

Hydrophobic α -Helices 1 and 2 of Herpes Simplex Virus gH Interact with Lipids, and Their Mimetic Peptides Enhance Virus Infection and Fusion

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Entry of herpes simplex virus into cells occurs by fusion and requires four glycoproteins. gD serves as the receptor binding glycoprotein. Of the remaining glycoproteins, gH carries structural and functional elements typical of class 1 fusion glycoproteins, in particular α -helix 1 (α -H1), with properties of a candidate fusion peptide, and two heptad repeats. Here, we characterized α -H2 and compared it to α -H1. α -H2 (amino acids 513 to 531) is of lower hydrophobicity than α -H1. Its deletion or mutation decreased virus infection and cell fusion. Its replacement with heterologous fusion peptides did not rescue infection and cell fusion beyond the levels exhibited by the α -H2-deleted gH. This contrasts with α -H1, which cannot be deleted and can be functionally replaced with heterologous fusion peptides (T. Gianni et al., *J. Virol.* 79:2931–2940, 2005). Synthetic peptides mimicking α -H1 and α -H2 induced fusion of nude lipid vesicles. Importantly, they increased infection of herpes simplex virus, pseudorabies virus, bovine herpesvirus 1, and vesicular stomatitis virus. The α -H1 mimetic peptide was more effective than the α -H2 peptide. Consistent with the findings that gH carries membrane-interacting segments, a soluble form of gH, but not of gD or gB, partitioned with lipid vesicles. Current findings highlight that α -H2 is an important albeit nonessential region for virus entry and fusion. α -H1 and α -H2 share the ability to target the membrane lipids; they contribute to virus entry and fusion, possibly by destabilizing the membranes. However, α -H2 differs from α -H1 in that it is of lower hydrophobicity and cannot be replaced with heterologous fusion peptides.

Viruses that enter cells by fusion with cell membranes carry fusion glycoproteins with distinctive features (13, 31, 34). A key element is the fusion peptide, a hydrophobic segment located N terminally in cleaved glycoproteins or internally in uncleaved glycoproteins. The sequential steps that lead to fusion execution include extensive refolding of the fusion glycoprotein, the exposure and relocation of the fusion peptide, and its insertion in the target cell membrane; this generates an intermediate simultaneously anchored to the virion envelope and the cell membrane. The insertion of the fusion peptide in cell membranes is thought to facilitate fusion through a destabilizing effect on the lipids. In class 1 fusion glycoproteins, a key step in the refolding process is the formation of a coiled coil between trimers of heptad repeat 1 (HR-1) and HR-2—also called a six-helix bundle, or hairpin. This relocates the fusion peptide and the transmembrane domain to the same end of the coiled coil, such that the viral and the cellular membranes are brought in close juxtaposition and fusion starts.

Recently, it has become evident that the ectodomain of certain class 1 fusion glycoproteins, including human immunodeficiency virus (HIV) gp41 and glycoproteins from some paramyxoviruses, exhibits additional elements that add to their complexities. HIV gp41 carries a membrane-proximal pre-

transmembrane domain, whereas the Sendai virus and other paramyxovirus fusion glycoproteins carry a hydrophobic segment downstream of HR-1, named internal fusion peptide, or HR-3 (14, 15, 24, 41, 52). The properties of these elements have been investigated mainly by means of mimetic peptides which, in most cases, induce fusion of model lipid membranes (16, 24, 52).

The molecular mechanism of entry of herpesviruses into the cell differs from that of well-known viruses, in that it requires a multicomponent fusion system. Herpes simplex virus (HSV), the paradigm of viruses encoding a multicomponent fusion machinery, enters target cells by fusion of the envelope with either the plasma membrane or the endocytic vesicle. Four viral glycoproteins—gD, gB, gH, and gL—are absolutely required for virus entry and cell-cell fusion (6, 9, 18, 35, 47, 51, 53). The role of gD is twofold. It serves as the receptor-binding glycoprotein able to interact with two alternative receptors: nectin1 and herpesvirus entry mediator (11, 23, 40). It also serves as the trigger of fusion. The current model envisions that gD action is exerted through conformational changes (10). The unliganded gD adopts a closed conformation where the ectodomain C terminus, which carries the profusion domain, folds back towards the N terminus. At receptor binding, gD adopts an opened conformation, where the profusion domain is displaced from its binding site on the N terminus and the latter becomes part of the receptor binding site (20, 33).

The role of the highly conserved gH, gL, and gB, which altogether are responsible for carrying out fusion in the *Herpesviridae* family, has remained elusive until recently, when

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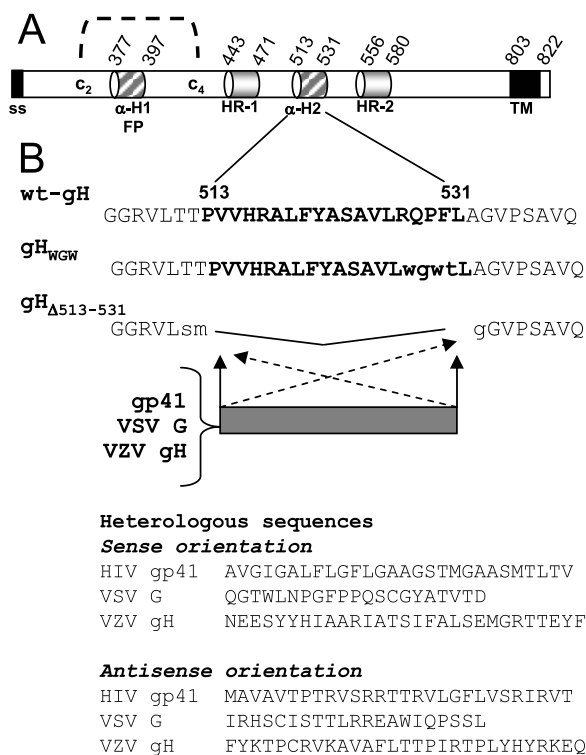


FIG. 1. (A) Linear map of HSV gH showing the amino acid positions of α -H1 (i.e., candidate fusion peptide; FP), α -H2, HR-1 and HR-2, signal sequence (SS), and the transmembrane (TM) domain. C2 and C4, cysteines 2 and 4. (B) Amino acid sequence of α -H2 and adjacent sequences in wt-gH, in gH_{WGW}, and in α -H2-deleted gH (gH _{Δ 513-531}). Heterologous sequences show the amino acid sequences of the fusion peptides of HIV gp41, VSV G, and the putative fusion peptide of VZV gH or the amino acid sequences obtained by cloning the DNA fragments in the antisense orientation. The heterologous sequences were cloned in gH _{Δ 513-531}, in the sense or antisense orientation, thus generating the plasmids gH- α H2_{VSVG}, gH- α H2_{AS-G}, gH- α H2_{gp41}, gH- α H2_{AS-gp41}, gH- α H2_{VZV}, and gH- α H2_{AS-VZV}.

structural and functional elements typical of fusion glycoproteins were identified in gH, in particular an α -helix (α -H1) and two HRs (Fig. 1) (21, 22, 25–27). The essential α -H1 is located between amino acid (aa) residues 377 and 397 and is positionally conserved in all the gH alleles examined, and in HSV-2 gH (25), α -H1 is located in an antigenic loop made of cysteines 2 and 4 (7, 8). It is highly hydrophobic, and when transplanted at the C terminus of a soluble glycoprotein (gD_{1-260t}), it renders the soluble gD membrane bound. Its replacement with heterologous fusion peptides partially rescues infection and fusion of the α -H1-deleted gH. Because of these properties, it has been proposed that α -H1 be considered the candidate fusion peptide. gH HR-1 and HR-2 are located downstream of the candidate fusion peptide and upstream of the transmembrane segment, respectively (Fig. 1) (26, 27). Their properties are as follows. Replacement of 2 aa in HR-1, predicted to abolish coiled coil formation, abolishes infection and cell-cell fusion. Replacement of 3 aa in HR-2, predicted to increase the coiled coil propensity, increases infection and cell-cell fusion. Synthetic peptides mimicking HR-1 and HR-2 inhibit virus infection and form a stable complex which exhibits a higher α -helix content than those of the two single peptides, suggesting that

HR-1 and -2 may form a six-helix bundle (22, 27). Functional HRs are present in gH of human cytomegalovirus and Epstein-Barr virus (36, 43).

In addition to α -H1, gH carries an α -H2 located between aa 513 and 531 (25) (Fig. 1). α -H2 exhibits a weaker predicted interaction with membranes than α -H1, and when transplanted at the C terminus of gD_{1-260t}, it confers to gD_{1-260t} only partial ability to become membrane bound. Synthetic peptides that include a portion of α -H1 or the entire α -H2 interact with lipid membranes, induce their fusion, and also adopt an α -helical conformation in buffers of decreasing polarity (21). Inasmuch as flexibility and ability to adopt different secondary structures are key properties of fusion peptides (34), these findings further support the contention that α -H1 may serve as the fusion peptide and raised the question of the functional significance of α -H2 in virus infection and cell fusion. An additional sequence of interest, located at gH aa 626 to 644, was predicted by its tendency to partition at the membrane interface. A peptide mimicking this sequence induces fusion of nude lipid vesicles with high efficiency and adopts an α -helical conformation in buffers of decreasing polarity (21). This sequence remains to be characterized in the context of gH and with respect to virus infection and fusion.

gL is a soluble glycoprotein that forms a heterodimer with gH (29) and is required in order for gH to adopt its correct conformation and to be transported from endoplasmic reticulum to the plasma membrane. It carries neutralizing epitopes (54). The significance of gB in fusion execution is unclear at the moment. Numerous lines of evidence indicate that gB is absolutely required for HSV to enter cells and for fusion to occur (6, 53). gB plays a role in virus attachment to the cell surface heparan sulfate (28). It also binds cells devoid of heparan sulfate in a saturable manner. A soluble form inhibits infection, suggesting that it may interact with a receptor (4).

The objective of this study was to characterize α -H2 and compare its ability to interact with membranes with that of α -H1. Deletion, mutational analysis, and substitution of α -H2 indicate that this is an important but nonessential domain. Synthetic peptides mimicking α -H1 and α -H2 not only induce fusion of nude lipid vesicles but also enhance virus entry in a species-unspecific manner as well as cell-cell fusion.

MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney (BHK), COS, Madin-Darby bovine kidney, F6, and J-nectin 1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% to 10% fetal calf serum. F6 cells are a stably transformed Vero cell line that expresses HSV-1 gH under the control of the HSV-1 gD promoter (18). The J11.1-2 cell line (J cells), a derivative of BHKtk⁻ cells with high resistance to HSV infection, and their derivative line, J-nectin 1, stably expressing human nectin1, were described previously (12). In the gH deletion mutant (Δ gH HSV) SCgHZ, the gH gene was replaced with a *lacZ* gene. SCgHZ was grown and titrated in the complementing F6 cells (18). R8102, a recombinant HSV-1 strain expressing *lacZ* under control of the α 27 promoter, pseudorabies virus (PrV), and the bovine herpesvirus type 1 (BoHV-1) recombinants carrying *LacZ* were described previously (3, 19, 38). The vesicular stomatitis virus (VSV), New Jersey serotype, was obtained from the American Type Culture Collection.

Plasmids. Expression plasmids for HSV-1 gD, gB, gH, and gL were described previously (2). EGFR2 Δ (named Erb-2) carries the extracellular domain and transmembrane sequences of rat HER-2/neu (nucleotides 25 to 2096) (GenBank accession no. NM_017003) and has had the tyrosine kinase domain deleted (48). Plasmid pCAGT7 contains the T7 RNA polymerase gene under control of the CAG promoter, and the pT7EMCluc plasmid expresses the firefly luciferase

under the T7 promoter (42, 46). Plasmid pcDNA 3.1(-) Myc-His/Lac vector (Invitrogen, Italy) constitutively expresses β -galactosidase (β -Gal).

Constructs. gH_{WGW}, carrying the RQPF527-530WGWT substitutions, was derived by site-directed mutagenesis with oligonucleotide 5'-CGCCTCGGCTG TCCTCTGGGGGTGGTACCTGGCTGGCGTCCCTCGGCG-3'. For the deletion of the sequence from aa 513 to 531, a SphI site was first introduced at aa 512 in pMTS-gH, by means of the oligonucleotide 5'-GGGGGCCGCGTGC TGAGCATGCCGTACCCACCGGGCGCTATTTAC-3', thus generating gH_{Δ512-SphI}. A second SphI site was introduced at aa 532 by means of the oligonucleotide 5'-GGCTGCTCCTCCGGCGCCGCGCATGCTGGCGTCCCT CGGCGG-3'. To generate gH_{Δ513-531}, the SphI fragment was then collapsed. The mutagenesis inserted the following substitutions: TT511-512SM and A532G. The gH- α H2_{VSVG}, gH- α H2_{AS-G}, gH- α H2_{gp41}, gH- α H2_{AS-gp41}, gH- α H2_{VZV}, gH- α H2_{AS-VZV} plasmids (carrying the sequences encoding the fusion peptide of HIV gp41, VSV, or putative fusion peptide of varicella zoster virus [VZV] gH in a sense or antisense [AS] orientation) were generated by ligation of the appropriate sequences in gH_{Δ513-531}, as previously detailed (25).

Fusion assay. The luciferase-based cell-cell fusion assay was performed in COS cells as detailed previously (39, 46), using the luciferase assay system from Promega (Florence, Italy). The total amount of transfected plasmid DNA was made equal by addition of Erb-2 plasmid DNA. All samples were run three times and in triplicates. Subconfluent cultures of BHK cells, grown on glass coverslips in 24-well plates, were transfected with DNA mixtures that contained the expression plasmids for gD, gH, gL, and gB, plus pcDNA 3.1(-) Myc-His/Lac vector (Invitrogen, Milan, Italy), for constitutive expression of β -Gal (80 ng of each plasmid). After incubation at 37° for 24 h in the presence or absence of the indicated synthetic peptides (pep.gH- α H1₂₁, pep.gH- α H2₁₉, and pep.gD₂₆₅₋₂₈₉), cells were fixed, and syncytia were detected by staining with 5-bromo-4-chloro-3-indolyl- β -galactopyranoside (X-Gal), as described previously (1).

Infectivity complementation assay. The complementation assay was performed as detailed in reference 10. Briefly, cells in T₂₅ flasks were transfected with the appropriate gH plasmid. The total amount of plasmid DNA transfected per flask was made equal by addition of Erb-2 plasmid DNA. Four hours later, cells were infected with a gH^{+/+} stock of SCgHZ (7 PFU/cell). Unpenetrated virions were inactivated by washing two times with phosphate-buffered saline (PBS), followed by a 1-min rinse with 40 mM sodium citrate, 10 mM KCl, 135 mM NaCl, pH 3. The monolayers were rinsed twice with PBS and overlaid with medium containing 1% fetal calf serum. Cells were incubated overnight at 37°C, and the progeny virus was titrated in F6 cells.

CELISA and IFA. Cell enzyme-linked immunosorbent assay (CELISA) was performed as described previously (25, 46). For indirect immunofluorescence assay (IFA), COS cells were grown on glass coverslips and transfected with the indicated plasmids by means of Polyfect (QIAGEN, Florence, Italy). After 24 h, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature, followed, when requested, by 0.1% Triton X-100 in PBS. Samples were incubated with the following monoclonal antibodies (MAbs): 52S and 53S to gH (44, 50) and anti-mouse immunoglobulin G (IgG)-fluorescein isothiocyanate (FITC)-conjugated antibody (Jackson ImmunoResearch). Samples were observed with a Zeiss microscope, and micrographs were taken with a Kodak DC290 digital camera.

Synthetic peptides. Lyophilized peptides pep.gH- α H1₂₁, pep.gH- α H2₁₉, pep.gH-HR1₂₅, pep.gH-HR1₃₅, pep.gH-HR2₂₅, and pep.gD₂₆₅₋₂₈₉, synthesized by Primm, San Raffaele Biomedical Science Park, Milan, Italy, and by the Peptide Synthesis Facility, University of Wisconsin Biotechnology Center, were dissolved in DMEM without serum at a concentration of 3 mM. The solutions were adjusted to neutral pH by addition of 1 M Tris HCl, pH 10.

Effect of synthetic peptides on virus infection and cell-cell fusion. The experiments were performed in 96-well plates with extracellular virions of R8102, PrV LacZ, or BoHV-1, at an input multiplicity of infection of 1 PFU/cell. Cells were incubated with increasing concentrations of the peptides and the viral inoculum for 90 min at 37°C. After removal of the inoculum and rinsing of the cells with DMEM containing 1% fetal calf serum, infected cells were incubated for 16 h. Infectivity was measured as β -Gal activity using *o*-nitrophenyl- β -D-galactopyranoside (ONPG) as a substrate (40). The absorbance at 405 nm was read in a Bio-Rad microplate reader. Serial 10-fold dilutions of VSV were allowed to adsorb to Vero cells in the absence or presence of the indicated amounts of peptides. After removal of the inoculum and rinsing of the cells with DMEM containing 1% fetal calf serum, infected cells were incubated for 16 h in the absence of peptides in medium containing methylcellulose. Plaques were scored 24 h later.

Liposome preparation. Asolectin (L- α -phosphatidylcholine from soybean) (Sigma-Aldrich, Milan, Italy) was dissolved in 3:1 chloroform-methanol and dried under a nitrogen stream to a thin film. The film was hydrated in 140 mM

NaCl-10 mM Tris HCl, pH 8.0, vigorously vortexed for 7 min, and subjected to 19 cycles of extrusion through two 200-nm-pore-size polycarbonate membranes, with a Liposofast syringe-type extruder (Avestin, Ottawa, Canada).

Fluorescence measurements. Fluorescence measurements were performed at 25°C with a Jasco (FP-777) spectrofluorimeter. An aliquot of liposomes was loaded with 3 μ M pyrene (Fluka, Milan, Italy) and mixed with nude lipid vesicles. The emission spectra excited at 340 nm was recorded in the 300- to 550-nm range. The fluorescence intensities of the monomer (I_M) (390 nm) and excimer (I_E) (470 nm) emission peaks were measured. The extent of fusion was expressed as the excimer/monomer fluorescence intensity (I_E/I_M) ratio (30). The peptide-induced fusion of lipid vesicles was performed by adding nude liposomes to pyrene-loaded liposomes (1:2 [wt/wt]) in the presence or absence of peptides (30 μ M for single peptides or 15 μ M each for mixtures of two peptides). To quantify the extent of fusion, a replicate lipid vesicle fusion assay was performed in the presence of 8×10^7 PFU of partially purified extracellular HSV-1(F) virions. Fusion induced by peptides was expressed as percentage relative to the fusion induced by virions. Relative to liposomes solubilized with Triton X-100, fusion induced by virions was in the range of 10 to 30%.

Sedimentation analysis of gH_t, gL preincubated with liposomes. The recombinant soluble forms of gH_t, gL (gH_{792t}, gL), gB_t (gB_{730Hist}), or gD_t (gD_{285t}) (5, 32, 45) (generous gift of G. H. Cohen and R. Eisenberg, University of Pennsylvania) were produced by means of baculoviruses, purified by affinity chromatography (49). The negative control consisted of IgG (Sigma-Aldrich, Milan, Italy). Aliquots of 1.5 μ g in 15 μ l were mixed with 200 μ l liposomes (10 mg/ml) in a ratio of 1 mg of protein to 900 to 1,600 μ M lipids. The mixtures were incubated for 5 to 10 min at 37°C and layered onto a discontinuous sucrose gradient containing 1 ml of 50% (wt/vol) sucrose, 1.5 ml of 25% sucrose, 1 ml of 15% sucrose, and 0.5 ml of 5% sucrose. Centrifugation was carried out for 22 h at 22,000 rpm at 4°C in a Beckman SW50.1 centrifuge. Four fractions, corresponding to each sucrose concentration, were collected. The amount of lipids in each fraction was measured by a quantitative determination of phosphorus, as described previously (37). The fractions were concentrated to 50 μ l by means of Amicon Y10 filters and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were identified by enhanced chemiluminescence with MAb H12 to gH, MAb VIII-62 to gL, MAb H1817 to gB, MAb H170 to gD, and anti-mouse IgG peroxidase.

RESULTS

Deletion and mutational analyses of α -H2. Figure 1A shows the position of α -H2 in the linear map of gH and its sequence. In order to investigate the properties of α -H2, the sequence was either mutagenized or deleted in an expression vector that contained gH under the immediate-early cytomegalovirus promoter. The substitutions introduced in α -H2 were aimed at decreasing the propensity to form the helix. The resulting mutant was named gH_{WGW} (Fig. 1B). In order to delete α -H2, two SphI restriction sites were inserted at aa 512 and 532 of the gH coding sequence (Fig. 1B). The SphI fragment was then collapsed, and the resulting plasmid was named gH_{Δ513-531} (Fig. 1B). The two gH mutants were tested in three functional assays: i.e., (i) proper folding and ability to traffic to the cell surface; (ii) ability to induce cell fusion when cotransfected with plasmids encoding gD, gB, and gL; and (iii) ability to complement the infectivity of a gH deletion HSV mutant (infectivity complementation assay).

gH dimerizes with gL, in order to achieve proper folding and to be trafficked to the plasma membrane (47). The ability of mutant forms of gH to form a heterodimer with gL and to be trafficked to the plasma membrane was determined by IFA of cells cotransfected with the mutant or wild-type gH (wt-gH) plasmid and a gL expression plasmid. MAb 53S recognizes a discontinuous epitope and strictly requires gL for reactivity. MAb 52S recognizes a gL-independent discontinuous epitope with critical residues at positions 536 to 537 (44, 50). Both gH_{WGW} and gH_{Δ513-531} maintained the IFA reactivity to MAb

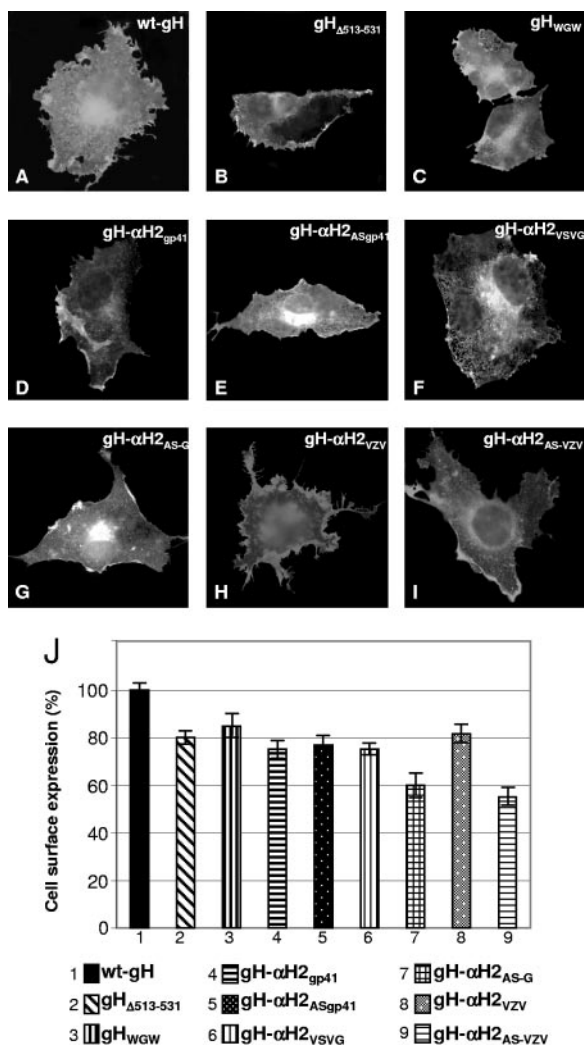


FIG. 2. Cell surface expression of wt-gH, gH $_{\Delta 513-531}$, gH $_{WGW}$, gH- α H2 $_{VSVG}$, gH- α H2 $_{AS-G}$, gH- α H2 $_{gp41}$, gH- α H2 $_{ASgp41}$, gH- α H2 $_{VZV}$, and gH- α H2 $_{AS-VZV}$. (A to I) Paraformaldehyde-fixed COS cells transfected with wild-type or mutant gH plasmids and a gL-encoding plasmid and reacted with Mab 53S. The positive reactivity denotes gH-gL heterodimer formation and trafficking to the plasma membrane. (J) CELISA quantification of cell surface expression of gH mutants in COS cells cotransfected with a gL plasmid. Each assay was performed in triplicate. The bars represent the mean percentage relative to wt-gH \pm standard deviation.

52S, suggesting no major defect in proper folding (data not show). The IFA reactivity of paraformaldehyde-fixed cells to Mab 53S indicates no major defect in trafficking to the plasma membranes and in heterodimer formation with gL (Fig. 2A to C). Cell surface expression, as quantified by CELISA, showed no significant decrease relative to wt-gH (Fig. 2J).

The cell-cell fusion was performed by transfection of effector COS cells with mutant or wt-gH plasmid plus plasmids encoding gD, gB, gL, and the reporter luciferase gene under the T7 promoter. The target cells were transfected with the T7 polymerase. Cells were mixed and allowed to fuse 24 h after transfection. The extent of fusion was quantified by means of the T7 promoter-driven reporter luciferase (46). As shown in Fig. 3 A,

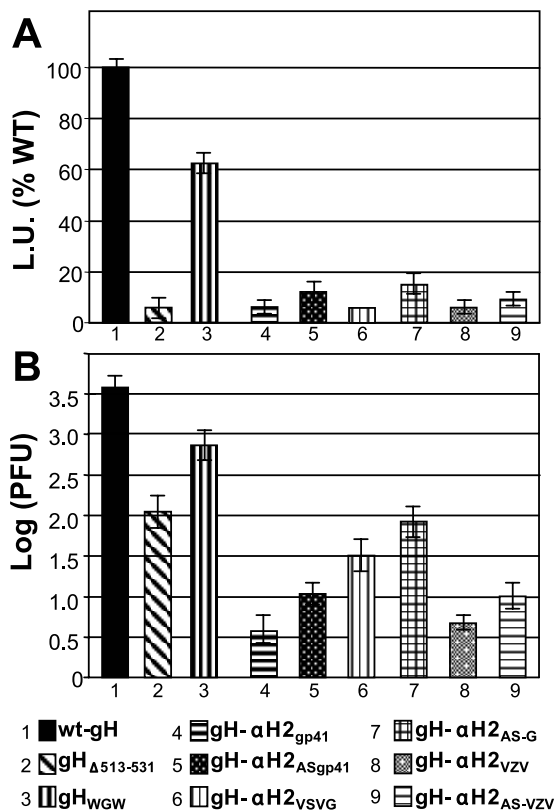


FIG. 3. (A) Cell-cell fusion and (B) infectivity complementation of mutant forms of gH. (A) Cell-cell fusion of COS cells transfected with the gH mutant plasmids plus plasmids encoding gL, gB, and gD. Fusion was quantified by means of a T7 promoter-driven reporter luciferase gene, as luciferase units (L.U.), according to reference 46, and is expressed as a percentage of fusion induced by wt-gH. Each assay was performed in triplicate. The bars represent the mean \pm standard deviation. (B) COS cells were transfected with mutant or wt-gH plasmids and superinfected with the gH deletion mutant virus SCgHZ 4 h later. Virus was harvested 24 h after transfection and titrated in F6 cells. The bars represent the mean of triplicates \pm standard deviation.

gH $_{\Delta 513-531}$ exhibited no fusion activity in COS cells; in contrast, gH $_{WGW}$ exhibited 60% activity relative to wt-gH.

In the infectivity complementation assay, cells were transfected with mutant or wt-gH and superinfected with the gH deletion mutant virus SCgHZ (18). The transgenic gH complements the deletion in the virus, and the complemented virions are or are not infectious, depending on whether gH is or is not functional. The results in Fig. 3B show that virions complemented with gH $_{\Delta 513-531}$ exhibited an about 4% infectivity, and virions complemented with gH $_{WGW}$ exhibited a 13% infectivity relative to wt-gH. (Note that the ordinate scales in Fig. 3A and B differ.)

Replacement of α -H2 with heterologous fusion peptides does not rescue virus infection and cell-cell fusion activity relative to α -H2-deleted gH. Previously, we showed that replacement of α -H1, the candidate fusion peptide with the fusion peptide of HIV gp41 or VSV G partially rescued HSV infectivity and cell-cell fusion (25). The corresponding antisense sequences did not. Here, we asked whether α -H2 could be substituted for with heterologous fusion peptides from HIV

gp41, VSV G, or the putative fusion peptide of VZV gH. To this end, the appropriate sequences were cloned into the α -H2-deleted gH (gH $_{\Delta 513-531}$) in a sense or antisense direction (Fig. 1B). The resulting constructs were named gH- α H2 $_{gp41}$, gH- α H2 $_{AS-gp41}$, gH- α H2 $_{VSVG}$, gH- α H2 $_{AS-G}$, gH- α H2 $_{VZV}$, gH- α H2 $_{AS-VZV}$, respectively. Preliminarily, we ascertained that the chimeric forms of gH were able to form a heterodimer with gL (seen as immunofluorescence reactivity to MAb 53S) and to traffic to the cell surface. The results in Fig. 2D to I indicate that all constructs reacted with MAb 53S and reached the cell surface with only minor decreases (Fig. 2J).

The chimeric gH constructs were assayed for cell-cell fusion activity and for infectivity complementation, as detailed above. The results illustrated in Fig. 3A show that none of the chimeric gH was capable of cell-cell fusion activity. The results of the infectivity complementation assay were essentially similar (Fig. 3B). Surprisingly, the chimeric forms of gH carrying the antisense sequences were somewhat more effective in rescuing infection than the corresponding forms of gH carrying the sense sequences. Altogether, the results of Fig. 3A and B indicate that α -H2 sequence is an important determinant for cell-cell fusion and for HSV infection. Its deletion or replacement of few amino acids drastically reduces both activities; the activity of the α -H2-deleted gH cannot be rescued by heterologous fusion peptides. The properties of α -H2 contrast sharply with those of α -H1, in that deletion or mutation of α -H1 completely abolished infection and fusion (25); the activities were rescued by heterologous fusion peptides.

Mimetic peptides with the sequence of α -H2 and α -H1 induce fusion of lipid vesicles. A wealth of studies have made use of synthetic peptides mimicking predicted hydrophobic sequences to characterize their properties: in particular, the ability to interact with lipid vesicles. To investigate further the properties of α -H2 and to compare them to the properties of α -H1, we derived synthetic peptides to the two sequences, named pep.gH- α H2 $_{19}$ and pep.gH- α H1 $_{21}$ (Fig. 4A), and measured their ability to induce fusion of lipid vesicles. Large unilamellar lipid vesicles were prepared as detailed in Materials and Methods. An aliquot was loaded with pyrene. Lipid vesicles were mixed with pyrene-loaded lipid vesicles in the absence or presence of pep.gH- α H1 $_{21}$, pep.gH- α H2 $_{19}$, or the unrelated peptide pep.gD $_{265-289}$ (30 μ M each). Lipid mixing was measured as the I_E/I_M ratio, as detailed in Materials and Methods (Fig. 4B). As a positive control and a quantification of the extent of fusion, we mixed the two populations of lipid vesicles in the presence of partially purified extracellular herpes simplex virions, which caused a readily quantifiable fusion. Fusion induced by the synthetic peptides was expressed as a percentage relative to fusion induced by herpes simplex virions. As can be seen in Fig. 4B, pep.gH- α H1 $_{21}$ induced a high extent of fusion; pep.gH- α H2 $_{19}$ was somewhat less effective. The effect was specific since the unrelated peptide pep.gD $_{265-289}$ and synthetic peptides to HR-1 (26) sequences did not exert any fusion activity. The synthetic peptide mimicking gH HR-2 sequence caused fusion of the lipid vesicles, in accordance with previous observation (21). Addition of cholesterol in a 1:1 molar ratio to lipids did not result in increase of the fusion activity of the peptides (data not shown).

To measure the effect of the simultaneous presence of pep.gH- α H1 $_{21}$ and pep.gH- α H2 $_{19}$, the peptides were added to

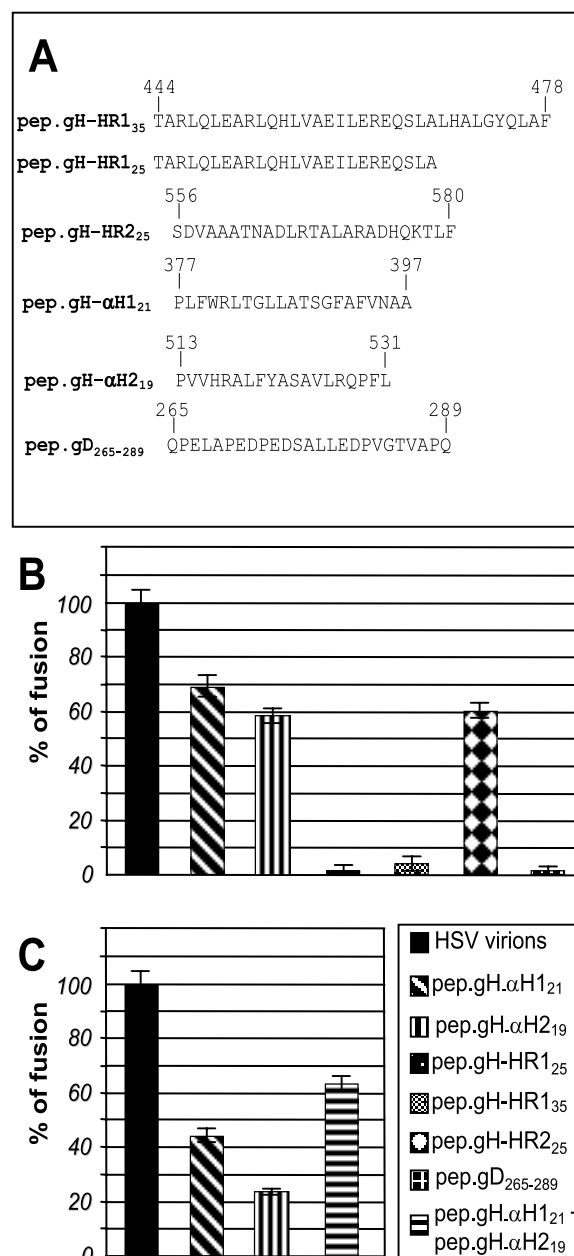


FIG. 4. (A) Sequence and coordinates of synthetic peptides to HSV-1 gH α -H1, α -H2, HR-1, HR-2, and gD. (B and C) Fusion of large unilamellar vesicles induced by mimetic peptides to α -H1 and α -H2, and, as a reference, by herpes simplex virions. Large unilamellar vesicles were mixed with pyrene-loaded vesicles in the presence of herpes simplex virions or the indicated peptides (30 μ M) in a 3:1 lipid/peptide ratio. Extent of fusion was measured as decrease of the excimer fluorescence emission (I_E) and increase in monomer fluorescence emission (I_M) and is expressed as percent I_E/I_M ratio. Fusion induced by synthetic peptides was expressed as a percentage relative to the fusion induced by herpes simplex virions. (B) Induction of lipid vesicle fusion by single peptides (30 μ M). (C) Induction of lipid vesicle fusion by a mixture of peptides mimicking α -H1 and α -H2 (each at 15 μ M). All assays were performed in triplicates. The bars represent the mean \pm standard deviation.

liposomes at lower concentration (15 μ M each, rather than 30 μ M). As can be seen from Fig. 4C, the mixture of the two peptides exerted a fusion activity somewhat higher than that exerted by the single peptides. No increase was seen when

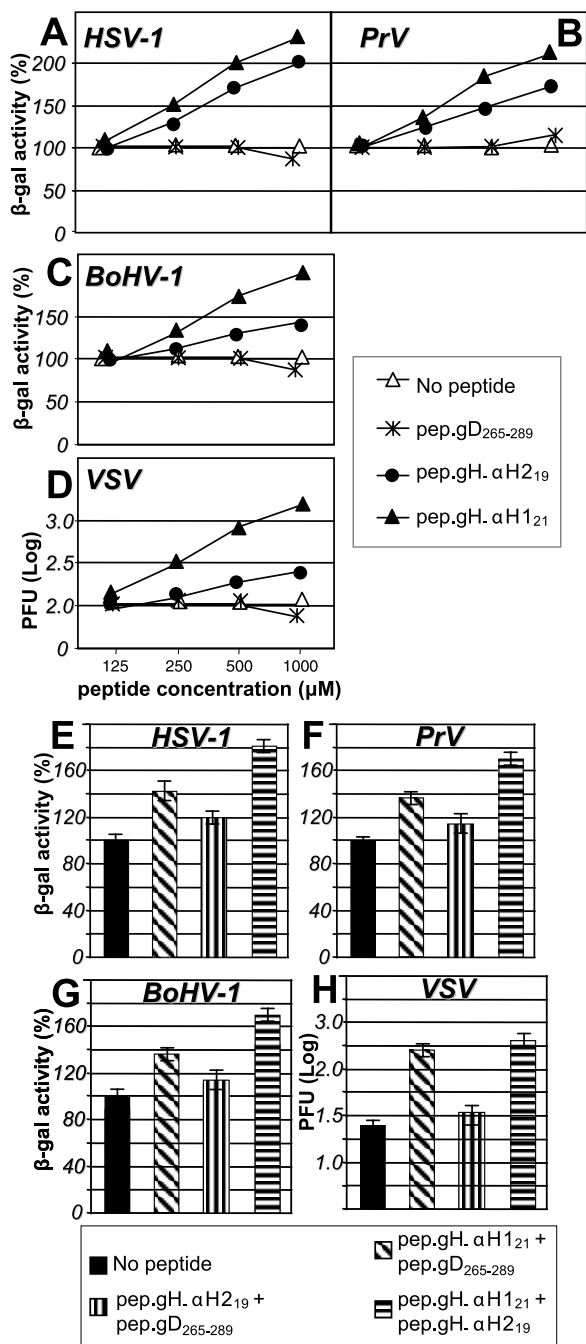


FIG. 5. Enhancement of HSV-1, PrV, BoHV-1, and VSV infection by synthetic peptides or a mixture thereof. (A to D) Effect of the peptides on infection with HSV-1(R8102) (A), PrV (B), or BoHV-1 (C) recombinants carrying the LacZ reporter gene and VSV (D). J-nectin1, Madin-Darby bovine kidney, or Vero cells were exposed to the indicated peptides from time zero (start of virus adsorption) until harvest. Infection was quantified as β -Gal activity (R8102, PrV, and BoHV) or as the number of plaques (VSV). The abscissa shows the μ M peptide concentration. In panel D, the scale is logarithmic. (E to H) Effect of simultaneous exposure to mixtures of mimetic peptides, each one present at a 200 μ M concentration, on HSV-1 (E), PrV (F), BoHV-1 (G), and VSV (H) infection. In panel H, the scale is logarithmic. The bars represent the mean of triplicates \pm standard deviation.

pep.gH- α H1₂₁ or pep.gH- α H2₁₉, was mixed with unrelated peptides (data not shown). We conclude that peptides mimicking α -H2, α -H1, and HR-2 exhibit an intrinsic ability to interact with lipids and to cause fusion of lipid vesicles.

Mimetic peptides with the sequence of α -H2 and α -H1 enhance HSV-1, PrV, BoHV-1, and VSV infection. The effect of α -H1 and α -H2 mimetic peptides on nude lipid vesicles prompted us to ask whether the fusogenic effect was exerted also in in vivo systems and, consequently, whether virus infection and cell-cell fusion were enhanced. The R8102 recombinant, carrying the Lac-Z reporter gene under the immediate-early α -27 promoter, was used in these experiments. In cells infected with this virus, β -Gal expression is a direct measure of the extent of infection. Surprisingly, we found that both peptides increased infection in a dose-dependent fashion. At a concentration of 1 mM, HSV infection was doubled. pep.gH- α H1₂₁ was somewhat more effective than pep.gH- α H2₁₉ (Fig. 5A). The effect was specific inasmuch as pep.gD₂₆₅₋₂₈₉ had no effect on HSV infection.

The fusion of nude lipid vesicles induced by α -H1 and α -H2 mimetic peptides suggested that the enhancing effect on infection was likely consequent to the effect on lipids. We reasoned that, if this were the case, the enhancing effect should not be restricted to HSV-1, but could be observed also with other viruses: e.g., PrV, BoHV-1, and VSV. For PrV and BoHV-1, recombinant viruses carrying the β -Gal reporter gene were used. For VSV, the effect was measured as an increase in the number of plaques. As shown in Fig. 5B and C, pep.gH- α H1₂₁ and pep.gH- α H2₁₉ exerted an enhancing effect on PrV and BoHV-1 infection, with somewhat lower increases than those observed with HSV. The increase in the number of VSV plaques was dramatic (15-fold at 1,000 μ M) (Fig. 5D). We also asked whether the simultaneous exposure to α -H1 and α -H2 mimetic peptides increased infection more than exposure to single peptides. Suboptimal concentrations (200 μ M) of each peptide were used. As shown in Fig. 5E to H, the simultaneous presence of α -H1 and α -H2 mimetic peptides increased infection of R8102, PrV, BoHV-1, and VSV to higher levels than those attained by the single peptides. The simultaneous presence of α -H1 mimetic peptide and pep.gD₂₆₅₋₂₈₉ did not increase infection.

Next, we examined whether the enhancing effect of the peptides was also detectable in the cell-cell fusion assay. BHK cells, cotransfected with plasmids encoding mutant or wt-gH, gB, gD, or gL, were incubated in the presence of pep.gH- α H2₁₉ or pep.gH- α H1₂₁, or a mixture of both. As can be seen from Fig. 6A, pep.gH- α H1₂₁ almost doubled the fusion induced by the HSV glycoproteins. pep.gH- α H2₁₉ exerted only a minor effect (typical examples are shown in Fig. 6C and D). The irrelevant peptide pep.gD₂₆₅₋₂₈₉ exerted no effect. The mixture of pep.gH- α H2₁₉ and pep.gH- α H1₂₁ increased fusion to a higher level than the single peptides (Fig. 6E). Thus, the effect of the peptides on fusion is similar to that observed on infections.

A soluble truncated form of gH₁gL interacts with lipid vesicles. The finding that peptides with the sequence of gH α -H2, and α -H1 have an intrinsic ability to interact with lipids prompted us to investigate whether a soluble truncated form of the glycoprotein carrying the gH ectodomain (gH₇₉₂-gL) associates with lipid vesicles. To control for specificity, the inter-

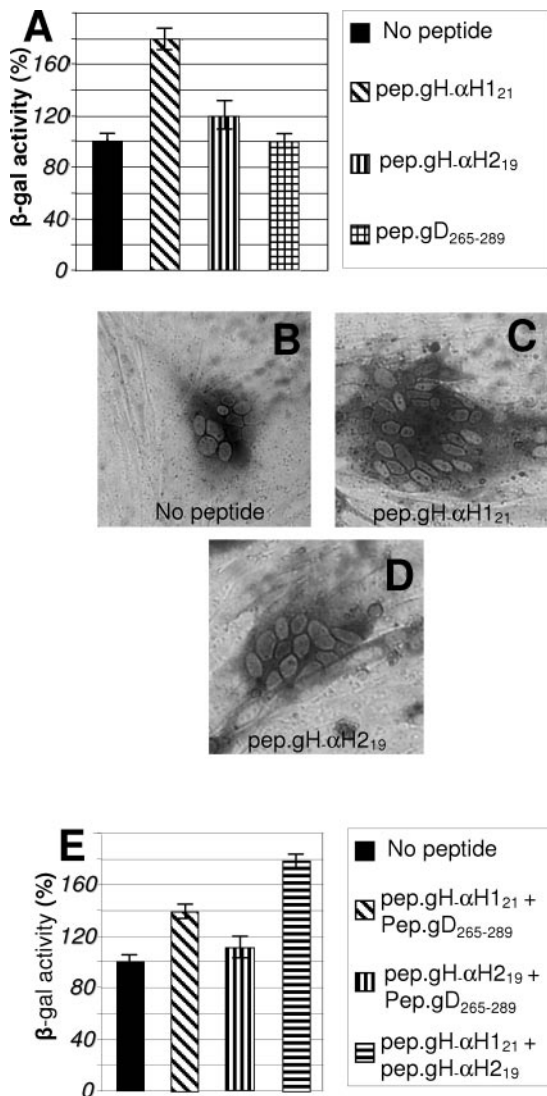


FIG. 6. Enhancement of cell-cell fusion by synthetic peptides or mixtures thereof. Cell-cell fusion in BHK cells transfected with plasmids encoding wt-gH, gL, gB, gD, and LacZ and exposed to the indicated peptides. (A and E) Fusion was quantified by means of β -Gal activity. (A to D) Peptides at 450 μ M each. (B to D) Representative examples of polykaryocytes formed in the absence of added peptides (B) or in the presence of the indicated mimetic peptides (C and D). (E) Additive effect on cell-cell fusion of mixtures of mimetic peptides, each one present at a 200 μ M concentration. The bars represent the mean of triplicates \pm standard deviation.

actions of soluble forms of gD and gB, carrying the ectodomains—gD_{285t} and gB_{730Hist}—or an unrelated glycoprotein, IgG, were analyzed in parallel. Aliquots (1.5 μ g) of the soluble glycoproteins were mixed with preformed lipid vesicles at 37°C for 5 to 10 min and then subjected to sedimentation through preformed discontinuous 5 to 15, 15 to 25, and 25 to 50% sucrose gradients. Under the centrifugation conditions, lipid vesicles float in the 5 to 15% sucrose layers, whereas soluble proteins sediment to the bottom of the tube. As can be seen in Fig. 7A, gH_{792t}gL specifically partitioned with the 5 to 15% sucrose gradient fractions, where the lipid vesicles partitioned. In contrast, gD_{285t}, gB_{730Hist}, and IgG partitioned with the 50%

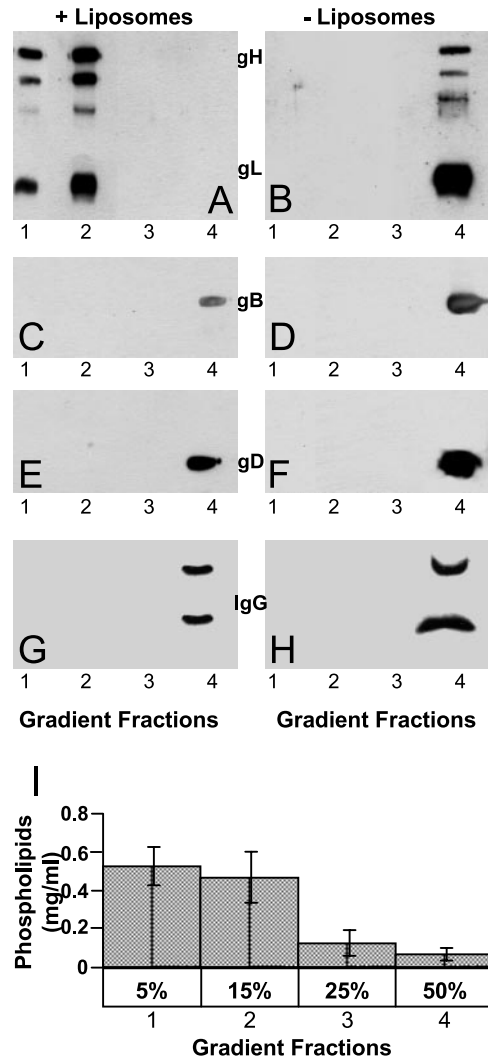


FIG. 7. Sedimentation analysis of soluble forms of gH_{792t}gL (A and B), gB_{730Hist} (C and D), gD_{285t} (E and F), and IgG (G and H), preincubated (A, C, E, and G) or not (B, D, F, and H) with liposomes, through discontinuous preformed sucrose gradients. Gradient fractions 1 to 4 contained 5, 15, 25, and 50% sucrose (wt/vol), respectively. After centrifugation, the fractions were concentrated by Amicon Y10 filters, and the proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and enhanced chemiluminescence. The liposome content in each fraction was determined by means of phosphorous determination. A typical example is represented in panel I. Vertical bars represent standard deviation.

sucrose layer and the pellet. We conclude that the ectodomain of gH_{792t}gL exhibits a specific ability to interact with lipids, not shared with the ectodomain of the other two HSV fusion glycoproteins.

DISCUSSION

We will discuss the key points to emerge from this study in view of the accumulating evidence that gH exhibits structural and functional elements typical of viral fusion glycoproteins. The salient features of our results are as follows.

(i) Deletion of α -H2, its replacement with heterologous fu-

sion peptides in a sense or antisense direction, or the mutation of 4 aa residues decreased infection to less than 10% relative to wt-gH and almost abolished cell-cell fusion. The results show that α -H2 constitutes an important functional domain of HSV gH, albeit a nonessential one.

(ii) α -H1 and α -H2 share important properties, underscored by properties of their mimetic peptides. Thus, synthetic peptides mimicking α -H1 and α -H2 interact with nude lipid vesicles in the absence of adjacent gH sequences and in the absence of membrane proteins in the vesicles (this study and reference 21). They induce fusion of the lipid vesicles, and, more importantly, increase virus infection and cell-cell fusion. Remarkably, the enhancement of infection was not restricted to HSV but was observed also with PrV, BoHV-1, and VSV. To our knowledge, enhancement of infection by mimetic peptides to hydrophobic sequences is novel and has not been described in any system. Taken together, these effects suggest that the interaction of α -H1 and α -H2 with the lipids possibly leads to lipid destabilization in the membranes and thus increases fusion and virus entry. The lipid destabilization may be critical for inducing the curvature of the membrane at the site of fusion and/or for decreasing the energy barrier. Interaction of gH with lipids may be critical also in guiding the glycoprotein refolding.

The synthetic peptides mimicking α -H1 and α -H2 differ from peptides mimicking HR-2 (27). All induce fusion of the lipid vesicles, denoting a certain ability to interact with lipids. However, the first two enhance infection and fusion, whereas the latter inhibits these activities. It is well known that HR-2 mimetic peptides block virus infection by forming a complex with the HR-1 located in the glycoprotein and thus hinder the glycoprotein refolding (17). Therefore, the different behavior of the two groups of mimetic peptides reflects the different molecules they target: i.e., the membrane lipids in the case of α -H1 and α -H2 mimetic peptides and gH itself in the case of the HR-2 mimetic peptide.

(iii) A soluble truncated form of gH, carrying the gH ectodomain, in the form of gH_tgL heterodimer, was capable of associating with lipid vesicles. This property was unique to gH_tgL and was not displayed by the ectodomains of soluble forms of gD_t or gB_t or by the irrelevant glycoprotein IgG. In the case of gB, which is clearly involved in fusion and virus entry, bioinformatic analyses predicted no candidate fusion peptide or hydrophobic segments and no strong HR sequence, and association with lipids was not expected. Hence, the ability of gH_tgL to interact with lipid membranes correlates with the presence of hydrophobic segments. The results imply that the ability of α -H1, α -H2, and perhaps other gH sequences to interact with lipids is maintained even when the segments are integral parts of gH. Remarkably, when α -H1 or α -H2 sequences were engineered downstream of the truncated soluble gD_{1-260t}, the truncated gD became membrane bound, showing that α -H1 and α -H2 do interact with cell membranes.

(iv) Notwithstanding the above similarities, α -H2 and α -H1 differ in a number of important properties. First, α -H1, but not α -H2, is positionally conserved in all of the gH alleles from different herpesviruses that were examined (25). Second, α -H1, but not α -H2, cannot be deleted without total loss of infection. Third, α -H1 can be functionally replaced with heterologous fusion peptides, whereas α -H2 cannot. Indeed, the

highest rescue of the α -H2-deleted gH was observed with constructs carrying the antisense inserts. Fourth, α -H2 is less hydrophobic than the candidate fusion peptide, both by bioinformatic prediction and measured as the ability to render the soluble gD_{1-260t} membrane bound (25). Fifth, the mimetic peptide to α -H1 was more effective than the mimetic peptide to α -H2 in inducing fusion of nude lipid vesicle and in enhancing infection and cell-cell fusion (this work and reference 21). These features differentiate α -H1 from α -H2. They reinforce the view that α -H1, but not α -H2, exhibits properties typical of fusion peptides. Collectively, the results reported here and elsewhere highlight gH as a structurally complex glycoprotein. It carries sequences able to interact with lipids, namely α -H1, with properties of a candidate fusion peptide, in addition to α -H2 and the pretransmembrane sequence with a tendency to partition at the membrane interface. It also carries two functional HRs. These elements are typical of class I fusion glycoproteins.

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