sought to identify cellular gene products that interact with UL56 in order to learn more about the function of this protein. Using a yeast two-hybrid screen, we identified human KIF1A as a UL56-interacting protein and investigated the nature of this interaction by mutagenesis of UL56. Whilst the mechanism of UL56 action needs to be resolved, the interaction of UL56 with KIF1A strongly suggests a role for UL56 in vesicular transport in infected cells.
METHODS

Cells. Vero cells, a stable line of African green monkey kidney cells, were grown in Eagle’s minimal essential medium supplemented with 5% calf serum. COS-1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum.

Yeast two-hybrid screen. A yeast two-hybrid screening system was used to isolate cDNAs encoding proteins interacting with UL56. Fragments encoding UL56 codons 1–217 were amplified by PCR and inserted into pGBKTK7 (Clontech) in frame with the GAL4 DNA-binding domain (BD) to give the fusion protein BD–UL56. The resulting ‘bait’ plasmid and a human fetal brain cDNA library (Clontech) were co-transformed sequentially into yeast strain Y190 (Clontech). Double transformants were selected on SD medium lacking tryptophan, leucine and histidine. Positive colonies were restreaked on the same medium and tested for β-galactosidase activity by filter assay. Blue colonies were grown in SD medium lacking leucine, and library plasmids were isolated and transformed into Escherichia coli XL-1 Blue. Bacterial transformants were selected on Luria–Bertani medium containing ampicillin (50 μg ml⁻¹) and the library plasmids were repurified. Purified plasmids were co-transformed with pGBKTK7 (Becton Dickinson), pGAD encoding murine p53 (BD–p53; Clontech) or pLAM5 encoding human lamin C (BD–LaminC; Clontech) into Y190 cells and transformants were assayed for β-galactosidase activity to eliminate false positives. ICP0 codons 543–775 fused with BD in pGBT9 (BD–ICP0) and EF-1α fused with the GAL4 transcriptional activation domain (AD) in pACT2 (AD–EF-1α) were used as positive controls for the β-galactosidase assay (Kawaguchi et al., 1997).

Plasmids. The plasmid pGEX-UL56 (Koshizuka et al., 2002) was used for the expression of glutathione S-transferase (GST) fused with HSV-2 UL56 wild-type protein and for the construction of UL56 deletion mutants. Fragments encoding UL56 codons 1–217, 56–217, 1–68/110–217 or 1–110 were amplified by PCR and inserted into pGEX4T-1 (Amersham Biosciences) in frame with GST. The resulting plasmids were designated pGEX-UL56ΔC, pGEX-UL56ΔN, pGEX-UL56ΔK and pGEX-UL56ΔK/N, respectively. The plasmid pRB4995, which encodes ICP0 codons 543–768, was used for expression of GST–ICP0 (Kawaguchi et al., 1997).

The mammalian expression plasmids of wild-type UL56 and the C-terminal deletion mutant UL56ΔR1 were as described previously (Koshizuka et al., 2002). To construct pcDNA-KIF1A, the mammalian expression construct of KIF1A, the full-length mKIF1A cDNA (Okada et al., 1995) was inserted into the BamHI and EcoRI sites of pcDNA3.1 (+) (Invitrogen).

Production and purification of GST fusion proteins. E. coli XL-1 Blue cells transformed with the plasmids encoding GST fusion proteins were induced with 0.1 mM IPTG. Harvested cells were lysed by sonication in PBS, and Triton X-100 was added to a final concentration of 1%. After cell debris had been clarified by centrifugation, GST fusion proteins were adsorbed to glutathione–Sepharose beads (Sigma) at 4°C, washed with PBS containing 1% Triton X-100 and eluted with 10 mM reduced glutathione (Sigma) in 50 mM Tris/HCl (pH 8.0). The proteins were separated by SDS-PAGE and quantified with Coomassie brilliant blue staining. BSA was used as a protein standard. Equal amounts of GST fusion proteins were used in GST pull-down assays.

Affinity precipitation with GST–UL56 fusion protein. COS-1 cells grown on 100 mm diameter dishes were transfected with pcDNA-KIF1A by using the DEAE-dextran method. After 2 days, transfected cells were lysed in PBS containing 1% Triton X-100, 1% deoxycholic acid, 5% glycerol and a protease-inhibitor cocktail (Sigma). Cell debris was removed by centrifugation at maximum speed in a microcentrifuge. Supernatant corresponding to 7 × 10⁶ cells was incubated with GST, GST–UL56 or GST–ICP0 protein immobilized on glutathione–Sepharose beads. After overnight incubation at 4°C with continuous mixing, beads were collected by brief centrifugation and washed four times with PBS buffer, and the bound protein complexes were subjected to SDS-PAGE, transferred to a PVDF membrane (Millipore) and reacted with a mouse monoclonal antibody to mouse KIF1A (Becton Dickinson).

Confocal microscopy. Vero cells grown on coverslips were transfected with pcDNA-UL56, pcDNA-KIF1A or control plasmid pcDNA3.1 (+) by using Lipofectamine (Invitrogen). At 24 h post-transfection, coverslips were washed with PBS, fixed with 4% paraformaldehyde at 4°C for 1 h and permeabilized with 0.1% Triton X-100 in PBS at 4°C for 45 min. The coverslips were incubated with 20% calf serum for 1 h at room temperature to reduce non-specific antibody binding. The coverslips were incubated for 30 min at room temperature with anti-UL56 rabbit polyclonal antiseraum (Koshizuka et al., 2002) and anti-KIF1A mouse monoclonal antibody (Becton Dickinson). After washing several times, the coverslips were treated with secondary antibodies in the same way. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG were used as secondary antibodies. After rinsing again with PBS, coverslips were mounted on PermaFluor (Immuron) and examined with a Zeiss LSM510 laser-scanning microscope.

RESULTS

UL56 interacts with a member of the kinesin superfamily, KIF1A, in a yeast two-hybrid assay

We used a human fetal brain cDNA library to identify putative UL56-interacting proteins by using a yeast two-hybrid screen. We constructed a bait plasmid truncated prior to the C-terminal transmembrane domain (TMD) as described in Methods to avoid association of the fusion protein with membranes. Among the positive clones, we were particularly interested in a partial cDNA encoding a kinesin-like protein, human KIF1A (hKIF1A), also known as ATSV (Furlong et al., 1996). hKIF1A is the human homologue of Unc-104/KIF1A kinesin and encodes a 1690 aa protein with 97% similarity to mKIF1A at the amino acid level (Furlong et al., 1996). KIF1A, a neuron-specific kinesin, is an anterograde motor that transports a subset of synaptic vesicle precursors and plays an important role in the axonal transport of pre-synaptic vesicles (Okada et al., 1995; Yonekawa et al., 1998). Our two-hybrid screen identified a clone containing hKIF1A codons 1502–1690, although it had a partial deletion between codons 1627 and 1638 (Fig. 1a). The GAL4 AD fusion protein was terminated by the hKIF1A stop codon. Therefore, the fusion protein that interacted with UL56 lacked 12 aa within the pleckstrin homology (PH) domain.

The positive clone, hKIF1A C terminus (KIF1A.C), was retransformed into yeast strain Y190 and tested more thoroughly for its putative interaction with UL56. Murine p53, human lamin C and the GAL4 BD alone were used as controls. Yeast expressing AD–KIF1A.C and BD–UL56 formed colonies on SD medium lacking tryptophan, leucine and histidine, but the control constructs did not
The combination of BD–ICP0 and AD–EF-1δ was used as a positive control (Kawaguchi et al., 1997). To further confirm the interaction of KIF1A.c and UL56, we performed a β-galactosidase filter assay (Fig. 1c). Only in the presence of a combination of BD–UL56 and AD–KIF1A.c did yeast colonies express a β-galactosidase activity greater than that of the positive control. These results suggested strongly that, in yeast, KIF1A.c and UL56 interact, and that examination of this interaction under different conditions was warranted.

UL56 interacts with KIF1A in GST pull-down and immunofluorescence assays

To verify and extend the binding data obtained in yeast, we performed GST pull-down experiments. GST fusion proteins were expressed in E. coli, and GST, GST–UL56 or GST–ICP0 bound to glutathione–Sepharose beads. The beads were pelleted, washed extensively, subjected to 8% SDS-PAGE, transferred to a PVDF membrane and immunoblotted with the KIF1A antibody. Molecular masses (kDa) are shown on the left.

Fig. 1. Identification of HSV UL56 interaction with the C terminus of hKIF1A by using a yeast two-hybrid system. (a) Schematic representation of the KIF1A coding sequence. FHA, Forkhead-associated domain; motor, N-terminal kinesin motor domain; PH, pleckstrin-homology domain. Numbers indicate amino acid positions. The horizontal black line marks the delination of the interaction domain of KIF1A with UL56. (b) The indicated combinations of plasmids were transformed into the yeast strain Y190. After growing transformants on SD medium lacking tryptophan, leucine and histidine, yeast colonies were restreaked on the same medium containing 20 mM 3-amino-1,2,4-triazole and growth was assessed. Plasmids used in this figure are described in Methods. AD–KIF1A.c indicates the yeast two-hybrid positive clone that encoded a fusion protein of GAL4 AD and KIF1A codons 1502–1626/1639–1690 in the pACT2 vector. (c) Protein–protein interactions were detected by using a β-galactosidase filter assay. The combination of BD–ICP0 and AD–EF-1δ served as a positive control.

Fig. 2. KIF1A is pulled down specifically by GST–UL56. (a) An anti-mKIF1A monoclonal antibody reacted with a single band of the expected molecular mass of mKIF1A. COS-1 cells transfected with pcDNA3.1 vector (V) or pcDNA-KIF1A (KIF1A) were harvested and cell lysates were separated by SDS-PAGE and subjected to Western blotting using an anti-mKIF1A monoclonal antibody. (b) Interaction of transfected KIF1A with GST, GST–UL56 or GST–ICP0. Lysates of transfected COS-1 cells were incubated with GST, GST–UL56 or GST–ICP0 C-terminal chimeric proteins immobilized on glutathione–Sepharose beads. The beads were pelleted, washed extensively, subjected to 8% SDS-PAGE, transferred to a PVDF membrane and immunoblotted with the KIF1A antibody. Molecular masses (kDa) are shown on the left.
monoclonal antibody and anti-UL56 polyclonal antibody. As reported previously (Koshizuka et al., 2002), UL56 localized to the Golgi apparatus and cytoplasmic vesicles in a fine speckled pattern (Fig. 4a). In cells expressing mKIF1A, mKIF1A localized to the cytoplasm and partially accumulated in a fine speckled pattern (Fig. 4b). In the presence of UL56, the number of mKIF1A speckles increased and mKIF1A co-localized with UL56 as punctate spots (Fig. 4d–f). As reported previously (Koshizuka et al., 2002), truncation of the C-terminal region of UL56, including the TMD, resulted in a protein product that localized to the nucleus, in particular the nucleolus (Fig. 4j); when co-expressed with KIF1A, no clear co-localization was observed (Fig. 4g–i). These data argue that UL56 interacts with KIF1A in the GST pull-down assay are shown. The arginine-rich region and C-terminal TMD are shown as hatched and filled boxes, respectively. Numbers indicate amino acid positions of UL56.

**DISCUSSION**

Whilst not required for virus replication in tissue culture, UL56 increases the pathogenicity of HSV. Despite a defined role in HSV biology, the molecular mechanism by which UL56 mediates its effects is unresolved. Here, we have presented evidence that UL56 interacts with a kinesin motor protein, KIF1A. We identified this protein by using a yeast two-hybrid screen and confirmed its interaction with UL56 by GST pull-down assays and *in vivo* immunofluorescence. Interestingly, the isolated yeast clone contained only the C-terminal 189 aa region of hKIF1A, including the PH domain, although it had a 12 aa deletion within the domain, suggesting that KIF1A interacts with UL56 through its restricted C-terminal region. Within UL56, aa 69–217 were responsible for its interaction with KIF1A in GST pull-down assays. This region of UL56 should face the cytoplasm, because UL56 should be anchored to cargo vesicles through its C-terminal TMD. Although the C-terminal TMD deletion construct interacted with KIF1A in a GST pull-down assay, we could not detect clear co-localization *in vivo* between these proteins. Membrane anchoring of UL56 may be required for the association with KIF1A *in vivo* and this may take place on the cytoplasmic face of cargo vesicles.

The C-terminal PH domain of KIF1A interacts with cargo vesicles by binding acidic phospholipids, especially phosphatidylinositolphosphates (PtdInsP₃) (Kavran et al., 1998; Lemmon, 1999). In *C. elegans*, the PH domain of Unc-104 binds phosphatidylinositol(4,5)bisphosphate [PtdIns(4,5)P₂] and Unc-104 docks onto cargo using the PH domain (Klopfenstein et al., 2002; Klopfenstein & Vale, 2004). Additionally, Unc-104/KIF1A-mediated vesicular transport requires both lipid and protein binding of components in/on the vesicular membrane. Klopfenstein et al. (2002) have shown that Unc-104 binds to PtdIns(4,5)P₂-associated lipid rafts by using *in vitro* assays. We have also shown that a portion of the UL56 protein in infected cells associates with lipid rafts (Koshizuka et al., 2002). Although it has yet to be determined, lipid-raft subdomains may play a role in the UL56–KIF1A interaction.

A number of genetic and biochemical studies have unravelled the protein–protein interactions required for kinesin-dependent transport events. Kinesin-interacting proteins can be divided into three classes: transmembrane proteins on the vesicle surface that are cargo-bound receptors, scaffold proteins that link kinesin indirectly to cargoes and regulatory proteins that phosphorylate the kinesin tail domain or, like HSP70, remove kinesin from the vesicle surface (Kamal & Goldstein, 2002; Karcher et al., 2002; Vale, 2003; Verhey & Rapoport, 2001). Our results suggest that UL56, a C-terminal-anchored transmembrane protein, may act as a viral receptor for the KIF1A motor. KIF1A has been shown to interact directly with liprin-α (Shin et al., 2003). Liprin-α is a multimodular scaffolding protein linking KIF1A to liprin-α-associated proteins. UL56 and liprin-α have partial similarity. A stretch of 34 aa within the KIF1A-interacting domain of liprin-α shared some similarity with the arginine-rich region of UL56, the common amino acid sequences being S-X-A-X₄-G-X₃-E-R-X₃-E-R-X₉-A-X₁₀-R-A-X₂-R. Liprin-α interacts with the central region of KIF1A, whereas UL56 interacts with KIF1A through its C-terminal region. Thus, it seems unlikely that the 34 aa stretch of UL56 is important for its interaction with KIF1A. However, further studies are required to evaluate the significance of this homology.

Homologues of UL56 are readily recognized among HSV-1, HSV-2 and herpes B virus (cercopithecine herpesvirus 1).
Although amino acid similarity between the HSV-1 and herpes B virus UL56 is about 40% (Ohsawa et al., 2003; Perelygina et al., 2003), the arginine-rich region and the N-terminal flanking region of the TMD have higher similarity. If the UL56 protein of herpes B virus associates with monkey KIF1A homologues during the virus life cycle, the UL56 regions with a higher degree of similarity are probably responsible for its interaction with KIF1A.

HSV is a neurotropic virus and the retrograde and anterograde axonal transport of virions is an essential process in its life cycle. UL56 may promote glycoprotein transport along the axon through its interaction with KIF1A. US11, a tegument protein of HSV, interacts with the conventional kinesin heavy chain and US11 may play an important role in the anterograde transport of unenveloped nucleocapsids (Diefenbach et al., 2002). US11 also associates with a microtubule-binding protein, PAT1 (Benboudjema et al., 2003). Additionally, US9, a C-terminal-anchored, type II membrane protein encoded by pseudorabies virus, is involved in the axonal transport of viral glycoproteins (Brideau et al., 2000; Tomishima & Enquist, 2001). US9 is conserved among alphaherpesviruses and is required for the transport of viral membrane proteins, but not capsids or tegument proteins, in the axon (Enquist et al., 2002; Tomishima & Enquist, 2001). Our data suggest that UL56, another C-terminal-anchored, type II membrane protein, may play a role in the axonal transport of vesicles containing viral envelope glycoproteins.

Alternatively, UL56 may lead to neuronal cell dysfunction by interfering with normal KIF1A function. Several viral proteins, including ICP34.5, envelope glycoproteins and virus-induced enzymes, promote neurovirulence in HSV-infected animals (Roizman & Knipe, 2001). Amino acids 56–217 of UL56 can interact directly with the C-terminal

Fig. 4. Subcellular distribution of UL56 deletion mutants and KIF1A. Vero cells were transfected with pcDNA-UL56 and vector (a), vector and pcDNA-KIF1A (b), vector only (c), pcDNA-UL56 and pcDNA-KIF1A (d–f), pcDNA-UL56R1 and pcDNA-KIF1A (g–i) or pcDNA-UL56R1 and vector (j). At 24 h post-transfection, cells were fixed and permeabilized as described in Methods. Samples were reacted with anti-UL56 polyclonal antibody and anti-KIF1A monoclonal antibody. Merged images are shown in (f) and (i).
region of KIF1A, near the PH domain, and it is possible that UL56 may interfere with PH-domain binding to cargo vesicles. Although point mutations in the PH domain of Unc-104 that interfere with binding to PtdIns(4,5)P₂ reduce the velocity of Unc-104 motors (Klopfenstein & Vale, 2004), unc-104 mutant worms display a normal neuronal anatomy and are viable (Hall & Hedgecock, 1991; Otsuka et al., 1991). However, mice genetically deficient in KIF1A exhibit defective pre-synaptic vesicle transport and die shortly after birth (Yonekawa et al., 1998). KIF1A-mediated axonal transport plays a critical role in the viability, maintenance and function of neurons, particularly mature neurons. The binding of UL56 to KIF1A might play a role in neuronal cell dysfunction. Whilst the functional significance of the interaction of UL56 with KIF1A has yet to be resolved, it is likely that this interaction is partly responsible for the neuropathology seen following HSV infection.

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