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Pseudorabies Virus Recombinants Expressing Functional Virulence Determinants gE and gI from Bovine Herpesvirus 1.1

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In the *Alphaherpesvirinae* subfamily, the gE and gI genes are conserved and encode membrane glycoproteins required for efficient pathogenesis (virulence). The molecular mechanism(s) responsible is not well understood, but the existence of similar phenotypes of gE and gI mutations in diverse *Alphaherpesvirinae* implies conservation of function(s). In this report, we describe construction of pseudorabies virus (PRV) recombinants that efficiently express the bovine herpesvirus 1 (BHV-1) membrane proteins gI and gE at the PRV gG locus. Each BHV-1 gene was cloned in a PRV mutant lacking both the PRV gI and gE coding sequences. All recombinant viruses expressed the BHV-1 proteins at levels similar to or greater than that observed after infection with parental BHV-1, and there were no observable differences in processing or ability to form gE-gI oligomers. The important observation resulting from this report is that the BHV-1 gE and gI proteins functioned together to complement the virulence defect of PRV lacking its own gE and gI genes in a rodent model, despite being derived from a highly restricted host range virus with a different pathogenic profile.

The *Alphaherpesvirinae* subfamily infects a broad spectrum of mammalian and avian hosts, causing distinct, but often overlapping pathology (28). A common theme in an alphaherpesvirus infection is the tendency to establish both productive and latent infections of the host nervous system. Many alphaherpesviruses have narrow host ranges, infecting primarily their natural host (e.g., bovine herpesvirus 1 [BHV-1], avian Marek's disease virus, and human varicella-zoster virus), while others have broader host ranges capable of productive infection of diverse vertebrate species (e.g., swine pseudorabies virus [PRV] and human herpes simplex virus type 1). Despite these different patterns of host range, the gene structures and overall organizations of the *Alphaherpesvirinae* genomes have marked similarities, and they are postulated to have evolved from a common ancestor (19). While comparative sequence analysis has been used to deduce the evolutionary history of this virus group, only a few studies have been performed in comparative molecular biology that directly ask questions concerning the conservation and divergence of molecular mechanisms for tropism, spread, and ability to cause disease. These experiments can be informative, as demonstrated by Kopp, Mettenleiter, Miethke, and colleagues, who compared functions of BHV-1 and PRV gB, a conserved membrane protein absolutely required for virus entry into cells (16, 22). While BHV-1 gB could complement a PRV gB null mutant for growth in tissue culture, PRV gB was unable to complement the BHV-1 gB null mutant.

In this report, we describe a system to compare functions of PRV and BHV-1 gE and gI (membrane proteins involved in tropism, cell-to-cell spread, and pathogenesis) by expressing each BHV-1 gene in a PRV vector. Our goal was to determine if gE and gI from BHV-1, a related but pathogenically distinct virus, could function as virulence determinants for PRV. In these studies, virulence is defined as the degree of pathogenesis in a rodent model.

We describe the construction and in vitro characterization of three recombinant PRV strains, all lacking the PRV gE and gI genes but carrying a single copy of the BHV-1, subtype 1, gE or gI gene. To facilitate the cloning and comparison of different gE and gI homologs to ascertain the function of the proteins, we used a common expression system based on the PRV gG early promoter (20) to ensure that the proteins were made at similar levels and in a similar context. As gE and gI interact physically and to facilitate analysis of different combinations of gE and gI homologs, we expressed each protein from separate viruses and relied on coinfection to ensure expression of both proteins in the same cell. As shown previously, coinfection can achieve in vivo complementation in the rodent model (10). All three recombinants produced BHV-1 gE and gI proteins that were indistinguishable from the proteins produced by the parental BHV-1 strain. We show that high-multiplicity, mixed infection by single recombinants expressing either gE or gI sponsored gE-gI complex formation as well as more efficient processing and export compared to infections by recombinants expressing the individual proteins. The coinfection strategy was then used to demonstrate that the BHV-1 proteins could complement the virulence defect characteristic of PRV gE-gI deletion mutants in rodents. These experiments show that despite considerable divergence of sequence, the ability to effect increased virulence is conserved between these genes of pathogenically diverse herpesviruses. We suggest that the gE-gI proteins are intrinsic virulence factors that affect virulence independent of virus and host.

MATERIALS AND METHODS

Virus strains and cells. Strain PRV-Becker and the isogenic strains PRV-99 and PRV-98 (with deletions in PRV gI and gE, or in gI alone, respectively) have been previously described (36). PRV-99Blue has a *lacZ* insertion in PRV gG and was constructed according to the method of Mettenleiter and Rauh, except that the *lacZ* gene was cloned in the gG gene of PRV-Becker rather than strain Ka (20). All PRV strains were propagated on PK15 cells. BHV-1 substrain 1 (Colorado) was provided by Leonard Bello (University of Pennsylvania) and was propagated on MDBK cells.

Antisera. The PRV-specific antisera have been described previously (27, 36). Rabbit, polyvalent anti-PRV gE serum was a generous gift from K. Bienkowska-Szewczyk (University of Gdansk). The rabbit, polyvalent anti-BHV-1 gI and gE sera have been described previously (37).

Cloning of BHV-1 gI and gE. The BHV-1 gE and gI open reading frames were obtained by PCR amplification of plasmid DNA derived from pBH145B and pBH144, respectively (37). The PCR primers introduced an *Eco*RI site imme-

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diately upstream of the first methionine and downstream of the stop codon and the putative poly(A) sequence of BHV-1 gI. BHV-1 gE was cloned with the first methionine and the stop codon but without a poly(A) signal. The PCR amplification was performed with a high-fidelity polymerase (cloned PFU polymerase; Stratagene), and the amplified as well as the nonamplified sequences were sequenced and compared with each other and with the reported BHV-1 sequences (26). Amplified sequences were transcribed and translated in vitro to ensure that proteins of the proper length and immunoreactivity were produced.

Construction of transfer vectors. The BHV-1 gE and gI open reading frames were engineered to be expressed from the PRV gG promoter as follows. We first built a transfer vector that allowed the in-frame insertion of either BHV-1 gE or gI open reading frames in the *Bam*HI site in the PRV gG gene that occurs after the seventh codon. Accordingly, a 400-bp *Bam*HI-*Pst*I fragment was removed from the gG gene from plasmid pPALM104 and a 400-bp *Bam*HI-*Eco*RI-*Pst*I linker was inserted. pALM104 contains a 6.1-kbp *Sph*I fragment from PRV-Becker (containing gG, gD, gI, and part of gE) cloned into the *Sph*I site of pGEM5Zf+. The resulting plasmid was called pAK44 and had a 400-bp internal deletion in the gG gene. The PCR-amplified 1.1- and 1.7-kbp open reading frames of BHV-1 gI and gE were isolated as described above, cleaved with *Eco*RI, and inserted into the *Eco*RI site of the pAK44 transfer vector in frame with the first 7 codons of PRV gG. (The natural translation termination codon of both genes was retained in the inserted fragment.) The resulting plasmids were named pAK45 and pAK46, respectively. As a result of this construction, the hybrid gG-gE or gG-gI genes had the first 7 codons of gG, followed by 3 new codons from the *Eco*RI linker (Arg, Ile, and Pro), followed immediately by the first Met of either BHV-1 gE or gI. These amino acids were not predicted to affect signal sequence function. Therefore, after removal of the signal sequence, the resulting proteins should be authentic BHV-1 gE and gI.

The BHV-1 gI open reading frame was engineered to be expressed from the PRV gI promoter as follows. We built a transfer vector that allowed the in-frame insertion of BHV-1 gI after the first 2 amino acids of PRV gI. The plasmid pALM93 (6.1-kbp *Pst*I-*Mlu*I fragment from PRV Becker *Bam*HI-7 fragment cloned into pGEM5Zf+) was cut with *Csp*I and *Xcm*I, sites located upstream of the start codon and at the stop codon of PRV gI, respectively, to remove PRV gI. We next inserted a *Csp*I-*Eco*RI-*Xcm*I linker that reconstituted the *Csp*I site to the first 2 amino acids of PRV gI. This plasmid was called pAK23. Next, we deleted the PRV gE gene from pAK23 by isolating an 800-kbp *Xcm*I-*Mlu*I fragment from pALM91 (4) that contains a gE deletion. This fragment was used to replace the *Xcm*I-*Mlu*I fragment from pAK23, and the resulting plasmid was called pAK32. The PCR-amplified fragment containing the BHV-1 gI open reading frame was isolated, cut with *Eco*RI, and inserted in the same orientation as PRV gI into the *Eco*RI site of pAK32. The natural translation termination codon of BHV-1 gI was retained in the inserted fragment. The resulting plasmid was called pAK36. As a result of this construction, the first 2 codons of PRV gI (Met and Met) are immediately followed by Arg, Ile, and Pro codons from the *Eco*RI linker and then the first methionine of BHV-1 gI. These additional amino acids were not predicted to affect the signal sequence function of the new BHV-1 gI protein, and when removed during protein export, the resulting protein should be authentic BHV-1 gI.

Selection for PRV recombinants carrying the BHV-1 gE or gI gene. A blue-white plaque screen for beta-galactosidase expression was used to identify recombinant viruses with gene insertions in the PRV gG locus. PRV-99Blue lacks both the PRV gE and gI genes and also carries the *lacZ* gene inserted in the gG locus (unpublished data). Therefore, we screened for replacement of the *lacZ* gene in PRV-99Blue after cotransfection of PRV-99Blue DNA and transfer plasmids with BHV-1 gE or gI in the gG gene. The desired recombinants should have a white-plaque phenotype after exposure to the beta-galactosidase substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), while PRV-99Blue will have a blue-plaque phenotype. PK15 cells were cotransfected with 1 μg of purified genomic PRV-99Blue DNA and 1 μg of pAK45 or pAK46 CsCl-purified plasmid DNA by the calcium phosphate precipitation method (12). Transfected cells were incubated until complete cytopathic effect was noted, at which time the medium and infected cells were harvested and frozen. Plaque-forming virus from each transfection were screened for expression of beta-galactosidase. LacZ-minus plaques (white after incubation with X-Gal) were purified three times as described previously (20). These putative recombinants were then grown up and tested for the presence of the appropriate BHV-1 gene.

We used the black-plaque assay as a screen for recombinant viruses carrying BHV-1 gI in the PRV gI locus (36). PRV-98 carries a deletion of the PRV gI gene but expresses immunoreactive gE protein on the surface of infected cells (36). Consequently, when rabbit antibody specific for gE is added to plaques followed by a peroxidase-linked anti-rabbit antibody, PRV-98 plaques turn black, while plaques of viruses lacking gE remain white after the appropriate peroxidase substrate is added. CsCl-purified pAK36 DNA (lacking the PRV gE gene but carrying the BHV-1 gI gene) was cotransfected with genomic PRV-98 DNA. The transfected cells were incubated until complete cytopathic effect was noted, and the medium and infected cells were harvested and frozen. Plaques were screened with a rabbit polyclonal anti-gE serum, and white plaques were picked and purified three times as previously described (36). These putative recombinants were then grown up and tested for the presence of the BHV-1 gI gene.

Immunoprecipitation and Western blot analysis. PRV-infected cells were labeled with [^{35}S]cysteine, and immunoprecipitations were performed essentially as described previously (36). For pulse-chase analyses, PK15 cells were infected at a multiplicity of infection (MOI) of 10, and at 5 h postinfection, the cells were incubated in cysteine-free medium for 20 min and then pulse-labeled for 10 min with [^{35}S]cysteine (NEN). After 10 min, the radioactive medium was removed and replaced with nonradioactive medium containing an excess of unlabeled cysteine and cystine. The first sample was taken immediately after the 10-min labeling period and is referred to as 0 min postpulse.

Nonradioactive Western blot analysis was performed as recommended by the manufacturer (Renaissance, NEN). Proteins were separated on sodium dodecyl sulfate (SDS)–12% polyacrylamide gel electrophoresis (PAGE) gels and transferred to nitrocellulose membranes.

Virion purification. PK15 cells were infected at an MOI of 5 PFU per cell, [^{35}S]cysteine was added 6 h postinfection, and the infected cells were incubated at 37°C for an additional 8 h. The medium fraction was harvested, and cells and debris were removed by low-speed centrifugation. Virions in the cleared medium were collected by pelleting through a 30% sucrose cushion in phosphate-buffered saline, pH 7.5 (SW50.1 rotor, 28,000 rpm, 90 min). The pellet was resuspended in 1× TNX (10 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100), RIPA buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1% Na deoxycholate, 0.1% SDS, 1% Nonidet P-40) was added, and the mixture was boiled and analyzed by immunoprecipitation as described above.

Single-step growth analysis. A series of dishes containing monolayers of PK15 cells were infected at an MOI of 5 PFU per cell. Virus was allowed to absorb to cells for 1 h at 37°C, the inoculum was removed by aspiration, and the cells were washed three times with warm phosphate-buffered saline. The cells were overlaid with prewarmed medium. Two plates were harvested at each time point. Before titration, intracellular virus was released from the cells by three freeze-thaw cycles. Extracellular virus was cleared of cells and debris by low-speed centrifugation prior to determination of virus titer.

Virulence determination. Sprague-Dawley male rats (300 g) were infected intravitreally with 2 μl of virus suspension (approximately 10^5 PFU) as previously described by Enquist et al. (10). Stocks of PRV-AK7, PRV-AK9, PRV-99, and PRV-99Blue were used in these experiments, and the titer, in PFU per milliliter, on PK15 cells was adjusted to 2×10^8 PFU/ml. For the coinfection experiments, a 1:1 mixture of PRV-AK7 and PRV-AK9 was prepared so that the titer of the mixture was 2×10^8 PFU/ml. Animals were examined at regular intervals for symptoms of PRV disease, which include nasal discharge, harderian gland discharge, labored breathing, hunched posture, scratching of the head, inappropriate motion, and lack of grooming behavior. Virulence was quantitated by determining the time at which distinct symptoms appeared for each animal as well as the time at which death was imminent. Animals were euthanized at this point. Virulence could also be determined qualitatively by visual inspection of the animals, because in wild-type virulent infections, not only do symptoms appear faster, they are more robust. 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.

RESULTS

Construction and analysis of PRV recombinants carrying BHV-1 gE or gI coding sequences. The unique short region of PRV encodes at least three membrane proteins not required for growth in tissue culture (gG, gI, and gE) (Fig. 1A). The gG locus has been exploited as a site for the insertion and expression of several foreign genes (20, 24, 30, 32, 35) and was used in this study to express the BHV-1 gE and gI genes. To compare levels of expression, we also inserted the BHV-1 gI gene at the PRV gI locus. The BHV-1 gI and gE coding sequences were inserted into two transfer vectors for gene replacement by homologous recombination into PRV as described in Materials and Methods. Both transfer vectors used a unique *Eco*RI site for constructing and marking the hybrid gene. As PRV has no *Eco*RI sites, successful introduction of the BHV genes into PRV can be monitored easily by digestion with *Eco*RI (11, 29). Recombinants at the gG and gI loci were screened with beta-galactosidase activity (blue-white plaques) and by immunohistochemical staining of plaques (black-white plaques) as described in Materials and Methods.

PRV-AK1 carried the BHV-1 gI gene inserted into the autologous region of PRV gI, while PRV-AK7 and PRV-AK9 carried the BHV-1 gI and BHV-1 gE genes, respectively, inserted into PRV gG (Fig. 1B). The fidelity of insertion was

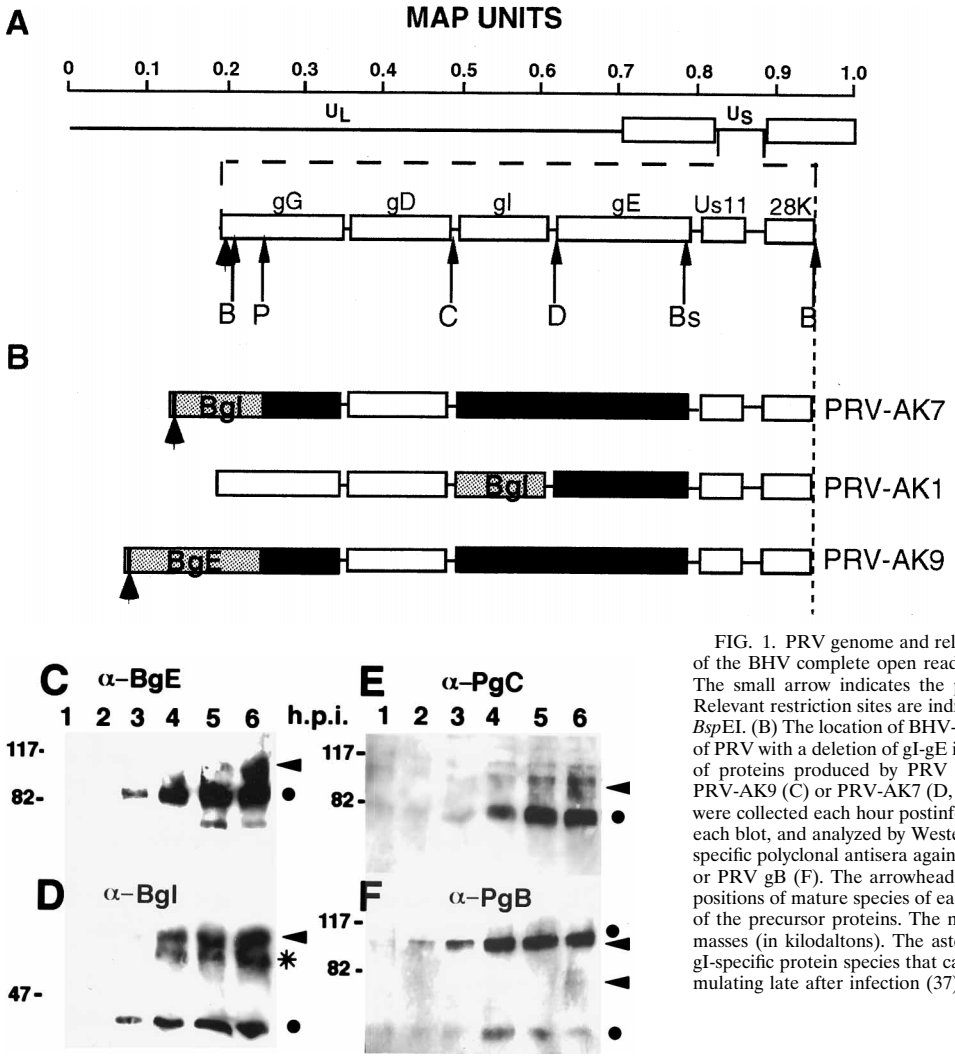

FIG. 1. PRV genome and relevant gene replacements. (A) Sites of insertion of the BHV complete open reading frame sequences within the PRV genome. The small arrow indicates the position of the first *Bam*HI site in PRV gG. Relevant restriction sites are indicated. B, *Bam*HI; P, *Pst*I; C, *Csp*I; D, *Dra*I; Bs, *Bsp*EI. (B) The location of BHV-1 gI and gE within the unique short (Us) region of PRV with a deletion of gI-gE is shown. (C, D, E, and F) Western blot analysis of proteins produced by PRV recombinants. PK15 cells were infected with PRV-AK9 (C) or PRV-AK7 (D, E, and F) at an MOI of 10. Whole-cell extracts were collected each hour postinfection (h.p.i) for 6 h, as indicated on the top of each blot, and analyzed by Western blots. The blots were incubated with monospecific polyclonal antisera against BHV-1 gE (C), BHV-1 gI (D), PRV gC (E), or PRV gB (F). The arrowheads on the right point to the expected migration positions of mature species of each glycoprotein. The dots indicate the positions of the precursor proteins. The numbers on the left of each blot are molecular masses (in kilodaltons). The asterisk in panel D indicates a presumed BHV-1 gI-specific protein species that can also be found in BHV-1-infected cells, accumulating late after infection (37).

confirmed by sequence analysis of the appropriate junctions and by Southern blot analysis (data not shown).

Detection of BHV-1 proteins by Western blot analysis after infection of PK15 cells by PRV recombinant viruses. The PRV gG promoter is a strong early promoter, and it was of interest not only to determine if the desired BHV-1 proteins were made after PRV infection but also to determine the time course of their synthesis. PK15 cells were infected at an MOI of 10 with either PRV-AK9 or PRV-AK7 expressing BHV-1 gE or gI, respectively, from the PRV gG promoter. Extracts were prepared every hour postinfection until 6 h postinfection, and proteins were fractionated by gel electrophoresis and analyzed for BHV-1 and PRV proteins on the Western blots as described in Materials and Methods. PRV gC and gB were used as internal controls to measure relative time of expression and quantity for comparison between samples. In a previous study, we determined that the BHV-1 gE 92-kDa protein was the mature species, while the 84-kDa species was the precursor species (37). Likewise for BHV-1 gI, the 62-kDa species is the mature form, and the 40-kDa species is the precursor of BHV-1 gI.

As shown in Fig. 1, the precursor forms of BHV-1 gE and gI were first detected in significant amounts at 3 h postinfection (Fig. 1C and D). The relative amount of BHV-1 gI made by PRV-AK7 was similar to the amounts of PRV gC (Fig. 1E) and PRV gB (a true late gene product) (Fig. 1F). The gC and gB profiles were identical in the PRV-AK9 infection (data not shown). Interestingly, while a substantial level of mature BHV-1 gE was only seen 6 h postinfection, mature BHV-1 gI was visible 2 h earlier (Fig. 1, compare 4 and 6 h postinfection in panels C and D). This delay in maturation most likely results from the absence of gI, which is required for efficient processing (see Fig. 2). With the PRV-AK1 recombinant that expresses BHV-1 gI from the PRV gI promoter, we observed a 15-fold-lower level of expression compared to that observed with PRV-AK7 (data not shown). Although these Western blot data are not quantitative, they establish that both BHV-1 genes are expressed at levels and times similar to those of major PRV membrane proteins.

Pulse-chase analysis of protein processing and export. It was important to establish the kinetics of synthesis and processing of BHV-1 gE and gI proteins made by PRV. We have previously noted that for PRV, expression of either gE or gI alone resulted in reduced kinetics of protein processing and export, while coexpression facilitated these processes. Facilitated processing and export after coinfection is therefore one measure of fidelity of expression and function.

Processing of gE and gI was first determined for the individual recombinants. PK15 cells were infected with each recombinant virus at an MOI of 10 as described in Materials and

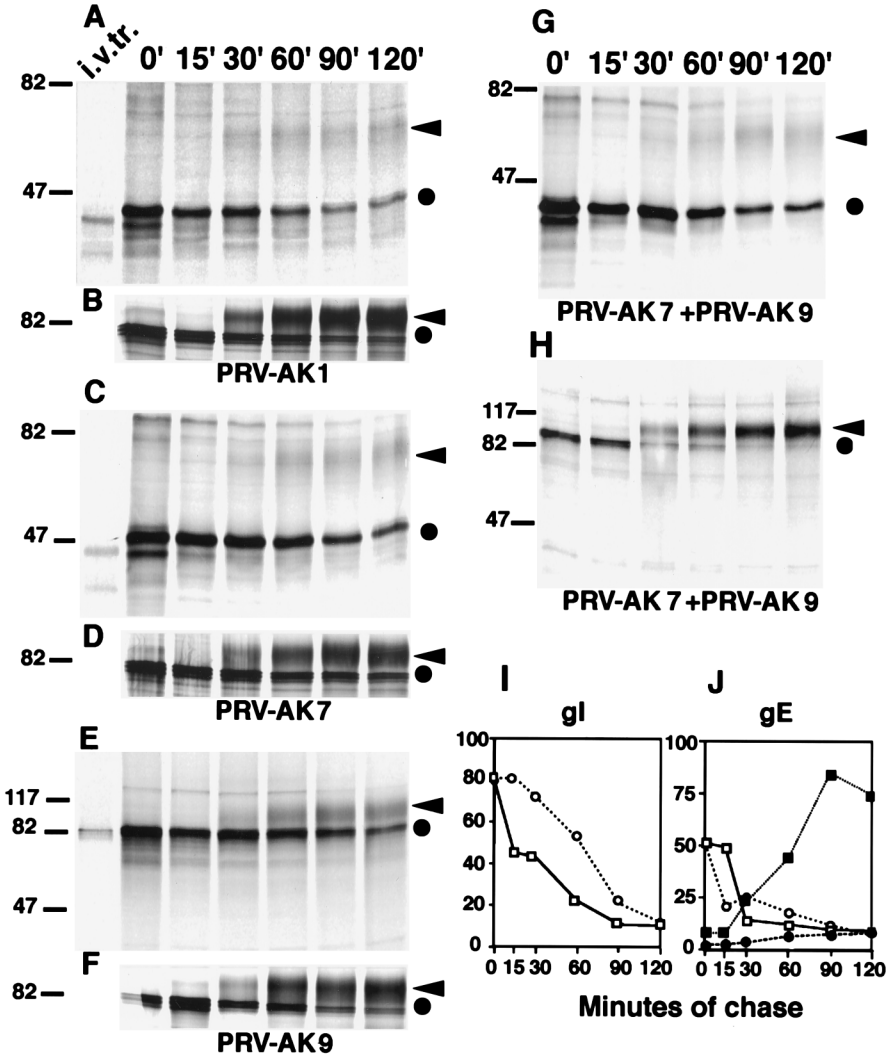

FIG. 2. Kinetics of protein modification and export. PK15 cells were infected with PRV-AK1 (A and B), PRV-AK7 (C and D), PRV-AK9 (E and F), or PRV-AK7 together with PRV-AK9 (G and H) at an MOI of 10 PFU per cell. Six hours postinfection, the cells were pulse-labeled with [^{35}S]cysteine for 10 min. After this labeling period, medium containing excess cold cysteine was added as described in Materials and Methods. The chase times (in minutes after the chase) are as indicated on the top of the figure. All protein samples were denatured in the presence of SDS and dithiothreitol before immunoprecipitation and analysis by PAGE. The first lane in each panel shows the products of BHV-1 gI and gE as obtained after in vitro transcription and translation to provide a marker for the unmodified proteins. In vitro-translated proteins were labeled with [^{35}S]methionine. Samples were immunoprecipitated with monospecific polyclonal antiserum specific for BHV-1 gI (A, C, and G), BHV-1 gE (E and H), and PRV gC (B, D, and F) and analyzed by SDS-PAGE. Molecular mass markers (in kilodaltons) are indicated on the left of each blot. The arrowheads point to the predicted mature species, and the dots indicate the positions of the precursor forms. Note that the autoradiographs shown in A and B were exposed for 3 days longer than the others. The autoradiograms resolving the precursor and mature glycoprotein forms of the BHV-1 gI- and gE-expressing recombinants PRV-AK7 and PRV-AK9 were scanned and quantitated with NIH Image Quant software (I and J). The graphs depict the chase kinetics of the precursor and mature forms. The data in panels C and G were used for the graph in panel I, and the data in panels E and H were used for the graph in panel J. Open circles, precursor glycoproteins in a single infection; open squares, precursor glycoproteins in a coinfection. Filled circles and squares, mature BHV-1 gE in a single infection and coinfection, respectively.

Methods. At 6 h postinfection, the infected cells were labeled for 10 min with [^{35}S]cysteine (pulse), and the radioactive medium was removed and replaced with medium containing excess unlabeled cysteine (chase). Samples were taken at 0, 15, 30, 60, 90, and 120 min after the pulse-label and analyzed by immunoprecipitation as described in Materials and Methods. The results are shown in Fig. 2. It is important to note three controls. First, in the left lane of panels A, C, and E, we show the in vitro translation (lanes i.v.tr.) product of the subcloned BHV-1 gI gene (A and C) and BHV-1 gE gene (E) to establish the apparent molecular mass of the nonglycosylated protein. Second, in the panels below panels A, C, and E (B, D, and F, respectively), we show the immunoprecipitation of the unrelated control protein gC from the same extract. In all cases, the rate of processing of the 74-kDa gC precursor to the 92-kDa mature species is qualitatively and quantitatively indistinguishable.

After infection with PRV-AK1, the 40-kDa gI precursor protein was detected immediately after the 10-min pulse-labeling (Fig. 2A, lane 0′). The gI precursor migrated slightly slower than the in vitro-translated BHV-1 gI, because it probably had acquired endoplasmic reticulum glycosylation modifications (Fig. 2A, lane i.v.tr.). During the 2-h chase period, the precursor decreased in intensity. By 30 min of chase, we detected the 62-kDa mature gI protein (see the legend to Fig. 2A).

A qualitatively similar result was obtained when cells were

infected with PRV-AK7 expressing gI from the gG promoter (Fig. 2C), except that the gel was exposed to film for 3 fewer days than the gel in Fig. 2A. When compared with the internal control PRV gC protein, the total amount of BHV-1 gI produced from PRV-AK7 was 15-fold higher than that produced from PRV-AK1, as determined by densitometry (data not shown). Despite the overproduction of gI by PRV-AK7, the kinetics of synthesis and processing were not significantly different than those found for PRV-AK1 or for wild-type BHV-1 in MDBK cells (37).

After infection with PRV-AK9, the 84-kDa gE precursor protein was labeled in the 10-min pulse, and the amount of immunoreactive protein then decreased with time of chase as it was converted to the mature form (Fig. 2E). By 30 min after the pulse, the 92-kDa mature gE species began to accumulate (Fig. 2E). In these experiments, the 92-kDa BHV-1 gE protein was sensitive to *N*-glycosidase F but resistant to endo-β-*N*-acetylglucosaminidase H (endo-H), and the BHV-1 gI protein of 62 kDa was sensitive to *N*-glycosidase F, while the smaller 40-kDa protein was also sensitive to endo-H (data reviewed but not shown). Finally, the protein species seen after infection with the PRV recombinants were indistinguishable from those made after BHV-1.1 wild-type virus infection of MDBK cells (data reviewed but not shown).

We had noted previously (36) that PRV gE was processed less efficiently in the absence of PRV gI. Therefore, we performed a coinfection experiment with PRV-AK7 and PRV-AK9 to determine if processing could be facilitated by coexpression of BHV-1 gE and gI in the same cell. Indeed, high-multiplicity coinfection of PRV-AK7 with PRV-AK9 resulted in a more rapid conversion of the BHV-1 gE 84-kDa precursor form to the 92-kDa mature form (compare Fig. 2E and H). The relative amount of precursor and mature species of BHV-1 gE and gI were quantitated and plotted as shown in Fig. 2I and J. Because the anti-BHV-1 gI serum reacts strongly with the precursor but weakly and nonquantitatively with mature gI, we can only draw indirect conclusions for the rate of maturation of BHV-1 gI by measuring the decrease in the amount of precursor form (Fig. 2C, G, and I). After coinfection, processing of BHV-1 gI and gE expressed from the PRV recombinant infections of swine cells mimics that observed for a BHV-1 infection of bovine cells.

We found more mature BHV-1 gI at an earlier time point than mature gE in single infections (for BHV-1 gI, compare Fig. 2B, lane 120′, with Fig. 1D, lane 6 [6 h postinfection]; for BHV-1 gE, compare Fig. 2E, lane 120′, with Fig. 1C, lane 6 [6 h postinfection]). This result suggests that maturation of BHV-1 gI is less dependent on gE than gE is on gI. It also demonstrates that high-multiplicity coinfection of cells by two viruses, one expressing BHV-1 gE and the other expressing BHV-1 gI, faithfully reproduces gE-gI processing of the wild-type infection in this assay.

BHV-1 gE localizes to virions. While BHV-1 gE and gI are found in the envelope of wild-type virions, it was important to determine if these heterologous proteins would be incorporated into a PRV envelope. To determine this, PK15 cells were infected at an MOI of 5, and cells were labeled with [^{35}S]cysteine for 15 h. The medium fraction was precleared of cell debris by low-speed centrifugation, and extracellular virions were centrifuged through a 30% sucrose cushion as described in Materials and Methods. gB and gC, two PRV glycoproteins with distinct precursor and mature protein species, were included as controls (Fig. 3, lanes 1 to 4). Because only the mature gB and gC glycoproteins are incorporated into virions, we would not expect to see any precursor forms in the enriched virion fraction. While the antibodies recognized a considerable amount of the 110-kDa gB precursor and the 74-kDa gC precursor in the whole-cell extract, no gB or gC precursors were detectable in the virion preparation, as predicted. The PRV gB 68- and 55-kDa proteins are cleavage products of the mature protein, are prominent bands in the virion preparation only, and are underrepresented in the whole-cell extract (Fig. 3, compare lanes 2 and 4). These results suggest that the virion preparation contained few, if any, cellular membranes containing precursor proteins. The same extracts were then analyzed with BHV-1 gE-specific antisera which readily detected mature BHV-1 gE in PRV virions (Fig. 3, lane 5). The weak reaction of the BHV-1 gI antiserum with mature BHV-1 gI made it difficult to confirm that BHV-1 gI was localized to virions (data not shown). No precursor species of BHV-1 gE or gI were detected in PRV virions. From this experiment, we conclude that BHV-1 gE can be incorporated into PRV virions.

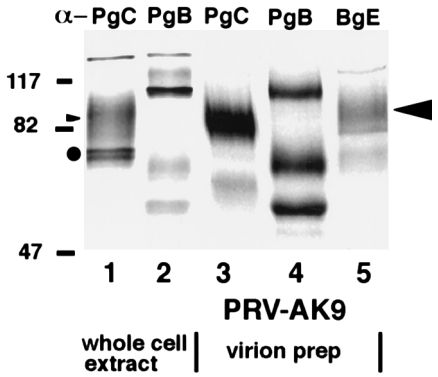

FIG. 3. Localization of BHV gE in extracellular virions. PK15 cells were infected at an MOI of 5 and labeled with ^{35}[S]cysteine for 15 h. The culture medium was clarified by low-speed centrifugation, and the supernatant was loaded onto a 30% sucrose cushion. After centrifugation, the pellet fraction was removed and solubilized, and proteins were analyzed by immunoprecipitation with antisera specific for PRV gC (PgC), PRV gB (PgB), and BHV gE (BgE) as indicated at the top of the figure. As a control, infected whole-cell extract was immunoprecipitated with the PRV gC- and PRV gB-specific antibodies. The absence of any precursor gC or gB forms in the virion preparations is an indication of the purity of the virion preparation.

Growth characteristics of recombinant viruses. As the PRV recombinants express heterologous gE and gI proteins from an ectopic site with a stronger promoter, it was important to verify that the overexpressed proteins were not adversely affecting viral growth. All three PRV recombinants formed small plaques on bovine cells that were indistinguishable from the small plaques formed by PRV-99, a gE-gI deletion mutant. Similar results were observed on PK15 cells, except that PRV-AK9 formed a distinctly smaller plaque than did the other recombinants.

As the recombinants formed small plaques, it was important to establish the kinetics of virus replication and release. Single-step growth curves were employed to determine the rates of intracellular and extracellular infectious virus formation. After infection of PK15 cells with each of the three recombinants at an MOI of 10, we harvested the cell and medium fractions at various times. The titers of cell-associated and cell-free virus were determined on PK15 cells and are plotted in Fig. 4. After an initial lag phase of 3 h, infectious virus began to accumulate for all recombinants. As expected, plaque-forming particles were first detected in the cell fraction, followed by a rise in titer in the medium fraction. Similar quantities of intracellular and extracellular infectious virus were produced by PRV-AK1 and PRV-AK7 (Fig. 4A and B). By contrast, PRV-AK9 released at least 10-fold-less plaque-forming virus into the medium than

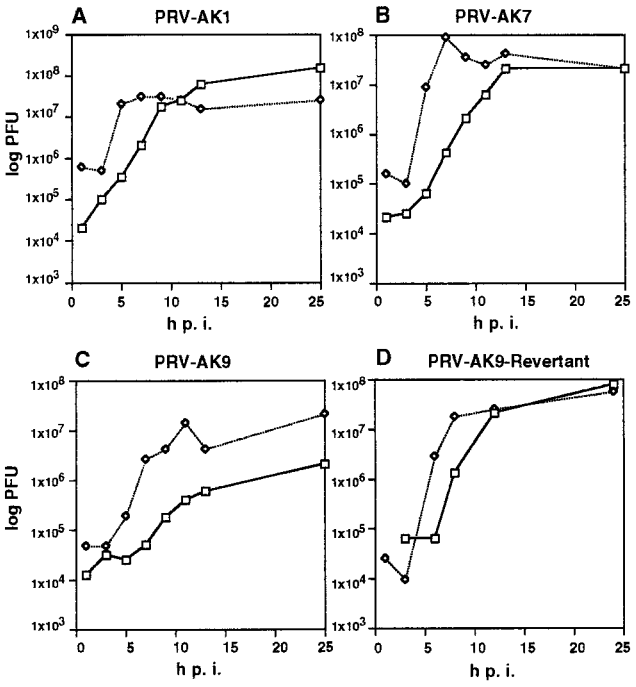

FIG. 4. Single-step growth analysis. A series of monolayers of PK15 cells were infected with PRV-AK1, PRV-AK7, PRV-AK9, and PRV-AK9-revertant at an MOI of 10 PFU per cell. At various times postinfection, plates were harvested and separated into medium and cell fractions as described in Materials and Methods. Plaque-forming virus titers were calculated after infection of PK15 cells. Squares, cell-free plaque-forming virus; diamonds, cell-associated plaque-forming virus. h.p.i., hours postinfection.

was produced in the cells. To determine whether this defect was associated with the insertion of BHV-1 gE or was a second-site mutation, we constructed a revertant by replacing BHV-1 gE sequences with *lacZ* sequences. The *lacZ*-expressing recombinant was normal for the release of plaque-forming particles into the medium, indicating that the release defect of PRV-AK9 was due to the insertion of the BHV-1 gE gene and probably reflects action of the BHV-1 gE protein in the PRV-infected cell (Fig. 4D).

Increased virulence after coinfection of rats with BHV-1 gI- and gE-expressing recombinants. We have previously shown that PRV strains with deletions of gI, gE, or both are markedly less virulent in rats than the parental PRV-Becker strain (4). To determine if the BHV-1 gE and gI gene products can complement the virulence defect of gE-gI-defective PRV, we performed the same experiment as that described by Card et al. (4), infecting rats by intravitreal injection. This paradigm facilitates rapid invasion of the central nervous system and produces a lethal infection in 100% of animals in a highly reproducible fashion. The animals were monitored at regular intervals, and virulence was quantitated by determining the time when symptoms first appeared as well as the time when death was imminent (see Materials and Methods). The data are shown in Fig. 5. Previous experiments defined the maximum virulence in this paradigm for the parental PRV-Becker strain (4). PRV-Becker-infected animals first show symptoms at about 60 h postinfection, with a mean time to death of 72 h, and no animals survived longer than 80 h postinfection. We performed two control experiments testing the virulence of PRV-99 (a strain with a gE-gI deletion) and PRV-99Blue (PRV-99 carrying a *lacZ* gene in gG). PRV99-Blue was constructed similarly to the PRV-AK7 and PRV-AK9 recombinants. PRV-99 was markedly less virulent than the parental PRV-Becker strain, because symptoms of infection did not occur until 93 h postinfection (33 h later than PRV-Becker-infected animals), with a mean time to death of 104 h, and two animals survived to 120 h. PRV-99Blue was tested with only two animals in this experiment and showed a reduced-virulence profile similar to that of PRV-99 (survival of 92 and 105 h for the two infected animals). Both PRV recombinants expressing either BHV-1 gE or gI (PRV-AK9 and PRV-AK7) appeared slightly more virulent than PRV-99, but this differ-

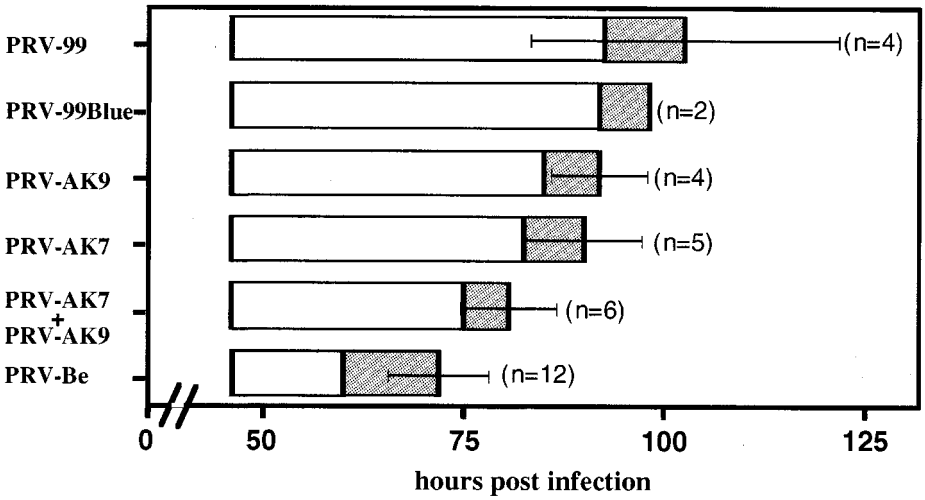

FIG. 5. Virulence of PRV recombinants. Time after injection in hours is shown on the horizontal axis, and the effective survival interval for animals injected with each strain of virus is represented by the horizontal bars. The length of each bar illustrates the mean time to death for each virus strain. Error bars are the standard deviation. 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. The mean time to death for each strain is as follows: PRV-Be, 72 ± 6.2 h; PRV-99, 104 ± 19 h; PRV-99Blue, 98.5 h (the values for two animals were 92 and 105 h); PRV-AK7, 89 ± 8 h; PRV-AK9, 92 ± 6 h; PRV-AK7 and PRV-AK9 (mixed infection), 81.5 ± 6 h. The statistical significance for each infection and that of PRV-99 was determined with a two-tailed Student's *t* test, assuming unequal variances. Only the PRV-Be infection and the coinfection of PRV-AK7 and PRV-AK9 were significant at the 95% confidence level.

ence was not statistically significant. However, the coinfection of both PRV-AK7 and PRV-AK9 resulted in an infection that was obviously more virulent than either virus alone and was significant at the 95% confidence level when compared to PRV-99. While mean time to death is a quantitative measure of virulence, it cannot convey the appearance of animals infected with vector alone (PRV-99), which is dramatically different than that of PRV-AK7–PRV-AK9-coinfected animals. Coinfection gives the rapid onset and robust appearance of all the symptoms described in Materials and Methods typical for wild-type PRV infection, while infection with either virus alone produces a visibly milder infection. We conclude that in this paradigm, BHV-1 gE and gI can at least partially complement the virulence defect caused by the deletion of PRV gE and gI.

DISCUSSION

The membrane proteins gE and gI are found in both cellular and virion membranes after infection by essentially all field isolates in the alphaherpesvirus subfamily. Their precise function has been elusive. The genes encoding these proteins can be deleted with modest to no effect on virus growth in tissue culture, but in cases where genetic analysis has been done, such mutants have reduced ability to spread between some cells in culture and some, but not all, neurons in vivo. Furthermore, these mutants cause less primary disease with reduced symptoms after infection of the natural host or a variety of laboratory animals. The relationship, if any, between these phenotypes is a matter of interest. For human herpesviruses herpes simplex virus and varicella-zoster virus, the gE-gI proteins can bind the Fc region of human immunoglobulin G antibody which is thought to interfere with host immune defenses. This mechanism cannot be invoked for the gE-gI homologs in PRV and BHV-1, as they do not bind human, porcine, or bovine antibody (references 6, 14, 18, 37, and 43 and unpublished observations). As the gE and gI genes are retained in wild-type alphaherpesvirus isolates and are usually lost in naturally attenuated strains, many argue that these genes offer a selective advantage in natural infections and postulate that the gE and gI homologs have conserved functions that govern virus spread, immune avoidance, and maintenance in natural hosts. The broad conservation of these gene sequences in the *Alphaherpesvirinae* and the common reduced-virulence phenotype of gE-gI mutants in diverse animal infections certainly are supportive of this idea, but until this report, there were no direct data to affirm or deny this implication.

Even though the DNA sequences of gE and gI are conserved, they have diverged considerably at both the DNA and protein levels. Indeed, analysis of the DNA sequence of seven gE-gI homologs reveals no significant homologies to host membrane proteins, nor does it show large blocks of conserved coding sequences that give immediate insight into the biological function of these conserved proteins (2, 12a). The gE and gI genes of PRV and BHV-1 represent two of the most highly conserved gene sets, yet the similarities between gE and gI homologs are 51% for gI and 54% for gE.

We have inserted the BHV-1 genes into PRV rather than inserting PRV genes into BHV-1 for two general reasons. First, we did not want to make a virus with the potential for expanded host range or virulence. BHV-1 is a narrow-host-range virus limited primarily to cattle and not swine, while PRV naturally infects and causes disease not only in swine but also in the natural host for BHV-1 (33, 38). PRV also has been associated with natural infections of a wide variety of other animals (25, 38). It seemed less likely that a PRV recombinant with a single BHV-1 gene would have an expanded host range. Second, not only does PRV have a broad animal host range, it also can be grown in a variety of cell types from various species. In particular, for analysis of BHV-1 genes, PRV grows well in cells of bovine origin. If we are correct in our speculation (see below) that gE and gI affect virulence via a common mechanism independent of virus or host, then being able to clone and express gE-gI homologs in a broad-host-range virus like PRV should facilitate analysis of any shared gE-gI functions.

We sought to minimize quantitative effects due to different promoter strengths and kinetic class by inserting both BHV-1 genes into the identical genomic locus of PRV gG. We also chose the gG locus because we anticipate comparing other gE-gI homologs from other viruses and a constant cloning locus and promoter facilitates constructions and comparisons. However, one might argue that such ectopic expression is artificial, as expression will not be under proper temporal control; therefore comparisons of function may be suspect. This is a reasonable concern, and any negative result with such a transcomplementing system would be difficult to interpret. However, positive results, of the kind we present in this report, indicate that the proteins per se are responsible and the site and extent of expression have little to no effect. We have shown, by all criteria employed so far, that the BHV-1 proteins produced by PRV were indistinguishable from those made by BHV-1 virus itself: we used Western blot analysis, pulse-chase radiolabeling experiments, endoglycosidase digestion, immunofluorescence, and in vitro translation to arrive at this conclusion (data reviewed but not shown in this paper). We did not analyze phosphorylation or sulfation, modifications that have been reported or predicted for other gE-gI homologs (7, 8, 25, 39, 40). We did insert the BHV-1 gI gene into the PRV gI locus, but we found that expression was considerably less efficient (15-fold lower than that from gG).

A common phenotype for gI-gE mutants of all alphaherpesviruses tested to date is that they form significantly smaller plaques on monolayers of some, but not all, cultured cells (5, 13, 42). Expression of BHV-1 gI had no effect on the plaque size of PRV-AK1 or PRV-AK7; both formed small plaques on PK15 or MDBK cells that were the same size as those made by PRV-99 (gE-gI deletion mutant). By contrast, expression of BHV-1 gE appeared to affect plaque size, as PRV-AK9 formed distinctly smaller plaques on PK15 or MDBK cells (data reviewed but not shown).

The single-step growth curve of recombinants expressing BHV gI was similar to those of the parent PRV-99 and the wild-type virus PRV-Becker. However, in accordance with the small-plaque phenotype on monolayers, the single-step growth curve of the gE-expressing strain PRV-AK9 was distinctive. The BHV-1 gE-expressing recombinant produced normal amounts of intracellular virus, but the amount of virus released into the medium was reduced (Fig. 4). As a *lacZ* revertant releases virus at normal levels, we conclude that the release defect is primarily due to the presence of BHV-1 gE and not to the absence of gG (data not shown). Perhaps overexpression of the heterologous glycoprotein interferes with PRV egress. Alternatively, this phenotype may reflect action of BHV gE in PK15 cells similar to that observed for PRV in chicken embryo fibroblasts where expression of PRV gE is deleterious for efficient virus release (41).

We could assess two functions of BHV-1 gE and gI in tissue culture by coinfection experiments where a high MOI ensured that single cells were infected with both gE- and gI-expressing viruses. One measure of function in coinfection is the formation of a BHV-1 gE-gI oligomer. Indeed, this oligomer appeared rapidly after coinfection, as it did in wild-type BHV-1 and PRV infections (data not shown) (36, 37). While the func-

tional significance of gE-gI complex formation is not fully understood, it provides a measure of another function of gE and gI: expression of both proteins in the same cell enables more efficient processing and maturation of either protein (23, 36, 37). We demonstrated that the export and processing of either gE or gI were facilitated by mixed infection (Fig. 2). In general, these results are significant because the coinfection analysis is critical for "mix-and-match" experiments where gE and gI homologs from any alphaherpesvirus would be analyzed for function by complementation.

A major objective in constructing these hybrid viruses was to determine if the BHV-1 gE-gI gene products could replace PRV gE-gI proteins in animal models of PRV pathogenesis. Perhaps a more fundamental question is the following: why would we expect that gE-gI from BHV-1 would affect virulence of PRV in rats? The basic hypothesis to be tested was that the gE-gI homologs have common functions that are independent of virus and host (they are intrinsic virulence factors). It is known that both BHV-1 gE and PRV gE are major determinants of virulence in young calves (15, 34) and swine (3), respectively. Moreover, PRV mutants lacking gE and gI also have reduced virulence after laboratory infection of rats and mice, as well as day-old chicks (4, 17, 21). The experiments whose results are presented in Fig. 5 demonstrate that the BHV-1 gE and gI gene products can, in the absence of any other BHV-1 genes, partially complement the virulence defect of a PRV gE-gI deletion mutant in rats, animals that cannot be infected by BHV-1. Given the diverse species where gE-gI affect virulence, the conservation of gE-gI in the *Alphaherpesvirinae* subfamily, and the apparent virus-independent action in virulence that we have observed between PRV and BHV-1, we speculate that these proteins are interacting in a similar fashion with highly conserved factors in the vertebrate lineage. The molecular nature of the gE-gI ligands remains to be elucidated.

Virulence, defined as the degree of pathogenesis, is a complex pathway involving both viral and host responses. gE-gI represent one set of viral genes in the process whose presence increases virulence upon primary infection. We know that the absence of gE and gI also affects anterograde spread of PRV in some, but not all, neurons in pigs, rats, mice, and chicken embryos (references 1, 9, 24, 31, and 36 and unpublished observations). It is not clear if the requirement for gE-gI in anterograde spread has any causal relationship with the reduced virulence seen for PRV gE-gI mutants or if virulence reflects other functions of these proteins. Experiments in several animal systems suggest that gE-gI mutants are not defective in retrograde transport and, indeed, spread like wild-type virus in the nervous systems of animals infected by this route yet continue to show reduced virulence (9, 13). Experiments are in progress to determine if BHV-1 gE and gI can complement the specific neurotropism defect of PRV gE-gI mutants.

A speculative prediction of our findings is that the gE-gI homologs all affect virulence by a common mechanism independent of the virus that expresses them or the host that is infected. If so, then studies focusing on the comparative biology of these proteins in more tractable animal models provided by the broad host range of PRV will be useful. In any case, an important objective is to understand the molecular mechanisms involved in the striking effects that gE and gI have on the ability of the alphaherpesviruses to be pathogenic in a variety of animals.

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