Mutations in the Cytoplasmic Tail of Herpes Simplex Virus Glycoprotein H Suppress Cell Fusion by a Syncytial Strain

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We have developed a complementation assay, using transiently transfected COS cells, to facilitate a molecular analysis of the herpes simplex virus type 1 glycoprotein gH. When infected by a gH-null syncytial virus, COS cells expressing wild-type gH generate infectious progeny virions and form a syncytium with neighboring cells. By deletion and point mutagenesis, we have found particular residues in the gH cytoplasmic tail to be essential for generation of a syncytium but apparently dispensable for production of infectious virions. This study emphasizes the different requirements for cell-cell and cell-envelope fusion and demonstrates that changes in the non-syn locus UL22-gH can reverse the syncytial phenotype.

Herpes simplex virus type 1 (HSV-1) infection follows fusion between the viral envelope and the cell plasma membrane (PM), or endosomal membrane. The HSV-1 envelope contains at least a dozen different membrane proteins, about 10 of which are known to be glycosylated, and virus entry is absolutely dependent on the activity of glycoproteins gB, gD, gH, and gL. The products of UL53 (gK; see reference 23), UL25, and an unknown number of other genes may also be required. HSV-1 is believed also to be capable of direct spread from cell to cell via intercellular junctions created by local fusion events between the PMs of infected and uninfected cells. More extensive cell fusion, resulting in polykaryocyte formation, occurs when cells are infected with syncytial (*syn*) mutants, which arise following repeated passage of virus in tissue culture (for a comprehensive overview, see reference 43).

At least four different *syn* loci have been defined; UL20 (2, 30), UL24 (24, 42), UL53-gK (3, 22, 31, 37, 38), and UL27-gB (7, 8, 15). It is unclear how such mutations result in formation of syncytia; however, at least some of the UL20 and UL24 *syn* mutations must result in failure to express the corresponding protein. These gene products are therefore not required for cell-cell fusion but may normally serve to down regulate it. In contrast, deletion studies suggest that UL27-gB is required for cell-cell fusion, as it is for envelope-PM fusion. Syncytial alleles of UL27 generate gB proteins with an altered cytoplasmic tail (15, 20) and range from alanine-to-valine substitutions (44) to severe truncations (1). Such amino acid substitutions or truncations presumably allow gB to participate in cell-cell fusion even in the presence of the UL20 and UL24 products. As *syn* mutations within UL53 are limited to missense mutations, gK, like gB, may be essential for syncytium formation.

Gene deletion and antibody inhibition studies have suggested that UL22-gH (18) and US6-gD (28, 36) are also essential for cell-cell fusion. However, no mutations conferring a syncytial phenotype have been described within either of these genes (a *syn* mutation initially assigned to UL1 has more recently been found to reside within UL53 [29, 41]). Thus, although there appears to be a common gB-gD-gH-gL-depen-

dent apparatus for cell-cell and cell-envelope fusion, it remains unclear how fusion occurs, how it is controlled, and to what extent the various fusion-catalyzing glycoproteins contribute to regulation.

Glycoprotein H is an approximately 110-kDa protein, predicted to have an N-terminal signal sequence and a single membrane-spanning hydrophobic region close to the C terminus (6, 11, 13, 14, 18). The amino-terminal bulk of gH is likely to reside on the outside of the cell or virus since this region contains all the consensus sites for N-linked glycosylation and all of the mutations which confer resistance to anti-gH neutralizing antibodies (16). In this orientation, a 14-amino-acid C-terminal tail (KVLRTSVPPFFWRRE in single-letter amino acid code) projects towards the cell cytoplasm or viral capsid. The external domain of gH associates with the 25-kDa glycoprotein gL (21), and assembly of the gH-gL complex is apparently essential for cell surface trafficking and envelope incorporation of both proteins (12, 19, 39, 41).

We have developed a transient cell expression assay to facilitate rapid molecular analysis of gH and have begun to investigate which regions of the protein are required for the various fusion events in which it participates. Surprisingly, modification of the cytoplasmic tail of gH almost completely abolishes the ability of gH to support syncytium formation by a UL27-gB *syn* mutant but has little effect on the yield of progeny virus or the rate at which mutant gH-containing envelopes fuse with cells.

MATERIALS AND METHODS

Viruses and cells. Vero, F6, and COS7 cells were grown in DMEM-FCS-PS: Dulbecco's modified Eagle's medium (GIBCO Laboratories) supplemented with 10% fetal calf serum (HyClone) and 1% penicillin-streptomycin (GIBCO). F6 cells are a stably transformed Vero cell line which express HSV-1 gH under the control of the HSV-1 gD promoter (13). HSV-1 gH⁻gB_{ANG} is a derivative of SC16 which carries a *syn* mutation in UL27 (44), resulting in an alanine-to-valine substitution in residue 855 of gB, and in which gH coding sequences have been deleted and replaced by the *Escherichia coli lacZ* gene. Construction of this virus will be reported elsewhere. Stocks of virus were prepared by low-multiplicity passage on F6 cells, and titers were determined by plaque assay on F6 cells.

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Transfection. COS7 cells were seeded into six-well plates to approximately 30% confluence (about 10^5 cells per well), grown for about 18 h, washed with prewarmed DMEM lacking serum, and then overlaid with 0.4 ml of prewarmed transfection mixture (34) containing 0.2 ml of Optimem (GIBCO)–0.2 ml of 1-mg/ml DEAE-Dextran (Pharmacia) in Tris-buffered saline (140 mM NaCl, 3 mM KCl, 25 mM Tris-Cl [pH 7.4]) and 1 μ g of plasmid DNA. Plasmid DNA was omitted when mock transfections were carried out. After 30 min at 37°C, the mixture was removed and cells were overlaid with 3 ml of DMEM-FCS-PS containing 100 μ g of chloroquine per ml. After 3 to 4 h at 37°C, this overlay was replaced by DMEM-FCS-PS and cells were incubated for 16 h prior to infection.

Transient expression assays. Transfected cells were infected with 2×10^6 PFU of HSV-1 gH[−]gB_{ANG} in 0.2 ml of DMEM-FCS-PS for 1 h at 37°C and then (with chilled solutions at all times) washed in phosphate-buffered saline (PBS), treated for 1 min with 100 mM glycine (pH 3.0) or 135 mM NaCl–10 mM KCl–40 mM citric acid (pH 3.0) (to inactivate unpenetrated virus), and returned to neutral pH by being washed with PBS and then a 1:1 mixture of PBS-DMEM. To screen for syncytial or nonsyncytial phenotypes, the cells were then overlaid with 2×10^6 Vero cells. For quantitative analysis, the washed COS7 cells were trypsinized, suspended, and then titrated onto preformed F6 or Vero monolayers. In both cases, after approximately 16 h (Vero platings) or 36 to 48 h (F6 platings) monolayers were fixed, permeabilized, stained to detect β -galactosidase expression, and counterstained with neutral red as previously described (13). To determine yields of infectious virus, a portion of the trypsinized, washed COS cells was replated into a tissue culture dish and incubated for a further 16 h to allow viral replication. The cells were subsequently collected by scraping, and a sonicated extract was titrated onto preformed F6 monolayers.

Rate of virus entry. A known number of PFU were incubated with preformed F6 monolayers for 2 h at 4°C to allow adsorption. At various times after being shifted to 37°C, unpenetrated virus was inactivated by acid treatment (as described in "Transient expression assays"), incubation was continued for 36 to 48 h, and plaques were scored. Total plaque number (100% penetration) was determined from samples which were not acid washed.

Western blotting (immunoblotting). Cell extracts were boiled in Laemmli buffer and then subjected to denaturing electrophoresis with a 7.5% polyacrylamide gel (27). Proteins were transferred to nitrocellulose at 125 mA for 1 h with a Hoefer semidry blotter and a transfer buffer identical to gel electrophoresis buffer but supplemented with 25% methanol. The filter was incubated for 1 h in blocking buffer (PBS–0.1% Tween 20–3% dried milk) and then for 2 h with a 1:2,000 dilution of rabbit anti-gH polyclonal serum (prepared as previously described [11]) in blocking buffer and washed for 20 min with four changes of PBS–0.1% Tween 20. After incubation with peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) diluted 1:4,000 in blocking buffer, the filter was washed as before, treated for 1 min with enhanced chemiluminescence reagent (Amersham), and exposed to Fuji X-ray film for between 1 and 30 s.

Plasmids and UL22 mutagenesis. Plasmid pgHlmpSC11 (5) was digested to release the HFEM gH gene as a 3.5-kb *Bgl*II fragment. Sticky ends were blunted with the Klenow fragment of DNA polymerase, and the DNA was ligated with *Eco*RV-digested plasmid expression vector SMH3 to yield the gH expression plasmid SMHgH1. Plasmid GEMH0 was constructed by ligating *Eco*RV-*Nsi*I-digested plasmid GEM5ZF(+) (Promega) with an *Xma*I-*Nsi*I synthetic linker (see below) and an

*Eco*RV-*Xma*I UL22 fragment derived from SMHgH1 and corresponding to nucleotides 43974 (*Sma*I-*Xma*I site) to 44247 (*Eco*RV site) in the GenBank HE1CG sequence file of HSV-1 strain 17. This region of UL22 encodes gH residues 1-713 to P-803 (single-letter amino acid code). The linker was prepared by annealing the complementary oligonucleotides 5' CCGG GATCGAATTCAGCATGCA and 5' TGCTGAATTCGATC and contains an internal *Eco*RI cleavage site to aid in identification of recombinants.

Modified UL22 genes were prepared by replacing the 3' end of UL22 in SMHgH1 with fragments generated by PCR with the oligonucleotide primers 5' GCCGCAATTGCGCCCGGG (a perfect match to the region of UL22 which includes the *Sma*I-*Xma*I site at position 43974) and a mutagenic primer. All mutagenic oligonucleotide primers begin with the sequence 5' GCGCATGCATGTCTAGAGAATTCCTATTA to append *Nsi*I, *Xba*I, and *Eco*RI sites to the end of the PCR fragment and encode two in-frame UL22 stop codons. For C-terminal deletion of gH, mutagenic primers continued CGGGACACT TGTCCGGAGAACCTTTAG or TGTCCGGAGAACCTT TAGGATGCCAGC, modifying the 3' end of UL22 to encode KVLRTSVP or KVLRT, respectively. For combined deletion and mutagenesis, primers continued with a derivative of the sequence CCGGACACTTGTCCGGAGAACCTTTAGGAT GCCAGCCAGGGCGGC (corresponding to the region of

FIG. 1. Plasmids for expression of wild-type and mutant gH proteins (not to scale). Plasmid SMHgH1 (top) contains the HFEM UL22 gene and 3' UTR flanked by the adenovirus major late promoter (pointed black bar) and HSV thymidine kinase 3' UTR (black rectangle). Positions and priming directions of mutagenic PCR oligonucleotides are shown by small stippled bars and arrowheads. The lower part of the figure indicates the structure of all mutant UL22 expression plasmids. The region of UL22 synthesized by PCR is indicated by heavy cross-hatching, and the region contributed by plasmid GEMH0 (see Materials and Methods) is indicated by light cross-hatching. Stop codons and relevant restriction sites are indicated.

TABLE 1. UL22 deletions and mutations

Name of gH derivative	Sequence of cytoplasmic tail
gH wild type.....	KVLRTSVPPFFWRRE
gHSVP.....	KVLRTSVP
gHΔSVP.....	KVLRT
gHSVA.....	KVLRTSVA
gHSAP.....	KVLRTSAP
gHAVP.....	KVLRTAVP

UL22 encoding gH residues A-818 to P-832), except that the underlined region was CGCGACACT, CGGGGCACT, or CGGGACAGC (encoding KVLRTSVA, KVLRTSAP, and KVLRTAVP, respectively). PCRs contained 20 pmol of each primer, 20 ng of SMHgH1 template DNA, 1 mM (each) deoxynucleoside triphosphate, and 0.5 U of *Taq* DNA polymerase (Cetus) in the manufacturer's recommended buffer in a final volume of 25 μ l. Reaction mixtures were overlaid with mineral oil; subjected to 25 cycles of 94°C for 0.5 min, 55°C for 1 min, and 72°C for 0.5 min; and chased for 5 min at 72°C, and then the products were gel purified and used to replace the corresponding region of wild-type UL22 in one of two ways. (a) PCR products were cut with *Nsi*I and *Xma*I and then ligated with *Nsi*I-*Xma*I-digested GEMH0, replacing the oligonucleotide linker and reconstructing the UL22 reading frame from the I-713 codon onwards. This UL22 region was released with

*Bst*XI and *Xba*I and then ligated with *Bst*XI-*Xba*I-digested SMHgH1. (b) PCR products were digested with *Xba*I and *Xma*I and then ligated with *Bst*XI-*Xba*I-digested SMHgH1 and the *Bst*XI-*Xma*I UL22 fragment from GEMH0. Both cloning strategies recreated a full-length mutated UL22 gene within the SMH3 vector. All PCR-derived regions were checked by DNA sequence analysis.

RESULTS

A *trans* complementation assay for wild-type and mutant gH proteins. To facilitate molecular analysis of gH, we developed a *trans* complementation assay, analogous to that described for study of other HSV glycoproteins (9, 10), in which gH and recombinant derivatives are expressed from a transfected plasmid and gH-null virions provide all other gene products. We chose COS7 monkey fibroblasts as the host cell, since they can easily be transfected to a relatively high frequency (20 to 70%). Plasmid SMHgH1 was constructed to express the UL22 open reading frame from the adenovirus major late promoter (Fig. 1). In SMHgH1, the UL22 gene retains about 1.0 kb of downstream DNA sequence, including all of the UL22 3' untranslated region (UTR) and polyadenylation signal.

We began our analysis of gH functional domains by testing the role of the short cytoplasmic tail in fusion. Using PCR and mutagenic oligonucleotide primers, we amplified modified versions of the 3' end of UL22 from SMHgH1. The PCR

FIG. 2. Result of seeding a confluent Vero monolayer onto transfected-infected COS fibroblasts. COS cells were mock transfected with no DNA (A) or with plasmids designed to express wild-type gH (B), gHSVP (C), or gHΔSVP (D). Infected cells and syncytia were stained for β -galactosidase expression (dark regions). For gHΔSVP, very small *syn*-like regions or examples of apparent viral spread to neighboring Vero cells were occasionally observed (e.g., in the stained cells immediately left of center in panel D). This was never observed in mock-transfected controls.

FIG. 3. Quantitative measurements of ICs, virus yield, and syncytium formation for the gH mutants. COS cells were transfected to express each of the forms of gH tested in Fig. 2 or were mock transfected as indicated below each graph. Cells from two wells of a six-well plate were pooled and processed as follows. (A) Cells were titrated onto F6 monolayers to determine the number of productively infected cells (ICs). Numbers are expressed as thousands of ICs per well of the six-well plate. (B) Cells were seeded into a tissue culture dish, and total virus yield was determined after 16 h. PFU are normalized to ICs. (C) Cells were titrated onto Vero cells to determine the number of individual syncytia formed. Results are expressed as the number of *syn* formed by each IC. Values reported are the means of data from duplicate transfection experiments.

product was then used to replace the corresponding wild-type region of UL22 in SMHGH1 (Fig. 1). In all recombinant UL22 expression plasmids, the 3' UTR was derived from the HSV-1 thymidine kinase gene rather than from UL22 itself; however, similar results were obtained for wild-type UL22 when followed by either its own 3' UTR or that of thymidine kinase (data not shown). UL22 deletions and mutations described in this study are listed in Table 1.

Healthy, subconfluent COS cells were transfected with gH expression plasmids by the DEAE-Dextran method and, after an overnight incubation, were infected with a gH⁻gB_{ANG} derivative of HSV-1 SC16 at a multiplicity of infection of 10 (the gH⁻gB_{ANG} virus was grown in F6 cells, able to provide wild-type gH protein in *trans*), and then unpenetrated virus was inactivated by acid treatment. To test for the ability of wild-type or mutant gH molecules to support syncytium formation, the COS cells were then overlaid with a sufficient number of Vero cells to generate a confluent monolayer, and incubation continued overnight. If the mutant gH expressed by the transfected cells is able to complement the gH deficiency in the infecting syncytial virus, those cells would be capable of fusing with surrounding Vero cells and giving rise to a syncytium.

A region of the cytoplasmic tail of gH is required to permit syncytium formation. COS-Vero monolayers were fixed and then stained for regions of LacZ expression to visualize infected cells and syncytia. Results are shown in Fig. 2. As expected, mock-transfected COS cells did not support syncytium formation (panel A) whilst cells expressing wild-type gH did (panel B). Panel C shows that deletion of the cytoplasmic tail to KVLRTSVP only slightly diminished the extent of syncytium formation (similar results were observed when the cytoplasmic tail was deleted to KVLRTSVPFFW [data not shown]). Strikingly, deletion of three further amino acid residues, leaving the sequence KVLRT, almost completely abolished syncytium formation (panel D).

To properly quantitate this effect, and to further investigate the properties of the mutated gH molecules, we prepared transfected-infected COS cells as before but then trypsinized them, prepared a suspension, and processed it in several ways. One aliquot was titrated onto preformed monolayers of F6 Vero cells, which provide wild-type gH in *trans*. Spread of even genotypically gH⁻ virions from COS to F6 thus results in a plaque and makes it possible to count the number of COS infectious centers (ICs) (i.e., those cells which had been productively infected and transfected). Figure 3A shows that

there were only slight variations (less than twofold) in the number of productively infected COS cells for each form of gH, demonstrating that each is able to mediate spread of infection from COS to F6 cells.

It was not possible to determine from the above experiment whether the mechanism or efficiency of viral spread was different for each of the forms of gH. We therefore next examined whether any of the gH mutants were defective in supporting the production of progeny virions. After seeding an aliquot of the COS cell suspension into a tissue culture dish, we allowed virus replication to proceed for 16 h and then titrated a sonicated cell extract onto F6 cell monolayers and scored plaque numbers. To take account of possible variations in the efficiency with which transfected-infected cells were recovered and replated after trypsinization, yield of progeny virus was

FIG. 4. Rate of entry experiment for progeny virions bearing mutant forms of gH. Progeny virions were prepared from the transient expression assay, equal numbers of PFU were adsorbed to F6 monolayers in the cold, and then infection was allowed to proceed at 37°C for various lengths of time (horizontal axis) before acid inactivation of unpenetrated virus. The vertical axis shows the number of plaques scored, expressed as a percentage of the number of PFU initially adsorbed (determined from non-acid-washed controls). Samples and the plaque number representing 100% penetration are as follows: gH (149 plaques) (solid circles), gHSVP (163 plaques) (solid squares), and gHΔSVP (117 plaques) (open triangles). All values are the means derived from duplicate experiments.

FIG. 5. Quantitation of gH derivatives subjected to alanine point mutagenesis in the SVP region. Measurements of ICs (A), virus yield (B), and syncytium formation (D) were as described in the legend to Fig. 3. The forms of gH tested were gHSVP, gHSVA, gHSAP, and gHAVP or a no-DNA control, as indicated below each graph. Rate of

normalized to the number of productive cells resulting from each transfection experiment. Figure 3B demonstrates that similar yields of infectious virus were generated by each productive cell for all the forms of gH tested.

Finally, to quantitate the frequency of syncytium formation we titrated the COS suspension onto preformed Vero cell monolayers to a sufficiently low density to count the number of individual syncytia formed. The results, again normalized to numbers of productive cells, are shown in Fig. 3C. As suggested by the results of the qualitative assay, gHSVP is greatly reduced in its ability to generate syncytia: wild-type gH and gHSVP expression supported generation of one syncytium for every three to five productively infected cells; however, only 1 in 200 gHSVP-expressing productive cells generated a clear syncytium.

Given that deletion of the SVP sequence had such a dramatic effect upon cell-cell fusion, we were surprised to observe only slight effects on virus yield (Fig. 3B). However, a limitation of these assays was that unpenetrated virus was not inactivated at the end of the 1-h infection period; even slowly penetrating virions would therefore have eventually given rise to plaques. We reasoned that if gHSVP was defective in fusion, we might observe differences between it and gHSVP or wild-type gH by examining the rate at which mutant gH-containing virions were able to penetrate the PM of target cells. To test this, we allowed virions containing various forms of gH to adsorb to F6 monolayers for 2 h at 4°C and then shifted them to 37°C for varying lengths of time before acid inactivation of unpenetrated virus. The results are shown in Fig. 4. The rate of entry of virions bearing gHSVP in their envelopes is only slightly lower than that of those bearing gHSVP or wild-type gH and suggests that gHSVP is not seriously compromised in catalysis of envelope-PM fusion.

Alanine mutagenesis of the SVP sequence. Mutant proteins gHSVP and gHSVP differ in both the sequence and the length of their cytoplasmic tail. To test which of these characteristics is important for syncytium formation, we constructed plasmids to express gH mutants bearing an alanine residue in place of the residue serine, valine, or proline (Table 1). These plasmids were transfected into COS cells and assayed as before for numbers of ICs, yield of progeny virus, rate of cell penetration, and *syn*-forming ability. The results are summarized in Fig. 5. As expected, substitution of any of the three terminal residues of gHSVP by alanine has little effect upon the number of ICs generated (panel A), the normalized yield of progeny virus (panel B), or rate of penetration during infection (panel C). However, unlike gH molecules terminating with SVP, SVA, or AVP, gHSAP is severely compromised in its ability to support syncytium formation (panel D): the number of productive cells able to form a syncytium falls from at least 1 in 8 (for gHSVP) to 1 in 120 for gHSAP. Figure 6 shows a field of the syncytia resulting from attachment of transfected-infected COS cells to Vero monolayers. We conclude that the gH cytoplasmic tail residue valine 831 is important for syncytium formation, at least when residues downstream of SVP are absent.

Expression of mutant gH molecules. We tested whether the steady-state levels of mutant gH proteins might differ. Trans-

entry of progeny virions (C) was determined as described in the legend to Fig. 4. In panel C, samples and the plaque numbers representing 100% penetration are as follows: gHSVP (442 plaques) (solid squares), gHSVA (474 plaques) (open triangles), gHSAP (302 plaques) (open circles), and gHAVP (402 plaques) (solid circles).

FIG. 6. Result of titrating transfected-infected COS fibroblasts onto Vero monolayers and visualizing as described in the legend to Fig. 2. COS cells were transfected with plasmids to express gH or gH derivatives as follows. (A) Mock transfection; (B) wild-type gH; (C) gHSVP; (D) gH Δ SVP; (E) gHSVA; (F) gHSAP; (G) gHAVP. For gH Δ SVP and gHSAP, very small *syn*-like regions or spread of virus to neighboring cells was occasionally observed, but syncytia resembling those in panels B, C, E, and G were never seen.

FIG. 7. Western blot of transfected-infected COS cells. Extracts were subjected to SDS-polyacrylamide gel electrophoresis, blotted to nitrocellulose, and probed with a rabbit anti-gH polyclonal antiserum. Extracts were from HSV-1 strain SC16-infected baby hamster kidney (BHK) cells (lane 1) or COS cells expressing no gH (lane 2), gHSVP (lane 3), gHSVA (lane 4), gHSAP (lane 5), or gHAVP (lane 6). The previously observed gH-related band is indicated by the lower arrow on the right of the figure. COS-specific forms of gH which run between the forms observed in BHK cells are indicated by the upper right-hand arrow. Arrowheads on the left of the picture represent the location of prestained molecular mass markers (Bethesda Research Laboratories) with masses of 105, 70, and 43 kDa.

fectected-infected cell extracts expressing gHSVP, gHSVA, gHSAP, or gHAVP, and containing between 1.9×10^6 and 2.6×10^6 PFU, were boiled in Laemmli buffer and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and then Western blotted and probed with an anti-gH polyclonal serum (Fig. 7). The forms of gH observed in all plasmid-containing samples resemble those detected by Gompels and Minson (19), including a band antigenically related to gH but migrating at approximately 85 kDa (lower arrow). Although appearance of this band is clearly dependent upon the presence of the gH gene (since it is absent in lane 2), its origin is unknown. Higher-molecular-weight forms of gH (indicated by the upper arrow), which run midway between the most abundant forms of gH expressed in BHK cells, are also visible, as previously shown (19). The amount of matured gHSAP polypeptide may be slightly reduced compared with the other forms of gH, but there are no gross differences in either the abundance or the molecular weight of each gH polypeptide.

DISCUSSION

In principle, syncytial strains of HSV-1 can be restored to a nonsyncytial phenotype by reversion of their *syn* allele or by complementation with sufficient dosage of the wild-type allele (23). Our studies suggest that the syncytial phenotype can also be suppressed by changes within a non-*syn* locus, even though other aspects of virus infectivity may be unaltered.

It is unclear why changes within the cytoplasmic tail of gH should affect its role in fusion. One possibility is that the tail may mediate multimerization of gH, interaction of gH with the cytoplasmic tails of other membrane proteins, or gH association with soluble proteins. Although gH has not previously been shown to multimerize or bind proteins other than gL, such interactions could be transient or unstable under the experimental conditions used. Alternatively, tail regions close to the membrane may interact with viral envelope or PM lipids, perturbing the inner leaflet of the bilayer and contributing to fusion. In this regard, it is interesting that, despite limited primary sequence identity, the gH proteins encoded by the

alphaherpesviruses HSV-1 and -2 (4), equine herpesviruses 1 and 4 (35, 40), bovine rhinotracheitis virus type 1 (33), varicella-zoster virus (25, 32), and pseudorabies virus (26) all contain two neutral, nonaromatic amino acids (ML or VL) in the most membrane-proximal region of their cytoplasmic tail. Moreover, all have a valine residue at a position equivalent to valine 831 in HSV-1, with the exception of varicella-zoster virus and pseudorabies virus, in which this residue is a serine. The corresponding gH regions in nonalphaherpesviruses are more diverged but do commonly contain hydrophobic or aromatic residues (see reference 17).

When downstream sequences are deleted, the motif SVP (and in particular the central residue, valine 831) appears to become essential for syncytium formation. It may appear surprising that deleting the SVP motif, or mutating valine 831 to the chemically similar amino acid alanine, should have such profound effects. However, similar changes in HSV-1 fusion glycoproteins are known to be sufficient to alter the syncytial phenotype: alanine-to-valine mutations convert nonsyncytial alleles of both UL53-gK (38) and UL27-gB (44) to syncytial ones. It is important to note, however, that in none of these cases were other regions of the gene product simultaneously deleted. It will be interesting to determine the effect of mutating valine 831 to alanine in the context of the full-length gH tail and of substituting structurally similar amino acids such as leucine at this position.

Why should a mutant gH molecule be much poorer at supporting one form of fusion than another? An obvious possibility is that cell-cell fusion and envelope-PM fusion require gH to perform different tasks and that these depend upon different residues in the gH tail. It is not unreasonable to suggest that these two fusion processes may have different requirements, since viral envelopes and cell surfaces differ in their degree of curvature and likely also in their lipid, cholesterol, and protein composition. It will be interesting to test whether nonsyncytial virions bearing gHΔSVP or gHSAP are diminished in their ability to spread directly from cell to cell (a process believed to occur by formation of limited fusion junctions between apposed PMs) or in their ability to induce polykaryocyte formation *in vivo*.

An alternative explanation for the observed results is that the ΔSVP and SAP mutations result in a generally defective or unstable gH polypeptide. There would then be insufficient active gH on the PM to allow syncytia to form but presumably enough to generate functional progeny: perhaps virions require relatively few active gH molecules in their envelopes to efficiently penetrate cell membranes. There were no apparent gross differences in the total steady-state levels of each gH polypeptide expressed in these cells, but we cannot exclude the possibility that the cell surface level of gHSAP is below some threshold critical for cell-cell fusion to occur. PM gH could also fall below this threshold level if the cytoplasmic tail mutations interfered with its intracellular trafficking, reducing the amount of gHΔSVP or gHSAP delivered to the cell surface. Recruitment of active gH into viral envelopes (believed to be derived from internal membranes) would be unaffected, leading to normal levels of fully infectious progeny. Although fluorescence immunocytochemistry using anti-gH antibodies suggested that all forms of gH reached the cell surface (data not shown), these studies were nonquantitative; a proper test of this possibility will require both quantitative cell-surface immunocytochemical studies and detailed examination of the rate of maturation of each recombinant form of gH.

This complementation assay will greatly facilitate a molecular analysis of gH and enable us to rapidly define those regions

of the molecule important for cell-cell and virus-cell fusion. It will also be interesting to test whether changes within gH can inhibit syncytial phenotypes resulting from *syn* mutations other than gB_{ANG} and to what extent. Different degrees of suppression might make it possible to group *syn* mutations in a particular locus into weak or strong alleles and also help distinguish among the roles of different *syn* loci in generation of the syncytial phenotype.

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REFERENCES

1. Baghian, A., L. Huang, S. Newman, S. Jayachandra, and K. G. Kousoulas. 1993. Truncation of the carboxy-terminal 28 amino acids of glycoprotein B specified by herpes simplex virus type 1 mutant *amb1511-7* causes extensive cell fusion. *J. Virol.* **67**:2396–2401.
2. Baines, J. D., P. L. Ward, G. Campadelli-Fiume, and B. Roizman. 1991. The UL20 gene of herpes simplex virus 1 encodes a function necessary for viral egress. *J. Virol.* **65**:6414–6424.
3. Bond, V. C., and S. Person. 1984. Fine structure physical map locations of alterations that affect cell fusion in herpes simplex virus type 1. *Virology* **132**:368–376.
4. Boursnell, M. (Cantab Pharmaceuticals Research Limited). 1994. Personal communication.
5. Browne, H., V. Baxter, and A. C. Minson. 1993. Analysis of the protective immune responses to the glycoprotein H-glycoprotein L complex of herpes simplex virus type 1. *J. Gen. Virol.* **74**:2813–2817.
6. Buckmaster, E. A., U. Gompels, and A. C. Minson. 1984. Characterization and physical mapping of an HSV-1 glycoprotein of approximately 115×10^3 M.W. *Virology* **139**:408–413.
7. Bzik, D. J., B. A. Fox, N. A. Deluca, and S. Person. 1984. Nucleotide sequence of a region of the herpes simplex virus type 1 gB glycoprotein gene: mutations affecting rate of virus entry and cell fusion. *Virology* **137**:185–190.
8. Cai, W., B. Gu, and S. Person. 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. *J. Virol.* **62**:2596–2604.
9. Cai, W., S. Person, C. Debroy, and B. Gu. 1988. Functional regions and structural features of the gB glycoprotein of herpes simplex virus type 1: an analysis of linker insertion mutants. *J. Mol. Biol.* **201**:575–588.
10. Chiang, H.-Y., G. H. Cohen, and R. J. Eisenberg. 1994. Identification of functional regions of herpes simplex virus glycoprotein gD by using linker-insertion mutagenesis. *J. Virol.* **68**:2529–2543.
11. Desai, P. J., P. A. Schaffer, and A. C. Minson. 1988. Excretion of noninfectious particles lacking glycoprotein H by a temperature sensitive mutant of herpes simplex type 1: evidence that gH is essential for virion infectivity. *J. Gen. Virol.* **69**:1147–1156.
12. Foa-Tomasi, L., E. Avitable, A. Boscaro, R. Brandimarti, R. Gualandri, R. Manservigi, F. Dall'Olio, F. Serafini-Cessi, and G. Campadelli-Fiume. 1991. Herpes simplex virus (HSV) glycoprotein H is partially processed in a cell line that expresses the glycoprotein and fully processed in cells infected with deletion or ts mutants in the known HSV glycoproteins. *Virology* **180**:474–482.
13. Forrester, A., H. Farrell, G. Wilkinson, J. Kaye, N. Davis-Poynter, and A. C. Minson. 1992. Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. *J. Virol.* **66**:341–348.
14. Fuller, A. O., R. E. Santos, and P. G. Spear. 1989. Neutralizing antibodies specific for glycoprotein H of herpes simplex virus permit viral attachment to cells but prevent penetration. *J. Virol.* **63**:3435–3443.
15. Gage, P. J., M. Levine, and J. C. Glorioso. 1993. Syncytium-inducing mutations localize to two discrete regions within the cytoplasmic domain of herpes simplex virus type 1 glycoprotein B. *J. Virol.* **67**:2191–2201.
16. Gompels, U. A., A. L. Carss, C. Saxby, D. C. Hancock, A. Forrester, and A. C. Minson. 1991. Characterization and sequence analysis of antibody-selected antigenic variants of herpes simplex virus show a conformationally complex epitope on glycoprotein H. *J. Virol.* **65**:2393–2401.
17. Gompels, U. A., M. A. Craxton, and R. W. Honess. 1988. Conservation of glycoprotein H (gH) in herpesviruses: nucleotide sequence of the gH gene from herpesvirus saimiri. *J. Gen. Virol.* **69**:2819–2829.
18. Gompels, U. A., and A. C. Minson. 1986. The properties and sequence of glycoprotein H of herpes simplex virus type 1. *Virology* **153**:230–247.
19. Gompels, U. A., and A. C. Minson. 1989. Antigenic properties and cellular localization of herpes simplex virus glycoprotein H synthesized in a mammalian cell experimental system. *J. Virol.* **63**:4744–4755.
20. Huff, V., W. Cai, J. C. Glorioso, and M. Levine. 1988. The carboxy-terminal 41 amino acids of herpes simplex virus type 1 glycoprotein B are not essential for production of infectious virus particles. *J. Virol.* **62**:4403–4406.
21. Hutchinson, L., H. Browne, V. Wargent, N. Davis-Poynter, S. Primorac, K. Goldsmith, A. C. Minson, and D. C. Johnson. 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.
22. Hutchinson, L., K. Goldsmith, D. Snoddy, H. Ghosh, F. L. Graham, and D. C. Johnson. 1992. Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion. *J. Virol.* **66**:5603–5609.
23. Hutchinson, L., F. L. Graham, W. Cai, C. Debroy, S. Person, and D. C. Johnson. 1993. Herpes simplex virus (HSV) glycoproteins B and K inhibit cell fusion induced by HSV syncytial mutants. *Virology* **196**:514–531.
24. Jacobson, J. G., S. L. Martin, and D. M. Coen. 1989. A conserved open reading frame that overlaps the herpes simplex virus thymidine kinase gene is important for viral growth in cell culture. *J. Virol.* **63**:1839–1843.
25. Keller, P. M., A. J. Davison, R. S. Lowe, M. W. Rieman, and R. W. Ellis. 1987. Identification and sequence of the gene encoding gpIII, a major glycoprotein of varicella zoster virus. *Virology* **157**:526–533.
26. Klupp, B. G., and T. C. Mettenleiter. 1991. Sequence and expression of the glycoprotein gH gene of pseudorabies virus. *Virology* **182**:732–741.
27. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
28. Ligas, M. W., and D. C. Johnson. 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β -galactosidase sequences binds to but is unable to penetrate into cells. *J. Virol.* **62**:1486–1494.
29. Little, S. P., and P. A. Schaffer. 1981. Expression of the syncytial (syn) phenotype in HSV-1, strain KOS: genetic and phenotypic studies of mutants in two *syn* loci. *Virology* **112**:686–697.
30. Maclean, C. A., S. Efsthathiou, M. L. Elliot, F. E. Jamieson, and D. J. McGeoch. 1991. Investigation of herpes simplex virus type 1 genes encoding multiply inserted membrane proteins. *J. Gen. Virol.* **72**:897–906.
31. McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor. 1988. The complete sequence of the long unique region in the genome of herpes simplex virus type 1. *J. Gen. Virol.* **69**:1531–1574.
32. McGeoch, D. J., and A. J. Davison. 1986. DNA sequence of the herpes simplex virus type 1 gene encoding glycoprotein H and identification of homologues in the genomes of varicella-zoster

- virus and Epstein-Barr virus. *Nucleic Acids Res.* **14**:4281-4291.
33. Meyer, A. L., E. A. Petrovskis, W. P. Duffus, D. R. Thomsen, and L. E. Post. 1991. Cloning and sequence of an infectious bovine rhinotracheitis virus (BHV-1) gene homologous to glycoprotein H of herpes simplex virus. *Biochim. Biophys. Acta* **1090**:267-269.
 34. Munro, S., and H. R. B. Pelham. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell* **48**:899-907.
 35. Nicolson, L., A. Cullinane, and D. Onions. 1990. The nucleotide sequence of an equine herpesvirus 4 gene homologous to the herpes simplex virus type 1 glycoprotein H gene. *J. Gen. Virol.* **71**:1793-1800.
 36. Noble, A. G., G. T.-Y. Lee, R. Sprague, M. L. Parish, and P. G. Spear. 1983. Anti-gD monoclonal antibodies inhibit cell fusion induced by herpes simplex virus type 1. *Virology* **129**:218-224.
 37. Pogue-Geile, K. L., G. T.-Y. Lee, S. K. Shapira, and P. G. Spear. 1984. Fine mapping of mutations in the fusion-inducing MP strain of herpes simplex virus type 1. *Virology* **136**:100-109.
 38. Pogue-Geile, K. L., and P. G. Spear. 1987. The single base pair substitution responsible for the syn phenotype of herpes simplex virus type 1, strain MP. *Virology* **157**:67-74.
 39. Roberts, S. R., M. P. deLeon, G. H. Cohen, and R. J. Eisenberg. 1991. Analysis of the intracellular maturation of herpes simplex virus type 1 glycoprotein gH in infected and transfected cells. *Virology* **184**:609-624.
 40. Robertson, G. R., N. A. Scott, J. M. Miller, M. Sabine, M. Zheng, C. W. Bell, and J. M. Whalley. 1991. Sequence characteristics of a gene in equine herpesvirus 1 homologous to glycoprotein gH of herpes simplex virus. *DNA Sequence* **1**:241-249.
 41. Roop, C., L. Hutchinson, and D. C. Johnson. 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.
 42. Sanders, P. G., N. M. Wilkie, and A. J. Davison. 1982. Thymidine kinase deletion mutants of herpes simplex virus type 1. *J. Gen. Virol.* **63**:277-287.
 43. Spear, P. G. 1992. Membrane fusion induced by herpes simplex virus, p. 201-232. *In* J. Bentz (ed.), *Viral fusion mechanisms*. CRC Press, Inc., Boca Raton, Fla.
 44. Weise, K., H. C. Kaerrner, J. Glorioso, and C. H. Schroder. 1987. Replacement of glycoprotein B gene sequences in herpes simplex virus type 1 strain ANG by corresponding sequences of the strain KOS causes changes in plaque morphology and neuropathogenicity. *J. Gen. Virol.* **68**:1909-1920.