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Molecular association of herpes simplex virus type 1 glycoprotein E with membrane protein Us9

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Abstract

Herpes simplex virus type 1 (HSV-1) glycoprotein E (gE), glycoprotein I (gI), and Us9 promote efficient anterograde axonal transport of virus from the neuron cytoplasm to the axon terminus. HSV-1 and PRV gE and gI form a heterodimer that is required for anterograde transport, but an association that includes Us9 has not been demonstrated. NS-gE380 is an HSV-1 mutant that has five amino acids inserted after gE residue 380, rendering it defective in anterograde axonal transport. We demonstrated that gE, gI and Us9 form a trimolecular complex in Vero cells infected with NS-gE380 virus in which gE binds to both Us9 and gI. We detected the complex using immunoprecipitation with anti-gE or anti-gI monoclonal antibodies in the presence of ionic detergents. Under these conditions, Us9 did not associate with gE in cells infected with wild-type HSV-1; however, using a nonionic detergent, TritonX-100, an association between Us9 and gE was detected in immunoprecipitates of both wild-type and NS-gE380-infected cells. The results suggest that the interaction between Us9 and gE is weak and disrupted by ionic detergents in wild-type infected cells. We postulate that the tight interaction between Us9 and gE leads to the anterograde spread defect in the NS-gE380 virus.

Introduction

The neurotropic alphaherpesviruses include the human pathogens herpes simplex virus type 1 (HSV-1), type 2 (HSV-2), varicella zoster virus (VZV) and the veterinary pathogens pseudorabies virus (PRV) and bovine herpesviruses. *In vivo* and *in vitro* studies have demonstrated that glycoprotein E (gE, also referred to as Us8), glycoprotein I (gI, also referred to as Us7), and Us9 are key viral proteins involved in anterograde axonal transport [1–13]. The genes encoding these proteins are adjacent to one another in the unique short segment of the HSV-1 genome [14]. Live cell imaging of fluorescently labeled viruses in neuronal cell cultures has demonstrated a role for gE, gI and Us9 in virus transport [7, 11–13, 15–17]. Glycoprotein E and gI form a heterodimer complex in BHV, HSV-1 and PRV; however, no direct molecular interaction has been demonstrated between Us9 and gE or gI despite the three proteins being involved in anterograde axonal transport [18–21].

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Compliance with ethical standards

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Conflict of interest Authors SA and HF declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Us9 is nonessential for virus replication, yet highly conserved among alphaherpesviruses. It is a 90-amino-acid, type II membrane protein with a short (four-amino-acid) ectodomain, a transmembrane domain, and a conserved acidic domain within the cytoplasmic region. The acidic domain directs Us9 to the *trans*-Golgi network (TGN) [5]. Us3 is a viral kinase that phosphorylates HSV-1 and PRV Us9 during *in vitro* infection, resulting in multiple variants ranging from 10–18 kDa [22–24]. Us9 is incorporated into virus particles and ubiquitinated during infection [24]. PRV Us9 associates with lipid raft microdomains on the surface of infected cells, which is required for efficient transmission of infection from neurons to epithelial cells [12].

HSV-1 gE and gI are type I membrane glycoproteins (552 amino acids and 383 amino acids, respectively, in HSV-1 strain NS) that form a heterodimer in the virus envelope and on the surface of infected cells, although gE and gI are also expressed as monomers [25]. Both gE and gI are non-essential for virus replication; however, they cooperatively promote epithelial cell-to-cell spread and neurovirulence in numerous alphaherpesviruses [26–34]. Glycoprotein E also interacts with tegument proteins UL11, UL16, and UL21 [35]. In addition to its role in axonal transport and cell-to-cell spread, gE has distinct domains that bind the Fc region of human IgG to evade effector functions of IgG [36].

NS-gE380 virus is an HSV-1 mutant strain constructed by insertion of an *Xho* I linker after gE amino acid 380, which resulted in the introduction of five amino acids, SARAD, into the gE protein [37]. The NS-gE380 mutant virus has lost IgG Fc binding activity and is defective in anterograde axonal transport in the mouse retinal infection model *in vivo*, and in rat superior cervical ganglion neuronal cell culture *in vitro* [1, 37]. The anterograde spread phenotype of the NS-gE380 mutant is similar to that observed for HSV-1 and PRV gE/gI deletion mutants and for PRV Us9null virus in neuronal cell cultures and the mouse retina infection model [1, 5, 6, 38, 39]. The extent of the anterograde spread defect of gE, gI and Us9 mutant strains varies depending on whether the assessments are performed in animal models or in neuronal cell cultures [8, 40, 41]. Here, we evaluate the molecular association of gE, gI and Us9 and demonstrate that gE, gI and Us9 form a tri-molecular complex in which gE binds to both Us9 and gI. We demonstrate the trimolecular complex using ionic and non-ionic detergents in cells infected with NS-gE380 mutant virus; however, the complex is only detected using nonionic detergents in cells infected with wild-type (WT) HSV-1.

Materials and methods

Cells, viruses and antibodies

Vero cells were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 5 % fetal calf serum. Mouse monoclonal antibodies (MAb) anti-gE 1BA10, anti-gI MAb Fd69, and rabbit polyclonal antibodies anti-gE UP575, anti-gI UP1925, anti-Us9 UP1991 and anti-glycoprotein C (gC) R118 were described previously, [6, 34, 37, 42]. Anti-TGN-38 and anti-calnexin were purchased from Novus Biologicals and Santa Cruz Biotechnology, Inc., respectively.

HSV-1 strain NS is a low-passage clinical isolate that was used as the WT virus [43]. NS-gEnull virus was derived from NS and has gE amino acids 124–510 replaced by a LacZ cassette [44]. NS-gE380 virus was also derived from NS and prepared by an in-frame *Xho I* linker inserted at amino acid 380 [37]. NS-Us9null virus was derived from NS and has the entire Us9 coding sequence replaced by an mCherry fluorescent marker [6]. HSV-1 gInull was provided by T. Minson (SC16 strain) [6, 34]. HSV-1 WT and mutant viruses were grown in Vero cells. Stock viruses were stored at -80°C .

Immunoprecipitation and western blots

Infected Vero cell extracts were prepared and immunoprecipitation experiments were performed using two different buffer systems. For our initial experiments, we used RIPA buffer containing both ionic and nonionic detergents, while for later experiments, we used RSB buffer containing only nonionic detergents. RIPA buffer contains 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 % NP-40 (nonionic), 1 % sodium deoxycholate (ionic), and 0.1 % SDS (ionic). The Vero cell lysates were incubated on ice for 5 minutes, passed through a 27-gauge needle four times, and pelleted at $14,000\times g$ in a microfuge. The supernatant fluids were incubated with anti-gE MAb 1BA10 or anti-gI MAb Fd69, immunoprecipitated using Protein G agarose, solubilized in Laemmli buffer containing 65.8 mM Tris-HCl, pH 6.8, 2.1 % SDS, 26.3 % glycerol, 0.01 % bromophenol blue, and 700 mM β -mercaptoethanol. The proteins were separated by 12 % SDS PAGE, transferred to PVDF membranes, blocked with 5 % non-fat milk and probed with antibodies to detect gE, gI, Us9, or gC. For immunoprecipitation experiments containing only nonionic detergents, infected Vero cells were lysed with RSB buffer containing 10 mM Tris, pH 7.2, 100 mM NaCl, 1 mM MgCl_2 , 2 mM DTT, and 1 % Triton X-100 (nonionic). The remainder of the immunoprecipitation procedure was otherwise as described above. To evaluate the effect of pH on the molecular association between gE and Us9, the immunoprecipitate containing agarose beads, antibodies and bound proteins was pelleted by centrifugation and washed three times in 0.1 % PBS-Tween (nonionic) buffer at pH 6.0 or pH 7.4.

Sucrose density gradients and fractionation

Five percent to 30 % sucrose density gradients were prepared in RSB buffer (nonionic) using a BioComp Gradient Master, Model 106 (New Brunswick). Cell extracts devoid of nuclei were prepared by resuspending HSV-1 WT- or NS-gE380-infected Vero cells in RSB buffer, passing through a 27-gauge needle five times, and pelleting the extracts at $1000\times g$ for 15 minutes. The supernatant fluids were layered on top of the gradient and centrifuged at $112,000\times g$ at 4°C for 42 h in a Beckman SW-28 rotor. Thirty-five 1-mL fractions were collected from the top at 4°C using a BioComp Model 150 Gradient Fractionator (New Brunswick) [45]. Alternate fractions were immunoprecipitated with anti-gE MAb 1BA10 using RIPA buffer containing both ionic and nonionic detergents.

Confocal microscopy

Vero cells were grown on glass coverslips and infected with WT or NS-gE380 virus at an MOI of 0.1. At 12 h postinfection, cells were fixed with 2 % paraformaldehyde in 20 mM Tris buffer, pH 7.2, and 0.2 % Triton X-100 (nonionic) and incubated with chilled acetone for 5 minutes at 4°C . Cells were washed and blocked with 10 % BSA in Tris buffer, pH 7.2,

with 0.2 % Tween-20 (nonionic) for 1 h at RT. Secondary fluorescent antibodies were added at a 1:200 dilution. Nuclei were stained using DAPI dye. Images were analyzed on a confocal laser-scanning microscope (model LSM 510, Zeiss) using a 100X oil immersion lens.

Fractionation of TGN and endoplasmic reticulum (ER) membrane-rich fractions by sucrose gradient centrifugation

Mock-infected Vero cells or cells infected with WT HSV-1 or NS-gE380 mutant virus were homogenized at 12 h postinfection using a glass homogenizer in a buffer without detergents containing 0.25 M sucrose (8.6 %), 10 mM Tris-HCl, pH 7.4, 1 mM MgAc₂, and a protease inhibitor mixture. Homogenates were pelleted at 1000×*g* for 15 min, and the supernatant fluids were gently placed on top of a step gradient comprised of 1 ml of 2 M sucrose (68.5 %), 4 ml of 1.3 M sucrose (44.5 %), 3.5 ml of 1.16 M sucrose (39.7 %) and 2.0 ml of 0.8 M sucrose (27.3 %). The sucrose solutions were prepared in a buffer without detergents containing 10 mM Tris-HCl, pH 7.4, and 1 mM MgAc₂. The gradients were centrifuged for 2.5 h at 100,000×*g* in a Beckman SW41 Ti rotor. Twelve 1-ml fractions were collected from the top of the gradient, and the proteins were separated by 4-to-12 % SDS PAGE, transferred onto PVDF membranes, and blotted with anti-TGN-38 and anti-calnexin antibodies as markers for TGN and ER membrane fractions, respectively [46]. The fractions were solubilized using Tris buffer, pH 7.4, containing 1 % Triton X-100 (nonionic) and a protease inhibitor cocktail. Immunoprecipitation by anti-gE MAb 1BA10 was performed without adding the ionic detergents SDS and sodium deoxycholate and analyzed for co-immunoprecipitation of Us9 by Western blot.

Results

Molecular interactions between gE, gI and Us9

Vero cells were infected with HSV-1 or mock infected, and cell lysates were immunoprecipitated with anti-gI MAb (Fd69) or anti-gE MAb (1BA10) to evaluate the molecular interactions between gE, gI and Us9. Anti-gI MAb Fd69 co-immunoprecipitated both gE and gI (Fig. 1A, lane 3), while anti-gE MAb 1BA10 only immunoprecipitated gE (Fig. 1B, lane 3). Neither antibody immunoprecipitated proteins from mock-infected Vero cell extracts (Fig. 1A and B, lane 2). The schematic provides an explanation for anti-gE MAb 1BA10 immunoprecipitating only gE in that the antibody binding may dissociate gI from the gE/gI complex (Fig. 1C). Alternatively, 1BA10 may only bind to gE monomer that has not formed a complex with gI. The different immunoprecipitation properties of these MAbs were useful in experiments described below to identify other binding partners of gE and the gE/gI complex.

HSV-1 WT and the NS-gE380 mutant strains were used to evaluate the molecular interactions between gE, gI and Us9, while NS-gEnull and HSV-1 gInull viruses served as controls for MAbs Fd69 and 1BA10. The advantage of NS-gE380 mutant virus is that it expresses the entire gE protein, yet it exhibits an anterograde axonal spread phenotype similar to HSV-1 gEnull [1, 6]. Immunoprecipitation was performed on mock- or HSV-infected cell extracts with anti-gI MAb Fd69, using RIPA buffer containing both ionic and

nonionic detergents, followed by Western blot to detect gE, gI and Us9 (Fig. 1D, lanes 2–6). An extract from WT-infected cells without immunoprecipitation was used as a positive control for antibody binding and identifying the gel positions for gE, gI and Us9 (Fig. 1D lane 1). Glycoproteins E and gI were both immunoprecipitated from WT-infected cells, but Us9 was not (Fig. 1D, lane 3). Importantly, gE, gI and Us9 were each immunoprecipitated from NS-gE380 infected cells, indicating that the proteins form a trimolecular complex in this strain (Fig. 1D, lane 5). Only gI was immunoprecipitated from NS-gEnull-infected cells (Fig. 1D, lane 4), and none of the proteins were immunoprecipitated from HSV-1 gInull-infected cells (Fig. 1D, lane 6). More gI was immunoprecipitated from NS-gE380-infected cells, suggesting either increased expression of gI or increased binding affinity of anti-gI MAb to gI of this mutant virus (Fig. 1D, lane 5). A dense, faster-migrating gE band was detected in NS-gE380-infected cells that likely represents altered post-translational modifications of the mutant gE380 protein (Fig. 1D, lanes 3 and 5).

Similar immunoprecipitation using RIPA buffer, containing ionic and nonionic detergents, was performed using anti-gE MAb 1BA10 (Fig. 1E). Lane 1 represents Western blots of WT-infected cell extracts that were not subjected to immunoprecipitation as controls. In lanes 2–6, the extracts were immunoprecipitated with anti-gE MAb 1BA10 prior to Western blotting. Anti-gE MAb 1BA10 immunoprecipitated gE without gI (Fig. 1E, lanes 3 and 5). The anti-gE MAb 1BA10 co-immunoprecipitated Us9 from NS-gE380 infected cells, supporting an interaction between gE and Us9 in NS-gE380-infected cells that does not require gI (Fig. 1E, lane 5). Only gE was immunoprecipitated from HSV-1 gInull-infected cells (Fig. 1E, lane 6), and none of the proteins were immunoprecipitated from NS-gEnull-infected cells (Fig. 1E, lane 4).

Sucrose gradient fractionation of the gE and Us9 complex

Sucrose density gradient fractionation allows protein-protein complexes to be identified in cell homogenates. To assess whether Us9 associates with gE380, we performed sucrose density gradient fractionation of WT- and NS-gE380-infected cell lysates. We postulated that if Us9 associates with the gE380 protein, Us9 would fractionate with gE380. The post-nuclear cell lysates from WT- or NS-gE380-infected cells were prepared in RSB buffer (nonionic detergent) and placed on a 5-to-30 % sucrose gradient. Thirty-five one-ml fractions were collected from the top, and alternate fractions were immunoprecipitated with anti-gE MAb 1BA10 using RIPA buffer containing both ionic and nonionic detergents. Use of alternate fractions enables sampling of a smaller number of fractions without introducing a selection bias. In cells infected with WT virus, gE was detected in fractions 3 to 19, while Us9 did not co-immunoprecipitate with gE (Fig. 2A). In cells infected with NS-gE380 virus, gE was detected in almost all fractions, including fraction 35, indicating that a larger complex formed in NS-gE380-infected cells (Fig. 2B). The faster-migrating gE380 band was again visible. Us9 co-immunoprecipitated with gE, most notably in fraction 19, but it was also visible in fractions 21, 23 and 31 (Fig. 2B). Faint Us9 and gE bands were also present in lane 3 and likely represent input cell lysate that did not properly enter the gradient (Fig. 2B).

Subcellular localization of the gE and Us9 complex

Confocal microscopy was performed 12 h after infection of Vero cells with WT and NS-gE380 virus. Glycoprotein E and Us9 co-localized mostly to the TGN in WT-infected cells, while gE380 and Us9 demonstrated a more diffuse distribution in the ER and perinuclear regions in NS-gE380-infected cells (Fig. 3).

Evidence of molecular association of WT gE with Us9 in TGN- and ER-enriched fractions in WT-and NS-gE380-infected cells

We used sucrose density gradients to fractionate TGN and ER membranes from mock-, WT- and NS-gE380-infected cells and examined the association of gE with Us9. Fractions from mock-infected cells were evaluated by Western blot with anti-TGN-38 and anti-calnexin antibodies to identify fractions enriched in TGN or ER membranes, respectively. Fractions 2 to 4 were enriched in TGN, while fractions 10 to 12 and, to a lesser extent, fraction 3 were enriched in ER membranes (Fig. 4A). Based in part on the fluorescent microscopy co-localization studies in Fig. 3, we postulated that an association between Us9 and gE may be apparent using milder immunoprecipitation conditions. Therefore, at 12 h postinfection, preparation of infected cell extracts and immunoprecipitation with anti-gE MAb 1BA10 were performed on WT- and NS-gE380-infected Vero cells using a nonionic detergent containing 1 % Triton X-100, followed by Western blotting for gE and Us9. Some gE immunoprecipitated in fractions enriched for TGN, while the majority of gE immunoprecipitated from fractions enriched for ER membranes (Fig. 4B and C). Us9 co-immunoprecipitated with gE in fraction 3, containing predominantly TGN, and more strongly in ER-enriched fractions 11 and 12 (Fig. 4B and C). The key observation was that the association of Us9 with gE was detected in cells infected with both WT and NS-gE380 virus using a nonionic detergent, Triton X-100 to solubilize ER and TGN membranes.

To assess the specificity of the immunoprecipitation, we used an unrelated IgG (rabbit anti-gC antibody R47) to immunoprecipitate TGN and ER fractions from infected cells that were prepared using nonionic detergents. Western blots were probed for gC using anti-gC R47, for gE using anti-gE MAb 1BA10, and for Us9 using rabbit anti-Us9 UP1991 (Fig. 4D and E). Anti-gC immunoprecipitation resulted in little or no gC in TGN- and ER-enriched fractions. An IgG heavy chain band was visible in the gC Western blot, since the same rabbit polyclonal antibody was used for both IP and the Western blot followed by an HRP-conjugated anti-rabbit IgG secondary antibody. Importantly, gE and Us9 were not immunoprecipitated from WT- or NSgE380-infected cells, indicating that these proteins did not bind nonspecifically to IgG or the beads, which supports the conclusion that the co-immunoprecipitation of gE and Us9 shown in Fig. 5B and C was specific. As an additional control, mock-infected and WT-infected whole-cell extracts (rather than TGN and ER fractions) were immunoprecipitated with the anti-gC antibody. Western blotting demonstrated that gC was immunoprecipitated from WT-infected cells (Fig. 4F), indicating that the anti-gC antibody is capable of immunoprecipitating WT gC.

Confirmation of a molecular association of WT gE with Us9 in WT- and NS-gE380-infected cells, using nonionic detergent

Immunoprecipitation was performed on mock- or HSV-infected cell extracts with anti-gI MAb Fd69, using RSB buffer containing nonionic detergent only (1 % Triton X-100) followed by Western blotting to detect gE, gI and Us9 (Fig. 5A, lanes 2–6). WT HSV-1-infected cell extract was added in lane 1 as a positive control (Fig. 5A, lane 1). Using nonionic detergents, gE, gI and Us9 were each immunoprecipitated from WT- and NS-gE380-infected cells (Fig. 5A, lanes 3, 5). Only gI was immunoprecipitated from NS-gEnull-infected cells (Fig. 5A, lane 4), and none of the proteins were immunoprecipitated using anti-gI MAb Fd69 from HSV-1 gInull-infected cells (Fig. 5A, lane 6). Similar experiments with nonionic detergents were performed using anti-gE MAb 1BA10. Western blots of WT-infected cell extracts served as a positive control (Fig. 5B, lane 1). In cells infected with WT or NS-gE380 virus, the anti-gE MAb 1BA10 immunoprecipitated gE without gI, (Fig. 5B, lanes 3 and 5). No proteins were immunoprecipitated from NS-gEnull infected cells (Fig. 5B, lane 4). The anti-gE MAb co-immunoprecipitated Us9 from WT- and NS-gE380-infected cells, indicating an interaction between gE and Us9 in WT- and NS-gE380-infected cells (Fig. 5B, lane 5). Only gE was immunoprecipitated from HSV-1 gInull-infected cells (Fig. 5B, lane 6). We conclude that gE is a binding partner for Us9, while gI is required for the interaction between gE and Us9.

Interaction of Us9 with gE is specific and dissociates at pH 6.0

Cells infected with HSV-1 WT or NS-Us9null virus were lysed at 12 h postinfection using a nonionic detergent, 1 % Triton X-100, and evaluated by Western blot without immunoprecipitation (Fig. 6A, lanes 1 and 2) or after immunoprecipitation with anti-gI MAb Fd69 (Fig. 6A, lanes 3 and 4). The anti-gI MAb co-immunoprecipitated Us9 in cells infected with WT virus, but not in cells infected with NS-Us9null virus. As a control, immunoprecipitates were probed for gC. No gC was detected (bottom gel, Fig. 6A, lanes 3 and 4), indicating that Us9 was not sticking nonspecifically to the beads or IgG used to immunoprecipitate gI. Enrichment of the slower-migrating gC band (fully processed) was evident in the NS-Us9null virus and likely represents differences in glycosylation of gC in WT and the US9null deletion viruses. We next evaluated whether Us9 dissociates from gE at low pH. WT-infected cell extracts were prepared using 1 % Triton X-100 (nonionic), followed by immunoprecipitation with anti-gE MAb 1BA10. The immunoprecipitate was suspended in PBS-Tween (nonionic) at pH 6.0 or pH 7.4, washed three times at pH 6.0 or pH 7.4, and probed for gE and Us9. The amount of gE immunoprecipitated was similar at pH 6.0 and 7.4 (Fig 6B, top gel, lanes 4 and 5); however, most Us9 dissociated at pH 6.0, while Us9 remained associated with gE at pH 7.4 (Fig. 6B, bottom gel, lanes 4 and 5).

Discussion

Glycoproteins E, gI and Us9 are nonessential for virus replication *in vitro*, yet for many alphaherpesviruses, they are essential for pathogenesis because they promote anterograde axonal transport of virus from the neuronal cell body to the axon terminus [1–13, 15, 18]. Molecular interactions of gE with gI have been investigated, and the role of gE, gI and Us9 in anterograde axonal transport has been evaluated, including by live cell imaging with

fluorescently tagged viruses [12, 13, 20, 47]. A molecular interaction between Us9 and the kinesin motor protein KIF1A was reported for PRV-infected PC12 cells at eight hours postinfection, and gE and gI were required for this interaction [47, 48]. More recently, the basic domain of HSV-1 Us9 was shown to recruit kinesin-1 and promote anterograde axonal transport and spread to non-neuronal cells [49]. In this report, using MAbs anti-gI Fd69 and anti-gE 1BA10, we demonstrated that Us9 forms a trimolecular complex with gE380 and gI in Vero cells infected with the NS-gE380 mutant virus. This association was apparent when cell extracts were prepared using both ionic and nonionic detergents and defined gE as the binding partner for Us9. In contrast, the association between Us9 and gE was only detected in cells infected with WT virus when nonionic detergents were used to prepare the cell extracts. In the presence of even low concentrations of SDS and deoxycholate (ionic detergents), Us9 dissociated from gE and did not co-immunoprecipitate when using anti-gE or anti-gI MAbs. Triton X-100 is a mild surfactant that breaks protein-lipid or lipid-lipid associations but does not disrupt the protein-protein interaction between Us9 and gE [50]. Non-ionic detergents such as Triton X-100 are often used to identify new molecular associations and larger protein complexes [51–53]. Others have demonstrated that HSV-1 gE forms a complex with three tegument proteins; therefore, the size of the gE molecular complex is likely to be much larger than trimolecular [35].

Confocal fluorescent microscopy indicated that the staining pattern of gE and Us9 in NS-gE380-infected cells was not identical to that of WT-infected cells; however, Us9 co-localized with gE in both WT-HSV-1- and NS-gE380- infected cells, which suggests that the conditions used to prepare the infected cell extracts might affect the ability to detect interactions between Us9 and gE. Us9 and gE were mostly identified in the TGN and ER in WT-virus-infected cells, while these proteins were partially redistributed to the periphery of the nucleus and ER in NS-gE380-infected cells. These results suggest that the tighter association between gE380 and Us9 may modify the cytosolic transport of these proteins. Biochemical enrichment of TGN and ER membranes from WT-HSV-1-infected cells confirmed the association of Us9 and gE in ER and TGN membranes. Others have also reported detecting HSV-1 Us9 antigen in the Golgi body and ER of retina ganglion cells [3]. Many tail-anchored proteins, such as Us9, are inserted into ER membranes before they are targeted to other organelles [54]. Studies with PRV-infected cells indicated that a PRV mutant virus deleted in the Us9 acidic domain and dileucine motif failed to localize to the TGN and did not recycle from the cell surface [55]. PRV Us9 predominantly co-localized with TGN membranes as assessed by immunofluorescence, which is similar to our findings for WT HSV-1, although biochemical fractionation performed in our study indicated that the association also occurred in ER membranes [55].

We demonstrated that WT Us9 dissociated from gE at acidic pH, suggesting pH-dependent modifications in conformation of their interacting domains. Changes in the stability of protein complexes at low pH are documented for viral proteins involved in membrane fusion [56, 57]. It is possible that pH-dependent dissociation of Us9 from gE may regulate the recycling of Us9 or gE from endosomes to the cell surface or cell junctions that are required for cell-to-cell spread of virus infection [58].

HSV-1 and PRV Us9 are homologs, with 33 % sequence identity in the 83-amino-acid overlapping sequences. When HSV-1 Us9 was inserted into a PRV Us9null virus, compensation was not observed for the loss of PRV Us9-mediated anterograde transport or spread of infection from neurons to epithelial cells [59, 60]. PRV gE exists as a heterodimer with gI in infected cells and in the virus [18]. Previously, in PRV-infected PC12 cells, a proteomics analysis of gE-interacting proteins identified gI, Us9, gC, gH, gM and U_L43 in addition to 40 cellular proteins. Following affinity purification of PRV gE, the enrichment of gI was 1.7-fold lower than that of gE, whereas that of Us9 was 14.8-fold lower, suggesting a stronger association between gE and gI than between gE and Us9 [48]. Studies done in PRV-infected polarized PC12 cells showed that Us9 is associated with lipid rafts that also include glycoproteins B, C, D and E. Reducing the ability of Us9 to be incorporated into lipid rafts abrogated the spread of PRV from neurons to epithelial cells [12]. It is noteworthy that while lipid rafts are rich in several viral membrane proteins, the association of HSV-1 gE with Us9 is specific, since Us9 co-immunoprecipitated with gE, but not with gC, which is another membrane protein localized to lipid rafts. Future studies evaluating the molecular interaction of PRV Us9 with gE/gI in infected PK-15 cells and transiently transfected cells using nonionic and ionic detergents will help determine whether these trimolecular associations are preserved in other alphaherpesviruses.

We demonstrated a stronger association between gE380 and Us9 than between WT gE and Us9. The sucrose gradient fractionation studies indicated that gE380 migrated as a complex with a higher molecular weight than the one containing WT gE, which is consistent with gE380 forming a complex with Us9. We cannot comment on the distribution of Us9 in the sucrose gradient, or whether the association of gE with Us9 modifies the distribution of Us9 in fractions, since immunoprecipitation was only done with gE antibody. The NS-gE380 virus encodes a complete in-frame gE protein that includes five additional amino acids. Future studies can assess whether gE380 has a dominant negative phenotype for redistribution of Us9 to the ER or TGN, or for axonal transport and IgG Fc binding. We have constructed an additional mutant with a gE linker insertion at amino acid position 210 that is defective in cell-to-cell spread but not in IgG Fc binding. It will be interesting to determine whether the gE210 protein associates with Us9 in this mutant strain [37]. We propose that the interaction between gE and Us9 is specific, but transient, and that it is essential for efficient anterograde axonal transport of virus. Future studies that identify the domains of interaction between gE and Us9 will help elucidate the importance of these interactions in anterograde transport.

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Fig. 1.

Immunoprecipitation of gE, gI and Us9 using ionic detergents. Vero cells (10^6) were infected with HSV-1 WT or mutant strains (MOI, 0.1). Twenty hours later, cell extracts were prepared using RIPA buffer containing both ionic and nonionic detergents, followed by immunoprecipitation with anti-gI MAb Fd69 (**A and D**) or anti-gE MAb 1BA10 (**B and E**). (**C**) Schematic model to explain Fd69 immunoprecipitation of both gE and gI and 1BA10 immunoprecipitation of only gE. Lane 1 in each gel represents a Western blot of an infected cell extract in which 25 % of the volume used for immunoprecipitation was loaded into the gel, while the remaining lanes represent immunoprecipitation followed by Western blotting. Equal volumes of cell extracts were loaded for immunoprecipitation. HSV-1 gE was detected using rabbit polyclonal antibody UP575; gI, using rabbit polyclonal antibody UP1925 in (B) or MAb Fd69 in (D and E); and Us9, using rabbit polyclonal antibody UP1991. gEnull, gE380 and gInull refer to NS-gEnull, NS-gE380 and HSV-1 gInull viruses. Positions of molecular weight markers are indicated on the left of each gel

Fig. 2.

Sucrose gradient fractionation and immunoprecipitation of gE and Us9 using ionic detergents. Post-nuclear cell extracts were prepared in RSB buffer (non-ionic detergent) from HSV-1 WT-virus- (A) or NS-gE380- mutant- (B) infected Vero cells and fractionated on a 5-to-30 % sucrose gradient. Alternate fractions were immunoprecipitated with anti-gE MAb 1BA10 in RIPA buffer containing ionic and nonionic detergents, followed by Western blotting using rabbit polyclonal anti-gE UP575 and anti-Us9 UP1991 antibodies. * The asterisk indicates the position of IgG heavy chains (A and B top gels) or light chains (A and B bottom gels) used for immunoprecipitation. The fractionation of gI is not shown, since anti-gE MAb 1BA10 does not co-immunoprecipitate gI. Molecular weight markers are indicated on the left

Fig. 3.

Confocal microscopy demonstrating that Us9 co-localizes with gE in HSV-1 WT infected cells and partially redistributes with gE380 to the ER and nuclear periphery in NS-gE380-infected cells. Vero cells were infected with WT or NS-gE380 virus (MOI of 0.1). At twelve hours postinfection, confocal images were collected and analyzed. A representative example is shown

Fig. 4.

Us9 and gE in HSV-1 WT-infected cells associate in TGN- and ER-enriched fractions when only nonionic detergent is used to prepare infected cell extracts. Cells were mock infected (A), or infected with WT HSV-1 (B) or NS-gE380 mutant (C). Post-nuclear fractions from mock-infected cells were prepared in the absence of detergents and separated on a sucrose step gradient. Fractions were run on two SDS PAGE gels to accommodate all fractions and treated identically. Western blots were probed for calnexin or TGN-38 to identify fractions rich in ER or TGN, respectively (A). Infected cell fractions were immunoprecipitated with anti-gE MAb 1BA10 using nonionic detergents, loaded on two gels to accommodate all samples, and probed with rabbit anti-gE (UP575) and rabbit anti-Us9 (UP1991) (B, C). As a control, rabbit anti-gC (R47) was used to immunoprecipitate infected cell fractions, followed by Western blotting using anti-gC R47, anti-gE MAb 1BA10, and rabbit anti-Us9 UP1991 (D, E). As an additional control, mock-infected and WT-infected whole-cell extracts that

were not further fractionated on a sucrose gradient were prepared using nonionic detergents and immunoprecipitated with anti-gC antibody followed by Western blotting for gC protein (**F**). * The asterisk indicates the presence of IgG heavy chains in the anti-gC gels D, E, F and IgG light chains in the anti-Us9 gel D. Molecular weight markers are indicated on the left

Fig. 5.

Immunoprecipitation using nonionic detergents demonstrating a gE, gI, Us9 trimolecular complex in HSV-1 WT- and NS-gE380-infected cells. Vero cells (10^6) were mock infected or infected with HSV-1 WT strain NS, NS-gEnull, NS-gE380 or HSV-1 gInull virus (MOI, 0.1). Twenty hours later, cell extracts were prepared using RSB buffer containing 1 % Triton X-100 (nonionic detergent), followed by immunoprecipitation with anti-gI MAb Fd69 (A) or anti-gE MAb 1BA10 (B). Lane 1 in each gel represents a Western blot of infected cell extract in which 25 % of the volume used for immunoprecipitation was loaded onto the gel, while lanes 2 to 6 represent immunoprecipitation with Fd69 or 1BA10 followed by Western blotting. Equal volumes of immunoprecipitates were loaded onto lanes 2 to 6. HSV-1 gE was detected using rabbit polyclonal antibody UP575; gI, using rabbit polyclonal antibody UP1925; and Us9, using rabbit polyclonal antibody UP1991. gEnull, gE380 and gInull refer to NS-gEnull, NS-gE380 and HSV-1 gInull viruses. Positions of molecular weight markers are indicated on the left of each gel

Fig. 6.

Immunoprecipitation using nonionic detergents demonstrates that the interaction of Us9 with gE is specific and dissociates at pH 6.0. **(A)** Lysates from cells infected with HSV-1 WT or NS-Us9null virus were prepared using a nonionic detergent, 1 % Triton X-100, immunoprecipitated with anti-gI MAb Fd69, and probed for Us9 (using rabbit antibody UP1991), gI (using MAb Fd69) and gC (using rabbit antibody R47). **(B)** Lysates were immunoprecipitated with anti-gE MAb 1BA10, and the precipitates were washed with a nonionic buffer at pH 6.0 or pH 7.4, followed by Western blotting for gE and Us9