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Site-Directed and Linker Insertion Mutagenesis of Herpes Simplex Virus Type 1 Glycoprotein H

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The gH-gL complex of herpes simplex virus type 1 (HSV-1) is essential for virion infectivity and virus-induced cell fusion, but functional domains of the gH molecule remain to be defined. We have addressed this question by mutagenesis. A set of linker insertion mutants in HSV-1 gH was generated and tested in transient assays for their ability to complement a gH-negative virus. Insertions at three sites in the C-terminal third of the external domain affected the ability of gH to function in cell-cell fusion and virus entry, while insertions at six sites in the N-terminal half of the external domain induced conformational changes in gH such that it was not recognized by monoclonal antibody LP11, although expression at the cell surface was unchanged. A recombinant virus in which a potential integrin-binding motif, RGD, in gH was changed to the triplet RGE entered cells as efficiently as the wild type, indicating that HSV-1 entry is not mediated by means of the gH-RGD motif binding to cell surface integrins. Furthermore, mutagenesis of the glycosylation site which is positionally conserved in all herpesvirus gH sequences in close proximity to the transmembrane domain generated a recombinant virus that grew in vitro with wild-type single-step kinetics.

Glycoprotein H (gH) is one of the three membrane-spanning glycoproteins that are found in all members of the herpesvirus family. Studies using herpes simplex virus type 1 (HSV-1) have shown that gH is essential for virus entry into cells and is required for the fusion of an infected-cell membrane with that of its neighbor (9, 10, 13), and it is likely that gH also plays an essential role in the entry of all herpesviruses. HSV-1 gH forms a heterodimer with the small, non-membrane-anchored glycoprotein gL. Complex formation with gL is required for authentic processing of gH (18) and this interaction, which is essential for viral infectivity, appears to be a general feature of all herpesvirus gH molecules (19, 21, 26, 37).

Although gH is required for virus entry and membrane fusion, the means by which gH interacts with cellular components and the roles of functional domains in the gH protein remain to be characterized. These questions have proved difficult to address, partly because comparisons of predicted gH primary sequences have shown that the molecule is poorly conserved between herpesvirus subfamilies (16). In fact, gH is one of the most divergent of the proteins which are common to all herpesviruses, and at the amino acid level, limited conservation is restricted to the C-terminal part of the molecule. It is therefore difficult to identify regions of likely functional importance based solely on criteria of primary sequence conservation. Characterization of potentially functional domains of HSV-1 gH has relied largely on the analysis of mutations which confer resistance to neutralization by gH-specific monoclonal antibodies, LP11 and 52S (15). These mutations give rise to amino acid substitutions which cluster at four independent sites in the external domain of the gH molecule. It has also been reported recently that specific amino acid residues in the cytoplasmic tail are required for efficient HSV-1 gH-mediated membrane fusion (5, 35).

We have taken two further approaches to investigate which regions of HSV-1 gH might play a functional role during virus entry and cell fusion. Site-directed mutagenesis of a predicted N-linked glycosylation site and of a potential integrin-binding motif was employed to investigate the requirements for these motifs in virus infectivity. In addition, a panel of gH linker insertion mutants was generated. These mutants provide a means of identifying epitopes required for recognition by gH-specific monoclonal antibodies, and they may also be tested for their ability to complement a gH-negative syncytial virus in cell-cell fusion and virion infectivity assays.

NGTV motif. Despite the poor degree of sequence conservation between gH proteins of the herpesviruses, one of the seven potential sites for the addition of N-linked glycans in HSV-1 gH (NGTV at residues 783, 784, 785, and 786), which lies close to the predicted membrane-spanning domain, is positionally conserved in all herpesvirus gH sequences (17, 20, 22, 28–31). Furthermore, a 23-amino-acid region of the external portion of human herpesvirus 6 (HHV-6) gH which includes this motif is a component of the epitope for a monoclonal antibody which inhibits cell fusion (25). We therefore tested the possibility that this site is conserved by virtue of its functional significance by mutating the threonine residue of this motif to alanine, thereby abolishing its capacity for modification by N-linked glycosylation, and introducing this mutation into a recombinant virus.

RGD motif. The rationale for performing mutagenesis of the RGD motif (amino acids 176, 177, and 178 in the external domain of HSV-1 gH) was based on the two independent observations that gH is known to play a key role during HSV entry (10, 13) and that several viruses, including adenovirus (34), foot-and-mouth disease virus (14), and coxsackievirus A9 (32), use attachment to integrin molecules by RGD-containing virus proteins as a means of mediating entry into cells. The integrin-binding activity of RGD motifs can be abrogated by mutating the aspartate residue of this triplet to glutamate (12). We therefore sought to investigate the requirement for this potential integrin-binding motif in HSV-1 gH by examining the ability of a mutant virus, in which this motif was changed to RGE, to bind to and penetrate cells in vitro.

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To complement these directed-mutagenesis approaches, we also investigated the properties of mutant gH molecules into which additional amino acid residues had been introduced at random throughout the protein sequence by means of linker insertion mutagenesis. This approach has proved successful for identifying functional domains of other HSV-1 glycoproteins, for example, gD (8) and gB (6), and mutants can be readily analyzed in transient assays by examining their ability to complement a gH-negative virus.

MATERIALS AND METHODS

Cells and viruses. The gH-negative virus used as a parental strain for generating gH mutant recombinants and for superinfection in transient complementation assays was SCgH-pABang (4). This virus contains a syncytial mutation in the gene encoding glycoprotein B, and the gH coding sequence is replaced by a cytomegalovirus immediate-early promoter-*lacZ* cassette. Vero, CR1, and Cos7 cells were grown in Glasgow's modified essential medium (MEM) containing 10% fetal calf serum. CR1 cells are a stably transformed Vero cell line expressing HSV-1 gH under the control of the HSV-1 gD promoter (2).

Plasmids and mutagenesis. The HFEM gH coding sequence was derived as a 2.6-kb *Hin*dIII-*Xba*I fragment from plasmid pSMH3gH (35) and was subcloned into *Hin*dIII-*Xba*I-digested pING14.2 (24). This plasmid is called pINGgH. The gL expression construct used in cotransfection experiments contains the gL coding sequence from strain 17 derived as a *Bam*HI-*Eco*RI fragment from pgHLmpSC11 (3) and cloned into pSMH3 (35).

(i) Site-directed mutagenesis. Mutagenesis of the RGD-encoding sequence to encode RGE and the NGTV-encoding sequence to encode NGAV was carried out on pINGgH according to the method of Kunkel (23), and the oligonucleotides used were designed to include diagnostic restriction enzyme recognition sites. The RGE mutation was introduced with synthetic oligonucleotide 5′ACG TTCCCGCGGGGTGAAAACGTGGCG3′, which includes a recognition site for *Hph*I. The NGA mutation was introduced with oligonucleotide 5′TTTCCA AACGGCGCCGTCATTCATT3′, which contains a recognition site for *Nar*I. Mutated gH coding sequences were subsequently transferred to shuttle vector pIMB52, which contains flanking sequences derived from either side of the gH gene (5). These plasmids are called pIMPL RGE and pIMPL ΔG.

(ii) Linker insertion mutagenesis. gH linker insertion mutants were generated according to protocols described by Digard et al. (11). Plasmid DNA (pINGgH) was linearized by digestion with restriction enzyme *Hae*III, *Cac*8I, or *Nla*IV, all of which cleave at many sites within the gH coding sequence. The digests were done in the presence of an empirically determined concentration of ethidium bromide sufficient to inhibit further digestion of linear plasmid DNA molecules generated by the first endonuclease cut. The digested plasmids were then linker tailed by ligation with an unphosphorylated 12-bp palindromic oligonucleotide, TGCATCGATGCA, generating linear DNA molecules containing a copy of the linker at both ends. To facilitate mapping of the insertion site, the oligonucleotide was designed to contain a *Cla*I restriction enzyme site, a sequence not present in the gH gene. Linear unit-length plasmid was gel purified from a 0.7% low-melting-point agarose gel, allowed to reanneal, and used to transform competent *Escherichia coli* by electroporation. In addition, oligonucleotide linkers were inserted at three restriction endonuclease sites (*Nru*I, *Stu*I, and *Hin*cII), all of which cleave once in the gH gene. All colonies were initially screened for linker insertion by restriction enzyme mapping, and the precise location of the linker was determined by nucleotide sequence analysis. For expression studies, mutant gH coding sequences were subcloned as *Hin*dIII-*Xba*I fragments into pCDNA3, which may be used to achieve high-level expression in Cos7 cells.

Construction of recombinant viruses containing modified gH genes. CR1 cells were cotransfected with 10 μg of SCgH-pABang-infected-cell DNA and 2.5 μg of either pIMPL RGE or pIMPLΔG according to the method of Chen and Okayama (7). Cotransfection progeny were plated on CR1 monolayers with an agarose overlay containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (as described in reference 9), and white plaques were picked and purified by limiting dilution. The presence of the appropriate mutation in working stocks of recombinant viruses was confirmed by Southern hybridization of infected-cell DNA and detection of the novel restriction enzyme site which had been introduced by the mutagenic oligonucleotide. The viruses are called SCRGE and SCΔG according to the pIMPL derivative which was used to generate the recombinant.

Single-step growth analysis. Dishes containing 3×10^6 Vero cells were infected at 10 PFU/cell for 1 h at 37°C, washed briefly in pH 3 buffer, and overlaid with MEM-newborn calf serum. At various times after infection cells were harvested, sonicated, and assayed by titration on Vero monolayers.

Measurement of adsorption and penetration rates. Monolayers of Vero cells were prechilled to 4°C for 1 h. Approximately 300 PFU of wild-type or recombinant virus was added to each dish in 0.5 ml of HEPES-buffered MEM. To measure adsorption rates, dishes were incubated for 5 to 120 min at 4°C. At each time point, inoculum was removed and cells were washed twice with cold medium and overlaid with medium containing carboxymethyl cellulose. To measure penetration kinetics, dishes were incubated at 4°C for 2 h to allow virus adsorption. Cells were then transferred to 37°C, and at various times after transfer to 37°C, monolayers were washed with a pH 3 citrate buffer before being overlaid with medium containing carboxymethyl cellulose. All dishes were incubated for 2 days, when plaques were stained and counted.

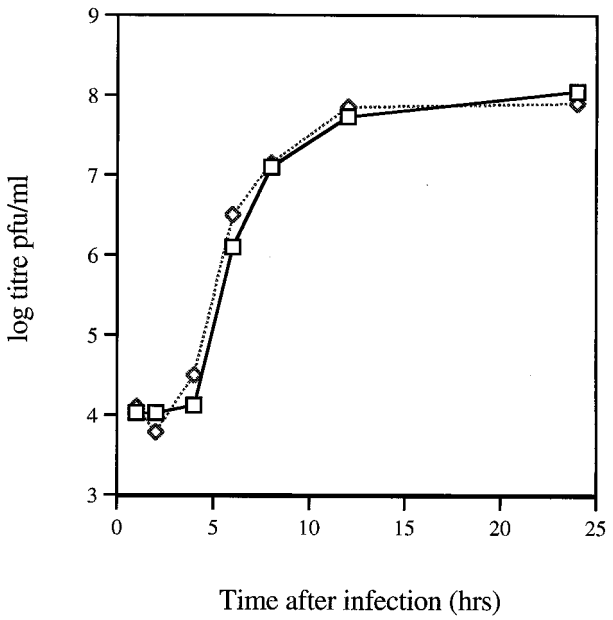

FIG. 1. Single-step growth kinetics of SCΔG and SC16. Monolayers of 3×10^6 BHK cells were infected at 10 PFU/cell with SCΔG (◇) or SC16 (□). Infected cells were harvested, resuspended in 1 ml of MEM, and sonicated, and virus titers were determined at various times after infection. Each point is the mean from duplicate samples.

Transfection of Cos7 cells. Cos7 cells were transfected with gH- and gL-expressing plasmids according to protocols described by Wilson et al. (35) and were incubated for 48 h prior to immunofluorescent staining or for 24 h prior to superinfection in complementation experiments.

Immunofluorescence. Transfected Cos7 cells (seeded on coverslips 24 h post-transfection) were fixed in 2% formaldehyde for 5 min. For internal staining, cells were permeabilized by incubation with a mixture of 1% Triton X-100, 10% sucrose, and 1% fetal calf serum for 5 min. Coverslips were incubated with monoclonal antibodies LP11, 52S, and 53S either singly or in combinations for 1 h. All antibodies were used as hybridoma supernatants and were diluted 1:3. Antibody binding was detected by incubation with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin (DAKO) diluted 1:50.

Transient complementation assays. Transfected cells (in six-well trays) were infected at 10 PFU/cell with SCgH-pABang, and after 1 h of adsorption at 37°C, unpenetrated virions were inactivated by being washed briefly with a low-pH buffer (35). To screen for syncytial phenotypes, cells were overlaid with 2×10^6 Vero cells and incubated for 12 h before being fixed in 0.5% glutaraldehyde and stained with neutral red. To determine yields of infectious virus, transfected and infected cells were incubated for 16 h to allow virus replication, and then harvested, sonicated, and assayed for infectious virus by titration on CR1 monolayers.

RESULTS

Mutagenesis of NGTV. The effect of mutating the predicted glycosylation site in gH at amino acid residues 783, 784, and 785 was determined by examining the in vitro phenotype of recombinant virus SCΔG. This mutant plaqued with equivalent efficiencies on both complementing and noncomplementing cell lines, and no discernible differences between SCΔG and wild-type virus in terms of plaque size were observed. Single-step growth kinetics were examined following high-multiplicity infection of BHK cells, and Fig. 1 shows that SCΔG grows as efficiently as wild-type virus under these conditions. These data indicate that mutagenesis of the threonine residue of this predicted glycosylation site to alanine has apparently no effect on virus viability and implies that the conservation of this motif in all herpesvirus gH sequences is unlikely to reflect a structural

or functional role, at least as a glycosylation site, during virus infection in vitro.

Mutagenesis of RGD. Preliminary characterization of recombinant viruses generated by cotransfecting gH-negative virus DNA with pIMPLgHRGE indicated that white-staining progeny viruses grew as well on Vero cells as they did on a gH-expressing cell line. This enabled stocks of the recombinant virus, SCRGE, to be propagated in Vero cells and tested for binding and entry on noncomplementing cells.

(i) Adsorption rates. We compared the rates at which SCRGE and wild-type strain SC16 adsorbed to cell monolayers at 4°C over a 120-min period and observed no significant difference between the two viruses (Fig. 2a). These data suggest that the RGD motif in gH is not important for initial stages of HSV entry, at least during the adsorption period.

(ii) Penetration rates. The effect of the RGE mutation on virus entry was assessed by comparing the rates at which mutant and wild-type virus became insensitive to low-pH inactivation following a 2-h adsorption period at 4°C and then a temperature shift to 37°C. Figure 2b shows that SCRGE penetrates cells with wild-type kinetics and indicates that HSV entry is not mediated by an integrin-gH interaction.

Construction and characterization of gH linker insertion mutants. (i) Construction of in-frame linker insertions. A 12-bp palindromic oligonucleotide linker was inserted randomly throughout the gene for HSV-1 gH. The sites of insertion were confirmed by DNA sequencing; Fig. 3 shows the locations of the 33 insertion mutations used in this study, and Table 1 lists the amino acids which are inserted at each site. The mutants are designated 1 to 33 according to the position at which the linker is inserted within the gH gene, and all the mutants generated in this study contain insertions in the external domain of the gH molecule. Before undertaking experiments to assess structural and functional properties of mutant gH proteins, the ability of each mutated gene to express a full-length gH polypeptide was examined by in vitro transcription and translation in rabbit reticulocyte lysate and by polyacrylamide gel electrophoresis under denaturing conditions (data not shown). All but two of the mutated gH genes tested encode a polypeptide of 85 kDa, which is the size of the predicted primary translation product of the gH open reading frame. Mutant 28 was expressed as a 65-kDa product, consistent with restriction mapping data which had identified the deletion of 500 bp of the 3′ end of the gH gene. Mutant 7, which restriction mapping had predicted to encode a full-length gH polypeptide, expressed a 43-kDa species, presumably as a result of additional mutations, and was not included in any further analyses.

(ii) Characterization of mutant gH proteins. We used transient expression of gH mutants in Cos 7 cells to characterize their phenotypes in two respects. (i) Structural integrity was investigated by determining reactivity with monoclonal antibodies against discontinuous epitopes of gH, and cellular localization was examined by immunofluorescent staining. (ii) Functional properties were assessed by measuring the ability of each mutant protein to complement a gH-negative virus in transient cell-cell fusion and infectivity assays.

Reactivity with monoclonal antibodies and cellular localization. To investigate whether the insertion of additional amino acids had induced global conformational changes in mutant gH proteins, we performed immunofluorescent staining of permeabilized Cos7 cells which had been cotransfected with mutant-gH- and wild-type-gL-expressing plasmids. A pool of monoclonal antibodies (52S, 53S, and LP11), all of which recognize discontinuous epitopes on gH, was incubated with transfected cells, and reactivity was detected with a fluorescein-conjugated secondary antibody. Each mutant was tested in at least two independent experiments, and the results of this analysis are summarized in Fig. 4a. The phenotypes of the mutant proteins fell into one of three groups: (i) mutants which reacted with the pool of monoclonal antibodies and gave fluorescent staining patterns similar to that of wild-type gH, (ii) mutants which

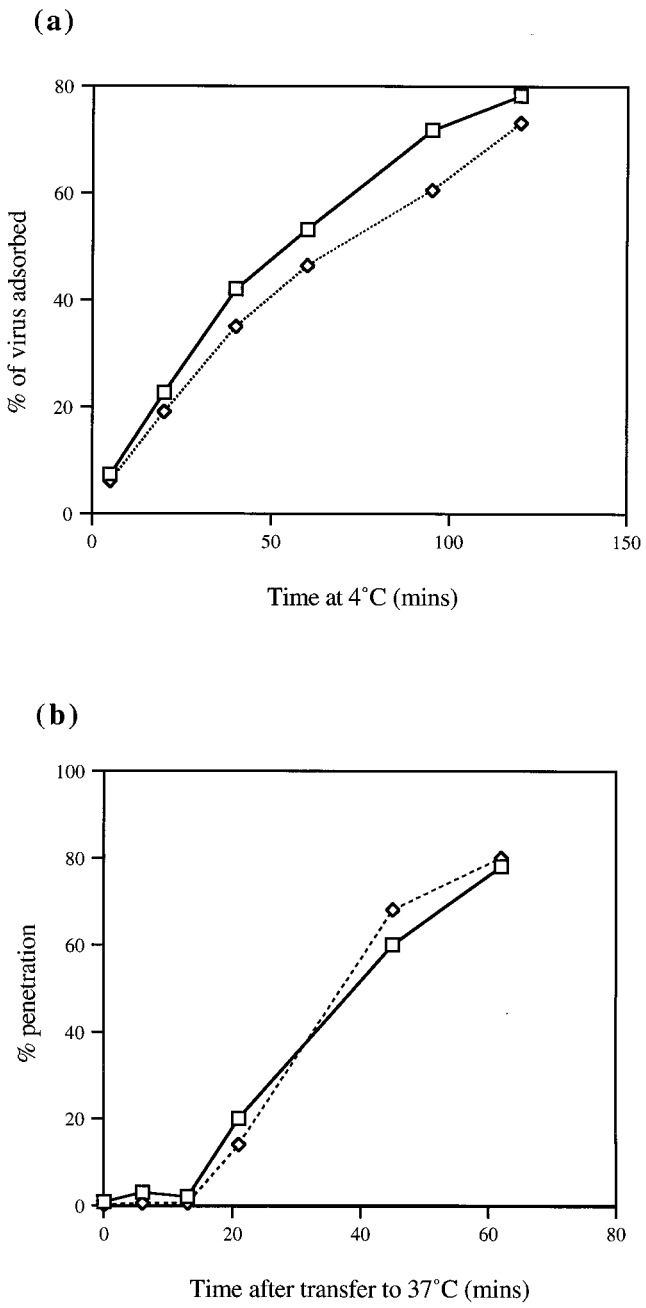

FIG. 2. Adsorption (a) and penetration (b) rates of SCRGE and SC16. □, SC16; ◇, SCRGE. (a) Vero cells (3×10^6) were infected at 4°C with approximately 300 PFU of virus. At various times monolayers were washed and transferred to 37°C, and plaques were counted after 2 days. (b) Vero cells (3×10^6) were infected with approximately 300 PFU of wild-type or recombinant virus at 4°C for 2 h. At various times after transfer of the cells to 37°C, virions which had not penetrated were inactivated with a pH 3 wash, and plaques were counted after 2 days.

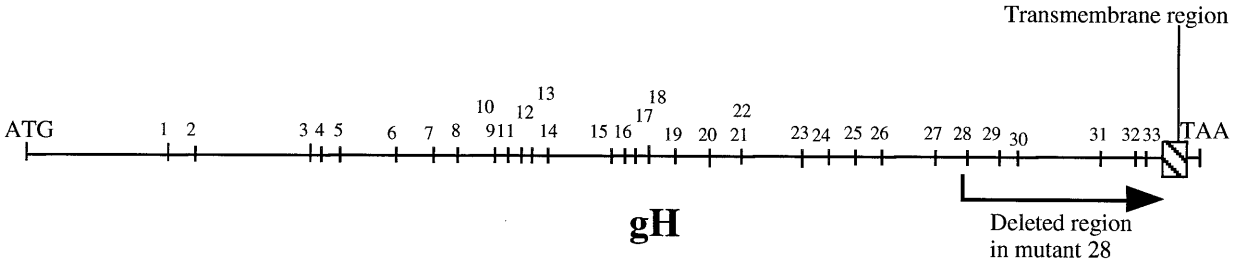

FIG. 3. gH linker insertion mutations. The mutations are numbered from the N to the C terminus of gH. Insertions either replace an amino acid residue or occur between two amino acid residues. The locations of the insertions as they occur within the gH gene are depicted schematically.

failed to react with the pool of monoclonal antibodies, and (iii) mutants which gave very weak immunofluorescent staining.

Cell surface expression of mutant gH proteins was monitored by immunofluorescence of transfected cells which had not been permeabilized. In these experiments, cells were stained either with a pool of 52S, 53S, and LP11 (as used above) or with LP11 alone, and the results are shown in Fig. 4a. Again, the phenotypes of the mutants fell into three groups: (i) mutants which express cell surface gH, as determined by reactivity with the LP11-52S-53S mix, and which are also recognized by LP11 alone; (ii) mutants which express cell surface gH, as determined by reactivity with the LP11-52S-53S mix, but which fail to react with LP11 alone; and (iii) mutants which gave no evidence of cell surface expression of gH (all the mutants in this group had failed to express a structurally authentic molecule as determined by immunostaining of permeabilized cells).

TABLE 1. gH linker insertion mutants

Mutant[a]	Position of insertion (amino acid residue)[b]	Amino acids introduced[c]
1	102	DASMH
2	121/122	CIDA
3	200/201	CIDA
4	201	VHRCT
5	226	VHRCS
6	265	RASMH
7	291/292	CIDA
8	300	VHRCT
9	312/313	CIDA
10	313	LHRCT
11	316/317	CIDA
12	325/326	CIDA
13	326	LHRCT
14	329	RASMQ
15	368/369	CIDA
16	373	GASMH
17	381	LHRCS
18	387	VHRCT
19	413	VHRCT
20	458	VHRCT
21	507	VHRCS
22	509	VHRCM
23	552	VHRCT
24	570	LASMQ
25	591	VHRCT
26	607	LASMQ
27	648	VHRCT
28	671/822	CIDA
29	691/692	CIDA
30	708/709	CIDA
31	765/766	CIDA
32	791	LASMQ
33	799	VHRCT

[a] The mutants are numbered from the N to the C terminus.
[b] Insertions either replace an amino acid residue or occur between two residues.
[c] Amino acids are named according to the single-letter code.

Taken together, the results of these fluorescence experiments highlight a cluster of mutants (mutants 4, 8, and 10 through 13) in which the epitope for recognition by LP11 has been disrupted, despite the fact that these molecules are transported to the plasma membrane efficiently. These studies also identified a number of mutations (mutations 5, 6, 9, 17, 23, 25, and 28) which disrupt the structural integrity of the gH protein such that it is no longer recognized by any of the three conformation-dependent monoclonal antibodies. Over half of the mutations (mutations 1 through 3, 14 through 16, 18 through 22, 24, 26, 27, and 29 through 33) had no discernible effect on the antigenic conformation of gH or its expression at the cell surface, a finding which was somewhat surprising since most of these mutants contained an additional cysteine residue as a result of linker insertion (Table 1) which might be expected to cause aberrant disulfide bonding.

Functional analysis of gH mutants. We tested the ability of mutant gH molecules to mediate cell-cell and virus-cell membrane fusion by examining their abilities to complement a gH-negative virus which contains a syncytial mutation in the gene encoding gB. Cos7 cells were transfected with plasmids expressing either wild-type or mutant gH proteins before infection with SCgH-pABang. Transfected and infected Cos7 cells were then either overlaid with Vero cells for 12 h (to determine the efficiency with which the gH mutants could mediate syncytium production) or incubated for 16 h before sonication and titration on CR1 monolayers (to assess the ability of mutant gH proteins to function during virus entry).

(i) Effect of mutations on syncytium production. The extent of syncytia produced by each mutant was compared with that induced by wild-type gH and was scored in arbitrary units from 0 to 5, where 5 was the score attributed to wild-type levels of fusion. Each mutant was tested in two independent experiments, and both experiments gave the same pattern of results. These results are summarized in Fig. 4b. Of the group of mutants which were not recognized by the pool of monoclonal antibodies to gH (mutants 5, 6, 9, 17, 23, 25, and 28), all failed to induce cell-cell fusion. All other mutants which had shown evidence of surface expression of gH, with the exception of 29 and 33, gave rise to reasonable amounts of fusion, with most mutants inducing levels similar to levels with the wild type. However, 29 and 33, both of which express cell surface gH, were unable to mediate polykaryocyte formation in this assay, and 32, which also expresses an antigenically authentic protein on the cell surface, gave very small polykaryocytes. We noted in addition that two of the mutants in which the LP11 epitope

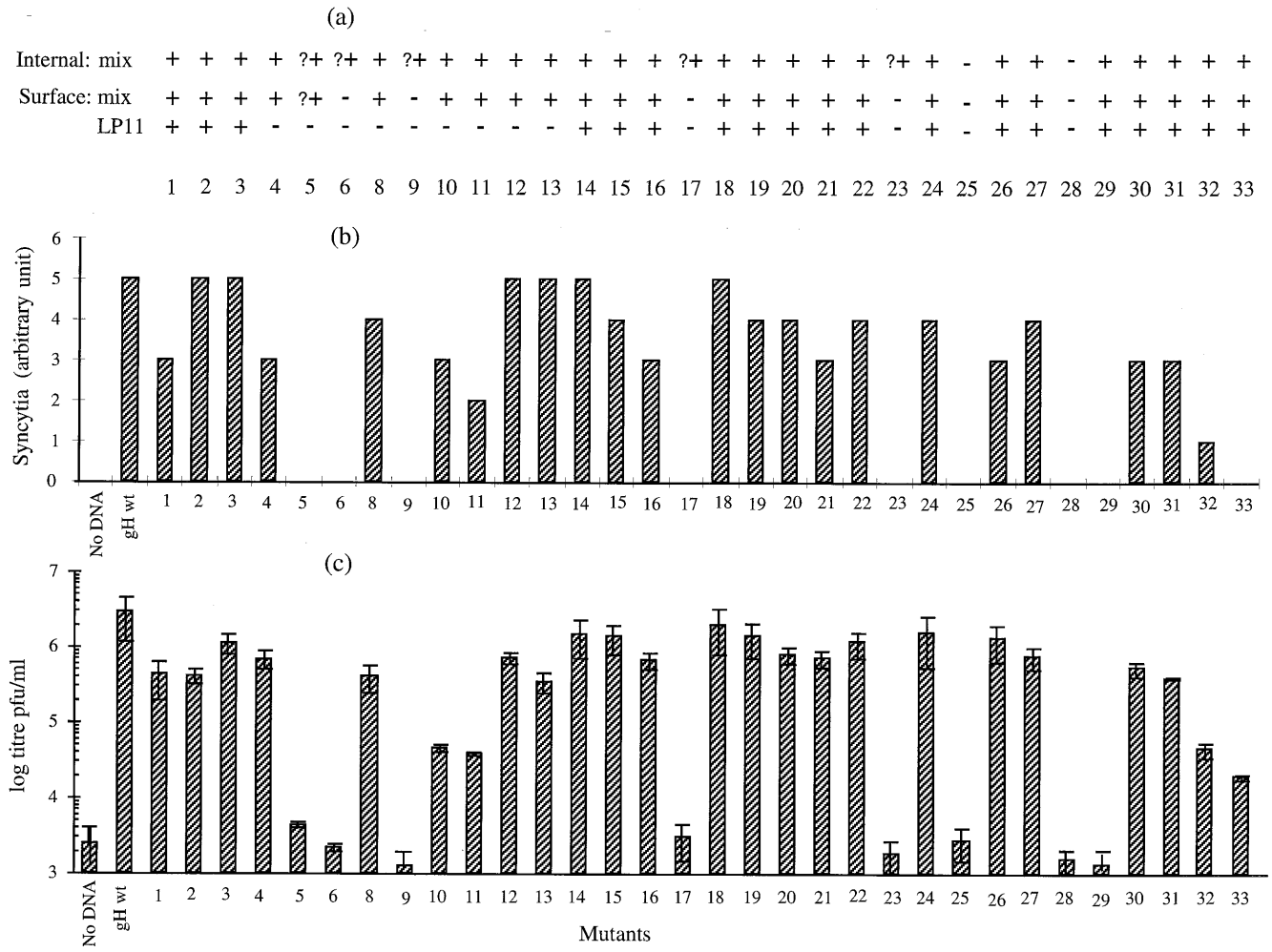

FIG. 4. Characterization of gH linker insertion mutants. (a) Immunofluorescence analysis of gH mutants in transfected Cos7 cells. Each mutant plasmid was cotransfected with a gL-expressing plasmid, and internal staining was examined with a mix of antibodies LP11, 52S, and 53S. Cell surface expression was assessed either with a mixture of LP11, 52S, and 53S or with LP11 alone. (b) Ability of mutant gH molecules to support cell-cell fusion. Transfected Cos7 cells were infected with gH-pABang, overlaid with Vero cells, and examined for evidence of syncytium formation after incubation for 12 h. (c) Ability of mutant gH proteins to function in virus infectivity. Transfected Cos7 cells were infected with gH-pABang and incubated for 16 h to allow virus replication. The cells were resuspended in 1 ml of medium, sonicated, and titrated on CR1 monolayers. The results shown are the means from duplicate experiments. wt, wild type.

is disrupted, 10 and 11, were also compromised in terms of their ability to mediate fusion.

(ii) Effect of mutations on virus-cell fusion. The infectivity of progeny virions produced after infecting transfected Cos7 cells with a gH-negative virus was determined by titration on CR1 cells. Cells transfected with wild-type gH DNA gave typical yields of 2×10^6 PFU/ml, and mock transfections, where no plasmid was added, gave background levels (reflecting input inoculum) of 2×10^3 PFU/ml. The yields of infectious virus obtained following transfection with the set of gH linker insertion constructs are shown in Fig. 4c. From these data, the effects of the mutations can be broadly classified into three categories: (i) mutations which have little or no effect on the ability of gH to mediate infectivity (mutations 1 through 4, 8, 12 through 16, 18 through 22, 24, 26, 27, 30, and 31) (we have included in this group any mutant which yields more than 10% of wild-type infectivity), (ii) mutations which abolish the ability of gH to mediate infectivity (mutations 5, 6, 9, 17, 23, 25, 28, and 29), and (iii) mutations which have a significant effect on the yields of infectious progeny and which give rise to viruses with 1% of the infectivity of the wild type (mutations 10, 11, 32, and 33).

The phenotypes of these mutants in infectivity assays closely correlate with the phenotypes observed in transient cell-cell fusion experiments. The group of mutations which cause global conformational changes in gH as judged by immunofluorescence and which abolish its ability to mediate cell-cell fusion not surprisingly fail to complement the infectivity of a gH-negative virus. Mutations which gave rise to progeny titers of between 5×10^5 and 5×10^6 PFU/ml encode a gH protein which is capable of mediating virus entry. This same group of mutations had little or no effect on cell-cell fusion or gH conformation. Two of the four mutants (mutants 10 and 11) which have lost the LP11 epitope and which gave reduced levels of cell-cell fusion produced low yields of infectious virus. In addition, the three mutations (mutations 29, 32, and 33) which induced no detectable effect on the conformation of gH yet which had abolished or significantly reduced its ability to mediate cell fusion also abolished or significantly reduced its ability to function in virus entry.

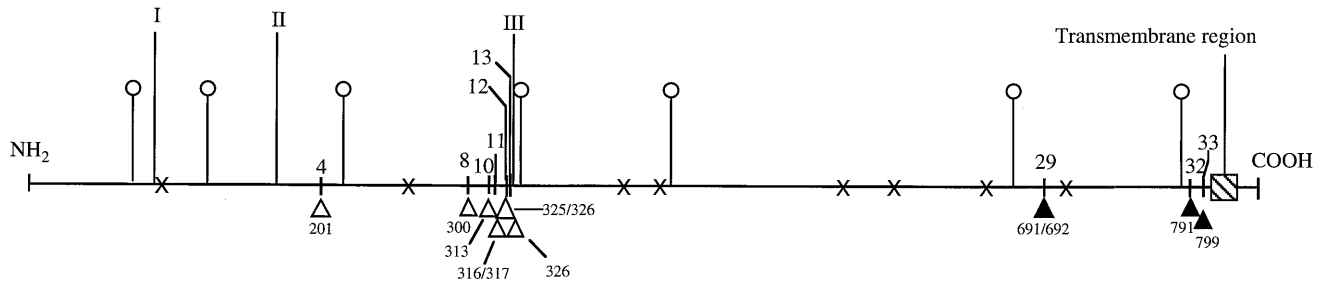

FIG. 5. Summary of locations of insertions in the gH gene which disrupt recognition by LP11 and affect membrane fusion. △, locations of insertions which affect recognition by LP11; ▲, locations of insertions which affect membrane fusion; I, II, and II, the three sites identified by Gompels et al. (15) at which mutations occurred in a group of LP11-resistant mutant viruses; ○, predicted sites for addition of N-linked glycans; ×, cysteine residues.

DISCUSSION

This report describes a series of experiments designed to identify regions of gH which are important for virus infectivity and membrane fusion. Site-directed mutagenesis was used to investigate the requirement for two consensus motifs in the gH primary sequence: a predicted site for the addition of asparagine-linked oligosaccharides (NGTV) and a potential integrin-binding peptide sequence of arginine-glycine-aspartate (RGD). These motifs were mutated from NGTV to NGA (to prevent the addition of N-linked glycans) and from RGD to RGE (to abolish potential integrin binding), and recombinant viruses expressing either of these mutations grew as efficiently in vitro as wild-type HSV-1.

The predicted site for N-linked glycosylation which we mutated is the only such site that is absolutely conserved in all known herpesvirus gH sequences. All herpesvirus gH proteins contain the motif NGTV at this position with the exception of HHV-6 gH, which contains NGSV. A motif which has been conserved over 200 million years of evolution must have structural or functional significance (27), but our data show that the absence of this predicted glycosylation site has no effect on the ability of gH to mediate virus infection in vitro. Whether SCΔG is attenuated in vivo and whether this motif is actually used as a site for addition of N-linked oligosaccharides during processing of gH remain to be determined.

Although it is well established that the initial stages of HSV entry involve an interaction with cell surface heparin sulfate proteoglycans (33, 36), subsequent steps in the pathway are not understood. Glycoproteins B, D, and HL of HSV-1 are all required for entry, but the means by which they interact with cellular components is unknown. The presence of an RGD motif in the primary amino acid sequence of gH suggested a mechanism by which gH could mediate virus penetration, and we tested the hypothesis that aspects of HSV entry may resemble adenovirus infection, where a high-affinity attachment to cell surfaces by the fiber protein is followed by internalization mediated by penton base-integrin interactions. A recombinant virus in which the potential integrin-binding capability of gH was abolished by mutating RGD to RGE bound to and entered cells with wild-type kinetics, implying that HSV-1 does not infect cells as a result of an interaction between plasma membrane integrin molecules and the RGD-peptide triplet in the external domain of gH.

Having failed to identify functional regions of gH on the basis of sequence conservation and similarities with other viral proteins involved in entry, we generated a panel of clones containing oligonucleotide linkers inserted randomly throughout the gH coding sequence. The effects of each mutation on recognition by conformation-dependent antibodies, on expression of gH at the plasma membrane, and on gH-mediated cell-cell and virus-cell fusion were determined in transient transfection-based assays. The aims of this approach were to identify sites of insertion which affect gH function without causing major structural disruption to the molecule and to identify regions of gH which are components of the epitopes for neutralizing monoclonal antibodies.

We generated and characterized 33 insertion mutants in gH, of which 8 expressed a conformationally abnormal molecule and failed to complement a gH-negative virus in functional assays. The majority of mutants expressed a biologically active protein which was expressed on the cell surface and was recognized both by a pool of monoclonal antibodies and by monoclonal antibody LP11 alone. All these mutants exhibited wild-type phenotypes in both virus entry and syncytium formation assays. However, three mutants (mutants 29, 32, and 33, with insertions at amino acid residues 691/692, 791, and 799, respectively) which expressed an apparently antigenically authentic protein on the plasma membrane were severely if not completely compromised in their ability to complement a gH-negative virus in both fusion and infectivity. These mutant proteins all contain insertions in a hydrophobic stretch spanning 108 amino acids which precedes the predicted transmembrane domain of the gH molecule, as indicated in Fig. 5, suggesting that this region of HSV-1 gH plays a functional role in mediating membrane fusion during cell-cell spread and virus penetration. It remains to be seen whether this region of gH is involved in the fusion process for all herpesviruses, but studies by Liu et al. (25) indicate that a 23-amino-acid region of the C-terminal domain of HHV-6 gH, which is within the region that we have identified, appears to function in cell fusion. Furthermore, the limited sequence conservation between predicted gH proteins of the herpesvirus subfamilies lies towards the C terminus of the molecule. Other domains in HSV-1 gH have also been implicated in the fusion process; certain amino acid residues in the cytoplasmic tail have previously been shown to be required for efficient polykaryocyte production and virus entry (5, 35). All the linker insertion mutants described in this report which failed to mediate cell-cell fusion were also compromised in virus entry assays. However, comparison of the results observed with mutant 33 with those obtained with mutants 10 and 11 suggests that there are subtle differences in the function of gH at the cell surface and in the virion, as proposed earlier by Gompels et al. from studies with LP11-resistant viruses (15).

An additional group of mutants (mutants 4, 8, and 10 through 13) expressed a protein which reached the plasma membrane and reacted with a mixture of monoclonal antibodies but which was not recognized by LP11. These mutants contain insertions at amino acids 201, 300, 313, 316/317, 325/326, and 326, and their locations are indicated in Fig. 5. It is

likely that these sites of insertion represent domains of gH which contribute directly or indirectly to the LP11 epitope. Studies by Gompels et al. (15), who sequenced the gH genes of a group of LP11-resistant viruses, identified mutations at three sites in gH (amino acids 86, 168, and 329), all of which were proposed to contribute to the conformationally complex LP11 epitope. This report identifies additional regions in the external domain of gH which may also contribute to this epitope, and we note that five of the linker insertion mutants in this group (mutants 8 and 10 through 13) map in close proximity to, i.e., within 29 amino acids of, the site III region described by Gompels et al. (15). Two of the mutant proteins which fail to react with LP11 (mutants 10 and 11, with insertions at amino acids 313 and 316/317, respectively) showed significant deficiencies in both cell fusion and virus entry assays, while the other LP11-resistant mutants appeared fully biologically active, as observed for the LP11-resistant mutants analyzed by Gompels et al. (15). The phenotypes of recombinant viruses which express mutant gH molecules which are not recognized by LP11 or which fail to mediate membrane fusion will enable further analysis of these potentially functional domains of HSV-1 glycoprotein H. None of the mutant gH molecules generated in this study yielded information concerning likely sites of interaction with gL; we did not obtain any mutants which expressed a protein which reacts with monoclonal antibody 52S, by immunofluorescence, yet which fails to reach the plasma membrane and to react with LP11 or 53S. However, although (as shown in Fig. 3) the 33 sites of linker insertion are distributed fairly evenly throughout the gH gene, we have no mutants with insertions in the sequence which encodes the first 100 amino acids of gH, and it has been reported recently by Anderson et al. (1) that the N terminus of HHV-6 gH contains a 230-amino-acid domain which is required for interaction with gL. Further mutagenesis of gH will be required to determine whether sequences in the N-terminal portion are involved in gH-gL interactions in HSV-1. Finally, we have not yet investigated the possibility that the mutations which affect the fusion properties of gH do so by influencing the amounts of glycoprotein which are incorporated into cellular and viral membranes. Analysis of the gH content of recombinant virions which express these mutant gH molecules will permit such distinctions to be made between quantitative and qualitative effects on the role of gH in membrane fusion.

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