Glycoprotein B Is a Specific Determinant of Herpes Simplex Virus Type 1 Neuroinvasiveness

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Herpes simplex virus type 1 strains ANG and KOS lack neuroinvasiveness when inoculated on the footpads of mice, and because the strains are able to complement each other, the genes associated with this phenotype differ. In this study, we used marker rescue techniques to show that at least two genes cloned from ANG are required to restore neuroinvasiveness to KOS. One of the two fragments required is the 6.3-kb BamHI-A/EcoRI-D fragment (0.15 to 0.19 map units). The second has been identified as the sequence encoding glycoprotein B (gB) (UL27). Analysis of ANG and KOS DNA sequences in the relevant region of the gB gene revealed two nucleotide differences which result in amino acid differences in the gB protein. One appears to be unique to the strain of KOS used in our laboratory. The second, at codon 523 of the mature gB protein, encodes a valine in KOS and an alanine in ANG. Recombinant KOS viruses which contained ANG sequences in this region were constructed, and two independently selected recombinants demonstrated increased neuroinvasiveness in mice. From these results, we conclude that gB significantly influences neuroinvasiveness. Mechanisms by which this might occur are discussed.

Herpes simplex viruses have a unique natural history and are intimately involved with the nervous system; in this relationship, the viral properties of neuroinvasiveness, neuroniruence, and latency are of fundamental importance. Viral genes specifically involved with each have been identified by ourselves and others (for review, see reference 24), and as might be expected, they display a wide range of functions. With respect to neuroinvasiveness, gene functions related to either hematogenous or neural spread are of interest, although the latter route probably represents the most significant route taken by the virus to the central nervous system.

In previous studies, we showed that the herpes simplex virus type 1 (HSV-1) strains KOS and ANG are nonneuroinvasive in mice (10, 25). Both viruses replicate efficiently in cell culture. After inoculation into mice, these viruses replicate well both in the central nervous system and at the site of infection on the body surface (the footpad), but they are restricted in travel between the footpad and the central nervous system. Since, in this model, peripheral nerves and ganglia are the conduit between the periphery and the central nervous system, neuroinvasiveness is related to the ability of the virus to traverse these tissues.

Coinfection of ANG and KOS viruses revealed that they are defective in different genes, since neuroinvasive recombinants were present in the central nervous system after inoculation of both viruses into mouse footpads (11, 22). Additional studies of the ANG virus showed that the relevant lesion occurred in the gene for glycoprotein D. When this area was sequenced in both viruses, a single-nucleotide change which predicts an amino acid change from alanine in ANG to glycine in ANG-path was found in the gene for glycoprotein D. All neuroinvasive recombinants derived from marker rescue experiments were found to contain this base change (10).

Using the same marker rescue techniques which were used for ANG, we found in our earlier studies of KOS that some portion of the HindIII-A fragment was at least partially responsible for the lack of neuroinvasiveness of KOS (25). The present investigation extends the earlier work and indicates that nucleotide sequences in at least two separate genes, one of which is contained in the HindIII-A fragment, are required to restore neuroinvasiveness to the KOS strain of HSV-1. Marker rescue experiments completed to date indicate that ANG sequences from both UL27 and the BamHI-A/EcoRI-D fragment are required to reconstruct the neuroinvasive phenotype in HSV-1 KOS. We have not yet identified the sequences in the BamHI-A/EcoRI-D fragment which are involved in this phenotype.

The UL27 open reading frame encodes glycoprotein B, a protein involved in viral fusion with the cell membrane after attachment of the virus and entry into the cell (5, 21). Mutations in this open reading frame are known to affect the formation of syncytia, certain pathologic characteristics of footpad infection, and the rate of entry of the virus into cultured cells (3, 7, 9). In addition, the protein is immunologically significant and is the target of both protective antibodies and cytotoxic T cells (8, 18).

In the present experiments, a 1.4-kb SalI-SalI fragment was found to be the critical region in glycoprotein B, and fragments derived from both virus strains were sequenced. Although several nucleotide sequence differences were found, only two were predicted to result in amino acid sequence changes in glycoprotein B. One of these differences is apparently unique to the KOS strain used in our laboratory, since it does not appear in the sequence of KOS strains used by other laboratories (4). The second encodes an alanine for the rescuing ANG strain and a valine for the KOS strain at amino acid 523 of the mature glycoprotein B. To further confirm the role of this mutation in neuroinvasiveness in transfection experiments, we constructed viruses containing ANG sequences in the 1.4-kb SalI-SalI fragment, which codes for the two amino acid differences in a KOS strain background. Those with insertions of the relevant ANG sequences were selected with an appropriate oligonu-
cleotide probe in vitro and shown to be significantly more neuroinvasive than the parental KOS viruses. From these results, we conclude that glycoprotein B plays an important role in determining the neuroinvasive capability of HSV-1. Since this is also true for glycoprotein D, we conclude that small changes in glycoproteins can significantly affect the invasive properties of herpes simplex virus.

**MATERIALS AND METHODS**

**Virus strains and tissue culture.** HSV-1 strains ANG and KOS have been described previously (14, 25). The procedures for tissue culture of rabbit skin cells and infection, propagation, and titration of viruses were as presented earlier (26).

**Transfections and virion DNA.** Virion DNA was purified for transfection by isopycnic centrifugation as described before (28). In all cases, viral DNA was transfected by calcium phosphate precipitation into rabbit skin cells by using full-length KOS DNA and cloned ANG or KOS DNA fragments and methods we have used for several years (27).

**Infection of mice and testing for neuroinvasiveness.** Outbred Swiss Webster mice approximately 4 weeks of age (Simonsen Laboratories, Gilroy, Calif., or Harlan Sprague Dawley, Indianapolis, Ind.) were infected on scarified rear footpads after saline treatment (6).

**DNA cloning and analysis.** Standard methods were used for DNA cloning and analysis (1), and Sanger dideoxy sequencing was performed with Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) according to the manufacturer’s directions. Oligonucleotide probes were labeled with polynucleotide kinase and hybridized in solution containing tetramethylammonium chloride (1).

**Plaque purification of recombinant viruses.** Transfection mixes were diluted, plated on rabbit skin cells, and overlaid with 0.7% agarose. Plaques were picked into 96-well plates containing medium, and a portion of this was used to infect a duplicate plate of rabbit skin cells. When cytopathic effects were complete, a portion of each well was blotted onto nylon paper and denatured (1), and the blot was then hybridized to oligomer probes spanning the sequence containing the nucleotide difference, as described above.

**RESULTS**

Identification of the ANG genomic region conferring neuroinvasiveness on KOS. In preliminary experiments, KOS DNA was transfected into rabbit skin cells with cloned XbaI and EcoRI fragments of ANG DNA that spanned virtually the entire genome. These were used either together or in pools of fragments, each pool comprising approximately one-third of the genome. When cytopathic effects were complete, virus was harvested and analyzed by infection of mouse rear footpads. A transfection mix which killed one or more of the animals in a group of four mice was presumed to contain recombinant viruses with increased neuroinvasiveness conferred by the cloned ANG fragments. Although earlier experiments (25) had indicated that the HindIII-A fragment confers increased neuroinvasiveness (as indicated by increased paralysis), we elected to analyze the entire genome, since the inability of the HindIII-A fragment to reliably produce lethal transfection mixtures suggested that more than one region of the genome was required to restore neuroinvasiveness to KOS. These experiments indicated that the cloned and pooled XbaI-F, EcoRI-D, and EcoRI-G fragments are sufficient to generate neuroinvasive KOS recombinants (data not shown).

To determine which fragments contained the relevant sequences, the clones comprising this pool were used both individually and in pairs for cotransfections. As indicated in Fig. 1, both the XbaI-F and EcoRI-D fragments of ANG are required to produce lethal transfection mixes. Since these two fragments do not overlap, it was concluded that sequences from at least two separate genes of ANG were required to restore neuroinvasiveness to KOS.

**Identification of an EcoRI-D subfragment involved in neuroinvasiveness.** The EcoRI-D clone of ANG was subcloned into three smaller clones: EcoRI-D/BamHI-C (9.1 kb), a small central BamHI fragment (1.4 kb) unique to ANG, and BamHI-A/EcoRI-D (6.3 kb). Marker rescue experiments with these fragments in combination with a subclone of the XbaI-F clone (both clones are required to produce lethal transfection mixes) indicated that the BamHI-A/EcoRI-D 6.3-kb fragment contained the sequences necessary to produce neuroinvasive recombinants (Fig. 2). This fragment contains part of open reading frames 9 and 15 and all of open reading frames 10 through 14. We have not yet localized the precise region in the 6.3-kb BamHI-A/EcoRI-D fragment which restores a fully neuroinvasive phenotype to KOS.

**Identification of an XbaI-F subfragment involved in neuroinvasiveness.** Since XbaI-F is over 22 kb in length, we tested a smaller cloned 7.8-kb fragment, BamHI-G, which lies entirely within XbaI-F. In several experiments, when used in combination with EcoRI-D, BamHI-G was found to confer neuroinvasiveness with an efficiency equivalent to that of XbaI-F. We also found that BamHI-G was able to generate lethal cotransfection mixes when transfected alone with KOS DNA, although at significantly reduced levels (Fig. 3). Finally, in order to definitively establish the requirement for both cloned fragments just identified, fragments cloned from either ANG or KOS were cotransfected in pairs with KOS DNA, and the viral progeny were tested for
neuroinvasiveness. As shown in Table 1, both cloned ANG fragments were required to confer neuroinvasiveness to the KOS virus.

Identification of the genetic defect in KOS DNA fragment BamHI-G. To identify more precisely the location of the genetic defect in the KOS BamHI-G fragment, several subclones of ANG BamHI-G were used in cotransfection experiments. Unless otherwise noted, in all of these transfections, either the ANG EcoRI-D or the BamHI-A/EcoRI-D 6.3-kb subclone was transfected along with the fragments to be tested. First, the KpnI-N and KpnI-N/BamHI-G subclones were used, and the KpnI-N fragment was found to contain the sequences conferring neuroinvasiveness on KOS (Fig. 4). This 4.7-kb fragment contains only two open reading frames—the complete UL27 (glycoprotein B) and part of UL28 (a virion structural protein). Since, as stated earlier, the neuroinvasive phenotype of ANG-path is referable to glycoprotein D, and since preliminary results indicated that KOS is more neuroinvasive in immunosuppressed mice (19a), we thought it quite likely that the relevant sequences were in UL27.

In preliminary experiments, we found that cotransfections with both necessary fragments became extremely inefficient in generating lethal recombinants when the transfected plasmid DNAs were less than approximately 4 kb in length. Therefore, to improve recombination frequency, clones constructed to test fragments smaller than 4 kb consisted of ANG sequences spliced into a larger KOS clone. For these specific experiments, a clone that contained a large portion of the ANG glycoprotein B open reading frame flanked by KOS sequences colinear to those removed from the ANG fragment was constructed. When tested, this construct was found to be as effective as the entire ANG KpnI-N fragment in producing lethal cotransfection mixtures (Fig. 5).

With similar ANG/KOS fusion clones, other experiments conducted at the same time indicated that the region from KpnI-N to SalI-H (2.1 kb from the left end of KpnI-N) could also produce lethal cotransfection mixtures (data not shown). Therefore, an ANG-KOS fusion clone which contained only 1.5 kb of ANG sequences between the SacI site

FIG. 2. Rescue of HSV-1 KOS neuroinvasiveness with subclones of the ANG EcoRI-D DNA fragment and the BamHI-G fragment. Cloned ANG fragments were transfected into cells with KOS DNA, and progeny virus was tested by inoculation on rear footpads of mice. Fractions indicate the number of cotransfection cultures that killed mice relative to the total number of cultures tested. The locations of the BamHI-G fragment within the XbaI-F fragment and the location of the XbaI-F fragment in the HSV-1 genome are also shown. ND, not done.

FIG. 3. Rescue of HSV-1 KOS neuroinvasiveness with the ANG BamHI-G fragment of the XbaI-F fragment and the EcoRI-D fragment. Cloned ANG fragments were transfected into cells with KOS DNA, and the progeny virus was tested by inoculation on rear footpads of mice. Fractions indicate the number of cotransfection cultures that killed mice relative to the total number of cultures tested. The locations of the BamHI-G fragment within the XbaI-F fragment and the location of the XbaI-F fragment in the HSV-1 genome are also shown. ND, not done.

FIG. 4. Rescue of HSV-1 KOS neuroinvasiveness with ANG KpnI-N fragment and the EcoRI-D or BamHI-A/EcoRI-D fragment. Cloned ANG fragments were transfected into cells with KOS DNA, and the progeny virus was tested by inoculation on rear footpads of mice. Fractions indicate the number of cotransfection cultures that killed mice relative to the total number of cultures tested. The locations of the KpnI-N and KpnI-BamHI 3.0-kb fragment within BamHI-G and the location of BamHI-G in the HSV-1 genome are also shown.
differences were found, but only two amino acid changes in glycoprotein B were predicted. These results are summarized in Fig. 7.

In vitro selection of KOS viruses containing ANG glycoprotein B sequences. To verify that the sequence difference just identified is significant and that no selection for neuroinvasive recombinant viruses occurred during passage in the mouse, we elected to select, in vitro, KOS viruses which carried ANG sequences in the region of interest. When tested in mice, these viruses should be significantly more neuroinvasive than the parental KOS strain. KOS virus DNA was cotransfected with the plasmid containing ANG SacI-SalI sequences in a KOS background (the EcoRI-D fragment was not cotransfected in this case), and viruses derived from two different transfection plates were plaque purified. Recombinant viruses were detected by hybridization to a 21-base oligomeric probe spanning the sequence coding for amino acid 523 of glycoprotein B (alanine versus valine amino acid difference). One recombinant from each plate was then tested via footpad inoculation to determine the PFU/50% lethal dose ratio and neuroinvasive phenotype. As shown in Table 2, a PFU/50% lethal dose ratio could not be calculated. However, both viruses killed a significant number of mice ($P = 0.001$) over a range of inoculation titers, indicating that the neuroinvasive phenotype had been restored. From these results, we conclude that a single base difference in glycoprotein B significantly enhances KOS neuroinvasiveness.

**DISCUSSION**

Our results indicate that at least two separate genes are responsible for the nonneuroinvasive phenotype of the HSV-1 KOS strain, and we have localized one of these to a 6.3-kb fragment of the genome, BamHI-A/EcoRI-D. The BamHI-A/EcoRI-D fragment contains the entire UL10, UL11, UL12, and UL13 and part of the UL9 and UL15 open reading frames. This region of the HSV-1 genome has not been well characterized with respect to function; however, among these open reading frames are those for alkaline nuclease (UL12), a hydrophobic structural protein (UL10), an origin-binding replication protein (UL9), a myristylated protein involved in nucleocapsid envelopment (UL11), and a putative protein kinase (UL13) (2, 15, 16, 23, 30). We do not yet know which of these open reading frames is responsible for the increased neuroinvasiveness of ANG-KOS recombinants.

The second area of the genome required to restore neuroinvasiveness to the KOS virus was localized to glycoprotein B, more specifically, to a region in which ANG differs from KOS by only two amino acid changes. One of these differences appears to be unique to the KOS strain used by our laboratory, since the published sequences for the KOS, 17syn+, and F strains of the virus are the same as the ANG sequence at this codon (4, 17, 20). The second amino acid difference, at amino acid 523 of the mature glycoprotein B, is a relatively conservative change (from a valine in KOS to an alanine in ANG). However, it was shown some time ago (3) that a mutant carrying a temperature-sensitive mutation in glycoprotein B (tsB5) actually demonstrated a more rapid rate of entry into the cell than the KOS strain. This difference was traced to the same amino acid difference which we have defined here and is not related to the temperature sensitivity of glycoprotein B in tsB5. Another analysis of monoclonal antibody-resistant mutants indicated that changes in this region (within 40 codons of that for amino
TABLE 2. Lethal effects of KOS-ANG glycoprotein B recombinants after footpad inoculation of mice

<table>
<thead>
<tr>
<th>PFU inoculated</th>
<th>KOS (n = 7)</th>
<th>GB1 (n = 8)</th>
<th>GB2 (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁴</td>
<td>0</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td>10³</td>
<td>0</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>10¹⁰</td>
<td>ND</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>10⁴</td>
<td>ND</td>
<td>0</td>
<td>13</td>
</tr>
</tbody>
</table>

Recombinant KOS viruses containing ANG sequences in the SacI to SaI region of the gB open reading frame (here named GB1 and GB2) and KOS were infected at various dilutions on the rear footpads of mice. Mice were observed for 20 days, and deaths were recorded.

ND, not done.

![Image](https://via.placeholder.com/150)

Acid 523 also produced viruses with altered rates of entry (9). Thus, it would appear that this region of glycoprotein B is involved in the entry process; changes affect the rate at which the virus is able to enter cells.

Glycoprotein B is a transmembrane glycoprotein required for infection of cells. Together with glycoproteins C and D, glycoprotein B appears to interact with cell surface components for attachment of virions to the cell surface (13). It is also known to function in the penetration step of infection (21). Various domains of the protein have been associated with the formation of syncytia (3), the pathobiology of infection, and, as just stated, the rate of cell entry. In a "negative" genetic experiment, replacement of glycoprotein B gene sequences in HSV-1 ANG-path with HSV-1 KOS glycoprotein B sequences was shown to reduce the pathogenicity of the ANG-path virus (29). This result is consistent with our results but cannot be considered definitive because the recombination process itself may have altered the biological functions of glycoprotein B, resulting in loss of gene function. In addition to these studies, two mutations in the carboxyl-terminal portion of glycoprotein B were found to produce increased peripheral inflammatory response and decreased neuropathy following footpad inoculation (7). All these results indicate that glycoprotein B is intimately involved in the pathogenicity of HSV-1. We show here that this general property is due in part to the role that glycoprotein B plays in neuroinvasiveness.

We had earlier shown that a single amino acid change in glycoprotein D is a principal determinant in the enhanced neuroinvasiveness demonstrated by ANG-path compared with its parent ANG (10). This resulted in a greater than 100-fold increase in the PFU/50% lethal dose ratio when virus was inoculated on the footpad. In the case of glycoprotein B, the changes that we observed, in particular the amino acid change which has been shown to alter the rate of entry into infected cells, could affect neuroinvasiveness by changing either (i) the ability of the virus to penetrate neurons or (ii) the immunogenicity of the virus. With respect to option i, it is of interest that in studies of the related pseudorabies virus, deletion mutants missing gp63 (a membrane glycoprotein homologous to HSV-1 gB) exhibit decreased virulence (12). This protein is involved in cell-to-cell transmission of the virus and in neurotropism (31, 32). Other studies of pseudorabies virus have also noted that, while glycoproteins involved in cell-to-cell transmission are involved in pathogenesis, those mediating viral attachment to host cells are not important for this property (19). The decreased rate of entry of KOS into cultured cells might extend to decreased capacity for virion penetration into
nerve terminals. Since KOS replicates efficiently in the brain, this phenomenon would have to be restricted to nerve terminals in the footpad or between the peripheral and central nervous systems; alternatively, this phenomenon would not extend to direct infection of neuronal cell bodies. As for immunologic effects, the differences in structure of glycoprotein B may make the ANG virus a less competent immunogen, or the faster rate of cellular entry by the virus may make it less subject to immune restriction. Our preliminary results indicate that the explanation involving immunologic differences may be the correct one, since ANG and KOS exhibit PFU/50% lethal dose ratios similar to those of ANG-path tested under identical immunosuppressive conditions. Furthermore, tissue sections of dorsal root ganglia from ANG- and KOS-infected mice show more mononuclear cell infiltrates than tissue sections from ANG-path-infected mice (19a).

Finally, our results indicate that glycoproteins B and D are very important in determining the neuroinvasiveness ability of HSV. These glycoproteins are significant both in the infective process and in inducing a protective immune response. Either or both of these attributes could be important in determining the neuroinvasiveness of the virus. Since neuronal transmission is the usual route taken by HSV-1 to the central nervous system, the ability of these glycoproteins to facilitate infection of the central nervous system from the periphery may be among the most important components of the virus which determine its ability to induce encephalitis.

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