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Characterization of Pseudorabies Virus Mutants Expressing Carboxy-Terminal Truncations of gE: Evidence for Envelope Incorporation, Virulence, and Neurotropism Domains

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Glycoprotein E (gE) gene of pseudorabies virus (PRV) is conserved among diverse alphaherpesviruses and therefore is predicted to be important for virus survival. gE contributes to viral spread from cell to cell in a variety of hosts and is responsible, in part, for increased virulence or pathogenesis of the virus. Virulence and spread mediated by gE are thought to be highly correlated. We initiated this study to explore the hypothesis that these two phenotypes might reflect separate functions of the gE protein. We did so by focusing on the role of the gE carboxy terminus in neuronal spread. Viruses harboring nonsense mutations affecting the expression of the gE cytoplasmic domain had several notable phenotypes. First, the truncated gE proteins expressed from these mutants are not found in virion envelopes. Second, the mutants retain the ability to spread to all retinorecipient regions of the rodent brain after retinal infection of rats. Third, the mutants have the reduced virulence phenotype of a gE deletion mutant in rats. Finally, the mutants have distinct plaque-size phenotypes on MDBK cells but not PK15 cells. Based on these observations, we suggest that gE-mediated virulence and spread may reflect separate functions that are not mediated by gE on virus particles.

Pseudorabies virus (PRV) is an alphaherpesvirus capable of infecting and causing disease in a variety of mammals and birds, as well as in swine, its natural host (2, 30). PRV is neurotropic in that it readily infects the peripheral nervous system and central nervous system (CNS) after primary infection (7, 11). In young pigs, PRV frequently causes a fatal encephalitis, whereas in adult pigs, PRV infection usually causes respiratory disease (13, 14). Animals that recover from primary infection maintain the virus in a latent but reactivatable state in neurons of sensory and autonomic ganglia that innervate the site of primary infection (32). Once PRV infects neurons at the primary site of infection, it spreads via retrograde or anterograde mechanisms to sensory and autonomic ganglia and, in some circumstances, to the CNS (3, 6, 13, 32). In the CNS, the virus spreads predominantly in functionally connected neurons and therefore has been used as a tracer of neuronal connections (7, 10, 20).

The PRV gE and gI proteins form an oligomer, probably a heterodimer, and contribute, in part, to viral pathogenesis (reviewed in reference 13). gE and gI null mutants have two robust phenotypes, restricted neurotropism and reduced virulence, in the rodent eye infection model established by Card et al. (3, 5, 6). PRV Becker (PRV Be) replicates in the cell body of primary neurons in the rat retina by 24 h after infection and then spreads to and replicates in retinorecipient neurons in the superior colliculus, the dorsal and ventral aspects of the lateral geniculate nucleus, and the circadian rhythm centers (composed of the suprachiasmatic nucleus and the intergeniculate leaflet of the brain). The virus reaches these retinorecipient regions through anterograde transport of the virus through the optic nerve (6). Infection of the superior colliculus and the dorsal and ventral geniculate but not the circadian rhythm centers requires the expression of the PRV membrane proteins gE and gI (5, 35). These two proteins are not required for entry into retinal ganglion cells that project to visual centers but, rather, are required for targeting or exit of PRV from these cells (4). The inability of PRV gE mutants to spread in some neuronal circuits and not others has also been documented in mice and swine by using nasal mucosal infection (1, 17). While gE and gI are required for anterograde spread in some neuronal circuits, these proteins appear to play no significant role in the retrograde transport of PRV (1, 13, 17, 19).

In addition to restricted neurotropism, gE and gI are required for full virulence of PRV in the rodent model, as they are in most susceptible hosts (5, 13–14, 21, 30, 32). Virulence is quantified in this model by the time to appearance of symptoms after infection or by the mean time to death (4). Animals infected with PRV gE or gI mutants live longer, develop symptoms later, and are symptomatic for a significantly shorter time than are animals infected with wild-type virus (5, 13, 18). In swine, PRV gE mutants are markedly reduced in virulence, more so than they are in rodents (13, 18, 32). Furthermore, in swine, gE mutants are less virulent than gI mutants are (13, 14, 17, 19).

The dual phenotypes of restricted neurotropism and reduced virulence are characteristic of PRV strains lacking gE or gI. However, the molecular basis for either of these phenotypes is not well understood. PRV gE-gI mutants are frequently cited to be less virulent, in part because they have a reduced ability to spread from cell to cell and from neuron to neuron (5, 14, 22, 35). While reduced cell-to-cell spread promoted by gE and gI may affect virulence, some observations indicate other factors contribute to gE- and gI-mediated virulence as well. For example, when the stomach musculature of rats is infected with the attenuated strain PRV Bartha, which lacks both gE and gI as well as several other proteins, the virus spreads via the vagus nerve throughout the brain, invading all known regions associated with stomach muscle control (4, 28; unpublished observations). Spread of PRV Bartha in the CNS is remarkably widespread, so that the brains of these animals contain far more infected neurons than do the brains of animals infected with wild-type virus, which rarely spreads beyond

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TABLE 1. Viruses used in this study

Virus	Description
PRV Be	Wild type
PRV 99R	Rescue of PRV 99, wild type
PRV 25	Am457 gE, anchored gE
PRV 26	Am428 gE, secreted gE
PRV 25R	Rescue PRV 25, wild type
PRV 26R	Rescue PRV 26, wild type
PRV 91	gE deletion
PRV 98	gI deletion
PRV 99	gE, gI deletion
PRV 1007	Am436 gC, secreted gC

the brain stem before the animals succumb (3, 4, 28). Despite this massive brain infection, the Bartha-infected animals remain remarkably symptom free and live several days longer than do the wild-type virus-infected animals (28). Similarly, in the rodent eye infection model, gE mutants are not completely defective in neuronal spread to the brain and infect the circadian rhythm centers with wild-type efficiency. Nevertheless, gE mutants are less virulent because infected animals have reduced symptoms and live 1 or 2 days longer than do animals infected with wild-type virus (5, 35). Consequently, we questioned the hypothesis that the virulence and neuronal spread phenotypes of PRV were intractably linked.

In this report, we provide evidence that the two phenotypes, virulence and spread, can be distinguished by mutations in the gE protein. Based on the premise that the carboxy-terminal cytoplasmic domain of gE was likely to be involved with virus transport and spread, we constructed two mutant viruses expressing carboxy-terminal truncated versions of the gE protein. One virus mutant expresses a gE protein that lacks the cytoplasmic domain, while the other mutant expresses a gE protein lacking both the transmembrane and cytoplasmic domains. We draw three conclusions from these studies: (i) the cytoplasmic domain is necessary for incorporation of gE into virus particles, (ii) the cytoplasmic domain is necessary for full expression of virulence but not neurotropism in the rat eye model, and (iii) the neurotropism phenotype in this model is not mediated by gE on virus particles. We suggest that gE is a multifunctional protein and that the virulence and neurotropism defects of gE mutants may indeed reflect separate functions.

MATERIALS AND METHODS

Virus strains and cells. PRV Be and the isogenic strains PRV 91, PRV 98, and PRV 99 (deletions in PRV gE, gI, or both, respectively) have been previously described (35). All PRV strains were propagated in PK15 (pig kidney) cells. Plaque size phenotypes were analyzed on MDBK (bovine kidney) cells.

Antisera. The PRV-specific antisera have been described previously (12, 29, 31, 35). Rabbit polyvalent gE serum was a generous gift from K. Bienkowska-Szewczyk (University of Gdansk).

Construction of mutant viruses. Table 1 includes a list of viruses used in this study. Oligonucleotide mutagenesis of PRV gE was performed with the Altered Sites kit (Promega). The multiple cloning site of the mutagenesis plasmid, pAlter-1, was modified by the addition of a conversion adapter containing three overlapping restriction sites (*Nco*I, *Bst*EII, and *Rsr*II), resulting in the plasmid pRT13. A 1,052-bp restriction fragment (*Bst*EII-*Sph*I) containing the transmembrane domain of gE was cloned into pRT13, resulting in pRT14. Amber mutations were introduced by site-directed mutagenesis at amino acid 457 (pRT19; anchored gE) or at amino acid 428 (pRT20; secreted gE). Mutations were confirmed by DNA sequencing with Sequenase (United States Biochemical). A transfer vector, pRT24, was constructed by cloning the *Sal*I-*Mlu*I restriction fragment from the *Bam*HI-7 fragment of PRV into a modified pGEM11Zf (Promega). The 1,052-bp restriction fragment from pRT19 or pRT20 containing the mutations was then introduced into pRT24, resulting in plasmids pRT25 (Am457) and pRT26 (Am428), respectively. PRV 99 DNA, which has the gE and gI sequences deleted (35), was then cotransfected by the calcium phosphate precipitation method into PK15 cells with either pRT24, pRT25, or pRT26 to enable the formation of recombinant virus (Fig. 1). After a complete cytopathic effect was observed, the infected cells were harvested, frozen, thawed, and replated onto PK15 cells. Recombinants were screened for gI expression by an immunoreactivity assay with a polyclonal antiserum to gI (35). These gI-plus recombinants must also contain the unselected gE mutations, as depicted in Fig. 1. Recombinants of PRV 25 (recombinant with pRT25) and PRV 26 (recombinant with pRT26) were picked and purified by three rounds of plaque purification.

PRV 25 revertants were constructed by cotransfection of PRV 25 DNA with a wild-type 1.4-kb *Sty*I restriction fragment that spans the transmembrane domain of gE. Wild-type recombinants were screened by plaque size phenotypes (large plaques) on MDBK cells. One isolate was plaque purified twice and was named PRV 25R. Rescue of the gE nonsense mutation in PRV 26 was done by cotransfection of PRV 26 DNA with the original 1,052-bp fragment from wild-type DNA used in the construction of pRT13. Wild-type recombinants produced black plaques when polyclonal antiserum to gE was used in a black-plaque analysis (35), while PRV 26 plaques display a distinctive gray color reactivity. One black plaque was picked, plaque purified, and named PRV 26R. PRV 99R is a revertant of PRV 99 made by screening for a gE/gI-plus recombinant after transfection with pRT24 and was used to confirm that no mutational events occurred during the generation of PRV 25 and PRV 26.

The presence or absence of the desired mutations in recombinant virus DNA was confirmed by Southern blot analysis as follows: the amber mutation in PRV 25 creates a novel *Bfa*I restriction site in the *Bam*HI-7 fragment of PRV, which alters a 6,400-bp fragment to 1,400- and 5,000-bp fragments following DNA digestion with *Bam*HI and *Bfa*I. The amber mutation in PRV 26 introduces a new *Alu*I restriction site changing a 1,458-bp restriction fragment to 1,300 bp following DNA digestion by *Bam*HI and *Alu*I. The rescued viruses, PRV 99R, PRV 25R, and PRV 26R, displayed wild-type restriction patterns as predicted.

Black-plaque and plaque size analysis. For black-plaque analysis, PK15 cells were infected and overlaid with Methocel until visible plaques were formed. A mixture of monoclonal antibodies against gE diluted in phosphate-buffered saline (PBS)–3% bovine serum albumin (BSA) was added to the plates and incubated for 1 h at room temperature. After three washes with PBS, the cells were incubated with horseradish peroxidase-conjugated anti-immunoglobulin G antibody (Kirkegaard & Perry Laboratories, Inc.) for 1 h at room temperature. Horseradish peroxidase staining was detected with 4-chloro-1-naphthol as a substrate. In this assay, plaques produced by virus containing wild-type gE become a distinctive black color (35).

MDBK cells were infected with sufficient virus to produce approximately 200 plaques per 28.3-cm^2 well and were overlaid with Methocel. At 48 h after infection, plaques were clearly visible. At this time, the cells were fixed in 3% formaldehyde for 20 min. Plaques were visualized by projecting the plates onto a screen, and the plaque diameters were measured manually. Forty random plaques away from the plate edges were measured for each virus under study. Several plates representing independent infections are represented in the analysis.

Immunoprecipitation and Western blot analysis. For steady-state experiments, PRV-infected cells were labeled with [^{35}S]cysteine plus [^{35}S]methionine

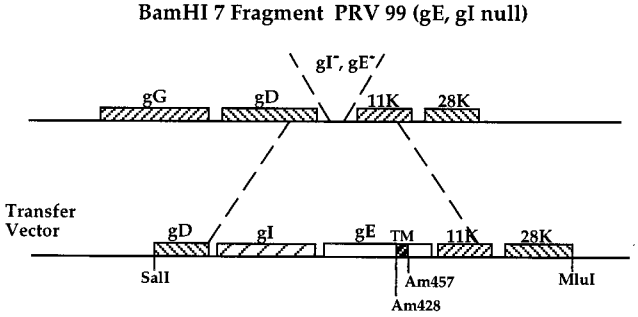

FIG. 1. Construction of mutants. The recombination events needed to produce gE-mutant/gI+ recombinant virus are shown. PRV 99 DNA, containing a deletion of both gE and gI coding sequences, was cotransfected with plasmid transfer vectors grown in *Escherichia coli* containing the mutated *Sal*I-*Mlu*I fragment of *Bam*HI-7 as described in Materials and Methods. Only the *Bam*HI-7 fragment of the PRV 99 genome is shown in the figure for simplicity. The transfer vectors used were pRT 24 (wild-type gE), pRT 25 (Am457 gE), and pRT 26 (Am428 gE). The transmembrane of gE is indicated (TM). Two homologous recombination events occurring between the homologous regions of PRV 99 and the transfer vector as indicated in the figure would produce a virus containing both gE and gI from the transfer vector. By screening for gI expression, recombinant viruses will contain the appropriate gE allele provided by the transfer vector. PRV 99R (wild-type gE), PRV 25 (Am457 gE; anchored gE) and PRV 26 (Am428 gE; secreted) were screened for gI and gE expression by black-plaque analysis. Southern blot analysis was done on viral DNA to verify the presence of the appropriate gE gene.

(NEN) and denatured, and native immunoprecipitations were performed as previously described (35). For pulse-chase analysis, PK15 cells were infected at a multiplicity of infection (MOI) of 10. At 5.5 h postinfection, the cells were incubated in cysteine- and methionine-free medium for 30 min and pulsed for 5 min with 125 μCi of label in 2 ml, and radioactive medium was removed and replaced with nonradioactive medium. Samples were taken immediately after the 5-min labeling period (time zero) and at times up to 4 h. As a control for sample loading onto gels and efficiency of infection, an unrelated glycoprotein, gC, was also analyzed in the same extracts.

For analysis of secreted proteins, the medium was removed from cells during the pulse-chase experiment. Virions were removed from the medium by centrifugation at 28,000 rpm for 90 min in an SW50.1 rotor. The supernatant was removed and analyzed by immunoprecipitation with various antisera as previously described (35).

For analysis of virion proteins, the medium was removed from infected PK15 cells (MOI = 10) at 12 h postinfection and clarified of cells and debris by low-speed centrifugation. Virions were isolated from the cleared medium by pelleting twice through a 5-ml 30% sucrose cushion in PBS (pH 7.5) at 23,000 rpm for 3 h in an SW27 rotor. The pellet was resuspended in PBS and electrophoresed through a sodium dodecyl sulfate–10% polyacrylamide gel. The proteins were transferred to nitrocellulose membranes, and Western blot analysis and enhanced chemiluminescence detection were performed as recommended by the manufacturer of SuperSignal (Pierce).

Indirect immunofluorescence microscopy. PK15 cells were grown on glass coverslips and infected at a MOI of 10. At 6 h postinfection, duplicate samples either were permeabilized with acetone and Nonidet P-40 and fixed with 3.7% formaldehyde or were fixed immediately with 3.7% formaldehyde (nonpermeabilized). The coverslips were immersed in anti-gE antibody (1:200 in PBS–3% BSA–2.5 mM $MgCl_2$) for 30 min in a 37°C humidified chamber, washed three times with PBS, overlaid with fluorescein isothiocyanate-conjugated secondary antibody (Pierce) (1:100 in PBS–3% BSA–2.5 mM $MgCl_2$), and incubated as described above. Following three washes with PBS, the coverslips were affixed to microscope slides with Testog as a mounting medium (Testog, Inc.).

Animal experiments, tissue processing, and immunohistochemistry. Adult male Sprague-Dawley rats weighing 200 to 250 g at the time of the experiment were used. Food and water were freely available during the course of the experiment, and the photoperiod was standardized to 14 h of light and 10 h of darkness (light on at 6 a.m.). Experimental protocols were approved by the Princeton University Animal Welfare committee and were consistent with the regulations stipulated by the American Association for Accreditation of Laboratory Animal Care and those in the Animal Welfare Act (Public Law 99-198). The animals were confined to a biosafety level 2 facility, and the experiments were conducted with specific safeguards as described previously (10).

For intraocular injections, 2.5 μl of virus suspension (6×10^8 PFU/ml) were injected into the vitreous humor of the right eye of an anesthetized animal. When symptoms of infection were overt, the animals were sacrificed and exsanguinated and the brains were removed as described previously (10). Immunohistochemical analyses of coronal brain slices have been described previously (10). Tissues were taken for analysis just prior to the estimated mean time to death.

RESULTS

Rationale. We focused on the gE carboxy-terminal domain for the following reasons. gE is a type I membrane protein and as such, the C-terminal domain is predicted to extend into the cytoplasm, where it may interact with cellular factors to promote anterograde transport of the virus. Indeed, the gE cytoplasmic domain is phosphorylated in PRV- and varicella-zoster virus (VZV)-infected tissue culture cells by cellular kinases (8, 9, 25). If this model is correct, gE mutants lacking the cytoplasmic domain may not be able to interact with cytoplasmic transport factors and may be defective in anterograde spread from the retina to visual centers—the same phenotype observed for gE null mutants. If virulence is a consequence of neuronal spread, these mutants will also have the same reduced virulence phenotype as the gE null mutant.

Rather than constructing deletions that might affect genome and mRNA structure and function in unpredictable ways, we created nonsense mutations that result in the translation of truncated proteins. Nonsense mutations were introduced immediately before and after the predicted gE transmembrane by site-directed mutagenesis (Fig. 1). PRV 25 (anchored gE) and PRV 26 (secreted gE) were constructed. While both truncated proteins lack the cytoplasmic domain, the former should produce a membrane-anchored form of gE and the latter should produce a secreted form of gE. Below, we describe the isolation of these mutants and revertants, characterization of the proteins produced by these viruses in tissue culture, and analysis of their neurotropism and virulence phenotypes in the rodent eye model.

TABLE 2. Plaque size on MDBK cells

Virus	Size (mm)[a]	SD (mm)	% Wild type	P^b
PRV Be	0.61	0.24	100	ND[c]
PRV 25	0.39	0.17	64	<0.005
PRV 26	0.51	0.26	84	0.039
PRV 25R	0.72	0.13	118	0.007
PRV 26R	0.65	0.12	107	0.821
PRV 91	0.39	0.12	64	<0.005

[a] Forty plaques from each virus representing infections on at least two plates were measured 48 h after infection.

[b] *P* was determined by Student's *t* test, comparing the indicated virus with PRV Be.

[c] ND, not determined; values were compared to those for this virus.

Black plaque and plaque size analysis. We determined the qualitative extent of gE expression on the cell surface by black-plaque analysis with a polyvalent, monospecific antiserum (35). After infecting PK15 cells with PRV 25, PRV 26, PRV Be, or the revertants, the cells were overlaid with Methocel to allow plaque formation. On PK15 cells, the mutant, revertant, and wild-type plaques were approximately the same size. The reaction of PRV Be and PRV 25 plaques with gE antiserum gave indistinguishable black plaques. By contrast, PRV 26 plaques exhibited a distinctly lighter colored gray plaque (data not shown). Although PRV 26 is predicted to encode a secreted gE protein, these observations suggest that some protein is present on the surface of the cells. One possible explanation for this result is that the secreted gE is capable of interacting with gI, thereby giving rise to weak staining in the black plaque assay. PRV 99R, PRV 25R and PRV 26R exhibited a black plaque phenotype indistinguishable from that of PRV Be.

Viruses that lack gE form small plaques on MDBK cells (13, 37). We observed that the plaques of both PRV 25 and PRV 26 were smaller than PRV Be plaques, but there was a significant difference in plaque size between the mutants (Table 2). PRV 25 formed consistently smaller plaques than PRV 26. The size of PRV 25 and PRV 91 plaques (gE deletion) were indistinguishable from one another, while the sizes of PRV 26 and PRV 91 plaques were visually and statistically different. The plaque size of the revertants, PRV 25R and PRV 26R, was comparable to that of wild-type PRV Be. If plaque size reflects the ability of viruses to spread from cell to cell, PRV 26 retains significant function, in contrast to PRV 25.

Single-step growth analyses on MDBK cells (data not shown) are consistent with the plaque size measurements. Both mutants form intracellular virus at the same rate and to the same extent as the parental virus, but PRV 25 releases significantly less virus into the media than does PRV 26. The amount of virus released by PRV 26 is roughly the same as that produced by the parental virus or the revertants. However, single-step growth analyses of the mutants on PK15 cells (data not shown) showed that their growth was identical to that of wild-type virus, PRV Be.

Steady-state levels and processing of PRV 25 and PRV 26 gE. Steady-state levels of the proteins produced by the gE mutants were assayed after virus infection of PK15 cells. Infected cells were labeled for 12 h beginning 5 h postinfection. Polyclonal antiserum against gE immunoprecipitated both pre-

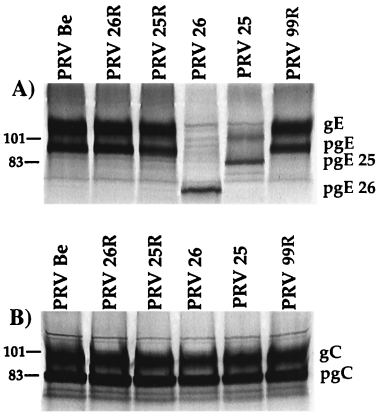

FIG. 2. Steady-state analysis of gE proteins. PK15 cells infected with either PRV Be (wild type), PRV 25 (anchored gE), PRV 26 (secreted gE), PRV 25R (wild type), PRV 26R (wild type), or PRV 99R (wild type) at an MOI of 10 were radiolabeled for 12 h, and cell lysates were collected 16 h postinfection. The cell lysates were denatured with SDS and dithiothreitol and immunoprecipitated with antisera specific for either gE (A) or gC (B). Polyclonal rabbit antiserum against gE immunoprecipitated both precursor (pgE) and mature forms (gE) of gE from the extracts. PRV 25 encoded gE precursor and mature forms of 80 and 101 kDa as predicted. PRV 26 encoded a gE precursor of approximately 65 kDa and a mature form of 82 kDa, although the latter was difficult to visualize during steady-state analysis. Faint bands, presumably reflecting different degrees of glycosylation, can be seen at around 82 kDa. The PRV 26 gE precursor and mature forms migrate with higher electrophoretic mobility than expected for the predicted molecular masses of 76 and 93 kDa, respectively. Polyclonal goat antiserum against gC (Ab 282) recognized the precursor (pgC; 82-kDa) and mature (gC; 90-kDa) forms of the gC protein. gC expression was unaltered in cells infected with PRV 25 or PRV 26. Positions of apparent molecular mass markers (kilodaltons) are indicated on the left.

cursor and mature forms of gE from extracts isolated from the infected cells. PRV Be, PRV 99R, PRV 25R, and PRV 26R produced identical species of gE. The gE precursor had a molecular mass of approximately 93 kDa and was further glycosylated to form the 110-kDa mature species (Fig. 2A). PRV 25 (anchored gE) encoded a gE precursor form of approximately 80 kDa and a mature form of approximately 101 kDa. The observed molecular masses of the proteins corresponded well to the predicted molecular masses of 79 and 96 kDa, respectively. PRV 26 (secreted gE) expressed a gE precursor with an apparent molecular mass of 65 kDa and a mature form of 82 kDa, smaller than the predicted molecular masses of 76 and 93 kDa for the precursor and the mature forms, respectively. We believe that this is an electrophoretic anomaly reflecting the amino acid composition and posttranslational modifications, because the deglycosylated PRV 26 proteins migrate with the expected electrophoretic mobility (data not shown). The mature species was barely discernible during steady-state analysis, presumably because it was secreted (see below). Faint bands around 82 kDa are probably glycosylated forms of the protein. In these experiments, the gE-specific antiserum immunoprecipitated less precursor and mature forms of gE from cells infected with either PRV 25 or PRV 26 than with the wild type, while gC protein levels immunoprecipitated from the same extracts were comparable to those immunoprecipitated from the wild type (Fig. 2B). The decreased accumulation of the truncated gE proteins suggests that there is a defect in protein production, processing, or stability. Endo-β-*N*-acetylglucosaminidase H (endo H) and endo F digestion of the immunoprecipitated proteins indicated that the glycosylated forms of PRV 25 and PRV 26 gE received modifications indistinguishable from wild-type gE (data not shown). Therefore, while the amount of immunoprecipitable gE protein was reduced in extracts from PRV 25- or PRV 26-infected cells, the extent of glycosylation of gE appeared to be equivalent to that for wild-type-infected cells.

Truncated gE proteins form a complex with gI. PRV Be gE forms a complex with gI in the endoplasmic reticulum shortly after its synthesis, and this complex facilitates the transport of both gE and gI to the surface of infected cells (35). We determined if the truncated gE proteins could associate with gI by performing coimmunoprecipitation of gI with gE antiserum from nondenatured extracts of infected cells following steady-state labeling. In experiments with undenatured extracts, the gE antiserum reacts predominantly with the gE precursor species and much less with the gE mature species. Antiserum to gE immunoprecipitated the 93-kDa precursor species of gE and coimmunoprecipitated the 65-kDa immature form of gI from PRV Be-infected cells as well as from cells infected with the revertants (Fig. 3). Mock-infected cells and cells infected with PRV 91, a gE-deleted virus, showed no immunoprecipitible protein (Fig. 3). As predicted, the gI protein was absent from extracts of cells infected with PRV 98, a virus lacking gI (Fig. 3). gI could be readily coimmunoprecipitated by gE antiserum from both PRV 25- and PRV 26-infected cells (Fig. 3). Although PRV 25 and PRV 26 express less gE, in these experiments the amount of gI bound to gE was similar to that bound by wild-type gE (PRV Be, PRV 25R, PRV 26R, and PRV 99R), because each gI protein band was of equal intensity. Coimmunoprecipitations of pulse-chase extracts also showed that gE from PRV 25 and PRV 26 formed a complex with gI immediately after synthesis, with similar kinetics to those for wild-type gE (data not shown). We conclude that the cytoplasmic tail of gE is not required for formation of a complex with gI. These results also indicate that the truncated gE proteins associate with gI at levels comparable to those of wild-type gE.

Pulse-chase analysis of gE protein processing and export. In experiments not shown, we found no difference in the amount of gE-specific RNA made by any gE mutant, revertant, or wild-type virus. However, because PRV 25 and PRV 26 expressed less gE protein during overnight labeling experiments, we examined if the deletion of the C terminus of gE had an effect on translation efficiency, protein processing, or stability in a pulse-chase experiment in PK15 cells. At 6 h after infection, cells were radiolabeled with [^{35}S]cysteine plus [^{35}S]methionine for 5 min, rinsed with PBS, and incubated with nonradioactive medium (chase) for the times indicated before

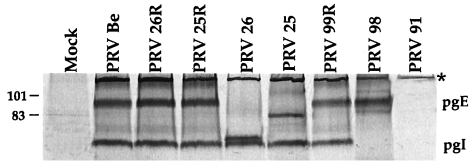

FIG. 3. Coimmunoprecipitation of gE and gI from infected cells. Cell lysates were obtained as described in Fig. 2, but the lysates were not denatured prior to immunoprecipitation with antiserum specific for gE. The lanes contain (from left to right) mock-infected cells, PRV Be (wild type), PRV 26R (wild type), PRV 25R (wild type), PRV 26 (secreted gE), PRV 25 (anchored gE), PRV 99R (wild type), PRV 98 (gI deletion), and PRV 91 (gE deletion). Mock-infected cells were included as a measure of the specificity of the antiserum. The polyclonal rabbit antiserum used reacts better with the precursor protein (pgE) of nondenatured extracts, and therefore no mature protein forms were seen. The apparent molecular masses of the precursor gE proteins (pgE) immunoprecipitated are described in the legend to Fig. 2. The labeled protein species denoted by an asterisk is not related to gE since it was seen in PRV 91-infected cell extracts. Apparent molecular mass markers (kilodaltons) are indicated on the left.

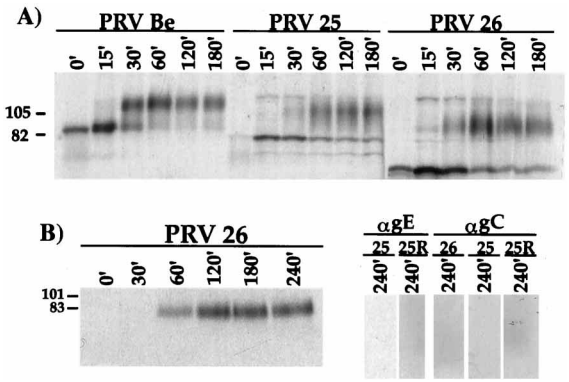

FIG. 4. Pulse-chase analysis. PK15 cells were infected at an MOI of 10 with PRV Be (wild type), PRV 25 (anchored gE), and PRV 26 (secreted gE) (A) and PRV 25, PRV 26, and PRV 25R (wild type) (B). The cells were pulse-labeled for 5 min with 125 μCi of [^{35}S]methionine plus [^{35}S]cysteine in 2 ml and chased for the times indicated with nonradioactive medium before collection of cell lysates and the medium. Cell lysates were denatured and immunoprecipitated with polyclonal antibody to gE (A), or the medium was cleared of virus, denatured, and immunoprecipitated with antiserum to either gE or gC as indicated (B). Apparent molecular mass markers (kilodaltons) are indicated on the left.

protein analysis. As controls for loading the gels and efficiency of infection, the unrelated glycoprotein gC was also analyzed in the same extracts (data not shown). The results are presented in Fig. 4A where the PRV Be infection is compared to that of either PRV 25 or PRV 26. In this experiment, the PRV Be and PRV 26 gE precursor proteins were labeled to approximately the same extent while the PRV 25 precursor incorporated less label in the 5-min labeling period. In other experiments (data not shown), the amount of label incorporated in the 5-min labeling period was approximately the same for mutant and wild-type viruses. In the experiment shown in Fig. 4A, the half-time for conversion of the wild-type gE (PRV Be) precursor to the mature species is between 15 and 30 min (Fig. 4A, PRV Be). We observed a delay in the processing of the gE precursors in a PRV 25 and PRV 26 infection. For both viruses, it took between 30 and 60 min to convert half of the mutant gE protein into the mature form. The extent of processing of the mutant precursor species also differed by comparison to wild-type gE. Wild-type gE precursor was completely converted to the mature form by 60 min of chase. By contrast, the gE precursor produced by PRV 25 and PRV 26 was not completely converted to the mature species by 180 min after the chase. In another set of experiments, both of these precursor forms were shown to remain after 240 min postchase (data not shown). Clearly, these protein species are stable during their transport and processing in an infected cell.

PRV 25 is predicted to express a membrane-anchored form of gE, while PRV 26 is predicted to express a gE protein lacking the transmembrane and membrane anchor. Therefore, we looked for evidence of gE secretion into the medium of PRV 26 infected cells (Fig. 4B). Medium was collected from the cells during pulse-chase experiments, and virions and cellular debris were removed by centrifugation before analysis. The resulting supernatant was assayed for secreted proteins by immunoprecipitation with gE or gC antiserum. The gC protein is not secreted and serves as a negative control. A complete pulse-chase time course is shown in the left panel, and samples taken after 240 min of chase are presented in the right panel. After 60 min of chase, mature gE with the predicted apparent molecular weight was found in the medium of PRV 26-infected cells, and levels of secreted gE plateaued at 120 min (Fig. 4B, left panel). No gC was detected in the medium of identical extracts even as late as 240 h postchase (Fig. 4B, right panel). Neither gE nor gC could be immunoprecipitated from the supernatant of cells infected with PRV 25 or PRV 25R, confirming that PRV 25 encodes a membrane-anchored form of gE and also confirming that virions were completely removed from the medium prior to analysis. These data are evidence that the hydrophobic sequence present in PRV 25 gE, but absent from PRV 26, is sufficient to prevent the secretion of the protein. The gE protein species produced by PRV 25 are stable during their transport and processing in an infected cell.

Cellular localization of truncated gE proteins by immunofluorescence microscopy. To confirm the expression of the truncated proteins on the surface of infected cells, infected nonpermeabilized PK15 cells were stained with a gE-specific monoclonal antibody, 1/14 (12). At 6 h postinfection, wild-type gE was expressed on the surface of cells (Fig. 5, PRV 99R, top row). Similarly, gE expressed by both PRV 25 and PRV 26 was observed on the cell surface with approximately equal intensity at 6 h postinfection. Interestingly, these experiments show that a portion of the gE made by PRV 26 remained associated with the plasma membrane. We suggest that this occurs presumably through its interaction with gI.

Permeabilized cells were also stained for gE at the 6-h time point (Fig. 5, bottom row). Wild-type gE showed a strong perinuclear staining on one side of the nucleus, characteristic of Golgi localization. By contrast, gE in PRV 25-infected cells displayed a perinuclear ring and no characteristic Golgi staining, indicative of localization in the endoplasmic reticulum. PRV 26 had an intermediate phenotype compared to wild-type gE and PRV 25 gE, with cells that showed mixed Golgi and endoplasmic reticulum staining. This corroborates the results of the pulse-chase experiment that indicated that the truncated gE proteins were delayed in processing.

Truncated gE proteins are not detectable in virions. While PRV 25- and PRV 26-infected cells contained significant amounts of intracellular gE, we were interested in determining if the gE proteins were incorporated into virions. Virions isolated from the medium of infected PK15 cells were analyzed for gE and gC proteins by Western blotting. To control for the purification of virions away from secreted protein and cell debris, we used PRV 1007, a gC mutant that expresses a secreted gC protein not incorporated into virions but retains wild-type gE expression (33). In the experiment shown in Fig. 6B, no gC protein could be detected in virions isolated from PRV 1007-infected cells, indicating that the virion preparation was not contaminated with cellular material. When the same PRV 1007 virions were probed for gE, a significant signal characteristic of the mature gE protein was observed, indicating that virions were indeed present (Fig. 6A). Unexpectedly, virions from PRV 25 and PRV 26, while containing the mature form of gC, showed no detectable gE protein—even when 10 times more total protein from the virion preparations was loaded on the gel (Fig. 6). Western blots of cellular fractions from these samples indicated that gE protein was made in these cells (data not shown). Due to the lack of an antibody that recognizes the mature form of gI, it is not possible to determine if gI is found in the gE-negative virions. These results indicate that although the mutant gE proteins are made in PK15 cells, they are not incorporated into viral particles.

Virulence of PRV 25 and PRV 26 in the rat eye model. Virulence of PRV can be assessed by determining the mean time to appearance of symptoms or mean time to death in

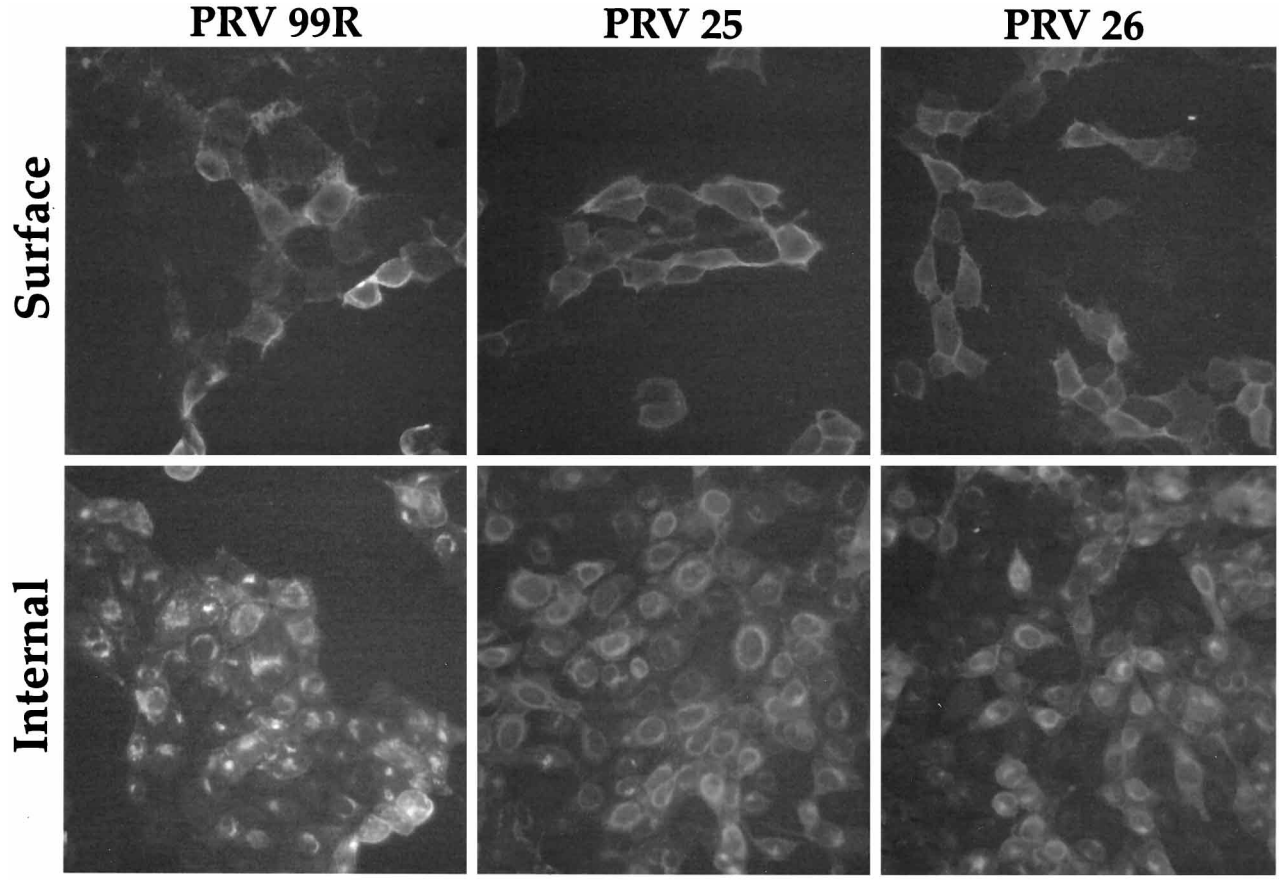

FIG. 5. Immunofluorescence of infected cells. PK15 cells were infected at an MOI of 10 with either PRV 99R (wild type), PRV 25 (anchored gE), or PRV 26 (secreted gE), fixed 6 h postinfection, and processed to detect surface (top row) or internal (bottom row) localization of gE protein by using monoclonal antibody 1/14 against gE. A fluorescein isothiocyanate-labeled anti-mouse immunoglobulin G secondary antibody was used to visualize bound monoclonal antibody.

animals infected with virus. PRV Be, a virulent strain, causes the death of rats approximately 68 h after intraocular infection (5). PRV 91 (with gE deleted) is less virulent, causing death at 110 to 115 h after infection by the same route (5). To establish the virulence of PRV 25 and PRV 26, 36 rats were infected intraocularly and observed for symptoms. Controls for this experiment included infections with the revertants PRV 25R and PRV 26R as well as PRV 91 and PRV Be. The time when signs of imminent death were obvious was recorded. The mean time to signs of imminent death for PRV 25 and PRV 26 was delayed, similar to that found for PRV 91 and distinctly different from the rapid appearance of symptoms after PRV Be infection (Table 3). Animals infected with PRV 25 survived to an average of 105.5 h, while those infected with PRV 26 survived to approximately 99 h. There is a statistical difference between PRV 25 and PRV 26 compared to PRV 91, suggesting that PRV 26 is slightly more virulent than PRV 25. Given the large standard error in these measurements, more animals must be infected to strengthen this conclusion. Compared to PRV Be, all gE mutants are significantly reduced in virulence.

The revertants PRV 25R and PRV 26R were considerably more virulent than the corresponding mutants and were indistinguishable from wild-type virus, since animals infected with these viruses survived no longer than an average of 67 h postinfection. We conclude that the reduced virulence phenotypes were due to the nonsense mutations carried by PRV 25 and PRV 26. Thus, although PRV 25 and PRV 26 encode the entire ectodomain of gE, they manifest a reduced-virulence phenotype in rats similar to that of PRV 91, a virus carrying a deletion of the entire gE gene. Clearly, the C ter-

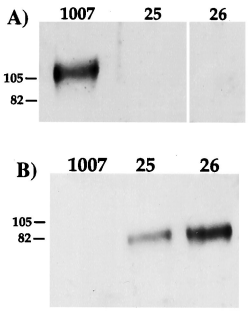

FIG. 6. Analysis of proteins in virions. PK15 cells were infected at an MOI of 10 with either PRV 1007 (secreted gC, not localized to the virion envelope), PRV 25 (anchored gE), or PRV 26 (secreted gE). Medium was removed from the cells 12 h postinfection. Virions released to the medium were collected by centrifugation through a 30% sucrose cushion prior to Western blot analysis. The blots were probes with either polyclonal rabbit antiserum against gE (A) or goat antiserum 282 against gC (B). (A) A 10-fold-higher level of total protein from the virion preparation was loaded in lanes labeled 25 and 26 as compared to 1007. Apparent molecular mass markers (kilodaltons) are indicated on the left.

TABLE 3. Virulence of PRV 25 and PRV 26 in the rat eye infection model

Viral genotype	No. of animals	Mean time to symptoms (h)	P^a with respect to:	
			PRV 91	PRV Be
PRV Be (gE^+)	8	68.5 ± 6.5	0.005	ND[b]
PRV 26 (gE Am428)	11	98.9 ± 16.8	0.05	<0.005
PRV 25 (gE Am457)	8[c]	105.5 ± 12.2	0.149	<0.005
PRV 91 (gE null)	3	115 ± 12.0	ND	0.005
PRV 26R (gE^+)	2	67.0[d]	ND[d]	ND[d]
PRV 25R (gE^+)	4	66.0 ± 4.2	0.008	0.778

[a] *P* was determined by Student's *t* test.
[b] ND, not determined.
[c] One animal died at 120 h and was excluded from this data set.
[d] No error or *P* value was calculated given that only two animals were used.

minus of gE is required for full virulence of PRV in the rodent model.

Spread of PRV 25 and PRV 26 in neurons of the rat visual system. Following intraocular infection, PRV Be infects all known retinorecipient regions of the brain (6, 35). Viral antigen is detected in the visual centers including the lateral geniculate complex (LGN; dorsal and ventral aspects) and the superior colliculus (SC), as well as the circadian rhythm centers such as the suprachiasmatic nucleus (SCN) and the intergeniculate leaflet (IGL) (6, 35). Infection of the superior colliculus and the LGN is dependent on gE and gI, because viruses lacking these genes have the capacity to spread to and infect only circadian rhythm centers after infection of the retina (35). We examined the spread phenotypes of PRV 25 and PRV 26 after intraocular infection. At times of imminent death after infection, the brains of infected animals were removed, sectioned, and analyzed for infection of retinorecipient areas. As shown in Fig. 7, both wild-type gE and the gE mutant viruses caused a robust infection in the SCN and the IGL indistinguishable from infection due to PRV 91 (gE deletion). However, in contrast to PRV 91, PRV 25 and PRV 26 retained the ability to spread to all visual centers, as evidenced by heavy staining in the SC and both the dorsal and ventral aspects of the LGN. Neither the cytoplasmic tail nor the transmembrane

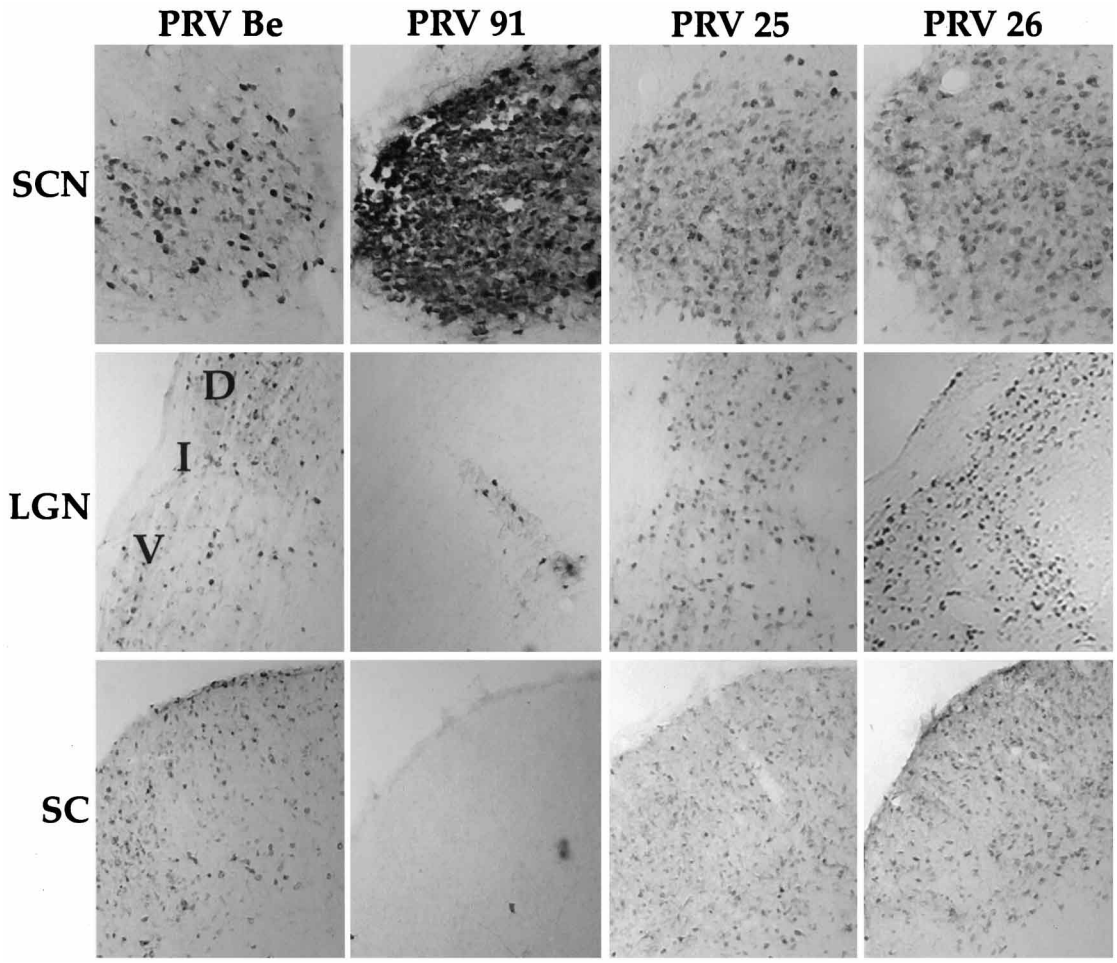

FIG. 7. Localization of viral antigen in brain sections. Animals were infected with either PRV Be (wild type), PRV 91 (gE null), PRV 25 (anchored gE), or PRV 26 (secreted gE) by intraocular injection as described in Materials and Methods. Upon signs of imminent death, the animals were sacrificed and the brains were removed and analyzed for viral antigen with a polyvalent rabbit antiserum generated against whole virus particles (Rb133) (10). Serial sections (30 μm) through the coronal plane were cut, processed, and mounted on slides. Representative sections containing the SCN, LGN including the dorsal and ventral aspects as well as the IGL, and the SC are pictured. In this assay, wild-type PRV Be spreads to all areas shown including the SCN, dorsal (D) and ventral (V) aspects of the LGN, the IGL (I), and the SC (column 1). PRV 91 spreads only to the SCN and the IGL and is never found in the dorsal and ventral aspects of the LGN or the SC (column 2). Both PRV 25 and PRV 26 spread to the SCN, the dorsal and ventral aspects of the LGN, the IGL, and the SC and are identical to PRV Be (columns 3 and 4). The dark reaction product in the SCN section of PRV 91 resulted from a longer exposure of tissue during processing and does not reflect more extensive infection.

domain of gE is required for PRV to infect the optic tectum or the dorsal and ventral aspects of the lateral geniculate nucleus after intraocular infection.

DISCUSSION

We initiated these experiments to test the role of the gE carboxy terminus in the spread of virus from the retina to the visual centers of the brain. We observed that viruses carrying either of two nonsense mutations, one at codon 428 (PRV 26) and one at codon 457 (PRV 25), had distinctive phenotypes in several assays, suggesting that gE is a multifunctional protein. Both mutants have a reduced virulence phenotype similar to a virus carrying a gE deletion, but both mutants spread like wild-type virus in the rat brain after retina infection. Moreover, neither mutant virus had gE in the virus envelope. These observations suggest that virulence and neurotropism, as measured in the rat eye model, reflect distinct functions of gE. Additionally, they provide evidence that gE on virus envelopes is not necessary for these phenotypes. Several possible interpretations are discussed below.

We assessed the contribution of gE to cell-cell spread by two methods: transneuronal infection of the visual centers from the retina, and plaque size on MDBK cells. We note at the outset that it is not yet clear if these two systems measure the same or different functions of gE and gI. PRV 25 and PRV 26 both exhibited a neural spread phenotype after retinal infection, similar to that of a wild-type virus. Upon intraocular injection, both mutants spread to and infected all visual and circadian rhythm centers of the brain (Fig. 7). This is in contrast to results found for viruses with gE deleted, which could infect only circadian rhythm centers. At the time of imminent death, staining of retinorecipient regions of the brains of infected animals was essentially identical for PRV Be, PRV 25, and PRV 26 in terms of the extent of infection and intensity of immunoreactivity. By contrast, the mean time to death for animals infected with either PRV 25 or PRV 26 was extended and was similar to that observed for a gE-null virus (PRV 91). Notably, the revertants of PRV 25 and PRV 26 regained wild-type virulence (Table 3). These experiments demonstrate that the N-terminal 428 amino acids of gE is sufficient to sponsor virus infection of all retinorecipient areas of the brain that the wild-type virus infects and also suggest that sequences necessary for the expression of gE-mediated virulence lie between amino acid 457 and the carboxy terminus.

It is commonly held that the small plaques of gE mutants on certain cell types like MDBK cells reflect the ability of virus to spread from cell to cell (reviewed in reference 13). We observed that both PRV 25 and PRV 26 formed small plaques on MDBK cells compared to the parental virus. PRV 25 plaques were indistinguishable from those of PRV 91, a gE deletion mutant; however, PRV 26 formed significantly larger plaques than PRV 25 did. This indicates that PRV 26 retained considerable wild-type function with respect to cell-cell spread in these cells. As both mutant viruses had the reduced virulence similar to PRV 91, our observations provide further evidence that spread (as determined by plaque size) and virulence are not strictly correlated and may represent separate functions of gE.

An alternative hypothesis must be considered since less gE protein is produced in PRV 25- and PRV 26-infected cells. A formal possibility is that the reduced virulence observed for these two viruses resulted from less protein and not from the cytoplasmic tail deletion. This interpretation implies that more gE protein is required for virulence than for spread in the CNS and for plaque formation in MDBK cells (for PRV 26 only). By contrast, this amount of gE is sufficient for normal spread of virus in the rat CNS after retinal infection. This idea can be tested by expressing the mutant gE proteins from stronger promoters (e.g., the gG promoter); if concentration only is responsible, we would predict that these viruses would regain virulence and the ability to form larger plaques on MDBK cells. What is important from our studies to date is that the phenotypes of spread and virulence manifested by gE can be distinguished by mutation. The reduced levels of expression of the gE proteins made by PRV 25 and PRV 26 remain to be explained. We have demonstrated that neither decreased mRNA stability (data not shown) nor decreased protein stability is responsible. One possibility to be examined is that transport of the mutant mRNA from the nucleus to the cytoplasm may be reduced, resulting in less mRNA available for translation.

An unexpected result was that neither truncated gE protein was found in virions. Previous results proved that a nonsense mutation truncating the cytoplasmic tail of the PRV gC gene (similar to the mutation in PRV 25) had no effect on incorporation of the gC protein into viral particles (34). Perhaps the cytoplasmic tail of gE contains sequences critical for incorporation into the virion envelope or for targeting gE to the site of envelopment. Alternatively, the cytoplasmic tail sequence of gE may play an indirect role in the incorporation of proteins into the virion. For example, the decreased expression of truncated gE proteins by PRV 25 and PRV 26 may have affected the amount of gE available for incorporation into mature virions. Thus, the truncated gE proteins may indeed be present in the virion envelopes but at a level undetectable by our methods. We do not know if an undetectable amount is the same as no gE or if a few molecules of gE may be enough for wild-type function. Further experiments to increase levels of truncated gE expression or decrease levels of wild-type gE protein could provide more insight into these questions.

As deduced from analyses of gD-deleted PRV strains, cell-cell spread and transneuronal infection are unlikely to be mediated by virus particles (23, 26, 27). Our results indicate that gE does not have to be in the virus envelope in detectable quantities to promote anterograde, cell-cell spread to all the retinorecipient regions of the rat brain after intraocular infection. If virus particles are not involved in this mode of transneuronal spread, spread of the virus between infected retinal ganglion cells and neurons of the visual centers might occur through the action of the external domain of gE at or near sites of synaptic contact of an infected neuron with an uninfected one. Similarly, in certain tissue culture cells, contact of an uninfected cell with an infected cell bearing gE on the cell surface could render the uninfected cell susceptible for passage of virions into the interior of the cell. This interaction may or may not involve gI, because both gE mutants retain the ability to interact with gI. We cannot say if gE must be anchored to the membrane because much of the secreted form of gE remains on the surface of infected cells, perhaps through interaction with gI (Fig. 5).

A common assumption is that gE mutants have reduced virulence because they do not spread well from cell to cell in an infected animal. By this interpretation, virulence is an indirect effect of the primary action of gE. While this proposal certainly has merit, especially when infection of mucosal surfaces and epithelial cells is considered, our results suggest that gE is multifunctional and that other ideas should be entertained. The eye infection model places the virus in an immunoprivileged site with direct access to neurons. As we have demonstrated, local cell-cell spread is minimal and local inflammatory responses do not occur after primary infection of the retina (4,

6, 35). Thus, pathogenic responses in this model are most probably produced after neuronal infection. It is noteworthy then that PRV 25 and PRV 26 spread reasonably well to the brain after infection of the rat eye and stomach muscles (unpublished observations) but have reduced pathogenic effects. This implies that gE may be an intrinsic virulence factor, which affects virulence directly, not indirectly by promoting virus spread. The gp120 envelope protein of human immunodeficiency virus type 1 is an example of a viral membrane protein with intrinsic toxicity. There is precedence for this situation in PRV from two lines of experimentation. Kost et al. (16) expressed glycoproteins from the PRV Us region in an attenuated vaccinia virus and noted that vaccinia virus recombinants expressing gI and gE had increased virulence in mice after intracranial but not intraperitoneal injection. Additionally, Knapp and Enquist (15) expressed the gE and gI homologs of the respiratory pathogen bovine herpes virus type 1.1 in a gE- and gI-deleted PRV vector and showed that despite the differences in virus tropism and pathogenesis between viruses, virulence in the rat eye infection model was restored to PRV. In addition, as first noted by Pereira and colleagues (21a, 27a), PRV gE has significant amino acid homology to the *Vibrio cholera* toxin called Zot (zona occludans toxin), which acts by initiating a signal transduction cascade, ultimately affecting tight junctions in polarized epithelial cells. PRV gE may act to promote pathogenic effects directly, perhaps by binding a ligand while on the surface of an infected cell (for example, it may bind a cell surface protein on a neighboring glial cell or astrocyte) and signaling through its cytoplasmic sequences to affect gene expression in the infected cell. Ligand binding and signaling may induce a variety of gene products, including biological mediators of inflammation (e.g., cytokines like interferon and tumor necrosis factor) or calcium fluxes that can lead to more global pathogenic effects. The carboxy-terminal domain of PRV gE can be phosphorylated in infected cells, and this may reflect such a signaling process (8). Perhaps PRV 25 and PRV 26 are less virulent because they cannot signal via the gE cytoplasmic sequences.

We also have evidence that the cytoplasmic tail of PRV gE may be involved in gE localization and trafficking, processes that may be critical for incorporation of gE in virion envelopes as well as for gE-mediated virulence. Two groups have reported that VZV gE undergoes internalization after expression on the surface of transfected cells in a manner analogous to receptor-mediated endocytosis (24, 36). A tetrapeptide motif (YXXL) located in the cytoplasmic tail of gE directs this internalization, and the tyrosine residue is critical (24). Furthermore, the same motif required for internalization is also believed to be a tyrosine phosphorylation site in VZV gE (25). Olson et al. (25) have shown that homodimers of VZV gE are tyrosine phosphorylated whereas monomeric forms of gE are serine/threonine phosphorylated by casein kinase II in cells transfected with the gE protein (25). Thus, control of phosphorylation may be involved in controlling gE internalization. In PRV, the cytoplasmic tail of gE contains two tetrapeptide motifs identified in VZV gE, and the PRV gE tail is also phosphorylated (8). Our preliminary experiments suggest that in infected cells, wild-type PRV gE is rapidly internalized after expression on the cell surface but the truncated gE protein produced by PRV 25 is defective in internalization (unpublished results). Thus, internalization of gE must also be considered when thinking about how PRV gE promotes spread and virulence. For example, internalization of the protein while it is bound to its ligand may elicit a pathogenic response in the infected cell. Loss of the cytoplasmic tail would prevent internalization, leading to reduced gE-mediated virulence. Further genetic and biochemical studies to identify domains and proteins that interact with the carboxy-terminal amino acids of gE will facilitate our understanding of the molecular mechanisms involved in gE-promoted virulence.

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