AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology

Functional Characterization of Glycoprotein H Chimeras Composed of Conserved Domains of the Pseudorabies Virus and Herpes Simplex Virus 1 Homologs

Sebastian W. Böhm,[a] Marija Backovic,[b] Barbara G. Klupp,[a] Felix A. Rey,[b] Thomas C. Mettenleiter,[a] Walter Fuchs[a]

Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Molecular Virology and Cell Biology, Greifswald-Insel Riems, Germany[a]; Institut Pasteur, Unité de Virologie Structurale, Département de Virologie and CNRS Unité Mixte de Recherches 3569, Paris, France[b]

ABSTRACT

Membrane fusion is indispensable for entry of enveloped viruses into host cells. The conserved core fusion machinery of the *Herpesviridae* consists of glycoprotein B (gB) and the gH/gL complex. Recently, crystal structures of gH/gL of herpes simplex virus 2 (HSV-2) and Epstein-Barr virus and of a core fragment of pseudorabies virus (PrV) gH identified four structurally conserved gH domains. To investigate functional conservation, chimeric genes encoding combinations of individual domains of PrV and herpes simplex virus 1 (HSV-1) gH were expressed in rabbit kidney cells, and their processing and transport to the cell surface, as well as activity in fusion assays including gB, gD, and gL of PrV or HSV-1, were analyzed. Chimeric gH containing domain I of HSV-1 and domains II to IV of PrV exhibited limited fusion activity in the presence of PrV gB and gD and HSV-1 gL, but not of PrV gL. More strikingly, chimeric gH consisting of PrV domains I to III and HSV-1 domain IV exhibited considerable fusion activity together with PrV gB, gD, and gL. Replacing PrV gB with the HSV-1 protein significantly enhanced this activity. A cell line stably expressing this chimeric gH supported replication of gH-deleted PrV. Our results confirm the specificity of domain I for gL binding, demonstrate functional conservation of domain IV in two alphaherpesviruses from different genera, and indicate species-specific interactions of this domain with gB. They also suggest that gH domains II and III might form a structural and functional unit which does not tolerate major substitutions.

IMPORTANCE

Envelope glycoprotein H (gH) is essential for herpesvirus-induced membrane fusion, which is required for host cell entry and viral spread. Although gH is structurally conserved within the *Herpesviridae*, its precise role and its interactions with other components of the viral fusion machinery are not fully understood. Chimeric proteins containing domains of gH proteins from different herpesviruses can serve as tools to elucidate the molecular basis of gH function. The present study shows that the C-terminal part of human herpesvirus 1 (herpes simplex virus 1) gH can functionally substitute for the corresponding part of suid herpesvirus 1 (pseudorabies virus) gH, whereas other tested combinations proved to be nonfunctional. Interestingly, the exchangeable fragment included the membrane-proximal end of the gH ectodomain (domain IV), which is most conserved in sequence and structure and might be capable of transient membrane interaction during fusion.

Membrane fusion is an essential process in pro- and eukaryotic organisms and occurs, e.g., during cell division, autophagy, and endo- and exocytosis. For entry into host cells, enveloped viruses depend on fusion of their envelopes with cellular membranes. Fusion can occur at the plasma membrane either immediately after attachment or after endosomal uptake of the enveloped virus particles. Members of the *Herpesviridae* have been shown to utilize both pathways, but herpes simplex viruses 1 and 2 (HSV-1 and HSV-2, respectively) and other members of the subfamily *Alphaherpesvirinae*, e.g., pseudorabies virus (PrV; suid herpesvirus 1), predominantly enter cells by direct penetration at the plasma membrane (1). In most alphaherpesviruses four conserved viral envelope glycoproteins are relevant for entry: the core fusion machinery consisting of glycoprotein B (gB), the gH/gL heterodimer, and the receptor-binding gD (1–3). Recent publications suggest that gB represents the actual fusion protein and that gD and gH/gL are required for activation of gB via direct gD-gH/gL and gH/gL-gB interactions (4–8).

In many alphaherpesviruses attachment is facilitated by binding of the nonessential glycoprotein gC to heparan sulfate of cell surface proteoglycans (9–11). In a second step, which is crucial for virus entry, gD binds to nectin-1 or other specific virus receptors and is then suggested to activate gH/gL by an yet unknown mechanism (5, 12). However, PrV gD is dispensable for induction of membrane fusion *in vitro* (13), and previous studies also revealed that gD-negative PrV mutants were still capable of plaque formation by direct cell-to-cell spread (14, 15). Moreover, after passage of cells infected with gD-deleted PrV, compensatory mutations were acquired in gH and gB which supported efficient gD-independent entry (16, 17). In line with these observations, several

Received 5 August 2015 Accepted 12 October 2015

Accepted manuscript posted online 21 October 2015

Citation Böhm S, Backovic M, Klupp BG, Rey FA, Mettenleiter TC, Fuchs W. 2016. Functional characterization of glycoprotein H chimeras composed of conserved domains of the pseudorabies virus and herpes simplex virus 1 homologs. J Virol 90:421–432. doi:10.1128/JVI.01985-15.

Editor: R. M. Longnecker

Address correspondence to Thomas C. Mettenleiter, thomas.mettenleiter@fli.bund.de.

alphaherpesviruses like varicella-zoster virus completely lack gD homologs (18), and gD is not conserved in other herpesvirus subfamilies.

In contrast, gH and gL homologs are present in all known members of the *Herpesviridae*, and at least gH is essential for induction of membrane fusion, virus entry, and cell-to-cell spread (19–21). Epstein-Barr virus (EBV) (22), human cytomegalovirus (HCMV) (23), and HSV-1 gH (24, 25) bind to integrin receptors in the host cell plasma membrane, but the general relevance of these interactions for attachment and fusion is unclear. Whereas in HSV-1 gL is required for maturation, virion incorporation, and function of gH (26), gH was found in virions of gL-deleted PrV, and the corresponding mutants also exhibited limited spread in tissue culture (27). Moreover, as with gD-negative PrV, *in vitro* passaging of gL-deleted PrV resulted in the accumulation of compensatory mutations which enabled gL-independent entry and spread (28). Interestingly, the most efficient compensatory mutation was a translocation of part of the gH gene to the gD locus, resulting in expression of a chimeric protein containing major parts of the gD ectodomain (amino acids [aa] 1 to 271) fused to a truncated gH lacking the N-terminal 96 residues. This chimeric gDH was able to substitute for gD, gH, and gL in fusion assays and virus replication (13, 28). The corresponding gH core fragment (gH^c) was crystallized, and its structure was solved (29). However, targeted construction of similar gD-gH chimeras of HSV-1 did not result in fully functional proteins (30).

Despite these differences, the general conservation of the mechanism of herpesvirus entry was demonstrated by heterologous complementation studies which showed that gB of PrV or simian alphaherpesvirus (saimiriine herpesvirus 1; SaHV-1) can substitute for the HSV-1 protein (31–33) and that gB of bovine herpesvirus 1 complements gB-deleted PrV (34). However, heterologous complementation by gD and gH/gL homologs has not been described, indicating that these proteins mediate species-specific interactions which might be relevant for tropism.

As a prerequisite for a more detailed understanding of gH/gL function, the structures of the corresponding heterodimeric protein complexes of HSV-2 and EBV, as well as of the core fragment of PrV gH, were determined by crystallography and compared (29, 35, 36). Despite a low level of overall amino acid sequence conservation (e.g., 27% conservation between PrV and HSV-2 gH proteins), the three gH homologs were found to share similar three-dimensional (3D) structures (Fig. 1). Four distinct domains were identified in EBV gH (36), and PrV gH^c, which lacked the first domain due to the N-terminal truncation, was composed of corresponding domains II, III, and IV (29). HSV-2 gH (35) was divided into three domains, with the last, C-terminal domain (H3) corresponding to domain IV in the PrV and EBV gH proteins. The remaining domains were grouped differently. For clarity reasons, in this paper we will apply the four-domain subdivision established for EBV and PrV gH also to the HSV-2 and HSV-1 gH ectodomains. Domain I of HSV-2 (Fig. 1A) and EBV gH interacts with gL to form the functionally active heterodimeric complex. Domain II contains a β-sheet ("fence"), which separates the gL-binding domain I from the rest of the molecule, and a syntaxin-like bundle (SLB) formed by three α-helices (Fig. 1C and D). The SLB is similar to structures found in eukaryotic fusion proteins and is relevant for PrV gH function since disruption or immobilization of this element impaired or abolished gH activity in fusion assays and in complementation studies of gH-deleted

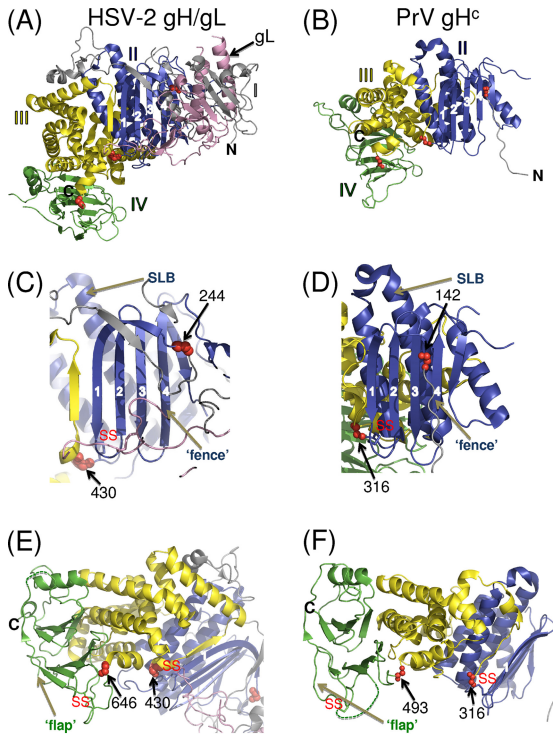


FIG 1 Structures of HSV-2 gH/gL (PDB accession number 3M1C) (A, C, and E), and of PrV gH^c (PDB accession number 2XQY) (B, D, and F). Amino and carboxyl termini of gH are indicated (N and C, respectively). gL is shown in pink in the HSV-2 structure, and gH domains are labeled with roman numerals and highlighted in gray (I), blue (II), yellow (III), and green (IV). Domain I is absent from the PrV gH^c fragment. The complete structures (A and B) and enlargements of domain II (C and D) are shown in the same orientation, whereas the enlargements of domains III and IV (E and F) were rotated ≈90° counterclockwise. The conserved syntaxin-like bundle of α-helices (SLB) and the β-sheet fence in domain II, as well as the flap in domain IV, are marked by gray arrows. The strands of the fence are numbered to serve as landmarks. Conserved disulfide bonds (SS) in domain IV and at the end of domain II are labeled. Red spheres marked by black arrows and numbers represent individual atoms of the indicated junction residues used for generation of the chimeric proteins (Fig. 2).

PrV (37, 38). Domain III consists of eight consecutive α-helices and contains a strictly conserved and functionally important disulfide bond (39). The membrane-proximal domain IV, which is followed by the transmembrane domain, encompasses an extended "flap" region (Fig. 1E and F) which masks an underlying patch of hydrophobic amino acids. Mutational analyses indicated that flap movement might be required to expose the hydrophobic patch for membrane interaction during fusion (40).

Although these structural features are preserved in the three gH homologs, it is unclear whether they are also functionally equivalent. Thus, we attempted to investigate the functional conservation of gH domains by generation of chimeric proteins containing domains of the PrV and HSV-1 homologs. Expression, maturation, and transport of the gH chimeras in the presence or absence of gL were determined by Western blotting and immunofluorescence tests of plasmid-transfected cells. Function of the chimeric proteins was analyzed by *in vitro* fusion assays including gB, gD, and gL of PrV or HSV-1. Functional gH chimeras were further investigated for *trans*-complementation of virus mutants with deletions of gH after establishment of stably expressing cell

TABLE 1 Primers for cloning of HSV-1 glycoprotein genes

Primer[a]	Site	Sequence[b]	Nucleotide position[c]
HHV1gB-F	BamHI	5′-*CACAGGATCC*GTCCCGCC**ATG**CGCCAG-3′	55702–55718 (R)
HHV1gB-R	EcoRI	5′-*CACAGAATTC*CCCCCCG**TCA**CAGGTCGTCC-3′	52989–53008
HHV1gD-F	EcoRI	5′-*CACAGAATTC*CGGT**ATG**GGGGGG-3′	138274–138289
HHV1gD-R	XhoI	5′-*CACACTCGAG***CTA**GTAAAACAAGGGCTGG-3′	139447–139465 (R)
HHV1gH-F	HindIII	5′-*CACAAGCTTCCA*CC**ATG**GGGAATGG-3′	46289–46301 (R)
HHV1gH-R	XhoI	5′-*CACACTCGAG***TTA**TTCGCGTCTCC-3′	43783–43796
HHV1gL-F	EcoRI	5′-*CACAGAATTC*CGCT**ATG**GGGATTTTGGGTTG-3′	9268–9288
HHV1gL-R	NotI	5′-*CACAGCGGCCGC*ATCA**TTA**GATGCGCCG-3′	9935–9952 (R)

[a] F, forward; R, reverse.
[b] Extensions are shown in italics, and restriction sites are underlined. Start codons and reverse stop codons are shown in bold letters.
[c] Nucleotide positions refer to the genome sequence of HSV-1 strain F (GenBank accession number GU734771). R, primers correspond to the reverse strand.

lines. A similar approach has recently been used to construct and analyze chimeric proteins composed of gH fragments of the closely related simplexviruses HSV-1 and SaHV-1 (41).

MATERIALS AND METHODS

Viruses and cells. The gH-deleted, green fluorescent protein (GFP)-expressing PrV recombinant pPrV-ΔgHABF has been described previously (37). A similar GFP-expressing gH-deleted HSV-1 mutant was prepared from the infectious plasmid clone pHSV-1ΔgJ (42) after the replacement of genomic BstEII/EcoRV fragments containing gH codons 95 to 712 by a kanamycin resistance gene according to standard protocols (43). Viruses were propagated in rabbit kidney (RK13) cell lines stably expressing authentic or chimeric gH of PrV and/or HSV-1 (see below) or gH and gL of PrV (44). Cells were grown at 37°C in minimum essential medium supplemented with 10% fetal bovine serum (FBS). For plaque assays the cells were inoculated for 2 h with serial virus dilutions and subsequently incubated under semisolid medium containing 6 g/liter methyl cellulose for 2 to 3 days.

Expression plasmids. The open reading frames (ORFs) encoding glycoproteins gB, gD, gH, and gL of HSV-1 strain F (kindly provided by David C. Johnson) were amplified from genomic DNA by PCR using *Pfx* DNA polymerase (Life Technologies) and specific primers which contained 5′-terminal extensions providing unique restriction sites (Table 1). These sites were used for cloning into correspondingly digested plasmid pcDNA3 (Life Technologies), which allows expression in eukaryotic cells under the control of the human cytomegalovirus immediate early promoter. Correct insertion in pcDNA-H1FgB, -H1FgD, -H1FgH, and -H1FgL was confirmed by DNA sequencing. To enhance surface expression and fusion activity of HSV-1 gB, pcDNA-H1FgB was doubly digested with BstEII and XbaI, treated with Klenow polymerase, and religated. The resulting plasmid pcDNA-H1FgBBX encoded a truncated gB lacking the C-terminal amino acids (aa) 878 to 904.

For prokaryotic expression of HSV-1 gH, a 2,258-bp AfeI/XhoI fragment containing codons 89 to 839 of pcDNA-H1FgH was recloned into SmaI/XhoI-digested pGEX-4T-3 (GE Healthcare). Subsequent double digestions of pGEX-H1FgH with NheI/XhoI and EcoRI/NruI, each followed by Klenow treatment and religation, led to pGEX-H1FgHC containing gH codons 388 to 792 3′-terminally fused to the glutathione *S*-transferase (GST) open reading frame (ORF). In a similar approach pGEX-H1FgH was shortened by subsequent double digestions with NruI/XhoI and BamHI/BsrGI, resulting in pGEX-H1FgHN which contained gH codons 147 to 386 fused to the GST ORF.

The eukaryotic expression plasmids for PrV glycoproteins gB, gD, gH, and gL used in this study have been described previously: pgB-008 (45), gDgI-CMV (46), pcDNA-gH, and pRc/CMV-gL (13).

Construction and expression of chimeric gH genes. A fusion PCR was used to generate gH genes encoding domains I to III of PrV and IV of HSV-1 ($P^{I–III}/H^{IV}$ gH), domains I and II of PrV and III and IV of HSV-1, or domain I of PrV and domains II to IV of HSV-1 (Fig. 2). The 5′-terminal parts of the PrV gH ORF were amplified from pcDNA-gH (13) using the vector-specific T7 primer and the desired reverse domain fusion primers (Table 2). The 3′-terminal parts of the HSV-1 gH ORF were amplified from pcDNA-H1FgH with the forward domain fusion primers and the vector-specific SP6-25 primer (Table 2). The PCR products were purified, and 10 ng of each of the matching products, which overlapped by ≥15 bp, was mixed and amplified in a final PCR using the T7 and SP6-25 primers. The chimeric products were doubly digested at terminal HindIII and XhoI sites and inserted into the correspondingly digested expression vector pcDNA3. The two parts of the complementary chimeric gH genes containing domains I to III of HSV-1 and IV of PrV, domains I and II of HSV-1 and III and IV of PrV, or domain I of HSV-1 and domains II to IV of PrV ($H^{I}/P^{II–IV}$) were also amplified from pcDNA-H1FgH or pcDNA-gH, respectively. However, the domain fusion primers used contained silent mutations introducing NheI or XhoI restriction sites (Table 2). Thus, the HindIII/NheI- and NheI/XhoI-digested or HindIII/XhoI- and XhoI-digested fragments could be inserted into HindIII/XhoI-digested pcDNA3 by three fragment ligations. All obtained expression plasmids were characterized by DNA sequencing of the complete chimeric gH ORFs. Geneious software, version 8.1.3 (Biomatters), was used for analysis and comparison of DNA and protein sequences.

Complementing cell lines. Cell lines stably expressing PrV, HSV-1, or chimeric gH were generated by transfection (X-tremeGENE HP reagent; Roche) of RK13 cells with the corresponding pcDNA3 expression plasmids. Two days after transfection the cells were trypsinized, and various dilutions in medium supplemented with 500 μg/ml G418 were seeded into 96-well plates. Resistant clones were tested for gH expression by Western blotting and indirect immunofluorescence (IIF) analyses with monospecific antibodies. Homogeneously gH-expressing cell clones were further propagated.

Antiserum preparation and IIF analyses. GST fusion proteins containing the N-terminal or C-terminal parts of the HSV-1 gH gene were expressed in *Escherichia coli* XL1 Blue MRF (Agilent Technologies) after transformation with pGEX-H1FgHN or pGEX-H1FgHC, isolated, and used for immunization of two rabbits as described previously (47). In IIF analyses the obtained antisera (anti-HSV-1 gHN and anti-HSV-1 gHC) and a similarly prepared PrV gH-specific rabbit serum (28) were used at dilutions of 1:200.

RK13 cells grown in 12-well plates were cotransfected (X-tremeGENE HP; Roche) with expression plasmids for native or chimeric gH and PrV gL, HSV-1 gL, or an irrelevant viral membrane protein (pORF25 of cyprinid herpesvirus 3). After 2 days the cells were fixed with 3% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 30 min at room temperature and subsequently permeabilized for 15 min with 0.5% Triton X-100 in PBS. For detection of surface-exposed gH the permeabilization step was omitted. After the cells were washed, they were blocked with 10% FBS in PBS and incubated with the rabbit antiserum diluted in blocking buffer for 1 h. Bound antibodies were detected by incubation for an additional hour with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Life

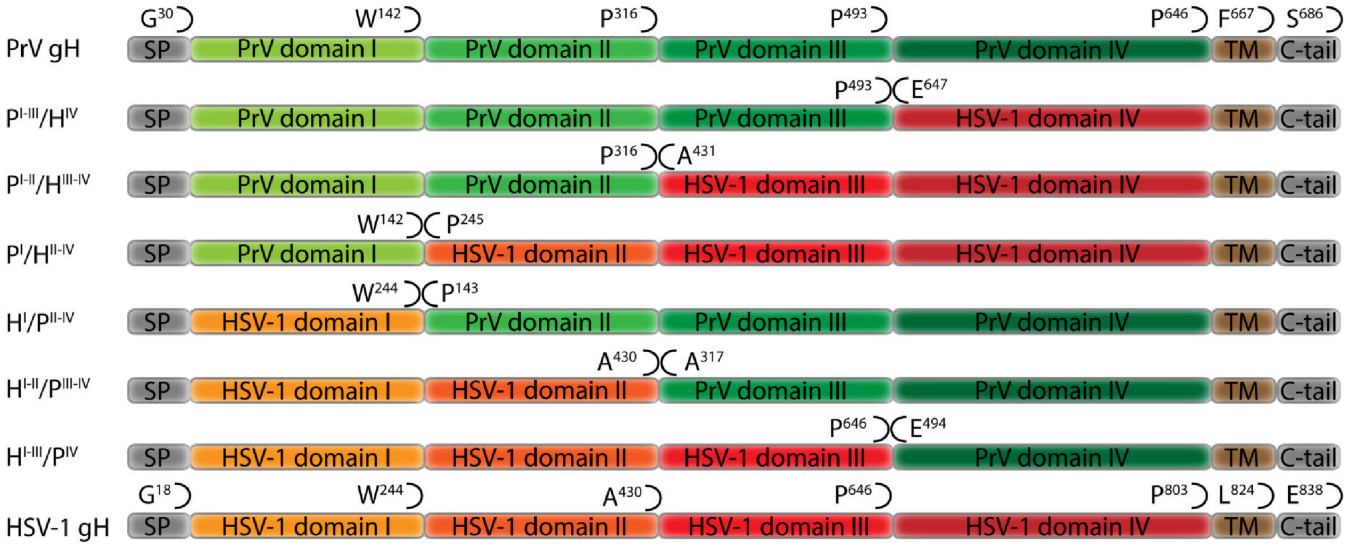


FIG 2 Diagram of the generated gH chimeras. The ectodomains of PrV and HSV-1 gH are shown in different shades of green and red, respectively. SP, signal peptide; C-tail, cytoplasmic tail; TM, transmembrane domain. Structurally conserved subdomains are indicated by roman numerals (I to IV). The last amino acids of the determined or predicted (Geneious) structural elements of PrV gH (GenBank accession number AAA47466) and HSV-1 gH (GenBank accession number AJE60179), as well as the last and first amino acids of the two components of chimeric proteins, are given above the respective bars. For clarity, the proteins were not drawn to scale.

Technologies) diluted 1:1,000 in PBS. After each incubation step the cells were washed repeatedly with PBS.

For semiquantitative analyses nonpermeabilized and permeabilized cells cotransfected with equal amounts of the same plasmid sets were incubated with a mixture of the three gH-specific antisera, and mean fluorescence intensities were measured by microscopy (Eclipse Ti-S with the software NIS-Elements, version 4.0; Nikon) at low magnification (4× objective) in six fields of view. Cells transfected with an irrelevant expression plasmid (see above) served as background controls. After background subtraction, the percentages of gH-specific surface fluorescence were determined for all tested protein combinations, and mean values as well as standard deviations were calculated from at least three independent experiments.

For subsequent photography, transfected cells incubated with the matching anti-gH serum were overlaid with 2.5% 1,4-diazabicyclo[2.2.2]octane in 90% glycerol containing 1 μg/ml propidium iodide for DNA staining.

Western blot analyses. Transfected RK13 cells were harvested after 48 h, and proteins were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Transfer to nitrocellulose membranes and subsequent incubation of the blots were done as described previously (48). The monospecific rabbit antiserum against PrV gH (28) was used at a dilution of 1:10,000, and the anti-HSV-1 gHN and anti-HSV-1 gHC sera were diluted at 1:20,000. Binding of peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) was detected (SuperSignal West Pico chemiluminescent substrate; Thermo Scientific) and recorded (VersaDoc 4000 MP; Bio-Rad).

***In vitro* fusion assays.** Monolayers of RK13 cells grown in 12-well plates were cotransfected (X-tremeGENE HP reagent; Roche) with equal amounts (300 ng each) of eukaryotic expression plasmids for C-terminally truncated gB, gD, and gL proteins of PrV or HSV-1, authentic or chimeric gH, and GFP (pEGFP-N1; Clontech). If single expression plasmids were omitted, the empty vector pcDNA3 (Life Technologies) was used instead. After 24 h or 48 h, the cells were fixed for 30 min with 3% PFA and repeatedly washed with PBS. Formation of polykaryocytes was analyzed by fluorescence microscopy (Eclipse Ti-S; Nikon). For each tested combination of plasmids, the areas of 50 syncytia from five random fields of sight were measured in three independent experiments. If no or

TABLE 2 Primers for construction of gH chimeras

Primer (site)[a]	Sequence[b]	Nucleotide positions or region[c]
PgHD3HgHD4-F	5′-*TCGCGCGCGCCGCGCCC**GAGGCCTCACATCGGTGC***-3′	PrV 62232–622451, HSV-1 44344–44361 (R)
PgHD2HgHD3-F	5′-*GGCCGGCTGCC**CCGCGGATTCTGTGTTTTTTAATG***-3′	PrV 61705–61717, HSV-1 44988–45011 (R)
PgHD1HgHD2-F	5′-*CGGCGGCGGT**GTGGCCCGTGGGCATCTG***-3′	PrV 6118261195, HSV-1 45554–45571 (R)
HgHD1PgHD2-F (NheI)	5′-**CCCCGTCGGCtagcAC***GTGGCCCG**CGGAGGTGGC***-3′	HSV-1 45564–45587 (R), PrV 61196–61209
HgHD2PgHD3-F (XhoI)	5′-**GCCGCtCGaGGGGCCGCGGGCTGTGC***CGCGG**GCTTCGCCGGGC***-3′	HSV-1 45006–45036 (R), PrV 61718–61733
HgHD3PgHD4-F (NheI)	5′-**GCGCCTTCGTCCCTGAG***ATCCCCGCCGAGGCGCTGCTaGCCCTG*-3′	HSV-1 44359–44375 (R), PrV 62249–62278
T7	5′-TAATACGACTCACTATAGGG-3′	pcDNA3 (upstream of gH)
SP6-25	5′-CTCTAGCATTTAGGTGACACTATAG-3′	pcDNA3 (downstream of gH)

[a] Designations of the domain fusion primers indicate the virus specificities of their 5′ and 3′ parts and the encoded gH domain ends or beginnings (where D3 indicates, e.g., domain III). P, PrV; H, HSV-1; F, forward.

[b] PrV sequences are shown in italics, HSV-1 sequences are shown in bold, and bold italics indicate identical sequence overlaps. Artificially introduced restriction sites are underlined, and nucleotides altered by silent mutations are shown in lowercase letters. Reverse domain fusion primers were complementary to the forward primers shown.

[c] Nucleotide positions refer to the genome sequences of PrV strain Ka (GenBank accession number JQ809328) and of HSV-1 strain F (GenBank accession number GU734771). R, sequences correspond to the reverse strand.

only very few syncytia were detectable, single cells were also measured to reach a value of 50. Mean sizes and standard deviations were calculated.

***In vitro* virus replication studies.** For plaque assays, nontransfected RK13 cells, RK13 cells expressing authentic or chimeric gH, or RK13-gH/gL cells were grown to monolayers and inoculated at a low multiplicity of infection (MOI) with phenotypically complemented gH-negative and parental gH-positive PrV or HSV-1 and further incubated under semisolid medium for 2 to 3 days at 37°C. Cells infected with the GFP-expressing viruses were visualized by fluorescence microscopy (Eclipse Ti; Nikon), and mean areas of 50 plaques per virus as well as standard deviations were calculated. For determination of final progeny virus titers, monolayers were infected at an MOI of 0.01, and after 2 h at 37°C nonpenetrated virus was inactivated by low-pH treatment (49). Incubation was continued under normal medium. After 4 days at 37°C the cells were scraped into the medium and lysed by freeze-thawing, and total progeny virus titers were determined by plaque assays on RK13-gH/gL (44) cells. The average of three experiments and standard deviations were calculated.

Statistical analyses. The significance of differences in gH surface expression, *in vitro* cell fusion activities, and virus replication properties was evaluated by Student's *t* tests.

RESULTS

Expression of gH chimeras. Based on the conserved domains identified by structural analysis of PrV gH (29), we constructed eukaryotic expression plasmids for chimeric proteins composed of individual domains of the gH homologs of PrV and HSV-1, which is closely related (79% identical amino acids) to the structurally characterized HSV-2 gH (35). In contrast, sequence identity between PrV and HSV-1 gH proteins amounts to only 27%. Figure 2 schematically shows the six chimeric gH constructs generated in this study and indicates the last and first amino acids of the PrV strain Kaplan (GenBank accession number AAA47466) (50) or HSV-1 strain F (GenBank accession number AJE60179) (51) proteins present at the junction sites. The N-terminal signal peptides (SP), the transmembrane (TM) domain, and the cytoplasmic (C-tail) domain always originated from the gH homolog providing domain I or domain IV. The transition sites (red spheres in Fig. 1) were chosen based on structure and sequence alignments of the two proteins and within stretches of identical or similar amino acid residues in HSV-1, HSV-2, and PrV gH (Fig. 2). Preservation of disulfide bonds (Fig. 1, SS) close to the domain boundaries was also taken into account.

Expression and transport of the chimeric proteins was tested by Western blotting and IIF analyses of transfected RK13 cells using rabbit antiserum raised against bacterial fusion proteins containing aa 88 to 632 of PrV gH (28) and aa 147 to 386 (anti-HSV-1 gHN) or aa 388 to 792 (anti-HSV-1 gHC) of HSV-1 gH. In Western blot analyses each of the chimeric proteins was abundantly detected by at least one of these antisera, and apparent protein masses were in the expected ranges (Fig. 3). Interestingly, the PrV gH-specific antiserum showed unambiguous reactions only with proteins containing domain III of PrV gH although a large fusion protein including major parts of the ectodomain had been used for immunization. The two HSV-1-specific antisera reacted as expected; i.e., the anti-gHN serum detected only chimeric proteins containing domain I and/or II, whereas the anti-gHC serum detected proteins containing domain III and/or IV of HSV-1 gH (Fig. 3).

Due to the considerable differences between PrV and HSV-1 in the sizes of the gH proteins as well as of individual domains (Fig. 2), the apparent molecular masses of the detected proteins were also different. Whereas the predominant glycosylated form

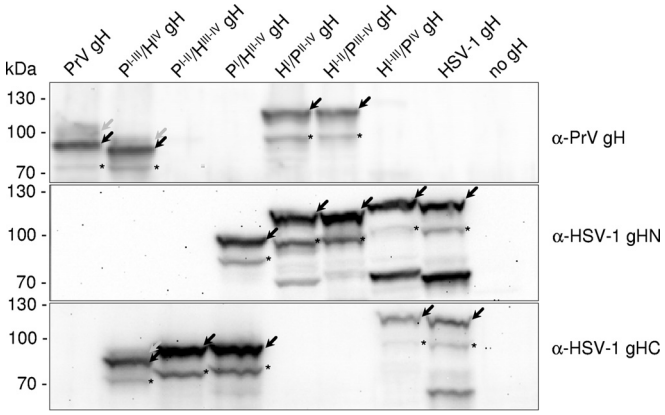


FIG 3 Western blot analyses of transfected RK13 cells. At 48 h after transfection with expression plasmids for the indicated gH variants, cell lysates were prepared and separated by SDS-PAGE. Blots were incubated with monospecific rabbit antiserum against PrV gH or the N-terminal and C-terminal parts of HSV-1 gH. The probable primary translation products (asterisks), as well as immature (black arrows) and mature (gray arrows) glycosylated forms of native or chimeric gH, are labeled. Molecular masses of marker proteins are indicated on the left.

of PrV gH (686 aa; calculated mass of the primary translation product, 72 kDa) migrated at approximately 80 kDa, the HSV-1 protein (838 aa, 90.5 kDa) exhibited an apparent mass of 120 kDa, and most chimeric proteins were in between (Fig. 3, black arrows). The apparent mass of only the chimeric protein containing domains I to III of PrV gH and domain IV of HSV-1 gH ($P^{I–III}/H^{IV}$ gH; 685 aa, 72 kDa) was reduced compared to that of PrV gH although the calculated sizes of the two proteins were almost identical. In contrast, the "reciprocal" protein, $H^{I–III}/P^{IV}$ gH (839 aa, 90.5 kDa), appeared larger than the authentic HSV-1 protein. Whereas various amounts of nonglycosylated gH precursors (Fig. 3, asterisks) and probable degradation products were found in all transfected cell lysates, presumably mature, fully glycosylated >90-kDa forms were detected of only the authentic PrV gH and the $P^{I–III}/H^{IV}$ chimeric protein (Fig. 3, gray arrows). Thus, in line with previous results (27, 37, 38), presence of the complex partner gL proved to be dispensable for maturation of PrV gH. In contrast, HSV-1 gL has been reported to be required for transport and final processing of gH (52), and an additional larger form of wild-type HSV-1 gH became visible after cotransfection of cells with gH and gL expression plasmids. However, coexpression of HSV-1 or PrV gL did not influence processing of any of the chimeric proteins (results not shown).

Transport of the gH variants to the plasma membrane was investigated by IIF analyses of RK13 cells fixed 48 h after plasmid transfection. In the absence of gL, only PrV gH and $P^{I–III}/H^{IV}$ gH were exposed at similar, readily detectable levels at the surface of nonpermeabilized cells, whereas all other chimeras as well as HSV-1 gH were barely observed at the cell surface (Fig. 4). However, in line with our Western blot results, abundant expression of all gH constructs was found in permeabilized cells. As described previously (52), cotransfection of cells with HSV-1 gH and gL expression plasmids substantially enhanced surface localization of gH (Fig. 4). Moderately increased amounts of PrV gH and $P^{I–III}/H^{IV}$ gH were also detected at the surface of cells after coexpression of PrV gL (Fig. 4). In contrast, coexpression of neither the gL matching the binding domain at the N terminus of gH (Fig. 4) nor

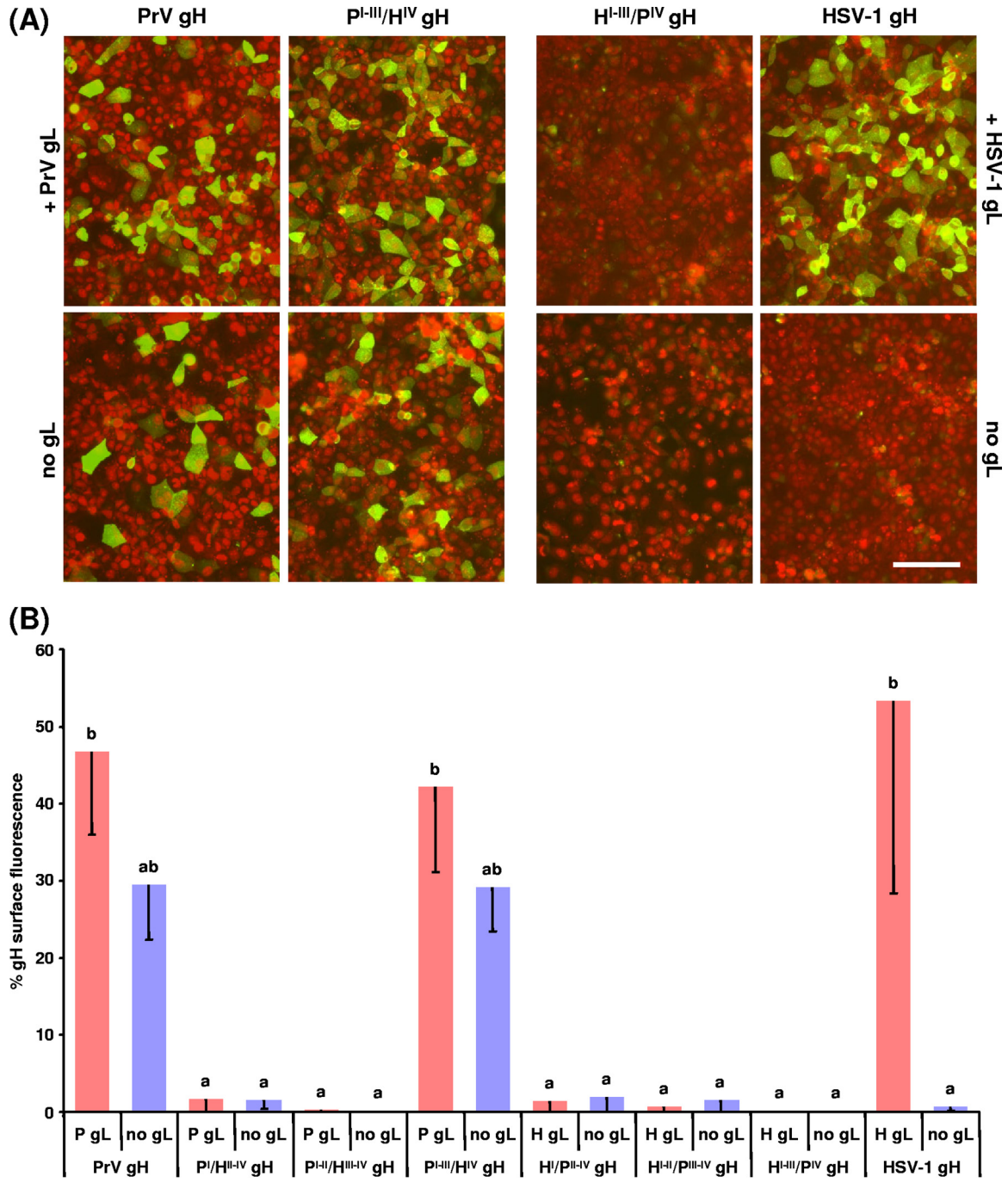


FIG 4 IIF analyses of transfected RK13 cells. At 48 h after transfection with expression plasmids for the indicated native or chimeric gH and, optionally, gL of PrV (P) or HSV-1 (H), the cells were fixed with 3% paraformaldehyde for detection of surface proteins or permeabilized with 0.5% Triton X-100 for determination of total protein. In panel A surface-located gH was detected using a monospecific antiserum against the PrV protein (left panels) or the N-terminal part of HSV-1 gH (right panels) and Alexa Fluor 488-conjugated secondary antibodies. Chromatin was counterstained with propidium iodide. Scale bar, 100 μm. In panel B gH-specific fluorescence intensities were compared between nonpermeabilized and permeabilized cells to evaluate the proportions of surface-exposed gH. Shown are the mean percentages and negative standard deviations from at least three independent experiments. Statistically significant ($P < 0.05$) differences to surface detection of PrV gH in the presence (a) and HSV-1 gH in the absence (b) of matching gL are indicated.

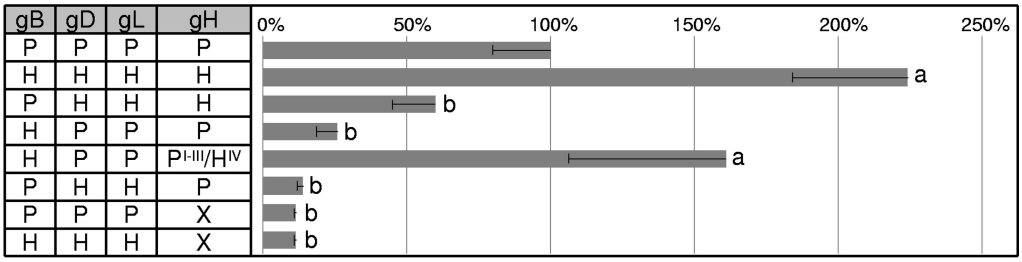
heterologous gL (data not shown) supported transport of any of the other gH chimeras.

***In vitro* fusion activity of chimeric gH proteins.** It has been demonstrated in previous studies that transient coexpression of HSV-1 or PrV gB, gH/gL, and gD induces the formation of syncytia in cell culture (13, 53). In contrast to the HSV-1 system, gD of PrV and, under certain conditions, also gL are not essential for *in vitro* cell fusion (13). However, in both systems deletion of C-terminal internalization signals of gB led to increased cell surface expression and enhanced *in vitro* cell fusion activity (13, 54, 55).

(A)

gB	gD	gL	gH							
			P	P^{I}/H^{II-IV}	P^{I-II}/H^{III-IV}	P^{I-III}/H^{IV}	H^{I}/P^{II-IV}	H^{I-II}/P^{III-IV}	H^{I-III}/P^{IV}	H	X
P	P	P	+++	-	-	++	-	-	-	-	-
H	H	H	-	-	-	-	-	-	-	++++	-
P	H	H	+	-	-	-	-	-	-	+++	-
H	P	P	++	-	-	++++	-	-	-	-	-
P	P	H	-	-	-	-	+	-	-	-	-
H	H	P	-	-	-	-	-	-	-	-	-
P	H	P	-	-	-	-	-	-	-	-	-
H	P	H	-	-	-	-	-	-	-	-	-
P	P	X	++			-				-	
H	H	X	-			-				-	
P	X	P	++			-				-	
H	X	H	-			-				-	
X	P	P	-			-				-	
X	H	H	-							-	

(B)



Relative syncytia sizes (% of PrV gp set)

(C)

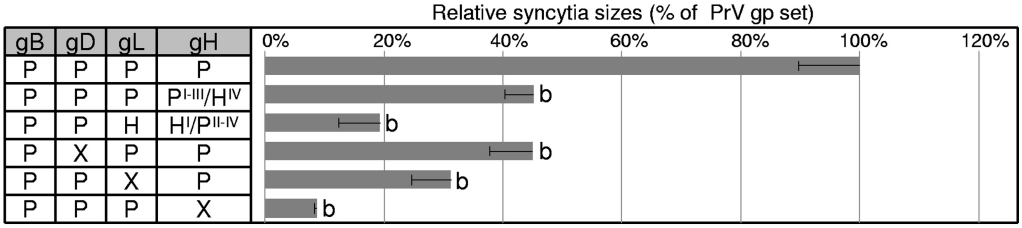


FIG 5 *In vitro* fusion assays. RK13 cells were cotransfected with different combinations of expression plasmids for GFP, C-terminally truncated gB, gD and gL of PrV (P) or HSV-1 (H), and native or chimeric gH as indicated. If expression plasmids for individual glycoproteins were omitted (X), they were replaced by the same DNA amount of the empty expression vector. After different times syncytia were detected and measured by fluorescence microscopy. (A) For all tested protein combinations, statistical significance ($P < 0.01$) of size differences to a negative control (PrV glycoprotein set without gB) was determined, and positive results were roughly quantified (+ to ++++). (B and C) Mean areas of syncytia induced by the fusogenic protein combinations or in the absence of gH were compared after 24 h (B) or 48 h (C) to the value obtained with the complete PrV set of proteins, which was set to 100%. All shown combinations formed significantly ($P < 0.001$) larger (a) or smaller (b) syncytia than the PrV positive control. Negative standard deviation is also indicated.

Therefore, C-terminally truncated forms of PrV gB (gB008 [45]) and HSV-1 gB (expressed from pcDNA-H1FgBBX) were used in this study to allow detection of even minimal *in vitro* fusion activity in the presence of the gH chimeras. The six gH chimeras, as well as the two native proteins, were tested in all possible combinations with gD, gL, and truncated gB originating from PrV and HSV-1 (Fig. 5A). In addition, single proteins were sometimes omitted. Because of considerably different fusion activities, several assays were evaluated after 24 h (examples are shown in Fig. 5B) to avoid early detachment of large syncytia, whereas most assays were incubated for 48 h (examples in Fig. 5C) after cotransfection of RK13 cells with expression plasmids. For clarity, only protein combinations yielding significant fusion activity are quantitatively displayed in the diagrams and compared to background fusion observed in the absence of gH.

In assays including gB008, gL, and gD from PrV, only wild-type PrV gH and the chimeric protein $P^{I–III}/H^{IV}$ gH induced significant cell fusion (Fig. 5C), whereas HSV-1 gH as well as all other gH chimeras showed no activity (Fig. 5A). The HSV-1 glycoproteins induced significantly more pronounced cell fusion than the homologous PrV proteins (Fig. 5B), which, however, was completely abolished after replacement of HSV-1 gH by the PrV homolog or any of the chimeras, including $P^{I–III}/H^{IV}$ gH or the reciprocal $H^{I–III}/P^{IV}$ gH (Fig. 5A).

Using PrV glycoproteins, replacement of authentic gH by $P^{I–III}/H^{IV}$ gH led to a reduction of syncytium size by approximately 50% but additional replacement of PrV gB008 by HSV-1 gBBX led to an increase to more than 150% of the size of syncytia induced by the original PrV proteins (Fig. 5B). In contrast, after single substitution of gB, only low fusion activity (approximately 25%) was retained, indicating that HSV-1 gB can only partly compensate for PrV gB in the presence of native gH (Fig. 5B). However, obviously the functional interaction of HSV-1 gB with chimeric gH $P^{I–III}/H^{IV}$ was much improved and almost comparable

to that obtained with authentic HSV-1 gH, gL, and gD proteins, which led to syncytia more than 200% of the PrV glycoprotein-induced size (Fig. 5B).

In reciprocal fusion assays including HSV-1 gD, gH, and gL, PrV gB008 could also partly compensate for HSV-1 gB, resulting in approximately 60% of the activity of the PrV glycoproteins (Fig. 5B). This was in line with previous results demonstrating that gB-deleted HSV-1 can be propagated in cell lines expressing PrV gB, but not vice versa (31).

In the HSV-1 system, substitution for or omission of gL led to a complete loss of fusion activity, whereas approximately 30% of activity was retained after omission of gL in the PrV glycoprotein set (Fig. 5C). However, replacing PrV gL with HSV-1 gL abolished this residual induction of syncytia by PrV gB008, gD, and gH (Fig. 5A). Interestingly, the gH chimera $H^{I}/P^{II–IV}$, together with gB008 and gD from PrV, exhibited an *in vitro* fusion activity of approximately 20% of the authentic PrV glycoproteins in the presence only of HSV-1 gL but not of PrV gL (Fig. 5A and C). This finding indicates that this gH chimera, although mainly containing PrV sequences, is strictly dependent on HSV-1 gL for function similar to wild-type HSV-1 gH.

In contrast to HSV-1, gD of PrV is dispensable for membrane fusion (13), and *in vitro* fusion activity of approximately 40% of that of the complete PrV glycoprotein set was retained when gD was omitted (Fig. 5C). Interestingly, activity of $P^{I–III}/H^{IV}$ gH in fusion assays was strictly dependent on the presence of PrV gD (Fig. 5A) while inclusion of HSV-1 gD abolished function of the PrV set of fusion proteins, and neither $P^{I–III}/H^{IV}$ gH nor any other gH chimeras supported cell fusion in the presence of HSV-1 gD (Fig. 5A), indicating that HSV-1 gD, like HSV-1 gL, impairs fusion induced by the core fusion proteins of PrV. Nevertheless, together with HSV-1 gD and gL, PrV gB008 and gH exhibited a minor, but statistically significant fusion activity (Fig. 5A and B).

***trans*-Complementation of gH-deleted PrV and HSV-1.** Since our *in vitro* fusion assays revealed that the gH chimeras $H^{I}/P^{II–IV}$ and $P^{I–III}/H^{IV}$ are at least partially functional, RK13-derived cell lines were prepared which stably expressed these proteins or authentic gH of PrV or HSV-1. Previous studies have shown that gH is essential for formation of infectious virus particles as well as for direct cell-to-cell spread in both PrV and HSV-1 (19–21). Therefore, it was attempted to propagate a phenotypically complemented, gH-negative PrV mutant (pPrV-ΔgHABF) (37) and a corresponding HSV-1 mutant (pHSV-1ΔgH) in the novel cell lines. Evaluation of these studies was facilitated by GFP expression of both virus mutants.

Whereas pHSV-1ΔgH exhibited almost wild-type-like virus titers and plaque sizes on cells expressing HSV-1 gH, it remained restricted to singly infected $H^{I}/P^{II–IV}$ gH- or $P^{I–III}/H^{IV}$ gH-expressing cells, and no infectious progeny virus was obtained even after 4 days at 37°C (data not shown). These results reflected the requirement for coexpression of PrV gD for *in vitro* function of both gH chimeras (see above).

The HSV-1 gL-dependent chimera $H^{I}/P^{II–IV}$ gH also failed to complement gH-deleted PrV in *trans* (results not shown). However, on RK13 cells expressing $P^{I–III}/H^{IV}$ gH, plaque formation of pPrV-ΔgHABF was restored (Fig. 6A). On RK13-$P^{I–III}/H^{IV}$ gH cells, mean plaque areas of gH-negative PrV reached approximately 21% of those of the parental virus pPrV-ΔgGG (40), whereas on cells expressing wild-type PrV gH (RK13-PgH), plaque areas of pPrV-ΔgHABF were restored to more than 50% of the wild-type size (Fig. 6B). Maximum infectious virus titers of pPrV-ΔgHABF on $P^{I–III}/H^{IV}$ gH-expressing cells were reduced only approximately 5-fold compared to titers for the same virus propagated on RK13-PgH cells or to pPrV-ΔgGG on cells expressing the chimeric gH (Fig. 6C). In contrast, neither productive replication nor spread of pPrV-ΔgHABF was observed on normal RK13 cells (Fig. 6A to C), demonstrating that $P^{I–III}/H^{IV}$ gH is functional in the viral context.

Remarkably, plaque sizes and titers of wild-type-like pPrV-ΔgGG were significantly larger on nontransfected RK13 cells than on cell lines expressing native or chimeric PrV-gH (Fig. 6A and C). Therefore, it is conceivable that the incomplete *trans*-complementation of gH-deleted PrV on these cells is also partly due to interference effects, as previously described for cells expressing HSV-1 gH (56).

DISCUSSION

The crystal structures of the gH homologs of HSV-2, EBV, and PrV revealed a conserved subdomain organization of the gH ectodomain (29, 35, 36). However, despite these structural similarities and an essential function of gH during herpesvirus entry and spread, no functional complementation between gH homologs of different virus species has been observed until now. This indicates highly specific interactions of gH with other viral proteins involved in membrane fusion. In contrast, functional *trans*-complementation between the gB homologs of several alphaherpesviruses has been described previously (31, 32, 34) and confirmed for gB proteins of PrV and HSV-1 in the present study.

Our main objective was to analyze whether individual domains of gH can be functionally exchanged between the alphaherpesviruses PrV (genus *Varicellovirus*) and HSV-1 (genus *Simplexvirus*). Therefore, we created six gH chimeras in which contiguous domains of PrV and HSV-1 gH were fused at homologous amino acid residues within structurally conserved stretches in order to avoid severe alterations, which might affect protein folding and stability.

While all tested gH chimeras were expressed and glycosylated after transient expression, final maturation and translocation to the cell surface were impaired in most cases. These defects could not be corrected by coexpression of the matching gL and, thus, were presumably due to improper folding of the chimeric proteins. Our previous studies revealed that introduction of artificial cysteine residues leading to the formation of disulfide bonds within a syntaxin-like bundle of α-helices in domain II and also between domains II and III severely affected maturation and function of PrV gH, indicating that flexibility and defined intramolecular interactions within and between these parts are critical (37). In contrast, the chimera containing PrV gH domains I to III and HSV-1 gH domain IV ($P^{I–III}/H^{IV}$ gH) showed maturation and surface expression similar to levels of wild-type PrV gH, even in the absence of gL (Fig. 3 and 4).

In *in vitro* fusion assays C-terminally truncated forms of PrV and HSV-1 gB (gB008 and gBBX, respectively) were used since they possess higher fusion activity due to deletion of conserved internalization signals from the cytoplasmic tail and/or altered membrane interactions (13, 45, 54, 55). Thus, even low cofusogenic activities of the gH variants should be detectable. However, only two out of six gH chimeras, $P^{I–III}/H^{IV}$ gH and $H^{I}/P^{II–IV}$ gH, induced membrane fusion in any of the tested protein combinations.

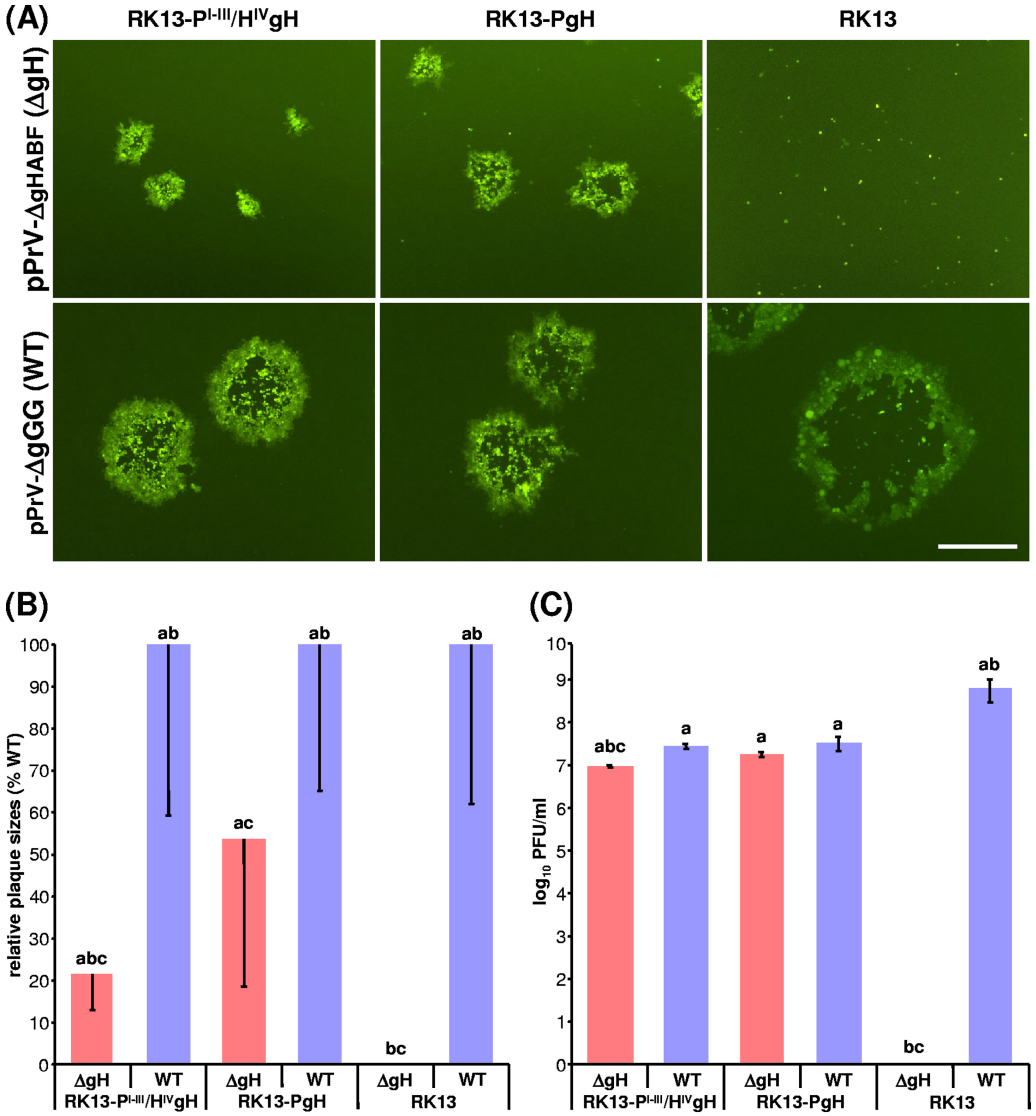


FIG 6 *trans*-Complementation of gH-deleted PrV. RK13, RK13-PgH, and RK13-P$^{I–III}$/H^{IV}gH cells were infected with phenotypically complemented gH-negative pPrV-ΔgHABF (ΔgH) or the parental gH-positive virus pPrV-ΔgGG (wild type, WT). (A) After 2 days (pPrV-ΔgGG on RK13) or 3 days (others) at 37°C under plaque assay conditions, autofluorescence of virus-expressed GFP was analyzed. Scale bar, 500 μm. (B) Mean areas of 50 plaques per virus and cell were calculated. To facilitate comparison, plaque sizes of pPrV-ΔgGG were set to 100% on each cell line. Negative standard deviations are indicated. (C) Mean progeny virus titers and standard deviations were determined 4 days after infection at an MOI of 0.01 in three independent experiments. Replication properties significantly ($P < 0.05$) different from those of pPrV-ΔgHABF on RK13 cells (a), pPrV-ΔgHABF on RK13-PgH cells (b), or wild-type PrV on the same cell line (c) are indicated.

In line with its wild-type-like maturation and cell surface localization P$^{I–III}$/H^{IV} gH, together with PrV gB008, gD, and gL, induced syncytia reaching approximately 50% of the size found with authentic PrV gH (Fig. 5). Surprisingly, activity of this gH chimera in fusion assays with HSV-1 gBBX and PrV gD and gL was substantially higher than that of even the wild-type PrV gH. This suggests that domain IV or the C terminus of gH specifically interacts with homologous gB to promote fusion although HSV-1 gBBX also exhibited moderate fusion activity together with wild-type gH, gL, and gD of PrV. Paralleling heterologous complementation of gB-deleted HSV-1 by PrV gB (31), *in vitro* fusion activity was also observed when PrV gB008 was combined with HSV-1 gH, gL, and gD. Fusion activity of P$^{I–III}$/H^{IV} gH was abolished by omission or replacement of PrV gL or gD by the homologous HSV-1 proteins (Fig. 5). Recent studies on gH chimeras between HSV-1 and SaHV-1 also revealed that domains HI and HII (corresponding to domains I to III in the PrV and EBV nomenclature) are crucial for essential species-specific interactions with gD and gL (41). In the presence of wild-type PrV gH, gD and gL are beneficial but not essential for membrane fusion (13). Thus, it remains to be elucidated whether the loss of function of P$^{I–III}$/H^{IV} gH in the absence of PrV gD and gL is due to additive defects or whether domain IV of PrV gH mediates gD and gL independence.

The chimeric protein containing domain I from HSV-1 gH and domains II to IV from PrV gH (H^{I}/P$^{II–IV}$) also exhibited limited *in vitro* fusion activity in the presence of HSV-1 gL and PrV gB008 and gD (Fig. 5). The requirement for HSV-1 gL is consistent with the structural information obtained for the EBV and HSV-2

gH/gL complexes (35, 36), in which an intimate network of contacts was observed between gH domain I and gL (Fig. 1A). The least conserved domain I varies significantly in length and sequence between different herpesviruses, and the landscape of gH-gL interactions is unique for each gH-gL pair. Authentic PrV gH did not require gL for *in vitro* function, but replacing PrV gL with the HSV-1 protein led to lower fusion activity than observed in the absence of any gL. It is unclear whether the heterologous gL impairs function of the PrV fusion machinery, e.g., by aberrant gH interactions, or whether abundant coexpression of a useless protein simply leads to reduced amounts of the active components in the same cells. Interestingly, a spontaneous recombination event during passage of a gL-negative PrV mutant led to the replacement of major parts of gH domain I by the gD ectodomain (28), and the resulting chimeric gDH exhibited enhanced and completely gL-independent *in vitro* fusion activity (13). In contrast, replacement of the gH domain I of PrV gH by the corresponding part of HSV-1 gH ($H^{I}/P^{II–IV}$ gH) abolished gL-independent as well as gL-dependent maturation. However, syncytium formation was restored to approximately 20% of the wild-type level in the presence of HSV-1 gL. Thus, although domain I of PrV gH is obviously not directly involved in triggering membrane fusion and is separated from the active part by the fence (Fig. 1), a properly formed N-terminal domain or the heterodimeric complex seems to be required for correct maturation and/or functional activation of gH.

To test whether the two *in vitro* fusion-active gH chimeras were also functional in the viral context, stably expressing cell lines were prepared and used for *trans*-complementation studies with gH-deleted PrV or HSV-1 mutants. In agreement with earlier observations, cell lines expressing wild-type gH of PrV or HSV-1 complemented only the homologous virus mutants. In a cell line expressing $H^{I}/P^{II–IV}$ gH, neither of the gH-deleted viruses exhibited any cell-to-cell spread or productive replication. This was anticipated since this gH chimera required the presence of HSV-1 gL and PrV gB and gD for *in vitro* function, which were never present together in the given settings.

Despite considerable *in vitro* fusion activity of $P^{I–III}/H^{IV}$ gH with HSV-1 gBBX, it did not complement gH-deleted HSV-1. Most likely, this was due to the absence of PrV gD and gL. However, despite decreased *in vitro* fusion activity with PrV gB008, cell lines expressing $P^{I–III}/H^{IV}$ gH supported replication and spread of gH-deleted PrV (Fig. 6). Although maximum virus titers were reduced approximately 5-fold and plaque sizes reached little more than 20% of the wild-type PrV size, these defects were less pronounced than those observed on noncomplementing cells for several PrV mutants possessing single amino acid substitutions within domain II, III, or IV of gH (37, 38, 40). Moreover, even cell lines abundantly expressing authentic PrV gH only partly *trans*-complemented gH-deleted PrV, and even wild-type PrV exhibited smaller plaques and lower titers on these transgenic cells. Thus, an excess of gH seems to affect PrV replication in general. Remarkably, a cell line which coexpresses PrV gH and gL at moderate levels supports almost wild-type-like replication of gH-negative PrV (44).

In summary, the present study confirms that gH, despite a high degree of structural and functional conservation (5, 29, 35, 36), exhibits multiple species-specific interactions with other proteins of the viral fusion machinery which impede functional substitution of the entire protein, or even of parts of it, between different herpesvirus species. However, in line with a recent publication showing that function was retained when only the membrane-proximal part of the gH ectodomain, including the membrane anchor and cytoplasmic tail, was exchanged between two closely related alphaherpesviruses of the genus *Simplexvirus* (HSV-1 and SaHV-1) (41), we demonstrate here that a similar fragment of HSV-1 gH can also substitute for the corresponding part of the phylogenetically more distant PrV, which is a member of the genus *Varicellovirus* (57). Moreover, we show that this chimeric gH not only is functional in transient *in vitro* assays but also complements gH-deleted PrV *in trans*. Interestingly, the exchangeable part of gH represents the most highly conserved domain IV, and it has been speculated that structural changes within this domain during initiation of membrane fusion may lead to movement of a conserved basic flap to unmask an underlying hydrophobic region for membrane interaction to distort the viral envelope and prime it for fusion (29). Our previous experiments supported this hypothesis for PrV gH (40), and apparently the function of domain IV is similar in other herpesviruses and partly independent from the preceding part of the protein. The present study also provides additional evidence for the relevance of the N-terminal gH domain I for species-specific interactions with gL and shows that a certain degree of gH function is retained when domain I is swapped together with the matching gL. In contrast, separate substitutions for gH domains II and III between HSV-1 and PrV always led to a complete loss of function, indicating that these mainly α-helical domains may form a highly interdependent functional unit. This was also suggested by our previous studies which showed that artificial disulfide bonds connecting domains II and III of PrV gH severely affected function in transient fusion assays, as well as during virus entry and spread (37). On the other hand, the interfaces between domains II and III are structurally different in the three characterized gH homologs (29) and might represent adaptations that allow optimal interactions with the homologous core fusion protein gB and other accessory fusion-promoting proteins like gD.

ACKNOWLEDGMENTS

The technical assistance of C. Ehrlich, B. Hammerschmidt, and A. Landmesser is greatly appreciated.

FUNDING INFORMATION

Deutsche Forschungsgemeinschaft provided funding to Thomas C. Mettenleiter under grant number Me 854/11-1.

REFERENCES

1. **Spear PG, Longnecker R.** 2003. Herpesvirus entry: an update. J Virol **77**:10179–10185. http://dx.doi.org/10.1128/JVI.77.19.10179-10185.2003.
2. **Connolly SA, Jackson JO, Jardetzky TS, Longnecker R.** 2011. Fusing structure and function: a structural view of the herpesvirus entry machinery. Nat Rev Microbiol **9**:369–381. http://dx.doi.org/10.1038/nrmicro2548.
3. **Roizman B, Knipe DM, Whitney RJ.** 2007. Herpes simplex viruses, p 2501–2601. *In* Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (ed), Fields virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
4. **Atanasiu D, Saw WT, Gallagher JR, Hannah BP, Matsuda Z, Whitbeck JC, Cohen GH, Eisenberg RJ.** 2013. Dual split protein-based fusion assay reveals that mutations to herpes simplex virus (HSV) glycoprotein gB alter the kinetics of cell-cell fusion induced by HSV entry glycoproteins. J Virol **87**:11332–11345. http://dx.doi.org/10.1128/JVI.01700-13.
5. **Eisenberg RJ, Atanasiu D, Cairns TM, Gallagher JR, Krummenacher C, Cohen GH.** 2012. Herpes virus fusion and entry: a story with many characters. Viruses **4**:800–832. http://dx.doi.org/10.3390/v4050800.
6. **Vanarsdall AL, Ryckman BJ, Chase MC, Johnson DC.** 2008. Human cytomegalovirus glycoproteins gB and gH/gL mediate epithelial cell-cell

fusion when expressed either in *cis* or in *trans*. J Virol **82:**11837–11850. http://dx.doi.org/10.1128/JVI.01623-08.
7. **Avitabile E, Forghieri C, Campadelli-Fiume G.** 2009. Cross talk among the glycoproteins involved in herpes simplex virus entry and fusion: the interaction between gB and gH/gL does not necessarily require gD. J Virol **83:**10752–10760. http://dx.doi.org/10.1128/JVI.01287-09.
8. **Gianni T, Amasio M, Campadelli-Fiume G.** 2009. Herpes simplex virus gD forms distinct complexes with fusion executors gB and gH/gL in part through the C-terminal profusion domain. J Biol Chem **284:**17370–17382. http://dx.doi.org/10.1074/jbc.M109.005728.
9. **Mettenleiter TC, Zsak L, Zuckermann F, Sugg N, Kern H, Ben-Porat T.** 1990. Interaction of glycoprotein gIII with a cellular heparinlike substance mediates adsorption of pseudorabies virus. J Virol **64:**278–286.
10. **Herold BC, WuDunn D, Soltys N, Spear PG.** 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J Virol **65:**1090–1098.
11. **Karger A, Saalmüller A, Tufaro F, Banfield BW, Mettenleiter TC.** 1995. Cell surface proteoglycans are not essential for infection by pseudorabies virus. J Virol **69:**3482–3489.
12. **Geraghty RJ, Krummenacher C, Cohen GH, Eisenberg RJ, Spear PG.** 1998. Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. Science **280:**1618–1620. http://dx.doi.org/10.1126/science.280.5369.1618.
13. **Klupp BG, Nixdorf R, Mettenleiter TC.** 2000. Pseudorabies virus glycoprotein M inhibits membrane fusion. J Virol **74:**6760–6768. http://dx.doi.org/10.1128/JVI.74.15.6760-6768.2000.
14. **Rauh I, Mettenleiter TC.** 1991. Pseudorabies virus glycoproteins gII and gp50 are essential for virus penetration. J Virol **65:**5348–5356.
15. **Peeters B, de Wind N, Hooisma M, Wagenaar F, Gielkens A, Moormann R.** 1992. Pseudorabies virus envelope glycoproteins gp50 and gII are essential for virus penetration, but only gII is involved in membrane fusion. J Virol **66:**894–905.
16. **Schmidt J, Klupp BG, Karger A, Mettenleiter TC.** 1997. Adaptability in herpesviruses: glycoprotein D-independent infectivity of pseudorabies virus. J Virol **71:**17–24.
17. **Karger A, Schmidt J, Mettenleiter TC.** 1998. Infectivity of a pseudorabies virus mutant lacking attachment glycoproteins C and D. J Virol **72:**7341–7348.
18. **Davison AJ, Scott JE.** 1986. The complete DNA sequence of varicella-zoster virus. J Gen Virol **67:**1759–1816. http://dx.doi.org/10.1099/0022-1317-67-9-1759.
19. **Babic N, Klupp BG, Makoschey B, Karger A, Flamand A, Mettenleiter TC.** 1996. Glycoprotein gH of pseudorabies virus is essential for penetration and propagation in cell culture and in the nervous system of mice. J Gen Virol **77:**2277–2285. http://dx.doi.org/10.1099/0022-1317-77-9-2277.
20. **Peeters B, de Wind N, Broer R, Gielkens A, Moormann R.** 1992. Glycoprotein H of pseudorabies virus is essential for entry and cell-to-cell spread of the virus. J Virol **66:**3888–3892.
21. **Desai P, Schaffer P, Minson A.** 1988. Excretion of non-infectious virus particles lacking glycoprotein H by a temperature-sensitive mutant of herpes simplex virus type 1: evidence that gH is essential for virion infectivity. J Gen Virol **69:**1147–1156. http://dx.doi.org/10.1099/0022-1317-69-6-1147.
22. **Chesnokova LS, Nishimura SL, Hutt-Fletcher LM.** 2009. Fusion of epithelial cells by Epstein-Barr virus proteins is triggered by binding of viral glycoproteins gHgL to integrins αvβ6 or αvβ8. Proc Natl Acad Sci U S A **106:**20464–20469. http://dx.doi.org/10.1073/pnas.0907508106.
23. **Wang X, Huang DY, Huong S-M, Huang E-S.** 2005. Integrin αvβ3 is a coreceptor for human cytomegalovirus. Nat Med **11:**515–521. http://dx.doi.org/10.1038/nm1236.
24. **Gianni T, Cerretani A, Dubois R, Salvioli S, Blystone SS, Rey F, Campadelli-Fiume G.** 2010. Herpes simplex virus glycoproteins H/L bind to cells independently of αVβ3 integrin and inhibit virus entry, and their constitutive expression restricts infection. J Virol **84:**4013–4025. http://dx.doi.org/10.1128/JVI.02502-09.
25. **Parry C, Bell S, Minson T, Browne H.** 2005. Herpes simplex virus type 1 glycoprotein H binds to αvβ3 integrins. J Gen Virol **86:**7–10. http://dx.doi.org/10.1099/vir.0.80567-0.
26. **Roop C, Hutchinson L, Johnson DC.** 1993. A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H. J Virol **67:**2285–2297.
27. **Klupp BG, Fuchs W, Weiland E, Mettenleiter TC.** 1997. Pseudorabies virus glycoprotein L is necessary for virus infectivity but dispensable for virion localization of glycoprotein H. J Virol **71:**7687–7695.
28. **Klupp BG, Mettenleiter TC.** 1999. Glycoprotein gL-independent infectivity of pseudorabies virus is mediated by a gD-gH fusion protein. J Virol **73:**3014–3022.
29. **Backovic M, DuBois RM, Cockburn JJ, Sharff AJ, Vaney MC, Granzow H, Klupp BG, Bricogne G, Mettenleiter TC, Rey FA.** 2010. Structure of a core fragment of glycoprotein H from pseudorabies virus in complex with antibody. Proc Natl Acad Sci U S A **107:**22635–22640. http://dx.doi.org/10.1073/pnas.1011507107.
30. **Cairns TM, Milne RS, Ponce-de-Leon M, Tobin DK, Cohen GH, Eisenberg RJ.** 2003. Structure-function analysis of herpes simplex virus type 1 gD and gH-gL: clues from gDgH chimeras. J Virol **77:**6731–6742. http://dx.doi.org/10.1128/JVI.77.12.6731-6742.2003.
31. **Mettenleiter TC, Spear PG.** 1994. Glycoprotein gB (gII) of pseudorabies virus can functionally substitute for glycoprotein gB in herpes simplex virus type 1. J Virol **68:**500–504.
32. **Eberle R, Tanamachi B, Black D, Blewett EL, Ali M, Openshaw H, Cantin EM.** 1997. Genetic and functional complementation of the HSV1 UL27 gene and gB glycoprotein by simian alpha-herpesvirus homologs. Arch Virol **142:**721–736. http://dx.doi.org/10.1007/s007050050114.
33. **Fan Q, Longnecker R, Connolly SA.** 2014. Substitution of herpes simplex virus 1 entry glycoproteins with those of saimiriine herpesvirus 1 reveals a gD-gH/gL functional interaction and a region within the gD profusion domain that is critical for fusion. J Virol **88:**6470–6482. http://dx.doi.org/10.1128/JVI.00465-14.
34. **Kopp A, Mettenleiter TC.** 1992. Stable rescue of a glycoprotein gII deletion mutant of pseudorabies virus by glycoprotein gI of bovine herpesvirus 1. J Virol **66:**2754–2762.
35. **Chowdary TK, Cairns TM, Atanasiu D, Cohen GH, Eisenberg RJ, Heldwein EE.** 2010. Crystal structure of the conserved herpesvirus fusion regulator complex gH-gL. Nat Struct Mol Biol **17:**882–888. http://dx.doi.org/10.1038/nsmb.1837.
36. **Matsuura H, Kirschner AN, Longnecker R, Jardetzky TS.** 2010. Crystal structure of the Epstein-Barr virus (EBV) glycoprotein H/glycoprotein L (gH/gL) complex. Proc Natl Acad Sci U S A **107:**22641–22646. http://dx.doi.org/10.1073/pnas.1011806108.
37. **Böhm SW, Eckroth E, Backovic M, Klupp BG, Rey FA, Mettenleiter TC, Fuchs W.** 2015. Structure-based functional analyses of domains II and III of pseudorabies virus glycoprotein H. J Virol **89:**1364–1376. http://dx.doi.org/10.1128/JVI.02765-14.
38. **Böhm SW, Backovic M, Klupp BG, Rey FA, Mettenleiter TC, Fuchs W.** 2015. The replication defect of pseudorabies virus induced by a targeted α-helix disruption in the syntaxin-like bundle of glycoprotein H (V275P) is partly corrected by an adjacent compensatory mutation (V271A). J Gen Virol **96:**2349–2354. http://dx.doi.org/10.1099/vir.0.000161.
39. **Schröter C, Klupp BG, Fuchs W, Gerhard M, Backovic M, Rey FA, Mettenleiter TC.** 2014. The highly conserved proline at position 438 in pseudorabies virus gH is important for regulation of membrane fusion. J Virol **88:**13064–13072. http://dx.doi.org/10.1128/JVI.01204-14.
40. **Fuchs W, Backovic M, Klupp BG, Rey FA, Mettenleiter TC.** 2012. Structure-based mutational analysis of the highly conserved domain IV of glycoprotein H of pseudorabies virus. J Virol **86:**8002–8013. http://dx.doi.org/10.1128/JVI.00690-12.
41. **Fan Q, Longnecker R, Connolly SA.** 2015. A functional interaction between herpes simplex virus 1 glycoproteins gH/gL domains I-II and gD is defined using α-herpesvirus gH and gL chimeras. J Virol **89:**7159–7169. http://dx.doi.org/10.1128/JVI.00740-15.
42. **Leege T, Fuchs W, Granzow H, Kopp M, Klupp BG, Mettenleiter TC.** 2009. Effects of simultaneous deletion of pUL11 and glycoprotein M on virion maturation of herpes simplex virus type 1. J Virol **83:**896–907. http://dx.doi.org/10.1128/JVI.01842-08.
43. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640–6645. http://dx.doi.org/10.1073/pnas.120163297.
44. **Klupp B, Altenschmidt J, Granzow H, Fuchs W, Mettenleiter TC.** 2008. Glycoproteins required for entry are not necessary for egress of pseudorabies virus. J Virol **82:**6299–6309. http://dx.doi.org/10.1128/JVI.00386-08.
45. **Nixdorf R, Klupp BG, Karger A, Mettenleiter TC.** 2000. Effects of truncation of the carboxy terminus of pseudorabies virus glycoprotein B on infectivity. J Virol **74:**7137–7145. http://dx.doi.org/10.1128/JVI.74.15.7137-7145.2000.
46. **Gerdts V, Jöns A, Mettenleiter TC.** 1999. Potency of an experimental

DNA vaccine against Aujeszky's disease in pigs. Vet Microbiol **66:**1–13. http://dx.doi.org/10.1016/S0378-1135(98)00300-9.
47. **Fuchs W, Klupp BG, Granzow H, Osterrieder N, Mettenleiter TC.** 2002. The interacting UL31 and UL34 gene products of pseudorabies virus are involved in egress from the host-cell nucleus and represent components of primary enveloped but not mature virions. J Virol **76:**364–378. http://dx.doi.org/10.1128/JVI.76.1.364-378.2002.
48. **Pavlova SP, Veits J, Keil GM, Mettenleiter TC, Fuchs W.** 2009. Protection of chickens against H5N1 highly pathogenic avian influenza virus infection by live vaccination with infectious laryngotracheitis virus recombinants expressing H5 hemagglutinin and N1 neuraminidase. Vaccine **27:**773–785. http://dx.doi.org/10.1016/j.vaccine.2008.11.033.
49. **Mettenleiter TC.** 1989. Glycoprotein gIII deletion mutants of pseudorabies virus are impaired in virus entry. Virology **171:**623–625. http://dx.doi.org/10.1016/0042-6822(89)90635-1.
50. **Klupp BG, Mettenleiter TC.** 1991. Sequence and expression of the glycoprotein gH gene of pseudorabies virus. Virology **182:**732–741. http://dx.doi.org/10.1016/0042-6822(91)90614-H.
51. **Szpara ML, Parsons L, Enquist LW.** 2010. Sequence variability in clinical and laboratory isolates of herpes simplex virus 1 reveals new mutations. J Virol **84:**5303–5313. http://dx.doi.org/10.1128/JVI.00312-10.
52. **Hutchinson L, Browne H, Wargent V, Davis-Poynter N, Primorac S, Goldsmith K, Minson A, Johnson D.** 1992. A novel herpes simplex virus glycoprotein, gL, forms a complex with glycoprotein H (gH) and affects normal folding and surface expression of gH. J Virol **66:**2240–2250.
53. **Turner A, Bruun B, Minson T, Browne H.** 1998. Glycoproteins gB, gD, and gHgL of herpes simplex virus type 1 are necessary and sufficient to mediate membrane fusion in a Cos cell transfection system. J Virol **72:**873–875.
54. **Chowdary TK, Heldwein EE.** 2010. Syncytial phenotype of C-terminally truncated herpes simplex virus type 1 gB is associated with diminished membrane interactions. J Virol **84:**4923–4935. http://dx.doi.org/10.1128/JVI.00206-10.
55. **Beitia Ortiz de Zarate I, Kaelin K, Rozenberg F.** 2004. Effects of mutations in the cytoplasmic domain of herpes simplex virus type 1 glycoprotein B on intracellular transport and infectivity. J Virol **78:**1540–1551. http://dx.doi.org/10.1128/JVI.78.3.1540-1551.2004.
56. **Scanlan PM, Tiwari V, Bommireddy S, Shukla D.** 2003. Cellular expression of gH confers resistance to herpes simplex virus type-1 entry. Virology **312:**14–24. http://dx.doi.org/10.1016/S0042-6822(03)00176-4.
57. **King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (ed).** 2011. Virus taxonomy: classification and nomenclature of viruses. Ninth report of the International Committee on Taxonomy of Viruses. Academic Press, London, United Kingdom.