Supporting Information

Han et al. 10.1073/pnas.1212900109

SI Materials and Methods

Cells, Virus Strains, and Antibodies. Vero cells were maintained in DMEM (Gibco) supplemented with 5% (vol/vol) FBS and 5% (vol/vol) FCS, penicillin, and streptomycin (131 μg/mL). HaCaT cells were cultured in DMEM supplemented with 10% (vol/vol) FBS and gentamycin. All viruses were derived from the KOS strain, the genome of which has been cloned into a bacterial artificial chromosome and provided by David Leib's laboratory (Dartmouth College of Medicine, Hanover, NH) (1). For infection assays, Vero cells were grown in DMEM supplemented with 2% (vol/vol) FBS, 25 mM Hepes buffer, glutamine (0.3 μg/mL), penicillin, and streptomycin.

Rabbit anti-GFP serum was raised against His_6-GFP and recognizes both GFP and the His_6 tag (2). UL16 antibodies were raised in rabbits against GST-UL16 (3, 4). Rabbit anti-UL11 serum was raised against GST-UL11 (5). Rabbit serum specific for UL21 was raised against GST-UL21 (6). The polyclonal antibody against gE was a gift from Harvey M. Friedman (University of Pennsylvania, Philadelphia, PA). Mouse monoclonal antibody (clone 3114) to glycoprotein E (gE) was provided by David Johnson (Oregon Health and Science University, Portland, OR). Rabbit antibodies to VP5 were provided by Richard Courtney (Pennsylvania State University College of Medicine, Hershey, PA).

Plasmids. Mammalian expression plasmids pUL16-GFP, pUL11, psUL11.HA, psUL11-GFP, psUL11(AC−).HA [acidic cluster minus (AC−)], and pUL21 have been described previously (5–7). All HSV genes in these plasmids are under control of CMV promoter in the vector pEGFP-N2. pHA.UL21 is a derivative of expression plasmid pEGFP-N2 that encodes a derivative of UL21 with an HA epitope tag at its N terminus. Similarly, psUL21 encodes a derivative of UL21 that has the Src membrane-binding peptide (MGSSKSKPKDAL) at its N terminus. The eukaryotic expression plasmid pCMV-gE has been described previously (8).

Construction of Mutant Viruses. A BAC containing the HSV-1 KOS strain genome was used to generate recombinant viruses by using the detailed procedures described elsewhere (1, 9, 10). The UL11-null mutant (ΔUL11) was created by deleting codons 31 to 96 without altering the overlapping and essential U_L12 gene (9). The revertant was constructed from the mutant ΔUL11 by replacing the nonessential U_L35 gene with the respective UL11 alleles (9). UL16- and UL21-null mutants (ΔUL16 and ΔU21) were created by deletion of the corresponding entire ORFs. The revertants were subsequently created from these null mutants by restoring the WT sequence to the original loci. The gE-null mutant (ΔgE) was made by replacing the first two codons of gE with stop codons (11). The gE cytoplasmic tail deletion mutant (gEΔCT) and its revertant have been described previously (10). To convert WT KOS into a syncytial strain, substitution (A855V) was introduced into the cytoplasmic tail of glycoprotein B (gB) to generate a Syn virus gBsyn. In the background of this virus, mutants ΔUL11/gBsyn, ΔUL16/gBsyn, ΔUL21/gBsyn, ΔgE/gBsyn, and gEΔCT/gBsyn were subsequently generated, and so were the corresponding revertants. Correct clones were verified by HindIII digestion, PCR analysis, and DNA sequencing of the relevant region. The resulting BAC plasmids were purified and then transfected into Vero cells with Lipofectamine 2000. After 3 to 4 d, transfected cells were harvested while showing cytopathic effects and used to infect new Vero cell monolayers to produce a viral stock.

Transfections. Vero cells grown on coverslips in six-well plates were transfected when 60% to 70% confluent with 2 to 5 μg DNA per well in mixtures of 7 to 8 μL of Lipofectamine in Opti-MEM. After incubation at room temperature for 20 min, the DNA–Lipofectamine complexes were added directly to cells in antibiotic-free culture medium supplement with 10% FBS. The cells were examined for expression with antibodies at 16 to 18 h after transfection.

Quantitative Analysis of Syncytia Formation of HSV Mutants. Vero or HaCaT cells grown on 100-mm plates were transfected with recombinant BAC plasmid DNA for various viral mutants. Virus-induced cytopathic effect was monitored daily. The frequency of virus-induced syncytia was measured by dividing the number of syncytia by the total number of infection foci, and, for each mutant, at least 200 infection sites for each mutant were counted.

Immunofluorescence Analysis. The immunofluorescence analysis procedures have been described elsewhere (10). Briefly, cells were fixed in 3.7% paraformaldehyde for 7 min, permeabilized for 10 min with PBS solution containing 0.1% Triton X-100 and 2% BSA, and then blocked with PBS solution containing 2% BSA and 20% porcine serum overnight at 4 °C. The samples were reacted with primary antibodies diluted in PBS solution containing 2% BSA and 20% porcine serum in a humid chamber. The gE mouse monoclonal antibody was used at a dilution of 1: 5,000. The UL11 rabbit polyclonal antibodies were used at a dilution of 1:1,000. The anti-HA epitope mouse monoclonal antibody was used in a 1:4,000 dilution. The UL21 rabbit serum was used in a 1:500 dilution. After incubation for 0.5 h at room temperature, the cells were then rinsed three times with PBS solution for 5 min each and reacted for another 30 min with Alexa Fluor 568-conjugated goat anti-mouse IgG $F(ab')_2$ fragment, Alexa Fluor 568-conjugated goat anti-mouse IgG $F(ab')_2$ fragment, Cy5-conjugated goat anti-rabbit IgG $F(ab')_2$ fragment, or a combination thereof. The cells were washed once with PBS solution. Nuclear DNA was stained with DAPI (Molecular Probes) for 5 min. After three rinses, the samples were mounted and examined with a Leica SP2 confocal microscope.

Immunoblot Analysis. Cells in 100-mm plates were infected with WT HSV KOS or mutants at a multiplicity of infection (MOI) of 5. At 16 to 24 h after infection, the cells were harvested and pelleted at 1,244 × *g* for 5 min. The cells were washed with PBS solution three times and 10% of the cells were dissolved in 150 μL 1× SDS/PAGE sample buffer, and equal quantities of proteins normalized to VP5 were loaded into an SDS-polyacrylamide gel for Western blot analyses. VP5 antibodies were used at a 1:15,000 dilution, and UL11, UL21, and UL16 antibodies were used at a 1: 2,000 dilution. Rabbit anti-gE and -gB polyclonal antibodies were used at a 1:6,000 dilution.

Flow Cytometry. Vero cells grown in 100-mm plates were infected with WT HSV KOS or mutants ΔUL11, ΔUL16, ΔUL21, or ΔgE at an MOI of 0.5. At 12 h after infection, the cells were washed three times with PBS solution without calcium and magnesium but containing 0.5% NaN3, incubated with 1 mL cell dissociation buffer at 37 °C for 10 min, and then dispersed in cold DMEM containing 0.5% NaN3. After being washed twice with cold PBS solution/0.5% NaN3, the cells were stained with mouse anti-gE monoclonal antibodies (clone 3114) at a dilution of 1:500 in PBS solution containing 1% BSA and 0.5% NaN3 on ice for 30 min. After one wash, the cells were then stained with FITC-conjugated F(ab′)2 fragment of goat anti-mouse IgG for another 30 min, followed by two washes with 0.5% NaN3 PBS solution. The cells

are fixed by 1% paraformaldehyde. All stained cells were analyzed on a FACSCalibur instrument (Becton Dickinson).

Membrane Flotation Assay. The membrane flotation protocol has been described previously (2, 12). Briefly, Vero cells grown to 65% to 70% confluence in 100-mm dishes were transfected with different combination of plasmids encoding UL11, UL16, UL21, or gE. At 16 to 20 h after transfection, cells were harvested, washed twice with cold NTE buffer (10 mM Tris·HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA), and then resuspended in 300 μL of hypotonic lysis buffer (10 mM Tris·HCl, pH 7.4, 0.2 mM $MgCl_2$) on ice for 20 min. Swollen cells were lysed on ice by 35 strokes with a Dounce homogenizer and then centrifuged at low speed to remove unbroken cells and nuclei. Postnuclear supernatants (~300 μL) were mixed with 1.7 mL of 65% sucrose (wt/wt), placed at the bottom of a Beckman SW55Ti tube, and sequentially overlaid with 2.5 mL of 45% and 0.5 mL of 2.5% sucrose. All sucrose solutions were made in NTE buffer. The samples were centrifuged for 20 h at 200,000 × *g* and 4 °C in a Beckman ultracentrifuge, and six equal-volume fractions (~833 μL each) were collected from the top. The membranes in each fraction were solubilized with 200 μL of 5× RIPA buffer, and immunoprecipitated with proper antibodies (rabbit anti-UL11, -UL16, -UL21, or -gE serum). Immunocomplexes were captured with Protein A agarose beads (Roche), washed three times with RIPA buffer, and dissociated in SDS/PAGE sample buffer. Samples were separated by SDS/PAGE followed by Western blot analysis using the appropriate primary antibodies and True-Blot HRP-conjugated secondary antibodies.

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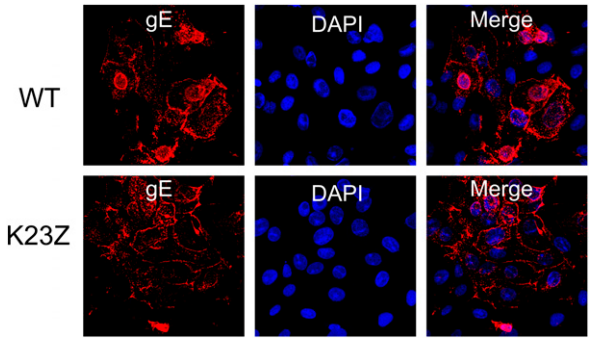

Fig. S1. gE accumulates on the cell surface independent of virion production. The K23Z mutant was created by deletion of the U_L18 gene, which encodes capsid protein VP23 and is required for capsid production, and rescued on a complementing cell line (G32). Vero cells were infected with this mutant or WT HSV at an MOI of 0.1. At 24 h after infection, the cells were fixed and stained with a mouse monoclonal antibody (Mab3114) to gE, followed by Alexa fluor 568-conjugated goat anti-mouse IgG before microscopy.

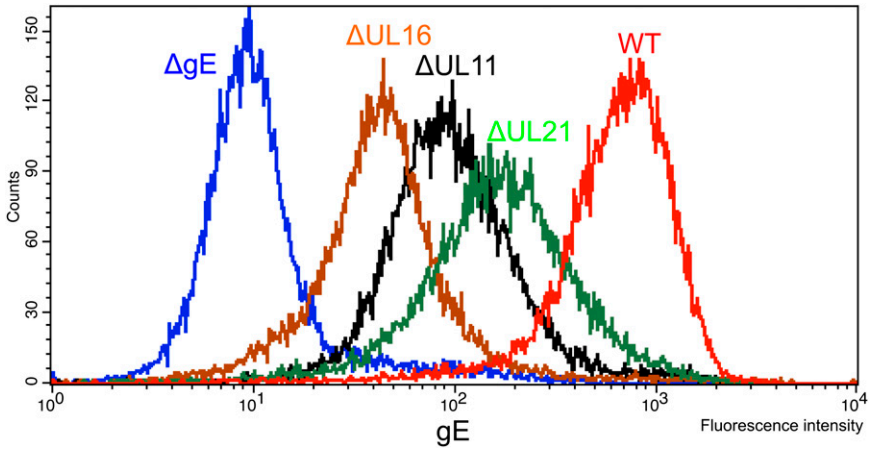

Fig. S2. FACS analysis of gE cell surface expression in infected Vero cells. Vero cells grown in 100-mm plates were infected with WT HSV or mutants ΔUL11, ΔUL16, ΔUL21, or ΔgE at an MOI of 0.5. At 12 h after infection, the gE cell surface expression was measured by flow cytometry as described in *Materials and Methods*. The *x*-axis represents the fluorescence intensity. Data are representative of two independent experiments, which generated identical results.

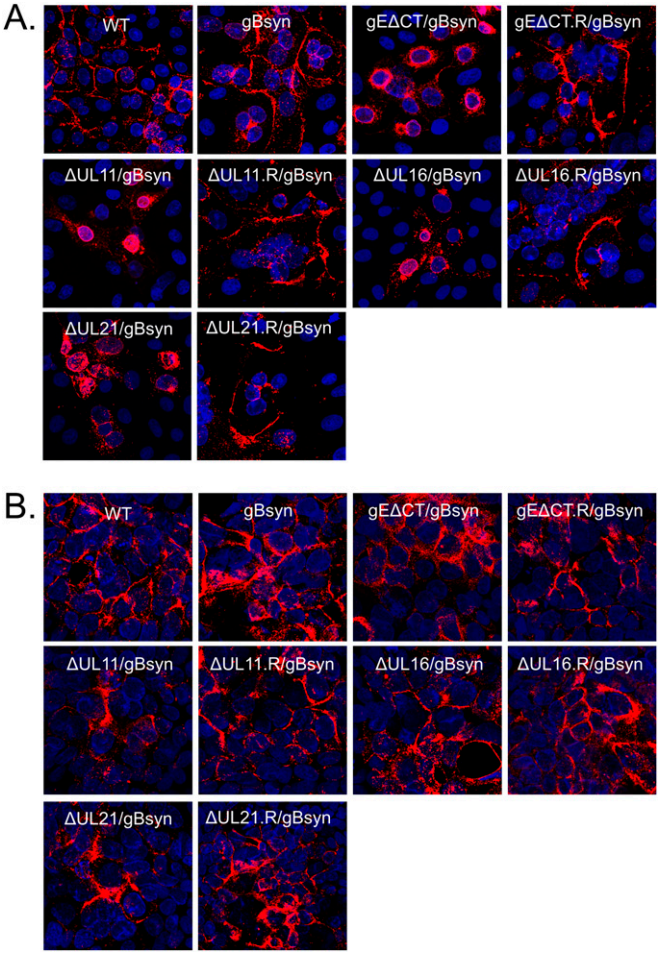

Fig. S3. The Syn substitution in the cytoplasmic tail of gB does not affect gE cell surface accumulation. Vero (*A*) or HaCaT cells (*B*) grown on coverslips were infected with the indicated viruses at an MOI of 0.01. At 24 h after infection, the cells were fixed, permeabilized, and stained with a mouse monoclonal antibody (Mab3114) to gE followed by Alexa fluor 568-conjugated goat anti-mouse IgG before microscopy.

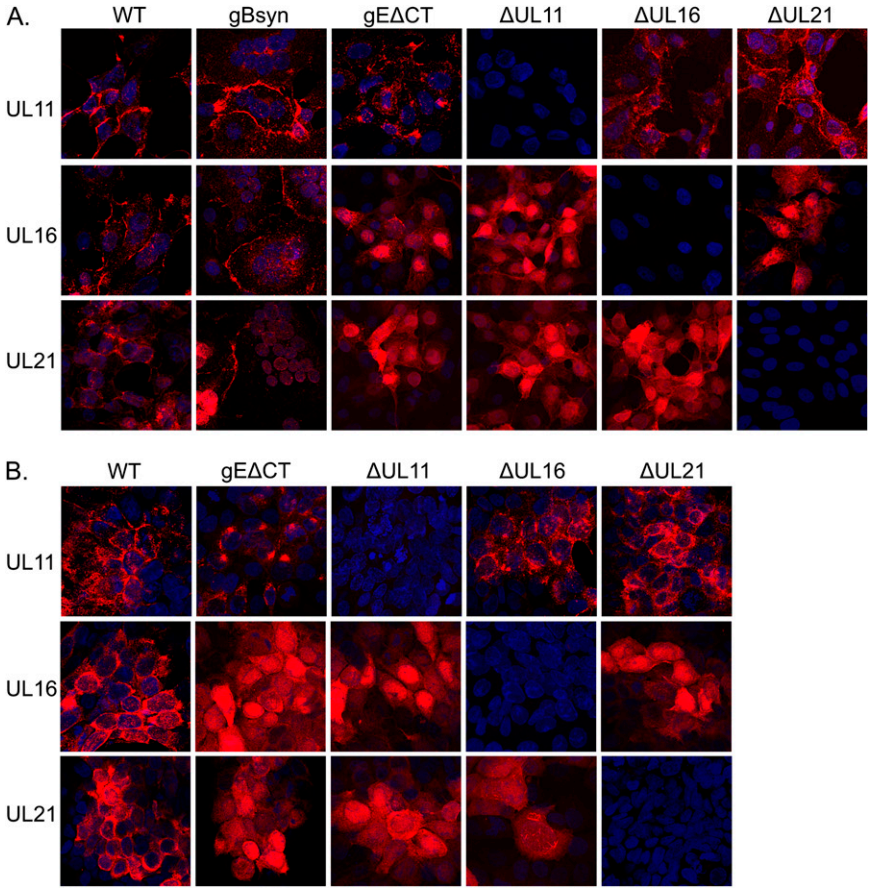

Fig. S4. UL11, UL16, and UL21 depend on gE and each other for proper localization in infected cells. Vero (*A*) or HaCaT cells (*B*) grown on coverslips in six-well plates were infected with the indicated viruses at an MOI of 0.01. At 24 h after infection, the cells were fixed, permeabilized, and blocked with PBS solution containing 1% BSA and 20% swine serum at 4 °C overnight. The cells were then stained with rabbit polyclonal antibodies to UL11, UL16, or UL21 at a dilution of 1:500 in PBS solution containing 1% BSA and 20% swine serum, followed by Alexa fluor 568-conjugated F(ab′)2 fragment of goat anti-rabbit IgG, before microscopy.