Locations of herpes simplex virus type 2 glycoprotein B epitopes recognized by human serum immunoglobulin G antibodies.

D E Goade, R Bell, T Yamada, G J Mertz and S Jenison

The herpes simplex virus (HSV) glycoprotein B (gB) is a transmembrane envelope glycoprotein that contributes to the penetration of virions into host cells (6, 15, 16, 22, 48, 53, 54). gB induces fusion of the virion envelope with the cellular cytoplasmic membrane, an essential function for virus entry (6, 15). Immunization of humans and animals with purified gB induces virus-neutralizing antibody responses, and recombinant gB proteins are being investigated as HSV vaccines (9–12, 15). Immunization of humans and animals with purified gB induces virus-neutralizing antibody responses, and recombinant gB proteins are being investigated as HSV vaccines (9–12, 15).

The HSV type 2 (HSV-2) gB (gB-2) polypeptide contains 904 amino acids (aa) (13, 56). The amino-terminal segment (22 aa) is a signal peptide that is cleaved from the mature protein during processing. aa 745 to 798 constitute a hydrophobic transmembrane anchor domain. The segment carboxy proximal to the anchor domain (aa 799 to 904) is cytoplasmic and appears to mediate membrane fusion (14–16, 22, 25, 50). The amino-proximal segment (aa 23 to 744) is extracellular and contains epitopes that are recognized by virus-neutralizing antibodies (27, 28, 46).

HSV-2 and HSV-1 are closely related viruses. Most of their homologous proteins are highly conserved (20, 42) and elicit cross-reactive antibody responses (2, 8, 41, 57). HSV-2 and HSV-1 infections elicit strong immunoglobulin G (IgG) antibody responses to the HSV-2 gB-2 and to the HSV-1 gB-1, respectively (4, 20, 42). gB antibodies are detectable early in acute infections, attain high titers, and persist for many years (2, 20, 23). gB-2 polyclonal antibody responses include antibodies that cross-react with gB-1, and gB-1 responses include antibodies that cross-react with gB-2 (2, 8, 41, 57). The antigenic relatedness of gB-2 and gB-1 is consistent with the marked overall conservation of their amino acid sequences (86% at the amino acid level) (13).

In contrast to most HSV-2 and HSV-1 homologous proteins, HSV-2 gG-2 and HSV-1 gG-1 have highly dissimilar amino acid sequences. Human gG-2 and gG-1 antibody responses induced by HSV-2 and HSV-1 infections, respectively, appear to be virus type specific (8, 40). Detection of reactivities to gG-1 and gG-2, either present as native viral proteins or produced as recombinant antigens, forms the basis for current HSV type-specific antibody assays (1, 4, 5, 47).

Although cross-reactive antibodies are represented in the human IgG responses to gB-2 and gB-1, respectively, virus type-specific gB antibodies may also be induced by HSV infections. Some murine monoclonal antibodies generated against gB-1 cross-react with gB-2, but others are virus type specific. Linear (continuous) epitopes that react with type-specific, virus-neutralizing murine monoclonal antibodies have been localized to the extracellular amino-terminal segment of gB-1 (21, 36–38, 46, 49, 51, 52). Within this amino-terminal segment, the amino acid sequences of gB-1 and gB-2 are highly diverged (51). Little information is available regarding the locations and type specificities of gB epitopes recognized by human HSV antibodies (21, 33, 52). If gB-2 epitopes that react with human HSV-2 antibodies in a virus type-specific manner exist and are distinguishable from gB-2 cross-reactive epitopes, then the
of HSV-2 vaccines. Serum samples were drawn from these subjects prior to the administration of HSV-2 vaccines. Additional gB-2 expression plasmids were made in order to further define antibody-reactive regions, pGB2-HA1 was constructed by digesting pGB2-SS2 DNA with HindIII and Apol, and the gB-2 fragment from nt 50 to 458 was ligated to pATH23 DNA (HindIII-EcoRI digest). pGB2-SP1 was generated by digesting pGB2-SS1 DNA with PstI, leaving the gB-2 fragment from nt 687 to 2711, and religating the plasmid DNA ends. pGB2-SP2 was constructed by digesting pGB2-SS1 DNA with XhoI and BamHI, and leaving the gB-2 fragment from nt 687 to 1879; the ends of the plasmid DNA were made blunt by digesting with DpnII, and the blunt-ended gB-2 fragment was ligated to pATH23 DNA (BamHI-XhoI digest). pGB2-SP2 was digested with Apol and PstI, leaving the gB-2 fragment from nt 228 to 503 ligated to pATH20 DNA (EcoRi-PstI digest). For pGB2-S1, pHS218 was digested with SacI and the gB-2 fragment from nt 687 to 2711 was ligated to pATH20 DNA (SacI-PstI digest).

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and the leucine residue immediately preceding the gb-2 stop codon as aa 904. gb-2 aa 1 to 22 is a signal sequence that is cleaved from the polypeptide during gb-2 protein maturation (56).

The samples were tested initially for the presence of HSV-1 and HSV-2 antibodies by using HSV-1- and HSV-2-infected cell lysates as antigen targets in Western blot assays (4, 5). Serum samples were classified as containing HSV-1 and HSV-2 antibodies on the basis of characteristic immunoblot banding patterns, including virus type-specific reactivities to gG-1 and gG-2. Samples included 23 samples from HSV-1/2 subjects, 23 samples from HSV-1/1 subjects, and 18 samples from HSV-1/2 subjects.

All (18 of 18) serum samples from HSV-1/2 subjects reacted strongly with the amino-proximal gb-2 segment (aa 18 to 228) and with the carboxy-proximal segment (aa 228 to 903); 8 of 18 HSV-1/2 subjects also reacted with the middle gb-2 segment (aa 154 to 503). None (0 of 23) of the HSV-1/1− subjects reacted with the amino-proximal gb-2 segment or with the middle gb-2 segment; all (23 of 23) of the HSV-1/2− subjects reacted with the carboxy-proximal segment (aa 228 to 903). No antibody reactivities to the pGB2-SS2, pGB2-AP2, or pGB2-SS1 proteins were detected in the serum samples from HSV-1/−2 subjects (n = 23). These findings suggested that HSV-2 infections elicit different antibodies that react with the amino-proximal and carboxy-proximal segments of gb-2. The data suggested further that HSV-1 infections induce antibodies that cross-react within the carboxy-proximal segment of gb-2 but do not induce antibodies that cross-react with linear epitopes located within the amino-proximal segment of gb-2.

Sixty-seven serum samples were then tested for gb2-SS2 and gb2-SS1 reactivities. These samples were obtained from participants in a phase III clinical trial evaluating the efficacy of a recombinant gb-2/gD-2 vaccine candidate. All subjects either had recurrent genital herpes or were the monogamous sex partners of subjects with recurrent genital herpes. Serum samples included 9 HSV-1/2− subjects, 16 HSV-1+/−2− subjects, and 42 HSV-2+/−2− subjects; among the 42 HSV-2+/−2− subjects, 21 samples contained HSV-2 antibodies only (HSV-1/−2+), and 21 samples contained both HSV-1 and HSV-2 antibodies (HSV-1+/−2+). No antibody reactivities were seen in the HSV-1/−2− subjects to any of the gb-2 recombinant proteins. Of the 16 HSV-1+/−2− subjects, 3 samples showed weak reactivity to the GB2-SS2 protein and reacted strongly with the GB2-SS1 protein. All serum samples from subjects with HSV-2 antibodies reacted strongly with both the GB2-SS2 and the GB2-SS1 proteins. The results for all serum samples tested are shown in Table 3.

### TABLE 2. Deletion constructs

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FIG. 1. gb2 recombinant Western blot assays. Serum samples from HSV-2+/−1− (left panel), HSV-2+/−1+ (middle panel), and HSV-2−/−1− (right panel) subjects were reacted with gb2 recombinant proteins in Western blot assays. Lanes contained recombinant bacterial proteins encoded by the pATH expression vector (V), the pGB2-SS2 plasmid (SS2), the pGB2-AP2 plasmid (AP2), and the pGB2-SS1 plasmid (SS1). A map of the gb2 coding region is shown at the bottom, and the horizontal bars represent the gb2 polypeptide segments expressed by the pGB2-SS2, pGB2-AP2, and pGB2-SS1 expression plasmids, respectively. The numbers at the left and right ends of the bars refer to the amino-terminal and carboxy-terminal boundaries of the polypeptide segments, respectively.
Localization of the gB-2 antibody-reactive region recognized preferentially by HSV-2 antibodies. The amino-proximal gB-2 segment contained within the GB-2-SS2 protein recognized by HSV-2 antibodies was further defined by reacting HSV-2 antibody-containing serum samples with nested sets of serially deleted gB-2 recombinant proteins. The carboxy-terminal boundary of this region was defined by using a nested set of recombinant proteins that contained carboxy-to-amino-terminal deletions in the pGB2-SS2 protein (Table 2).

Thirty-five HSV-1−/−/2+ serum samples were tested. All 35 serum samples reacted with pGB2-SS2-CEx227 protein (aa 18 to 75). Three of the 35 serum samples had either no reactivity (or greatly reduced reactivity) to the pGB2-SS2-CEx210 protein (aa 18 to 70), whereas 32 of 35 serum samples retained reactivity. By the next smaller deletion, the pGB2-SS2-CEx194 protein (aa 18 to 64), only two serum samples retained strong reactivity. No serum samples reacted strongly with the pGB2-SS2-CEx161 protein (aa 18 to 53). Representative Western blot assays of the deletion series proteins are shown in Fig. 2.

Therefore, the major amino-proximal linear epitope (or epitopes) recognized by HSV-2 human IgG antibodies lies within the gB-2 segment from aa 18 to 75 (Fig. 2). There is 46% amino acid sequence homology between this region of gB-2 and the corresponding region of gB-1 (HSV-1 Patton strain) (13).

In 12 of 35 HSV-1−/−/2+ samples, persistent but markedly diminished reactivity was observed in deletion constructs smaller than pGB2-SS2-CEx194 (aa 18 to 64). These samples were tested with more extensively deleted constructs in order to further define this minor reactivity. In 12 of 12 samples, antibodies reacted with the pGB2-SS2-CEx144 protein (aa 18 to 48) but did not react with the pGB2-SS2-CEx132 (aa 18 to 44) protein and more extensively deleted proteins. Therefore, this minor amino-proximal gB-2 specificity recognized by HSV-2 antibodies is contained within the gB-2 segment from aa 18 to 48.

Localization of a carboxy-terminal gB-2 segment recognized by both HSV-2 and HSV-1 antibodies. The major carboxy-terminal segment recognized by HSV-2 antibodies and by cross-reactive HSV-1 antibodies was further localized. Serum samples were tested for reactivity with a nested set of carboxy-to-amino-terminal deletion constructs derived from the pGB2-SS1 (aa 228 to 903) protein. HSV-1−/−/2+ samples that reacted with pGB2-SS1 protein did not react with the largest deletion construct pGB2-SS1- CEx2568 (aa 228 to 856) and more extensively deleted constructs. This observation suggested that the cross-reactive epitope(s) recognized by HSV-1 antibodies lies close to the gB-2 carboxy terminus. The gB-2 carboxy-terminal construct pGB2-SMB (aa 819 to 904) was tested for reactivity with HSV-1 and HSV-2 antibody-containing serum samples. All HSV-1 antibody-containing samples tested (23 of 23) and all HSV-2 antibody-containing samples tested (42 of 42) reacted strongly with this segment (Fig. 3). These observations indicate that the major epitope(s) of pGB2-SS1 (aa 228 to 903) recognized by HSV-2 antibodies and by HSV-1 cross-reactive antibodies is also contained within the gB-2 segment (aa 819 to 904) encoded by pGB2-SMB. This strongly antibody-reactive region between aa 819 and 904 is contained within the gB-2 cytoplasmic domain (56).

Localization of a second gB-2 cross-reactive region recognized by some HSV-2 antibodies and by some HSV-1 antibodies. Testing of HSV-2 and HSV-1 antibody-containing samples with the pGB2-SS1 deletion constructs indicated the presence of a second antibody-reactive segment. This segment was mapped by using nested sets of carboxy-to-amino-terminal deletions and

### Table 3. Human antibody reactivity to proteins encoded by expression constructs or to mapped immunoreactive regions

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<th>GB2-SS2 (%)</th>
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<th>Minor type-specific region F (%)</th>
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<td>1+/−/2−</td>
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<td>1/33 (3.0)</td>
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* Results are given as total number of samples with detectable reactivity by Western blot divided by the total number of samples tested. The GB2-SS2 protein includes aa 18 to 228, GB2-AP2 includes aa 154 to 503, GB2-SS1 includes aa 228 to 903, and GB2-SMB includes aa 819 to 904. Region F refers to the region recognized by some HSV-2 antibodies as displayed in Fig. 4; region G refers to the region recognized by most HSV-2 antibodies and approximately 50% of HSV-1 antibodies as displayed in Fig. 4.

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**FIG. 2.** Mapping of the GB2-SS2 epitope recognized by HSV-2 antibodies. (A and B) Western blots generated with identical proteins that were reacted with serum samples from two different HSV-1−/−/2+ subjects. Lane V contains the protein expressed by the pATH expression vector. Lane SS2 contains the GB2-SS2 protein. The remaining lanes contain proteins with carboxy-to-amino-terminal deletions in the GB2-SS2 protein; the numbers above the lane refer to the amino acid coordinate of the carboxy-terminal gB-2 amino acid in that construct (see Table 2). For the construct in lane X, the coordinate of the carboxy terminus was not determined. The numbers to the left of the panels refer to the mobilities of protein molecular mass standards (in kilodaltons). The map at the bottom depicts the gB-2 amino acid sequence from aa 18 to 228. The arrows above the amino acid sequence mark the carboxy terminus of the selected deleted GB2-SS2 proteins used in the Western blot assays, and the numbers below the amino acid sequence refer to the amino acid coordinates of the carboxy termini. The (+) and (−) symbols indicate that the antibodies in panel B reacted with (+) or did not react with (−) the GB2-SS2-deleted protein.
We identified an amino-terminal region of the HSV-2 gB-2 (aa 18 to 75) that contains a linear epitope, or epitopes, recognized by human HSV-2 IgG antibodies. The gB-2 segment from aa 18 to 75 reacted strongly with antibodies from all serum samples that contained HSV-2 antibodies \((n = 58)\). This gB-2 segment also reacted weakly with antibodies present in 3 of 33 HSV-1+ and HSV-2- samples. The serum samples were initially categorized as containing HSV-2 and HSV-1 antibodies on the basis of the results of an HSV lysate Western blot assay.

Our data suggest that the gB-2 segment from aa 18 to 75 contains an epitope (or epitopes) that reacts with HSV-2 antibodies in a virus type-specific manner. The discordant results obtained in three samples using the assay for gB-2 aa 18 to 75 compared with the HSV lysate Western blot assay suggest three possibilities. (i) The gB-2 segment from aa 18 to 75 cross-reacts with gB-1 antibodies induced in some individuals by HSV-1 infections. (ii) The assay for gB-2 aa 18 to 75 reacts with HSV-2 antibodies in a virus type-specific manner but gave false-positive results in these three HSV-1+/- serum samples. (iii) The assay for gB-2 aa 18 to 75 reacts with HSV-2 antibodies in a virus type-specific manner and is more sensitive in detecting HSV-2 antibodies than is the HSV-2 Western blot assay. Clinical and demographic data support the third possibility. Two of the three HSV-2+/1+ subjects who reacted with the gB-2 aa 18 to 75 protein were the regular sex partners of HSV-2-seropositive people with a history of recurrent genital herpès; one of these samples contained both IgG and IgM antibodies that reacted with the gB-2 segment from aa 18 to 75, suggesting that an acute infection was present at the time the sample was obtained. The third subject had a history of recurrent genital herpès but was classified as HSV-1+/- on the basis of the HSV lysate Western blot assay. In these three subjects, it is possible that HSV-2 antibodies were present that were detected by the assay for gB-2 aa 18 to 75 but were not detected by the HSV lysate Western blot assay.

The serodiagnosis of acute HSV-2 infections in patients who have been infected previously with HSV-1 has been problematic because of an anamnestic response to HSV-2 and HSV-1 type-common antigens \((4, 42)\). In this situation, detection of antibodies to HSV-2 type-specific antigens is obscured by the strong antibody responses to type-common antigens \((1)\). Difficulties in detecting HSV-2 type-specific antibodies by using the HSV lysate Western blot assay in subjects previously infected with HSV-1 may have resulted in the misclassification of samples reactive to gB-2 aa 18 to 75 as HSV-1+/-.

Therefore, the gB-2 segment from aa 18 to 75 may contain virus type-specific epitope(s) recognized by human HSV-2 antibodies. The homologous gb-1 amino-terminal segment contains virus type-specific linear epitopes recognized by mouse monoclonal antibodies; some of these monoclonal antibodies have virus-neutralizing activity \((14, 18, 21, 35, 36, 51)\). Algorithms that model protein antigenicity suggest that the amino-terminal segments of gb-2 and gb-1 are potentially the most antigenic regions of the proteins \((7)\). The complete gb amino acid sequences are 86% homologous, but gb-2 and gb-1 amino acid homology is 46% within the amino-terminal \((aa 18 to 75)\) segment. Therefore, it is plausible that this gb-2 segment is a type-specific antigen that is recognized by HSV-2 antibodies and not by HSV-1 antibodies.

The gb-2 carboxy-terminal segment between aa 819 and 904 contains a dominant epitope(s) that reacted strongly with all HSV-2 and all HSV-1 antibody-containing samples. This carboxy-terminal segment is contained entirely within the gb-2
cytoplasmic domain. The strong reactivities of HSV-1 antibodies with this gB-2 segment account, at least in part, for the observation that HSV-1 antibodies cross-react with native gB-2.

A third immunoreactive region recognized by HSV-2 antibodies lies within the segment between aa 564 and 626, immediately amino proximal to the membrane-spanning domain (Fig. 4). This segment reacted with antibodies from most (85%) HSV-2-seropositive subjects tested and cross-reacted with antibodies from some but not all (52%) HSV-1-seropositive subjects tested.

Our analysis of gB-2-reactive antibodies is restricted to the characterization of antibodies that recognize linear (or continuous) protein-binding sites. The recombinant gB-2 polypeptides were subjected to SDS-polyacrylamide gel electrophoresis under fully denaturing conditions before being transferred to nitrocellulose blots. Therefore, gB-2 antibodies that are dependent on native protein conformation for binding would not be detected. gB-2 regions that contain linear antibody-binding sites were defined by using nested sets of serially deleted recombinant proteins. The resolution of this method for localizing linear epitopes is dependent on the spacing of the serially deleted proteins. The deletion-mapping technique defines a segment of gB-2 that contains one or more linear antibody-binding sites but does not define the minimum epitope recognized by those antibodies. This strategy has been used extensively by our group to localize human antibody-reactive segments of the Four Corners hantavirus and human papillomavirus antigens (29–32, 59).

In acute human HSV infections, glycoprotein antibody reactivities appear sequentially, beginning at approximately 4 days after infection. In HSV-2 infections, gB-2 and then gD-2 antibodies appear first (2). Seroconversion to all antigenic determinants requires at least 21 days after infection in most subjects tested.

A gB-2 virus type-specific antibody test could complement gG-based assays. Because gB-2 antibodies generally appear in the serum before gG-2 antibodies, gB-2 virus type-specific antibody assays may provide earlier diagnosis of acute HSV-2 infections. Identification of a type-specific epitope within HSV-1 gB-1 would provide a similar capability for defining HSV-1 antibody responses. Because gG-1 antibody detection appears to be a relatively less sensitive indicator of HSV-1 infections, a gB-1 virus type-specific antibody assay could be a useful tool in the serodiagnosis of HSV-1 infections.

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REFERENCES


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