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Pseudorabies Virus Envelope Glycoprotein gI Influences both Neurotropism and Virulence during Infection of the Rat Visual System

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We previously demonstrated that intraocular injections of virulent and attenuated strains of pseudorabies virus (PRV) produce transneuronal infection of functionally distinct central visual circuits in the rat. The virulent Becker strain of PRV induces two temporally separated waves of infection that ultimately target all known retinorecipient neurons; the attenuated Bartha strain only infects a functionally distinct subset of these neurons. In this study, we demonstrate that deletion of a single viral gene encoding glycoprotein gI is sufficient to reproduce both the novel pattern of infectivity and the reduced neurovirulence of the Bartha strain of PRV. Glycoprotein gIII, a major viral membrane protein required for efficient adsorption of virus in cell culture, has no obvious role in determining the pattern of neuronal infectivity, but appears to function with gI to influence neurovirulence. These data suggest that neuroinvasiveness and virulence are the products of an interaction of viral envelope glycoproteins with as yet unidentified cellular receptors.

Pseudorabies virus (PRV) is an alphaherpesvirus belonging to the family *Herpesviridae* that causes an infection in swine similar to that induced by herpes simplex virus in humans (1a, 7, 34). These DNA viruses possess a membrane envelope containing at least seven different glycoproteins with differing but significant homology to glycoproteins in other herpesviruses and appear to serve similar functions (see reference 13 for a recent review on PRV). Three of the PRV glycoproteins (gp50, gII, and gH) are classified as essential because they are required for viral replication in cell culture. The remaining four (gI, gIII, gX, and gp63) are not required for replication of the virus but have been implicated in important viral functions such as target cell recognition, efficient viral release from infected cells, interaction with the host immune system, and virulence (34).

Virulent strains of PRV are highly neurotropic and produce lytic infections of central nervous system (CNS) neurons following either central or peripheral inoculation (2, 5, 6, 30). In contrast, an attenuated strain of the virus (Bartha [PRV-Ba]) remains neuroinvasive but does not induce the pronounced lytic infections characteristic of the virulent strains (3, 4, 12, 25, 26, 30, 31, 32). The fundamental mechanisms underlying these differences in attenuation have not been established with certainty, but substantial evidence points to alterations in the glycoprotein constituents of the viral envelope as one of the principal causes.

PRV-Ba is a well-known attenuated vaccine strain that is the basis for the Duvaxyn vaccine sold in Europe (20, 33). Although PRV-Ba grows very well in cell culture, it harbors mutations in genes for three of the seven envelope glycoproteins. Most notably, a large deletion in the unique short (U_S) segment of the viral genome removes coding sequences for a series of genes, including those for glycoproteins gI and gp63 (14, 19). Zuckermann et al. (35) have demonstrated that these two glycoproteins form a complex necessary for the full expression of virulence. In addition, other genetic alterations also contribute to the reduction in virulence exhibited by this strain (16). Two prominent mutations in the unique long (U_L) segment of PRV-Ba have been implicated in this effect. These include a signal sequence mutation within the gene for gIII that substantially reduces the concentration of this glycoprotein in the viral envelope (9, 22), and mutations in the *Bam*HI-4 fragment, encoding at least four genes thought to be involved in capsid assembly (10, 16). These investigations demonstrated that the major contributor to the reduced virulence in PRV-Ba was the large deletion in the U_S region of the genome, but that full virulence could only be restored through marker rescue of gIII and genes in the *Bam*HI-4 fragment. Similarly, Mettenleiter et al. (15) reported that combined deletion of gI and gIII produced a strain of virus that was avirulent when injected intracerebrally in chickens. Collectively, these studies indicate that two or more glycoproteins were involved in the expression of PRV virulence.

Our recent in vivo analyses in rats support the contention that the altered expression of envelope glycoproteins in PRV-Ba reduces virulence (2, 3). In addition, these studies have shown that intraocular injection of PRV-Ba produces a pattern of neuronal infectivity that differs substantially from that observed when the Becker strain of PRV (PRV-Be) is injected in the same manner. PRV-Be ultimately produces transneuronal infection of neurons in all areas in the CNS that receive direct retinal projections, but PRV-Ba only infects a functionally distinct subset of these neurons. Since viral glycoproteins are known to be involved in target cell recognition and virulence (13, 34), we hypothesized that one or more of the glycoprotein deficits in PRV-Ba was responsible for the altered pattern of CNS infectivity. In the present study, we have tested this idea by creating a series of defined mutant strains of virus and examining their infectivity and virulence in our rodent eye model. The data demonstrate unequivocally that the absence of a single PRV glycoprotein, glycoprotein gI, leads to the altered neurotropism produced by PRV-Ba. The data also provide insight into the role of individual glycoproteins in viral pathogenesis and virulence.

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MATERIALS AND METHODS

Animals. A total of 150 adult male Sprague-Dawley rats, weighing 200 to 350 g at the time of sacrifice, were used in these analyses. Food and water were freely available throughout the course of each experiment, and the photoperiod was standardized to 14 h of light and 10 h of dark (light on at 0600 h). Experimental protocols were approved by the Animal Welfare Committee and were consistent with the regulations of the American Association for Accreditation of Laboratory Animal Care and those in the Animal Welfare Act (Public Law 99-198). All animals were confined to a Biosafety Level 2 laboratory throughout the course of each experiment; specific safeguards necessary for operation of this laboratory have been published previously (2).

Virus. A total of 11 PRV strains were evaluated in this study. Eight mutants were constructed from three parental strains. The parental strains used were Becker (PRV-Be), Ka (PRV-Ka), and Bartha (PRV-Ba). Five strains were constructed in the PRV-Be background and three were constructed in the PRV-Ba background by marker rescue with PRV-Ka DNA. A diagram of the genetic organization of relevant portions of the PRV genome and the deletions constructed in this work are shown in Fig. 1. Not shown in this figure are three mutant strains of virus constructed in the PRV-Ba background by marker rescue with PRV-Ka DNA (1, 10). In PRV4325A, the U_S segment was rescued with PRV-Ka, but the U_L segment of the genome still contained all the PRV-Ba mutations, including the defective gIII gene and the mutant *Bam*HI-4 region. PRVD was identical to PRV4325A, but a deletion of the gI gene was introduced by genetic engineering. In PRV4325AB4, the *Bam*HI-4 fragment of PRV-Ka was used to rescue the *Bam*HI-4 mutation present in PRV4325A. Tamar Ben-Porat (Vanderbilt University) generously provided strains PRV-Ka, PRV4325A, PRV4325AB4, and PRVD.

Each strain of virus was grown in pig kidney fibroblasts as described previously (2). Virus titers, in plaque-forming units per milliliter, were as follows: PRV-Be, 6×10^8; PRV-Ba, 5×10^8; PRV91, 3×10^8; PRV4, 7×10^7; PRV4/91, 5×10^7; PRV22, 3×10^8; PRVBaBe, 2×10^8; PRV4325A, 1×10^9; PRVD, 5×10^8; and PRV-Ka, 8×10^8.

Construction of viral mutants. PRVBaBe, PRV91, and PRV4/91 were constructed in the following manner. To create PRV91, the *Bam*HI-7 fragment of PRV-Be (approximately 6.5 kb) was cloned into the *Bam*HI site of a bacterial plasmid vector (pBluescript II SK$^+$; Stratagene) by standard recombinant DNA techniques. Restriction enzyme mapping analysis was used to isolate a plasmid containing the PRV *Bam*HI-7 fragment (designated pALM94). The sequence encoding the gI gene was deleted by partial digestion of pALM94 with *Dra*I and then complete digestion with *Bsp*EI. The DNA termini were repaired with the Klenow fragment of *Escherichia coli* DNA polymerase and all four nucleotide triphosphates. The DNA was then religated and transformed into *E. coli* NF1829 (23), and a plasmid containing a 1,702-bp deletion (designated pALM91) that extended from 46 bp upstream of the ATG codon for gI to 75 bp upstream of the TAA stop codon for gI was isolated. DNA sequence analysis confirmed the anticipated deletion but also indicated that a C residue had been deleted during the cloning process.

In order to construct a virus strain carrying the gI deletion, PRV-Be DNA was cotransfected with pALM91 DNA as previously described (23). Virus progeny from this transfection were grown on PK15 cells and screened in a black-plaque immunoassay with a gI-specific monoclonal antibody (23). A nonreactive white plaque was isolated, purified three times, and designated PRV91. The absence of the gI gene was verified by Southern blot analysis of viral DNA and immunoprecipitation.

PRVBaBe was constructed by a similar strategy. The *Bam*HI genomic DNA fragment encompassing approximately 3.3 kb of the U_S region of PRV-Ba was cloned into pBluescript II SK$^+$. A plasmid (designated pALM95) containing this DNA fragment was isolated, and its identify was verified by restriction enzyme analysis. Subsequently, PRV-Be DNA was cotransfected with pALM95 DNA, and virus progeny were screened in the black-plaque assay described above. A white plaque was isolated and purified three times, and the presence of the deletion in the U_S region was verified by Southern blot analysis and immunoprecipitation of specific glycoproteins after infection.

The virus strain PRV4/91 was constructed by infecting PK15 cells with an equal number of infectious units of PRV4 and PRV91 at a multiplicity of infection of 2 PFU/cell. Resultant virus progeny were isolated and screened in a black-plaque assay with a mixture of monoclonal antibodies generated against gIII and gI. Recombinant viruses were identified as white plaques. One such white plaque was purified, and Southern blot analysis and immunoprecipitation confirmed that the virus contained both the PRV91 and PRV4 mutations carried by the parental viruses.

PRV4 has been described in detail by Ryan et al. (27). It is a double mutant containing a nonsense codon (TAG) at codon 157 and a deletion extending from codons 157 to 290 in the gIII gene. The virus expresses a truncated amino-terminal fragment of gIII that is secreted and not found in virion envelopes. In addition, the construction and characterization of PRV22 have been described by Robbins and collaborators (22).

Antisera. Three rabbit polyclonal antisera (Rb 132, Rb 133, and Rb 134) were used to localize both strains of virus. These antisera were generated by injecting rabbits with acetone-inactivated PRV-Be. Comparative localization of virus with each antiserum in adjacent sections from the same animal revealed that all of the antisera produced identical patterns of immunoreactivity. Additionally, immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of [^{3}H]glucosamine-labeled, PRV-Be-infected PK15 cells demonstrated that all of the major envelope glycoproteins were recognized by these antisera. However, in the majority of experiments, we used rabbit antiserum Rb 134, which we previously characterized in our analyses of the neural transport of PRV-Be and PRV-Ba (2, 3).

Experimental paradigm. Animals were anesthetized by intramuscular injection of ketamine and xylazine and injected with 2 μl of virus in the vitreous body of the eye as described previously (3). Each animal was killed by transcardiac infusion of buffered aldehyde solutions, followed by removal of the brain and injected eye for immunohistochemical localization of virus. The brains were sectioned serially in the coronal plane at 35 μm per section with a freezing microtome. These sections and whole-amount preparations of the retina were incubated in one of the rabbit polyclonal anti-PRV antisera, and virus in the tissue was localized with the avidin-biotin modification (8) of Sternberger's immunoperoxidase procedure (29) as described previously (2). Tissue sections were subsequently mounted on gelatin-coated glass slides, cleared, coverslipped for light microscopic analysis, and photographed with interference contrast optics on a Reichert Optiphot photomicroscope.

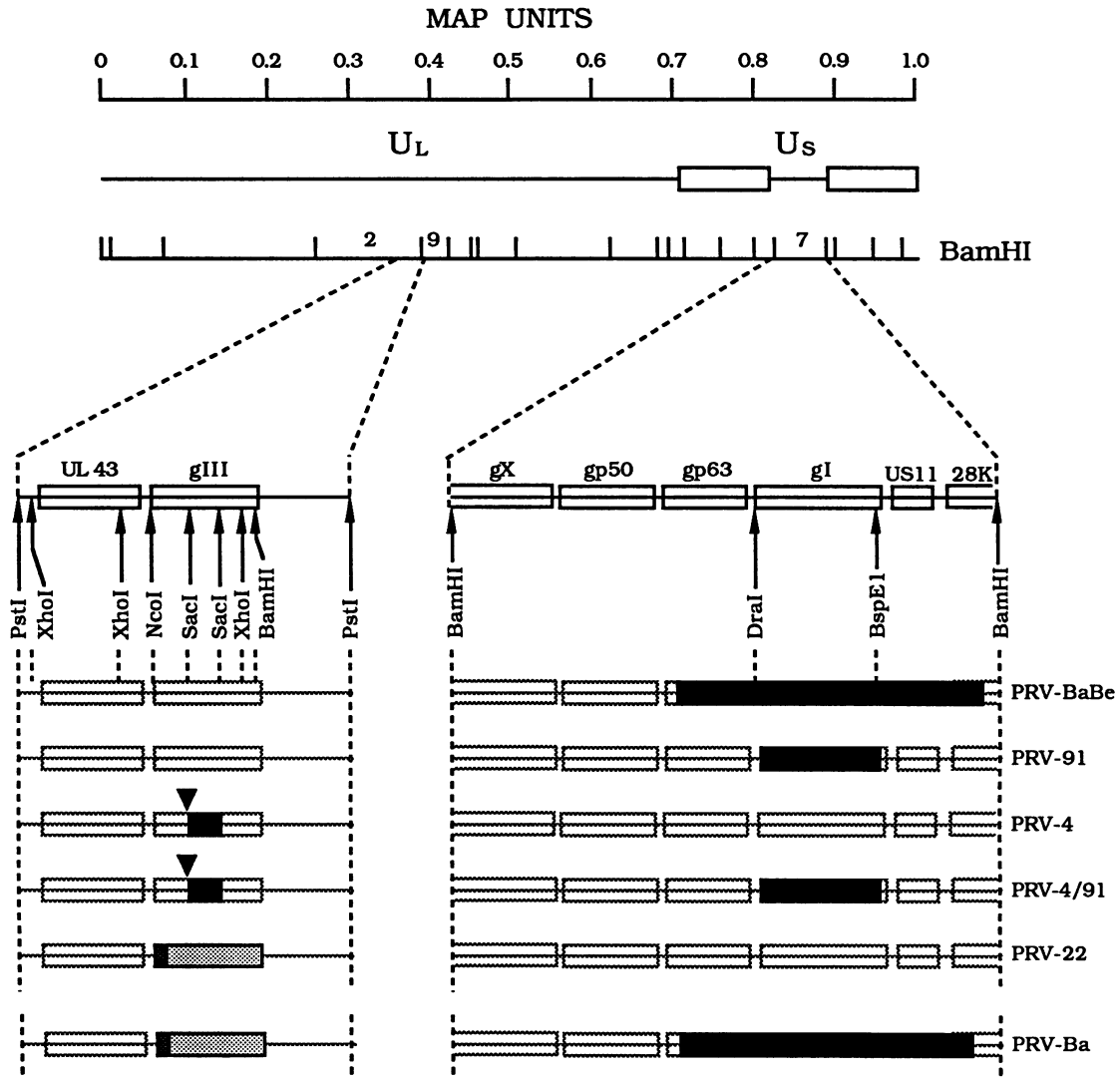

FIG. 1. PRV genome and map of relevant mutations. The genetic map of PRV is indicated at the top of the figure; the first line indicates arbitrary map units, and the second line illustrates the PRV genome with the unique long (U_L) and unique short (U_S) regions. The boxes in line 2 represent inverted repeat segments bracketing the U_S region (1a). The third line represents the *Bam*HI restriction map of PRV-Be and PRV-Ka (sizes of the fragments are not drawn to scale). The *Bam*HI-2,-4,-9, and -7 fragments are indicated because they are relevant to the genes examined in this study. An expansion of two relevant areas of the PRV genome in the next line shows the relative orientations of the genes encoding proteins U_L43, gIII, gX, gp50, gp63, gI, U_S11, and 28K. Mutations in PRV-Ba, an attenuated PRV strain central to this report, are indicated on the last line of the figure. They include a large deletion (black box) removing gI, U_S11, and large portions of gp63 and 28K. In addition, the PRV-Ba gIII gene contains several mutations (shaded box), including a point mutation in the signal sequence that substantially reduces the concentration of gIII in the viral envelope. The genetic organizations of five other mutants used in this study are also shown. The deletion from the U_S region of PRV-Ba was transferred to PRV-Be to make strain PRVBaBe. PRV91 was made by deleting a *Dra*I-*Bsp*EI fragment from PRV-Be. PRV4 is a gIII mutant with two mutations: a 134-codon deletion (codons 157 to 290), and an insertion of a short DNA linker containing a stop (TAG) codon at codon 157. PRV4/91 is a recombinant virus made by crossing PRV4 with PRV91. The solid inverted triangle in the gIII gene of PRV4 and PRV4/91 marks the location of the TAG stop codon. In PRV22, the PRV-Be gIII gene is replaced with the defective gIII gene from PRV-Ba.

RESULTS

Rationale. Full appreciation of the infectivity patterns and virulence observed in our studies requires an understanding of the mutations present in each strain of the virus and the organization of the visual projection systems in which they were tested. We used three approaches to map the defects in PRV-Ba responsible for its neurotropism and neurovirulence phenotypes. First, we cloned segments of PRV-Ba DNA that contained known mutations and transferred these mutations to PRV-Be by marker rescue techniques (Fig. 1). Specifically, we crossed the large deletion in the PRV-Ba U_S region to PRV-Be to create PRVBaBe. Similarly, the defective gIII gene of PRV-Ba was transferred to PRV-Be to create PRV22. Second, we made defined mutations in single PRV-Be glycoprotein genes; the gI gene was deleted to produce PRV91, a nonsense mutation in the gIII gene was introduced to create PRV4, and a double gI-gIII mutant was isolated by recombination of PRV91 and PRV4 to yield PRV4/91. This method produced a set of isogenic PRV-Be strains with the full spectrum of PRV-Ba glycoprotein gene

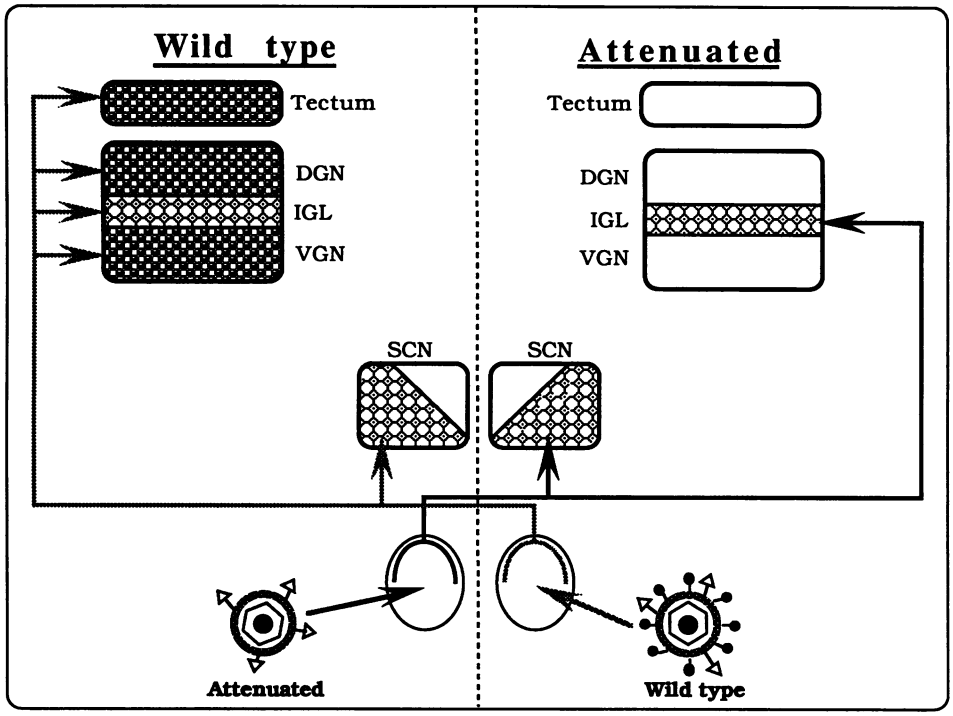

FIG. 2. Visual circuitry and viral infectivity. The organization of the central visual projection systems and the different patterns of infectivity resulting from intraocular injection of virulent virus (PRV-Be) and an attenuated strain (PRV-Ba) are illustrated. Only the crossed component of the central visual projection system is shown so that the two patterns of viral infectivity can also be represented. Retinal ganglion cells in the eye give rise to processes which project through the optic nerve, chiasm, and tract to innervate the suprachiasmatic nucleus (SCN), ventral and dorsal geniculate nuclei (VGN and DGN), intergeniculate leaflet (IGL), and tectum (VGN, DGN, and IGL constitute the geniculate complex). PRV-Be injected intraocularly infects neurons in all of these areas in two temporally separated waves (shown by the two patterns on the diagram). PRV-Ba only infects a functionally distinct subset of these neurons.

mutations as well as specific mutations in two of these PRV glycoproteins (Fig. 1). Finally, we obtained an isogenic set of PRV-Ba strains (generously provided by Tamar Ben-Porat) in which specific PRV-Ba mutations were repaired with DNA from PRV-Ka. PRV-Ka is a standard laboratory strain closely related to PRV-Be (1a). Specifically, the entire U_S region of PRV-Ba was replaced with DNA from PRV-Ka to produce strain PRV4325A, which was then used to construct two additional mutants. The *Bam*HI-4 fragment of PRV4325A was replaced with PRV-Ka DNA to produce PRV4325AB4, and PRVD was made by replacing the U_S region of PRV4325AB4 with DNA from PRV-Ka that contained a defined deletion of the gene for gI. We also included PRV-Ka in the studies as a control for the PRV-Ba/PRV-Ka hybrid viruses. Specific details on the construction and characterization of the mutations in each of these strains are provided in Materials and Methods and in Fig. 1.

The neuroinvasiveness and virulence of each of the above strains of virus were characterized by injecting 2 μl of virus (approximately 10^6 PFU) into the vitreous body of the eye and examining the resulting pattern of infection of the visual circuitry (for details, see Materials and Methods). In each case, the eyes were examined at several postinoculation time intervals, and the results were compared with the patterns of infectivity produced by injection of PRV-Be and PRV-Ba. Several aspects of the visual circuits make this an ideal system for these studies. First, injection of the virus intraocularly largely eliminates concerns related to nonspecific spread of virus from the site of injection; the initial infection involves retinal ganglion cells, and infection of the brain occurs via anterograde, transneuronal transport of virus through the axons of these cells. When virus leaked into the orbit, we noted infection of both motor neurons innervating the orbital muscles and the sympathetic component of the autonomic nervous system projecting to the orbit. However, these circuits do not overlap the primary retinorecipient fields in the brain and therefore did not compromise the analyses. Second, retinal projections to the CNS have been characterized quite rigorously and are known to terminate in functionally distinct and nonoverlapping areas of the brain (see reference 18 for a recent review). As we have shown, this functionally segregated distribution of retinal terminals provides an ideal system for defining differences in the infectivity of different virus strains (Fig. 2) (3).

Retinal infectivity. Immunohistochemical analysis of retinal whole-mount preparations demonstrated that each strain of virus infected retinal ganglion cells. Two general patterns of infectivity were observed. PRV-Be infected large numbers of small oval neurons, and at longer survival times, viral immunoreactivity was also noted in larger multipolar ganglion cells (Fig. 3A). The majority of these cells were confined to the peripheral two-thirds of the retina. PRV-Ba immunoreactivity was preferentially concentrated in the large multipolar population of ganglion cells, but also appeared in a limited number of the smaller cells (Fig. 3B). The infectivity produced by the remaining strains of PRV fell into one of these categories, with the determining factor being the presence or absence of the gI gene. Strains lacking gI (PRVBaBe, PRV91, PRV4/91, and PRVD) reproduced the

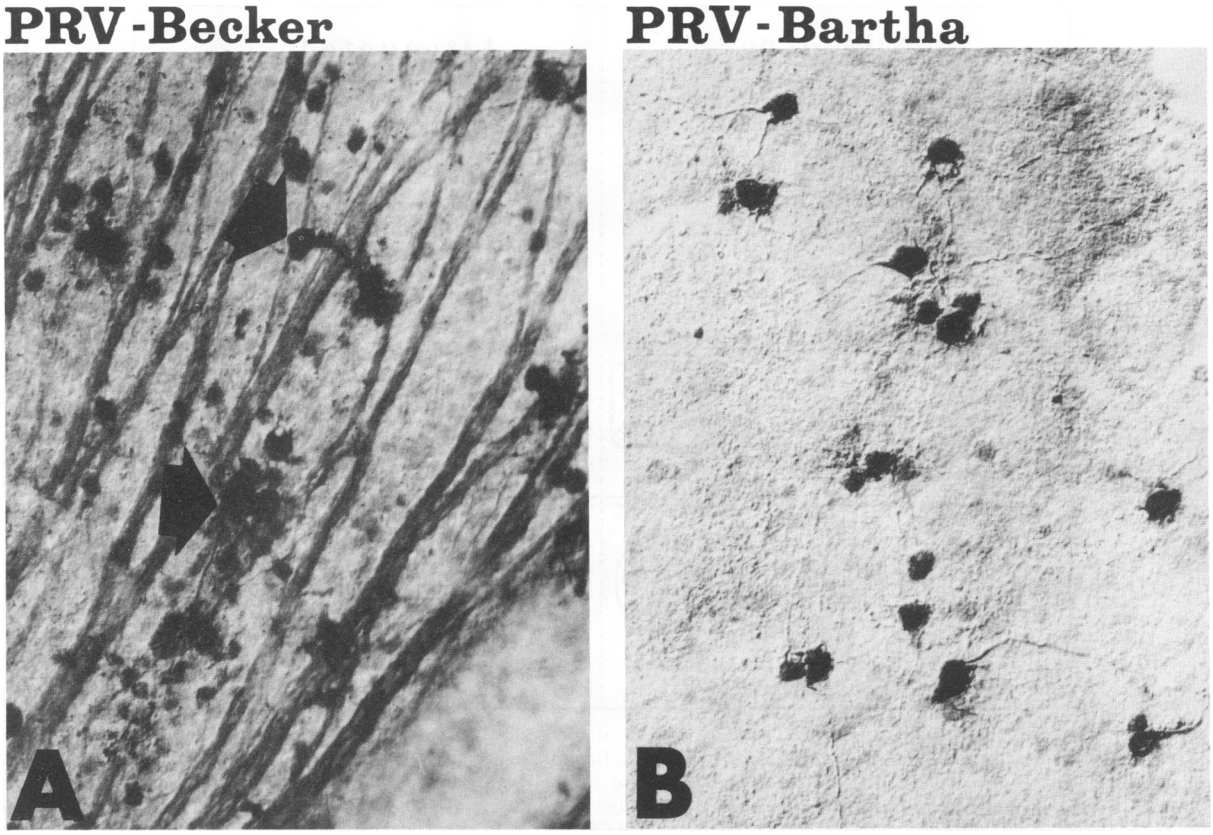

FIG. 3. Retinal infectivity. The morphology of retinal ganglion cells infected by PRV-Be (A) and PRV-Ba (B) is shown. Pronounced immunoreactivity is also apparent in optic axons in the PRV-Be preparation. The arrows in panel A designate cells infected by PRV-Be that are similar in morphology to those infected by PRV-Ba.

PRV-Ba pattern; all other strains produced infectivity characteristic of PRV-Be.

PRV-Be forebrain infectivity. Consistent with our previous observations, intraocular injection of PRV-Be produced two, temporally separated waves of infection which ultimately targeted neurons in all retinorecipient areas of the forebrain (Fig. 4A through C). The first wave of infection became evident in the dorsal and ventral geniculate nuclei (DGN and VGN) and the tectum approximately 48 h following inoculation, and the number of infected cells in these regions increased progressively with advancing time (Fig. 4B and C). The suprachiasmatic nuclei (SCN) of the hypothalamus and intergeniculate leaflet of the thalamus (IGL) were spared during the first wave of infection but were the principal target of the second wave. Infected cells in both the SCN and IGL were first apparent at 72 h postinoculation, and the number increased with advancing survival time (Fig. 3A and B). The same pattern and temporal aspects of the infection were produced with PRV-Ka, a well-characterized virulent laboratory strain (data not shown).

PRV-Ba forebrain infectivity. PRV-Ba infection of central visual circuits was dramatically different from that produced by PRV-Be (Fig. 4D through F). Neurons targeted by the first wave of PRV-Be infection were not infected with PRV-Ba, even at survival times extending to 120 h (compared with 48 h with PRV-Be). However, PRV-Ba infected the SCN and IGL (targets of the second wave of PRV-Be infection) in a temporal sequence identical to that induced by PRV-Be.

Identification of the gene locus responsible for the PRV-Ba infectivity phenotype. Our strategy focused primarily on two regions of the PRV-Ba genome containing known mutations in viral envelope glycoproteins. Glycoprotein gIII is involved in the efficient attachment of PRV to cells in culture, and the gI glycoprotein is known to have significant effects on virulence and virus exit from cultured cells (13, 34). Despite the variety of viral mutants tested, each individual strain produced only one of the two distinct patterns of visual circuit infectivity, either the extensive PRV-Be pattern or the restricted PRV-Ba pattern (Fig. 2). It was readily apparent that glycoprotein gIII had little influence on the pattern of infection; infection produced by two PRV-Be gIII mutants (PRV4 and PRV22) was indistinguishable from that produced by PRV-Be. These strains induced the two-phase, temporally separated waves of infection that ultimately targeted all retinorecipient neurons in the forebrain (data not shown).

In contrast, when the large deletion from the U_S region of PRV-Ba was transferred to PRV-Be (PRVBaBe), the resulting pattern of infectivity was identical to that produced by PRV-Ba. Infected neurons were restricted to the SCN and IGL and were never observed in the dorsal geniculate nuclei or tectum at survival times extending to 96 h (Fig. 5A through C). Furthermore, the time course of infection of this circuit with PRVBaBe was identical to that resulting from injection of PRV-Ba. Further evidence that the PRV-Ba deletion in the U_S region is responsible for the infectivity phenotype was obtained from experiments with PRV4325A.

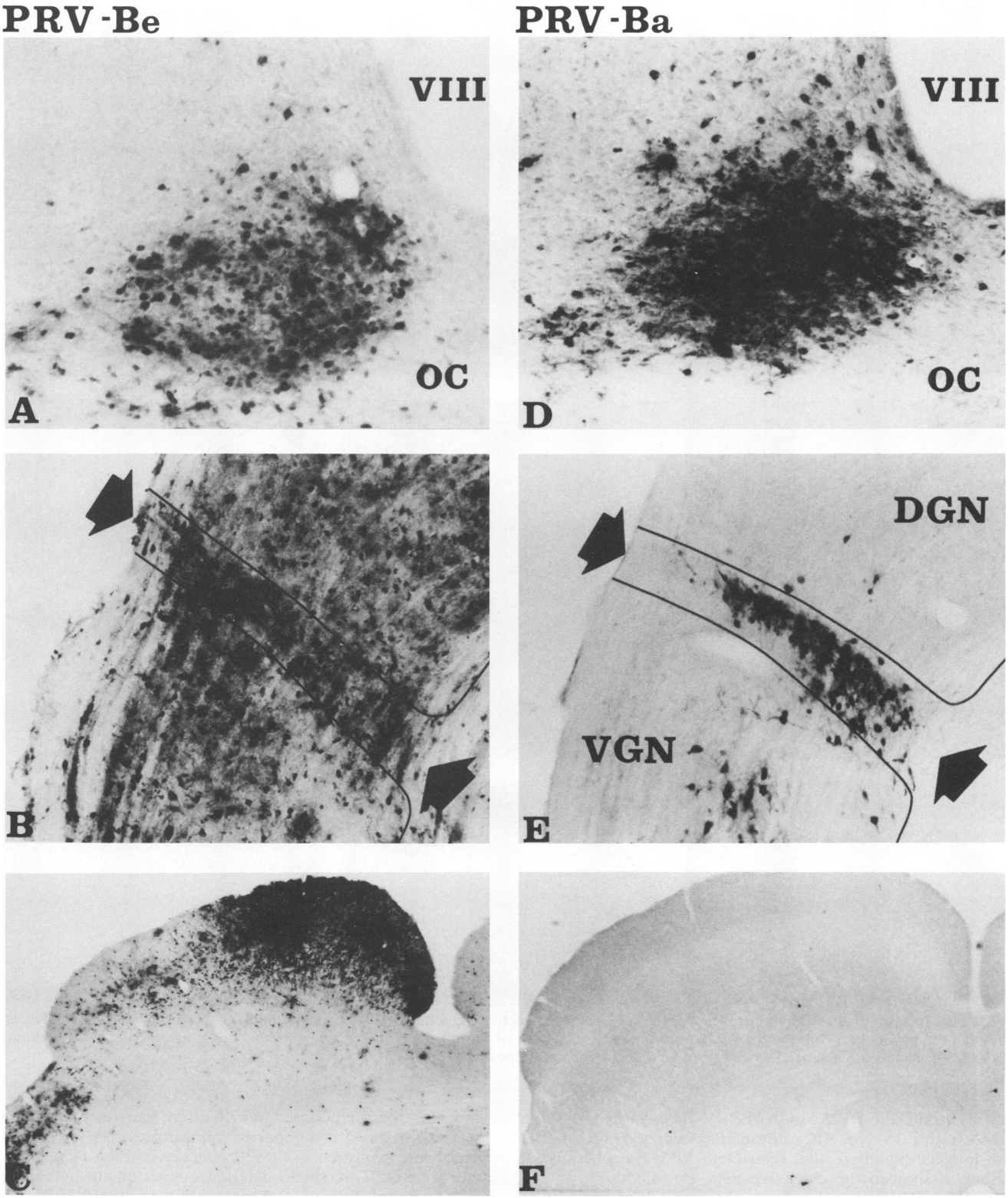

FIG. 4. Differential patterns of PRV-Be and PRV-Ba infectivity. The patterns of infectivity produced by intraocular injection of PRV-Be and PRV-Ba are shown. Panels A and D are through the SCN, panels B and E are through the geniculate complex, and panels C and F are through the tectum. Note that PRV-Be infects neurons in all visual centers, while PRV-Ba only infects neurons in the SCN and IGL (arrows) subdivision of the geniculate complex.

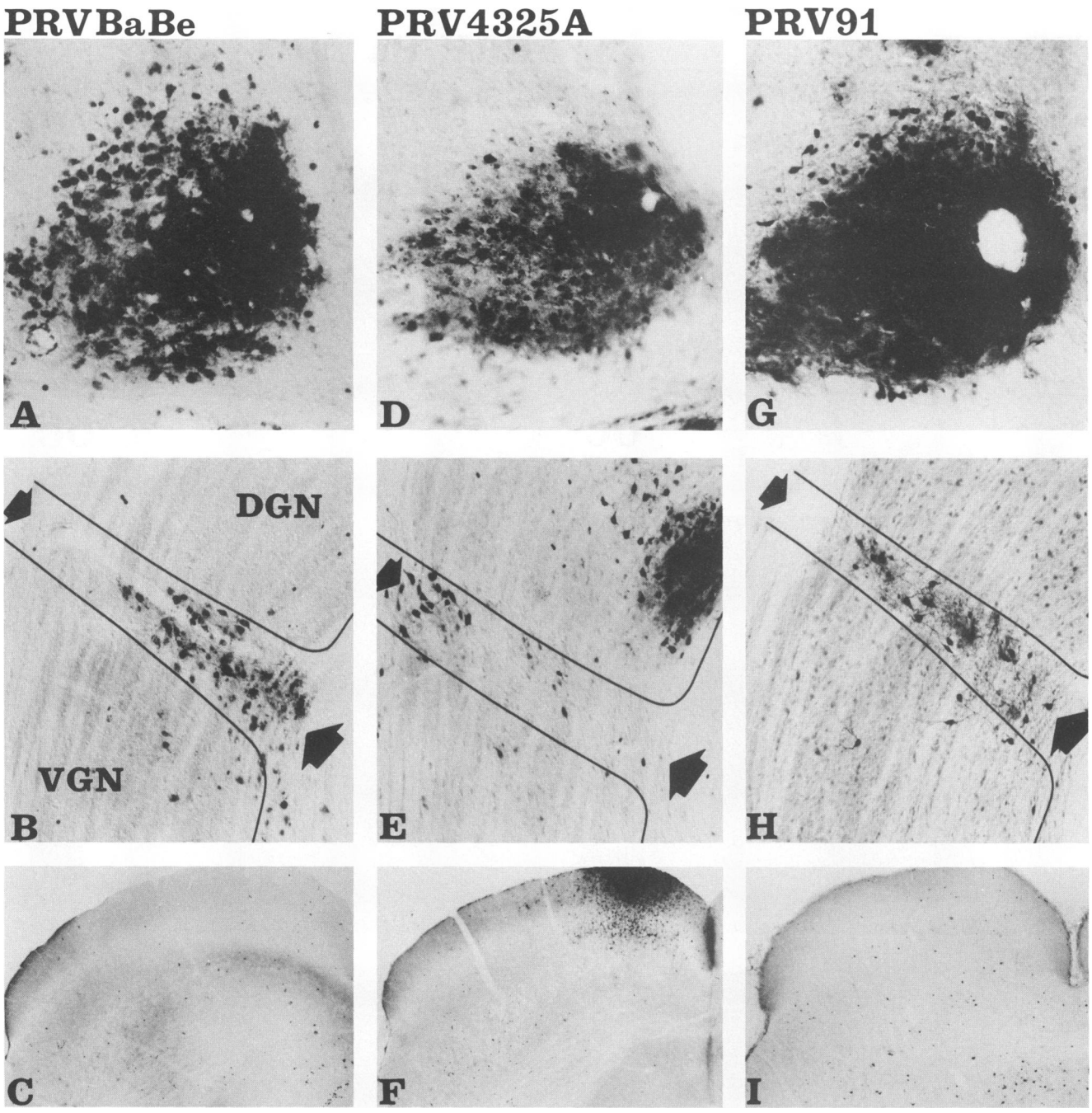

FIG. 5. Infectivity of viral mutants. The patterns of forebrain infectivity resulting from intraocular injection of PRVBaBe, PRV4325A, and PRV91 are shown. PRVBaBe and PRV91 both possess a deletion of the gI gene, and this segment of the genome has been restored in PRV4325A. Panels are arranged as in Fig. 4. Arrows designate the position of the IGL.

In this strain, the U_S deletion of PRV-Ba was restored with DNA from PRV-Ka. After intraocular injection, PRV4325A no longer exhibited the restricted PRV-Ba phenotype; rather, it showed the extensive PRV-Be infectivity phenotype (Fig. 5D through F). Thus, the inability of PRV-Ba to infect visual circuits targeted by the first wave of PRV-Be infection results from the deletion of one or more of the genes (gp63, gI, US11, or 28K) in the U_S segment of the genome.

Which gene(s) affected by the PRV-Ba deletion is responsible for the phenotype? We focused first on the gI gene because work in a number of laboratories has stressed its major role in virulence. PRV91 contains a precise deletion of the gI gene. This single deletion converted the infectivity phenotype of PRV-Be to that exhibited by PRV-Ba and PRVBaBe (Fig. 5G through I). A similar experiment was done in the PRV-Ba background. PRVD contains a deletion of the gI gene from PRV4325A, a PRV-Ba strain in which the U_S deletion was rescued with PRV-Ka DNA. This single mutation converted the infectivity phenotype to the PRV-Ba

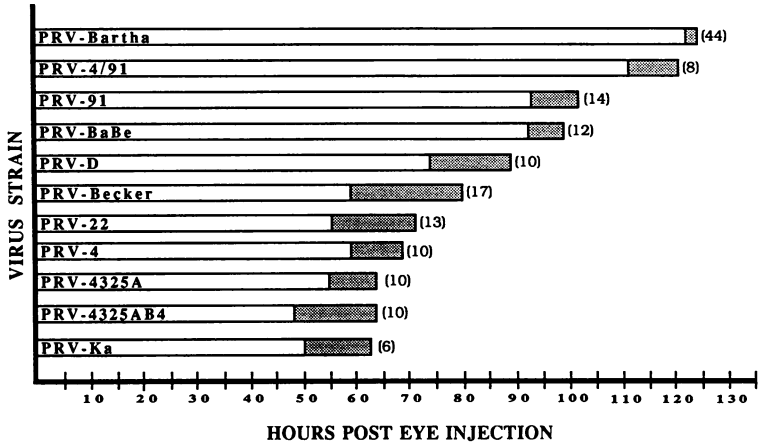

FIG. 6. Neurovirulence of PRV strains. The virulence of each of the strains of virus used in this analysis is illustrated. The postinoculation interval is shown on the horizontal axis, and the effective survival interval for animals injected with each strain of virus is represented by the horizontal bars. The length of each bar illustrates the postinoculation survival achieved with each strain of virus. The open portion of each bar reflects the period when there were few or no overt signs of infection; the shaded portion represents the period when symptoms of infection were pronounced. The number of experimental animals in each group is indicated at the end of each bar. Note that PRV-Ba and the viruses containing a deletion of glycoprotein gI are the least virulent.

pattern (data not shown). We found that all mutants lacking gI, including a gI-gIII double mutant (PRV4/91), infected neurons in the SCN and IGL but did not infect neurons in the dorsal geniculate nuclei and tectum at survival times ranging from 70 to 120 h. Other mutations in PRV-Ba have no apparent effect on the infectivity pattern, since PRV4325A, PRV4325AB4, PRV22, and PRV4 exhibit the PRV-Be infectivity phenotype.

Neurovirulence. Examination of the survival intervals observed after injection of this set of deletion mutants and their parental viruses also localized the PRV genes involved in virulence and cellular pathogenesis. In contrast to the two distinct patterns of infectivity produced by the viral mutants, considerable variation was noted in both the pathology and overt symptoms displayed by animals injected with the various strains of PRV. The laboratory strains (PRV-Be and PRV-Ka), the PRV-Ba/PRV-Ka hybrids (PRV4325A and PRV4325AB4), and the defined gIII mutant strains (PRV22 and PRV4) were extremely virulent and produced pronounced illness within 50 to 60 h of injection (Fig. 6). We note that PRV-Ka is apparently more virulent than PRV-Be in this model. The limited number of animals permitted to reach this stage of survival displayed increased lethargy and oral-nasal secretions indicative of a lung infection. Although the infected neurons in the forebrains of these animals were confined to retinorecipient projection fields, many of these cells were necrotic and surrounded by infected glia. In contrast, virulence and neuronal lysis were substantially reduced for all strains containing a deletion of the gI gene (PRV-Ba, PRVBaBe, PRV91, PRV4/91, and PRVD; Fig. 6). Animals inoculated with mutants lacking gI survived 100 h or longer, and the mutant with the combined deletion of gI and gIII (PRV4/91) was less virulent than the mutants with either of these deletions alone (PRV91 and PRV4). Furthermore, the behavioral symptoms of infection in these animals were essentially eliminated, and the lytic nature of the neuronal and glial infectivity was substantially reduced. A limited number of neurons that were the initial target of infection exhibited pronounced pathogenesis in association with infected glia. However, the majority of neurons exhibited normal morphology, with viral infectivity confined to neuronal perikarya and processes.

DISCUSSION

Previously, we demonstrated that PRV-Be and PRV-Ba produced two distinct patterns of infection in rat visual circuitry (3). We now report the mapping of the PRV genetic locus responsible for this phenotype by using defined mutant and hybrid viruses. The specific infection of a functionally distinct component of the visual circuitry by PRV-Ba was duplicated by deleting the viral gene encoding glycoprotein gI from the PRV-Be genome. Any virus lacking glycoprotein gI was incapable of infecting the dorsal geniculate and tectum but maintained the ability to infect circadian circuits (SCN and IGL) within a temporal framework identical to that for PRV-Ba. Furthermore, this defect could be reversed by replacing the mutant gI allele with the gI gene from the virulent strain PRV-Ka. Our data also indicate that glycoprotein gIII is not required for infection of either population of ganglion cells but does influence virulence in conjunction with gI.

It is well established that herpesvirus envelope glycoproteins play an important role in target cell recognition and neuroinvasiveness (see references 13 and 24 for recent reviews). Seven envelope glycoproteins (gI, gII, gIII, gp50, gp63, gX, and gH) have been characterized in PRV. The herpes simplex virus homologs of these glycoproteins are gE, gB, gC, gD, gI, gG, and gH, respectively. In PRV, gII, gIII, and gp50 are known to be involved in attachment and penetration of the host cell. Adsorption of virions to a heparan sulfate proteoglycan moiety on the host cell surface in the initial stages of virus attachment is mediated by gIII, whereas gII and gp50 are thought to be involved in penetration of the host cell (17, 21). Our data indicate that gI also plays a role in host cell recognition and/or viral invasiveness of the rat visual circuitry. This is supported by the clear demonstration that four mutants lacking the gI gene (PRV-Ba, PRVBaBe, PRV91, and PRV4/91) never infected ganglion cells projecting to the dorsal geniculate and tectum, but

efficiently infected a subset of retinal ganglion cells and their target neurons that project to the SCN and IGL.

In our previous study (3), we suggested that the different patterns of infectivity produced by PRV-Be and PRV-Ba could be explained by interaction of virus with two receptors that were differentially distributed on projection-specific classes of ganglion cells. In this model, virulent virus would see both receptors, but the mutations in the glycoproteins present in PRV-Ba would affect the ability of the virus to recognize the receptor on ganglion cells projecting to the dorsal geniculate and tectum. We continue to favor this hypothesis, but other possibilities must be considered. For example, similar patterns of infectivity would be produced if gI were required for anterograde transport or release of virus from a single class of ganglion cell, or if loss of this glycoprotein resulted in a defect in viral replication in certain cell types. Previous in vitro analyses of PRV mutants lacking the genes for gI and gp63 have demonstrated that these glycoproteins are necessary for virus release from rabbit kidney cells (16, 28, 35). However, several observations suggest that the inability of gI mutants to infect a subset of the central visual projection field in the present analysis is related to a defect in target cell recognition. First, our previous analysis (3) and data from the present set of experiments clearly demonstrated that, even at the longest survival times, PRV-Ba and all gI mutants tested only infected a subpopulation of ganglion cells. If the inability to achieve transneuronal infection of neurons in the dorsal geniculate and tectum were related to a release defect, one would expect that all ganglion cells would be infected. Furthermore, the retinal ganglion cells and their neuronal targets in the SCN and IGL showed no defect in replication or transport of the gI mutants through a multisynaptic pathway. Thus, one would have to postulate that defects in transport, replication, or release are unique to a subset of ganglion cells. Considering that gI mutants could be detected only in a subset of retinal ganglion cells, it seems more likely that the inability to infect a functionally distinct class of these cells is due to a defect in neuroinvasiveness caused by the absence of gI.

Whether gI acts alone or in conjunction with other viral glycoproteins cannot be determined from the present set of experiments. Zuckermann and collaborators (35) have shown that full expression of virulence and efficient release of virus from rabbit kidney cells were dependent upon the formation of a noncovalently linked complex between gI and gp63 proteins. The function of this complex is unknown, but Zuckermann et al. (35) demonstrated that, unlike the homologous herpes simplex virus gE-gI complex, the PRV gI-gp63 complex is not a receptor for human immunoglobulin G. However, because gI and gp63 do physically interact, the complex itself may be a functional unit necessary for infection of visual circuits. Experiments to test this hypothesis are in progress.

The mode of entry of PRV-Ba and other gI mutants into ganglion cells projecting to the SCN and IGL also remains to be established. Our data suggest that glycoprotein gIII is not involved in this process, since two gIII mutants had an infectivity pattern indistinguishable from that of PRV-Be. Zuckermann and colleagues (36) have postulated a gIII-independent route of viral infectivity that is slower and less efficient than the gIII-dependent process. Perhaps PRV uses this mode of infection when entering the retinal circuits. Further work is necessary to define the viral and cellular proteins necessary for virus attachment and entry in this circuit.

The collection of mutants used in our experiments also exhibited significant alterations in virulence and overt symptomology. We noted a marked decrease in virulence after deletion of gI from PRV-Be (PRV91) or from PRV4325A (PRVD), and this effect was further enhanced when combined with defects in gIII (compare PRV91 and PRV4 with PRV4/91). This is entirely consistent with the demonstration that strains of PRV defective in gI and gIII are less virulent than strains with either mutation alone (10, 16). In our in vivo studies with a rat model, decreased virulence was manifested by a reduction in the overt symptoms displayed by the infected animal as well as a marked decrease in the lytic effects of infection on neurons. The principal symptom of infection associated with the more virulent strains of PRV is oral-nasal excretions suggestive of lung involvement. These symptoms are largely eliminated in animals infected with gI and gI-gIII mutants and paralleled a similar reduction in neuronal lysis in the brain. PRV infection of lungs in mice has been reported after intravenous, intraperitoneal, and intranasal inoculation (6). The precise role of gI and gIII in these processes is unclear at present. However, the additive effect of combining the two deletions clearly indicates that both glycoproteins contribute to the virally induced pathogenesis. The role that each of these glycoproteins plays in the generation of neuropathogenesis awaits a systematic examination of the response of nonneuronal cells (i.e., astrocytes and microglia) to neuronal infection induced by mutant strains of virus lacking each of these glycoproteins.

In conclusion, we have demonstrated that a single viral envelope glycoprotein can exert a profound influence upon both the neuroinvasiveness and the virulence of neuronal infectivity produced by PRV. In addition, our findings reaffirm that functionally distinct classes of neurons are differentially susceptible to infection by different mutant strains of PRV and imply that more than one viral envelope glycoprotein can mediate this infectivity. All of these findings have important implications for the use of herpesviruses for mapping multisynaptic pathways in the nervous system and provide further insight into the fundamental mechanisms directing neuronal infectivity and the CNS response to viral infection.

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REFERENCES

1. **Ben-Porat, T.** Personal communication.
1a. **Ben-Porat, T., and A. S. Kaplan.** 1985. Molecular biology of pseudorabies virus, p. 105–173. *In* B. Roizman (ed.), The herpesviruses. Plenum Publishing Corp., New York.
2. **Card, J. P., L. Rinaman, J. S. Schwaber, R. R. Miselis, M. E. Whealy, A. K. Robbins, and L. W. Enquist.** 1990. Neurotropic properties of pseudorabies virus: uptake and transneuronal passage in the rat central nervous system. J. Neurosci. **10:**1974–1994.
3. **Card, J. P., M. E. Whealy, A. K. Robbins, R. Y. Moore, and L. W. Enquist.** 1991. Two α-herpesvirus strains are transported differentially in the rodent visual system. Neuron **6:**957–969.
4. **Dolivo, M., E. Beretta, V. Bonifas, and C. Foroglou.** 1978. Ultrastructure and function in sympathetic ganglia isolated from rats infected with pseudorabies virus. Brain Res. **140:**111–123.
5. **Field, H. J., and T. J. Hill.** 1974. The pathogenesis of pseudorabies in mice following peripheral inoculation. J. Gen. Virol. **23:**145–157.
6. **Fraser, G., and S. P. Ramachandran.** 1969. Studies on the virus of Aujesky's disease. I. Pathogenicity for rats and mice. J. Comp. Pathol. **79:**435–444.

7. **Gustafson, D. P.** 1975. Pseudorabies, p. 209–223. *In* H. W. Dunne and A. D. Leman (ed.), Diseases of swine. The Iowa State University Press, Ames, Iowa.
8. **Hsu, S. M., L. Raine, and H. Fanger.** 1981. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J. Histochem. Cytochem. **29:**577–580.
9. **Lomniczi, B., L. M. Blankenship, and T. Ben-Porat.** 1984. Deletions in the genome of pseudorabies virus vaccine strains and existence of four isomers of the genome. J. Virol. **49:**970–979.
10. **Lomniczi, B., S. Watanabe, T. Ben-Porat, and A. S. Kaplan.** 1984. Genetic basis of the neurovirulence of pseudorabies virus. J. Virol. **52:**198–205.
11. **Lomniczi, B., S. Watanabe, T. Ben-Porat, and A. S. Kaplan.** 1987. Genome location and identification of functions defective in the Bartha vaccine strain of pseudorabies virus. J. Virol. **61:**796–801.
12. **Martin, X., and M. Dolivo.** 1983. Neuronal and transneuronal tracing in the trigeminal system of the rat using herpes virus suis. Brain Res. **273:**253–276.
13. **Mettenleiter, T. C.** 1991. Molecular biology of pseudorabies (Aujesky's disease) virus. Comp. Immunol. Microbiol. Infect. Dis. **4:**151–163.
14. **Mettenleiter, T. C., N. Lukàcs, and H.-J. Rziha.** 1985. Pseudorabies virus avirulent strains fail to express a major glycoprotein. J. Virol. **56:**307–311.
15. **Mettenleiter, T. C., C. Schreurs, F. Zuckermann, T. Ben-Porat, and A. S. Kaplan.** 1988. Role of glycoprotein gIII of pseudorabies virus in virulence. J. Virol. **62:**2712–2717.
16. **Mettenleiter, T. C., L. Zsak, A. S. Kaplan, T. Ben-Porat, and B. Lomniczi.** 1987. Role of a structural glycoprotein of pseudorabies virus in virus virulence. J. Virol. **61:**4030–4032.
17. **Mettenleiter, T. C., L. Zsak, F. Zuckermann, N. Sugg, H. Kern, and T. Ben-Porat.** 1990. Interaction of glycoprotein gIII with a cellular heparinlike substance mediates adsorption of pseudorabies virus. J. Virol. **64:**278–286.
18. **Parnavelas, J. G., A. Dinopoulos, and S. W. Davies.** 1989. The central visual pathways, p. 1–164. *In* A. Bjorklund, T. Hokfelt, and L. W. Swanson, (ed.), Handbook of chemical neuroanatomy, vol. 7: integrated systems of the CNS, part III. Elsevier Science Publishers, New York.
19. **Petrovskis, E. A., J. G. Timmins, T. M. Gierman, and L. E. Post.** 1986. Deletions in vaccine strains of pseudorabies virus and their effect on synthesis of glycoprotein gp63. J. Virol. **60:**1166–1169.
20. **Quint, W., A. Gielkens, J. Van Oirshot, A. Berns, and H. T. Cuypers.** 1987. Construction and characterization of deletion mutants of pseudorabies virus: a new generation of live vaccines. J. Gen. Virol. **68:**523–534.
21. **Rauh, I., and T. C. Mettenleiter.** 1991. Pseudorabies virus glycoproteins gII and gp50 are essential for virus penetration. J. Virol. **65:**5348–5356.
22. **Robbins, A. K., J. P. Ryan, M. E. Whealy, and L. W. Enquist.** 1989. The gene encoding the gIII envelope protein of pseudorabies virus vaccine strain Bartha contains a mutation affecting protein localization. J. Virol. **63:**250–258.
23. **Robbins, A. K., M. E. Whealy, R. J. Watson, and L. W. Enquist.** 1986. Pseudorabies virus gene encoding gIII is not essential for growth in tissue culture. J. Virol. **59:**635–645.
24. **Roizman, B., and A. Sears.** 1990. Herpes simplex viruses and their replication, p. 1795–1841. *In* B. N. Fields et al. (ed.), Virology. Raven Press, Ltd., New York.
25. **Rouiller, E. M., M. Capt, M. Dolivo, and F. De Ribaupierre.** 1986. Tensor tympani reflex pathways studied with retrograde horseradish peroxidase and transneuronal viral tracing techniques. Neurosci. Lett. **72:**247–252.
26. **Rouiller, E. M., M. Capt, M. Dolivo, and F. De Ribaupierre.** 1989. Neuronal organization of the stapedius reflex pathways in the rat: a retrograde HRP and viral transneuronal tracing study. Brain Res. **476:**21–28.
27. **Ryan, J. P., M. E. Whealy, A. K. Robbins, and L. W. Enquist.** 1987. Analysis of pseudorabies virus glycoprotein gIII localization and modification by using novel infectious viral mutants carrying unique *Eco*RI sites. J. Virol. **61:**2962–2972.
28. **Schreurs, C., T. C. Mettenleiter, F. Zuckermann, N. Sugg, and T. Ben-Porat.** 1988. Glycoprotein gIII of pseudorabies virus is multifunctional. J. Virol. **62:**2251–2257.
29. **Sternberger, L. A.** 1979. The unlabeled antibody peroxidase-anti-peroxidase (PAP) method, p. 104–169. *In* L. Sternberger (ed.), Immunohistochemistry. John Wiley & Sons, Inc., New York.
30. **Strack, A. M., and A. D. Loewy.** 1990. Pseudorabies virus: a highly specific transneuronal cell body marker in the sympathetic nervous system. J. Neurosci. **10:**2139–2147.
31. **Strack, A. M., W. B. Sawyer, J. H. Hughes, K. B. Platt, and A. D. Loewy.** 1989. A general pattern of CNS innervation of the sympathetic outflow demonstrated by transneuronal pseudorabies viral infections. Brain Res. **491:**156–162.
32. **Strack, A. M., W. B. Sawyer, K. B. Platt, and A. D. Loewy.** 1989. CNS cell groups regulating the sympathetic outflow of the adrenal gland as revealed by transneuronal cell body labeling with pseudorabies virus. Brain Res. **491:**274–296.
33. **Van Oirschot, J. T., and A. L. H. Gielkens.** 1984. Intranasal vaccination of pigs against pseudorabies: absence of vaccinal virus latency and failure to prevent latency of virulent virus. Am. J. Vet. Res. **45:**2099–2013.
34. **Wittmann, G., and H.-J. Rziha.** 1989. Aujeszky's disease (pseudorabies) in pigs, p. 230–333. *In* G. Wittmann (ed.), Herpesvirus diseases of cattle, horses and pigs. Kluwer Academic, Boston.
35. **Zuckermann, F., T. C. Mettenleiter, C. Schreurs, N. Sugg, and T. Ben-Porat.** 1988. Complex between glycoproteins gI and gp63 of pseudorabies virus: its effect on virus replication. J. Virol. **62:**4622–4626.
36. **Zuckermann, F., L. Zsak, L. Reilly, N. Sugg, and T. Ben-Porat.** 1989. Early interactions of pseudorabies virus with host cells: functions of glycoprotein gIII. J. Virol. **63:**3323–3329.