Identification of Functional Domains in Herpes Simplex Virus 2 Glycoprotein B

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Glycoprotein B (gB) is one of four membrane proteins that are essential for the entry of herpes simplex viruses (HSV) into cells, and coexpression of the same combination of proteins in transfected cells results in cell fusion. The latter effect is reminiscent of the ability of virus infection to cause cell fusion, particularly since the degree of fusion is greatly increased by syncytial mutations in gB. Despite intensive efforts with the gB homologs of HSV and other herpesviruses, information about functionally important regions in the 700-amino-acid ectodomain of this protein is very limited at present. This is largely due to the misfolding of the majority of the mutants examined. It was shown previously that the percentage of correctly folded mutants could be increased by targeting only predicted loop regions (i.e., not alpha-helix or beta-strand), and by using this approach new functional domains in HSV-2 gB have now been identified.

MATERIALS AND METHODS

Cells, viruses, and antibodies. COS7 cells were obtained from the American Type Culture Collection and were grown in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum. D6 cells, an HSV-1 gB-expressing line, were obtained from Stanley Person and were grown in Dulbecco modified Eagle medium containing 5% fetal bovine serum and 0.2 mg of G418/ml (6). The gB-minus HSV-1 mutant K082 was obtained from Stanley Person and was propagated in D6 cells as described previously (6). The anti-HSV-2 gB polyclonal antibody R90 and monoclonal antibody (MAb) DL16 were kindly provided by Gary Cohen and Roselyn Eisenberg (1). Anti-gB MAb SB3 has been described previously (24).

Plasmids and mutagenesis. Plasmids pMM245, pMM346, pMM349, and pMM350, which express HSV-2 gB, glycoprotein D (gD), glycoprotein H (gH), and glycoprotein L (gL), respectively, were described previously (21). Plasmids pMM147, pHC138, and pCMV3gL, which express HSV-1 gD, gH, and gL, respectively, were obtained from Gary Cohen and Roselyn Eisenberg. For mutagenesis of HSV-2 gB, the QuikChange procedure (Stratagene) was used, using the enzyme Pfu.

Combining experimental data with a secondary structure prediction made by the PHD neural network method (32), based on a multiple sequence alignment of 19 alphaherpesvirus gB sequences, it was shown previously that no mutants with insertions in predicted alpha-helices or beta-strands folded correctly (24). In contrast, 50% of mutants with insertions in predicted loops, rather than alpha-helices or beta-strands, did fold correctly. Thus, it seemed likely that the possibility of identifying functional domains in gB by insertion mutagenesis would be increased by confining the mutations to predicted loops, although this would inevitably mean that any functional domains not in loops would be missed. HSV-2 gB has now been subjected to this targeted mutagenesis approach. Insertions were made at 18 positions within the ectodomain of the protein, of which 2 were identified as functionally important regions and 1 as important for efficient oligomerization.

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with plasmid pMM245, which expresses wild-type gB. Complementation with a mutant is then defined by the following formula: 
\[
\frac{\text{titer with mutant plasmid}}{\text{titer with carrier DNA}} \times \frac{\text{titer with pMM245}}{\text{titer with carrier DNA}} \times 100.
\]

Transfection-fusion assay. This assay was performed as described previously (21). On day 1, sparsely seeded COS7 cells were cotransfected with plasmids expressing gD, gH, gL, and either wild-type or mutant gB. On day 2, the cells were overlaid with an excess of freshly trypsin-treated untransfected cells, followed by Giemsa staining and photography on day 4. Quantitation of cell fusion was performed by the method of Pasieka et al. (26), with modifications. For each dish of cells, digital photographs of 25 different fields were taken at 100 magnification; to avoid the potential for user bias, movement of the computer-driven microscope stage was controlled by preprogrammed coordinates. The area of fused cells in each photograph was then determined with the Measure function of NIH Image software, and the total for the 25 fields was calculated. The total fused area obtained with wild-type gB, gD, gH, and gL was defined as 100%.

Immunofluorescence assay. Conditions for cell surface staining of cells in Nunc Labtek chamber slides were as described previously (21). The primary antibody was R90, a polyclonal anti-HSV-2 gB antibody, and the secondary antibody was goat anti-rabbit immunoglobulin G (IgG)-fluorescein isothiocyanate (Southern Biotechnologies).

Confocal microscopy. Labeling conditions were similar to those used for conventional microscopy, except that fixation with paraformaldehyde was performed after incubation of the cells with an anti-gB MAb for 45 min at 4°C, rather than before. After fixation, the cells were incubated with goat anti-mouse IgG-Alexa 488 (Molecular Probes) for 30 min at room temperature and then washed and mounted in 0.1% p-phenylenediamine in glycerol. Fluorescence was examined with a Bio-Rad Radiance 2000 confocal microscope, and images taken at 0.5-μm steps were combined into a projection by using LaserSharp 2000 software.

RESULTS

Construction of a panel of gB insertion mutants. The ectodomain of HSV-2 gB is predicted to be either 703 or 749 amino acids long, depending on whether the protein has three or only one transmembrane span (3, 27, 30). In the present study, two-amino-acid insertions were made in regions of the ectodomain identified as loops by a secondary structure prediction based on the PHD neural network procedure (Fig. 1).
Because the mutation frequency was low (usually less than 5%), we focused on the loops with at least five residues, and of those 21 loops we were able to isolate mutants in 15. For each of the remaining six loops, minipreps of at least 100 colonies were screened, but no mutants were obtained. In addition to the initial target sites, three others were arbitrarily chosen. Two were at different positions within loops already mutated, and one was a predicted loop of only four residues. Therefore, insertions were made at a total of 18 sites. After initially making insertions of Ser-Arg, which provided a unique NruI restriction site to aid in screening, we switched to Ala-Ser (providing a unique NheI site) in an unsuccessful attempt to increase the mutation frequency. Therefore, 3 mutants have Ser-Arg insertions and 15 have Ala-Ser. The structural and functional characteristics of each mutant were then assessed in a number of assays.

**Function in virus entry.** To determine whether the mutations affect the ability of gB to function in virus entry into cells, each mutant was tested for the ability to complement a gB-minus virus. As previously (10), we used the HSV-1 K082 mutant, as we have not found an equivalent HSV-2 mutant that works in this assay. The results are shown in Table 1 and Fig. 2. Seven mutants had activities in the range of 36 to 168% compared to wild-type HSV-2 gB; therefore, these seven mutations do not identify functional domains, based on our somewhat arbitrary cutoff point of 20%. The other 11 mutants had activities of 0 to 17%. There are several possible explanations for low activity in this assay, including misfolding (examined below) and failure of a mutant protein to be incorporated into virions. The latter possibility proved impossible to rule out, primarily because more than 95% of the progeny virions remained attached to the cell surface rather than being released into the medium and could not be cleanly separated from cell membranes containing gB on sucrose gradients (data not shown). Scale-up of the assay followed by centrifugation of the medium on a 30% sucrose cushion produced a pellet containing wild-type gB; therefore, gB is released from cells in membranous structures even in the absence of infection. Subsequent attempts to prevent or overcome cell surface attachment included (i) the use of a virus lacking gC, as well as gB, for the complementation assay; (ii) incubation of the cells with cytochalasins B and D; and (iii) incubation of the cells with a pH 3.0 glycine buffer.

**FIG. 2.** Quantitation of virus entry (complementation) and cell fusion by gB mutants. For the complementation assay, cells were transfected with a plasmid expressing a form of gB and then infected with the gB-minus virus K082; virus was harvested 24 h later and titers were determined on D6 cells. For the cell fusion assay, cells were cotransfected with plasmids expressing gD, gH, gl, and a form of gB and then overlaid with untransfected cells; at 48 h after overlay, the cells were stained with Giemsa. The horizontal line at 20% indicates the cutoff point for defining normal functional activity.

### Table 1. Activity of gB mutants in virus entry (complementation)

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Complementation No. of PFU&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean % ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (wild-type gB)</td>
<td>749,000</td>
<td>749,000</td>
</tr>
<tr>
<td>26AS27</td>
<td>693,300</td>
<td>680,000</td>
</tr>
<tr>
<td>82AS83</td>
<td>125,300</td>
<td>813,300</td>
</tr>
<tr>
<td>92AS93</td>
<td>347</td>
<td>600,000</td>
</tr>
<tr>
<td>103AS104</td>
<td>240,000</td>
<td>720,000</td>
</tr>
<tr>
<td>109SR110</td>
<td>290,700</td>
<td>600,000</td>
</tr>
<tr>
<td>163SR164</td>
<td>480</td>
<td>680,000</td>
</tr>
<tr>
<td>227SR228</td>
<td>264,000</td>
<td>600,000</td>
</tr>
<tr>
<td>255AS256</td>
<td>827</td>
<td>720,000</td>
</tr>
<tr>
<td>277AS278</td>
<td>353,300</td>
<td>826,700</td>
</tr>
<tr>
<td>303AS304</td>
<td>627</td>
<td>973,300</td>
</tr>
<tr>
<td>310AS311</td>
<td>440</td>
<td>720,000</td>
</tr>
<tr>
<td>331AS332</td>
<td>2670</td>
<td>613,300</td>
</tr>
<tr>
<td>354AS355</td>
<td>613,300</td>
<td>906,700</td>
</tr>
<tr>
<td>376AS377</td>
<td>693</td>
<td>693,300</td>
</tr>
<tr>
<td>456AS457</td>
<td>853,300</td>
<td>573,300</td>
</tr>
<tr>
<td>522AS523</td>
<td>413</td>
<td>720,000</td>
</tr>
<tr>
<td>566AS567</td>
<td>56,800</td>
<td>573,300</td>
</tr>
<tr>
<td>655AS656</td>
<td>547</td>
<td>560,000</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each value is an average from three independent experiments, together with the average obtained for wild-type gB in the same three experiments. These are the values after subtraction of the background obtained with carrier salmon sperm DNA (generally about 500 PFU).
None of these attempts was successful (data not shown). Therefore, a second assay of gB function was used, namely, the ability to trigger cell fusion when coexpressed with additional viral glycoproteins.

**Function in cell fusion.** As described previously, cell fusion can be induced by coexpression of HSV-1 or HSV-2 gB, gD, gH, and gL in transfected cells (21, 38). The effects of substituting the mutants for wild-type gB in an HSV-2 background are shown in Fig. 3. Quantitation of cell fusion was performed by a procedure adapted from that used by Pasieka et al. (26) for varicella-zoster virus, in which the area of syncytium formation is measured from digital images. The data are shown in Fig. 2. Eight of the mutants had activities greater than 20% of that obtained with wild-type gB, and seven of these eight had an activity of 36% or greater in the virus entry assay, confirming that these seven mutations do not disrupt functional domains. The exception was the 82AS83 mutant, which had 17% activity in the virus entry assay but 67% activity in the cell fusion assay. It cannot be ruled out that the difference in effect results from a difference in the mechanisms of virus-cell and

FIG. 3. Effect of mutations in gB on cell fusion. Cells were cotransfected with plasmids expressing gD, gH, gL, and a form of gB and then overlaid with untransfected cells and stained with Giemsa at 48 h after overlay. The images were assembled with Photoshop software.
cell-cell fusion, but equally likely explanations for the discrepancy include (i) reduced incorporation of the mutant protein into virions or (ii) different interactions of the mutated protein with HSV-1 glycoproteins in one assay and HSV-2 glycoproteins in the other. The latter possibility was eliminated by showing that the cell fusion activity of the 82AS83 mutant in a background of type 1 gD, gH, and gL was 56% ± 7% of that obtained with wild-type type 2 gB in a type 1 background (i.e., similar to the 67% activity of the mutant in a type 2 background).

The 10 mutants that had less than 20% activity in the cell fusion assay also had low activity in the virus entry assay and therefore were candidates for defining functional domains.

**Analysis of gB folding.** Trivial explanations for a lack of functional activity are the lack of synthesis or the inability to fold correctly. All of the mutants were expressed, as shown by...
immunofluorescence of fixed and permeabilized COS7 cells with R90, a polyclonal antibody that recognizes native and denatured epitopes (data not shown). Two characteristics of wild-type gB are its transport to the cell surface and its formation of homo-oligomers; any mutant that differs from the wild-type protein in either respect is likely to be misfolded in some way and therefore not informative about functional domains. Cell surface expression in transfected COS7 cells was examined by immunofluorescence without permeabilization, using antibody R90, and the results are shown in Fig. 4. Mutants 26AS27, 82AS83, 103AS104, 109SR110, 227SR228, 277AS278, 331AS332, 354AS355, 376AS377, 456AS457, and 655AS656 produced surface staining similar to that for wild-type gB, whereas 92AS93 and 566AS567 had reduced staining, and 163SR164, 255AS256, 303AS304, 310AS311, and 522AS523 had none. Note that the nuclei were not fluorescent; their visibility is an artifact of converting the original color images to black and white. The results eliminated mutants 163SR164, 255AS256, 303AS304, 310AS311, and 522AS523 from consideration as indicative of functional domains and also suggested that 92AS93 and 566AS567 are probably not correctly folded. Of the 10 mutants with reduced activity in both functional assays, the remaining candidates were therefore 331AS332, 376AS377, and 655AS656. These three, together with 92AS93, 566AS567, and wild-type gB, were examined for their ability to form oligomers.

Oligomerization was analyzed by immunofluorescence of unpermeabilized transfected COS7 cells using MAb DL16, which recognizes oligomeric but not monomeric gB (1); the control was MAb SB3, which is not oligomer specific. The cells were examined by confocal microscopy, and all images with a given antibody were obtained with the same laser and detector settings. Because staining with SB3 was considerably brighter than with DL16, laser power and detector gain were both increased twofold for the latter. Each image shown in Fig. 5 is a projection of a series of scans made in the z-plane. The fluorescence intensity for mutants 331AS332 and 376AS377 was similar to that of wild-type gB with both MAbs, and thus these mutations do not interfere with oligomerization. Mutant 655AS656 stained normally with SB3, but its fluorescence with DL16 was lower than that of wild-type gB, suggesting that the mutation adversely affects oligomerization. Mutants 92AS93 and 566AS567 had either low or no staining with SB3 and none with DL16, confirming their misfolding. Note that the appearance of cell nuclei in Fig. 4 but not in Fig. 5 is because they are visible by conventional immunofluorescence but not by confocal microscopy. In summary, the assays for cell surface transport and oligomerization indicate that 7 of the 10 mutants with severely disrupted functional activity are misfolded, 1 (655AS656) folds correctly as a monomer but oligomerizes inefficiently, and 2 (331AS332 and 376AS377) fold and oligomerize normally.
FIG. 6. Stick figure of gB ectodomain, with numbering for HSV-2 gB, beginning with the first residue after the signal peptide. Open arrowheads indicate insertions that did not disrupt folding or function; those above the line are for HSV-2 gB (the present study), and those below the line are for HSV-1 gB (5, 24, 29). Filled arrows indicate residues that are important for protein function: above the line for HSV-2 gB (the present study) and below the line for HSV-1 gB (9, 40). The double gray line (18) and gray arrow (the present study) indicate residues involved in oligomerization, for HSV-1 gB and HSV-2 gB, respectively. The dotted line indicates residues that align with a predicted heptad repeat sequence in BHV-1 gB (25).

DISCUSSION

Evidence of various kinds indicates that the gB homologs of HSV-1 (1, 4, 6, 19, 33, 38), HSV-2 (8, 21), varicella-zoster virus (20), Epstein-Barr virus (13), human cytomegalovirus (HCMV) (22), Kaposi’s sarcoma herpesvirus (28), bovine herpesvirus type 1 (BHV-1) (11, 39), equine herpesvirus 1 (23), Marek’s disease virus (34), and pseudorabies virus (16, 31), are involved in postattachment events of virus entry and/or cell-to-cell spread. However, the exact role of the protein in these processes remains to be discovered.

As described in the introduction, current knowledge of functionally important regions in the ectodomain of gB is quite limited. In addition to those studies, it has been proposed that residues 477 to 510 of BHV-1 gB (equivalent to residues 485 to 518 of HSV-2 gB) form a heptad repeat, and a synthetic peptide corresponding to this region interfered with virus spread but not with penetration (25). For HCMV gB and BHV-1 gB, mutagenesis of the highly basic proteolytic cleavage site that is found in some but not all gB homologs showed that cleavage is not essential for protein function (2, 37).

The more global approach of linker-insertion mutagenesis was used with HSV-1 gB (5, 24, 29) and with HCMV gB (36). For the HCMV protein, several mutants that retained correct folding were identified, but no functional assay was available to test their activity (36). For the HSV-1 protein, all mutants except one either retained functional activity or were misfolded, so only one candidate functional domain was identified. This HSV-1 gB mutant, with a two-amino-acid insertion between residues 159 and 160 (corresponding to residues 162 and 163 in HSV-2 gB) had no activity in virus entry but had normal carbohydrate processing, suggesting correct folding (5). Unfortunately, without an assay to demonstrate incorporation into virions or the cell fusion assay as an alternative, the data remain inconclusive. Nevertheless, the combined data from the three independent studies of HSV-1 gB indicated that the chances of success would be increased by making insertions only in regions predicted to be loops (i.e., not in α-helices or β-strands) (24). Consequently, a panel of 18 insertion mutants was made, targeting the longer loops. Eleven mutants had activities of 17% or lower in virus entry compared to the wild-type protein. Ten of these eleven had activities of 13% or lower in cell-cell fusion compared to the wild-type protein. The most likely explanation for the ability of the 82AS83 mutant to function efficiently in cell-cell fusion but not in virus entry is poor incorporation into virions.

Of the mutants that were defective in virus entry and in cell-cell fusion, two were correctly folded, as judged by cell surface transport and oligomerization, one was transported to the cell surface at wild-type levels but did not efficiently oligomerize, two were transported to the cell surface at a low level but did not oligomerize, and five were clearly misfolded. Therefore, we have identified two regions of HSV-2 gB where small insertions disrupt the function of the protein but do not prevent normal folding. These regions (residues 331 to 332 and residues 376 to 377) fall within loops 329 to 335 and 371 to 383, neither of which have previously been implicated in gB function. It remains possible that one or more of the six loops where insertions had no effect do in fact harbor functional domains but that the insertions were not deleterious. This would be a consequence of using conservative insertions (only two residues, and with amino acids having a low probability of being α-helix or β-strand formers), to minimize the chance of misfolding. The results obtained with a soluble form of HSV-1 gB suggest the possible existence of a cell surface receptor (1), and it will be interesting to test the 331AS332 and 376AS377 mutants for binding if a receptor is identified.

In comparison to previously published results, several further observations can be made. (i) It was somewhat surprising that the 277AS278 mutant functions normally. Mapping of the binding sites of neutralizing MAb’s had indicated that residues 276 to 301 may be important for gB function (14, 17), and therefore it was anticipated that an insertion in this region might disrupt function. However, the fact that this was not the case serves to emphasize the benefits of using a variety of approaches. (ii) Because the 163SR164 mutant was misfolded, our data do not help in evaluating the 159LT160 HSV-1 gB mutant (equivalent to 162LT163 in HSV-2 gB) of Cai et al. (5). (iii) Residue 523 of HSV-1 gB (equivalent to residue 528 in HSV-2 gB) has previously been found to influence the rate of virus penetration into cells at 30°C (9). Unfortunately, we have no useful mutations in that vicinity, since an insertion in the nearest predicted loop (522AS523) disrupted protein folding. (iv) We made no mutations in the putative heptad repeat region (25), since it corresponds to a long α-helix in the sec-
ondary structure prediction, or in the juxtamembrane region targeted by Wanas et al. (40). The high hydrophobicity of the latter is incompatible with an accurate secondary structure prediction by the PHD procedure (32), and consequently no loops could be identified there. (v) The relevance of residues 655 to 656 to oligomerization is consistent with an earlier and more extensive analysis of HSV-1 gB oligomerization. In that study, residues corresponding to amino acids 601 to 628 and 629 to 650 in HSV-2 gB were found to be independently able to allow oligomerization (18).

The relative positions of the regions identified by us and others as important for functional activity and oligomerization of HSV-1 and -2 gB are shown in Fig. 6, along with the positions of insertions that did not disrupt folding or function. It is possible that two or more of these regions are actually close together in the folded form of the protein, forming a conformational structure involved in gB function; this will be revealed if and when the three-dimensional structure is determined.

Finally, it is interesting to note the degree of sequence conservation for residues 331/332, 376/377, and 655/656 within the alphaherpesvirus gB homologs. (i) Residues 331 and 332 are Pro-Lys not only in HSV-2 gB but also in the corresponding alphaherpesvirus gB homologs. (ii) Residues 331/332, 376/377, and 655/656 within the herpesvirus gB map in separate domains. (iii) The residue positions in 15 of the other 18 homologs used in our alignment. (iv) Residues 331 and 332 are important for functional activity and oligomerization of HSV-1 gB and -2 gB shown in Fig. 6, along with the positions of insertions that did not disrupt folding or function. It is possible that two or more of these regions are actually close together in the folded form of the protein, forming a conformational structure involved in gB function; this will be revealed if and when the three-dimensional structure is determined.

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