A Glycine-Rich Bovine Herpesvirus 5 (BHV-5) gE-Specific Epitope within the Ectodomain Is Important for BHV-5 Neurovirulence†

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The bovine herpesvirus 5 (BHV-5) gE ectodomain contains a glycine-rich epitope coding region (gE5 epitope), residues 204 to 218, that is significantly different from the corresponding gE region of BHV-1. Deletion of the gE epitope significantly reduced the neurovirulence of BHV-5 in rabbits. Pulse-chase analyses revealed that the epitope-deleted and wild-type gE were synthesized as N-glycosylated endoglycosidase H-sensitive precursors with approximate molecular masses of 85 kDa and 86 kDa, respectively. Like the wild-type gE, epitope-deleted gE complexed with gI and was readily transported from the endoplasmic reticulum. Concomitantly, the epitope-deleted and wild-type gE acquired posttranslational modifications in the Golgi leading to an increased apparent molecular mass of 93-kDa (epitope-deleted gE) and 94-kDa (wild-type gE). The kinetics of mutant and wild-type gE processing were similar, and both mature proteins were resistant to endoglycosidase H but sensitive to glycopeptidase F. The gE epitope-deleted BHV-5 formed wild-type-sized plaques in MDBK cells, and the epitope-deleted gE was expressed on the cell surface. However, rabbits infected intranasally with gE epitope-deleted BHV-5 did not develop seizures, and only 20% of the infected rabbits showed mild neurological signs. The epitope-deleted virus replicated efficiently in the olfactory epithelium. However, within the brains of these rabbits there was a 10- to 20-fold reduction in infected neurons compared with the number of infected neurons within the brains of rabbits infected with the gE5 epitope-reverted and wild-type BHV-5. In comparison, 70 to 80% of the rabbits exhibited severe neurological signs when infected with the gE5 epitope-reverted and wild-type BHV-5. These results indicated that anterograde transport of the gE epitope-deleted virus from the olfactory receptor neurons to the olfactory bulb is defective and that, within the central nervous system, the gE5 epitope-coding region was required for expression of the full virulence potential of BHV-5.

Bovine herpesvirus 5 (BHV-5) is an alphaherpesvirus that causes fatal encephalitis in calves and is a significant viral pathogen in South America (6, 18). Bovine herpesvirus 1 (BHV-1) is associated with abortions, respiratory infections (subtype 1.1), and genital infections (subtype 1.2) in cattle (42) but does not usually cause encephalitis. BHV-1 and BHV-5 proteins have 82% predicted amino acid homology (14), and both viruses establish latency in the trigeminal ganglion following intranasal and conjunctival inoculation (2, 35). In a rabbit seizure model, BHV-1.1 and BHV-5 infections are distinguished by their differential neuropathogenesis (10). When rabbits are inoculated intranasally, BHV-5 invades the central nervous system via the olfactory pathway and produces acute neurological signs that are comparable to those seen in calves (6). However, BHV-1 does not invade the brain of infected rabbits and neurological signs do not develop (29).

The gE and gI homologues in alphaherpesviruses, including BHV-1 and BHV-5, form a noncovalently linked hetero-oligomer complex which is required for gE and gI maturation, cell-to-cell spread, and neurovirulence (3, 16, 22, 31, 40, 41, 43). In pseudorabies virus (PRV) and herpes simplex virus type 1, gE or gI null mutations significantly reduced neurovirulence

and significantly reduced the ability to infect second- and third-order neurons after nasopharyngeal or ocular infection (8, 16, 22, 24, 27, 28, 32, 33). Herpes simplex virus type 1 gE is predicted to bind a putative receptor molecule in the intercellular junction that promotes cell-to-cell spread (13, 15).

In our rabbit seizure model, gE-deleted BHV-5 has restricted anterograde transport from the olfactory receptor neurons to the bulb (second-order neurons) and does not infect second- and third-order neurons efficiently (11). BHV-1 gE did not complement BHV-5 gE with respect to neuroinvasion and neurovirulence (11). These results indicated that BHV-5 gE plays an important role in the differential neuropathogenesis of BHV-5.

The BHV-5 gE ectodomain contains a glycine-rich region (residues 204 to 218) that is significantly different from the corresponding gE region of BHV-1 (11). Rabbit polyclonal antibody raised against a peptide containing these residues reacted specifically with BHV-5 gE but not with BHV-1 gE on immunoblots (11). Additionally, the antibody specifically immunoprecipitated BHV-5 gE and not BHV-1 gE (11). Thus, the glycine-rich peptide sequence represents a BHV-5 gE-specific epitope (designated the gE5 epitope).

In this study, we investigated the significance of the gE5 epitope (glycine-rich BHV-5 gE ectodomain region) in BHV-5 neuropathogenesis. We generated recombinant BHV-5 viruses with the gE epitope coding region deleted and compared their neuropathogenicity in rabbits with that of the gE-deleted BHV-5, gE epitope-reverted BHV-5, and wild-type BHV-5.

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FIG. 1. Schematic illustration of the construction of a glycine-rich gE epitope-deleted BHV-5. (A) Schematic of the BHV-5 gE gene with the locations of the epitope coding region (residues 204 to 218), cysteine clusters (regions C1 and C2), N-linked glycosylation sites (N), transmembrane domain (TM), acidic domain (AD), and YXXL motifs shown. The positions of primer pairs P1-P2 and P3-P4 are indicated. (B) Scheme of the construction of the gE epitope deletion plasmid. In plasmid pBHV-5 gE Δ 204-218/temp, restriction sites for BamHI and KpnI created by primers P2 and P3, respectively, flank the deleted epitope coding region (15 amino acids). As described in Materials and Methods, the reading frame after the deletion point was changed. However, the reading frame was restored by digesting the plasmid DNA with KpnI and BamHI, followed by blunt ending with mung bean nuclease and religation. (C) Primer P1 and P4 sequences, used with P2 and P3, respectively, are shown with the restricted sites created.

Furthermore, the biosynthesis and maturation of epitope-deleted gE and its interaction with gI were analyzed.

MATERIALS AND METHODS

Virus strains and cell lines. The BHV-5 TX-89 strain (18) was used in this study. The virus was propagated and titrated in Madin-Darby bovine kidney (MDBK) cells grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum.

Construction of a recombinant BHV-5 gE epitope deletion vector. PCR amplification was performed with an XL-Long PCR kit (Applied Biosystems, Foster City, Calif.). The reaction was carried out in a 50- μ l volume containing 1 $\times$ PCR buffer, 1.6 μ l of 25 mM magnesium acetate, 4 μ l of a 10 mM deoxynucleoside triphosphate mixture, 500 pmol of each primer, 200 ng of the template DNA, and 5% dimethyl sulfoxide. Samples were heated for 1 min at 97°C, followed by addition of 1.5 μ l of rTth DNA polymerase (4 U) for each sample. Amplification consisted of 17 cycles of denaturation (95°C, 30s), annealing (55°C, 30s), and extension (72°C, 2 min). Then another 17 cycles were carried out with the same denaturation and annealing parameters but extension was for 3 min at 72°C. Upon completion of the last cycle, the samples were further incubated at 72°C for 10 min.

Plasmid construction. The BHV-5 gE gene is flanked upstream by the gI gene and downstream by the Us9 gene. Plasmid pBHV-5 gE5'3' containing gE and its upstream and downstream gene sequences (4.2 kb) was previously constructed in our laboratory (11). BHV-5 gE amino acid residues 204 to 218 (GGE GEGKG GRGA AK) represent a glycine-rich epitope. To delete residues 204 to 218 and to create an in-frame fusion of residues E₂₀₃ to P₂₁₉ within the gE open reading frame coding sequence, DNA sequences upstream of BHV-5gE amino acid residues 204 and downstream of BHV-5 gE amino acid residue 218 were ampli-

fied by long PCR with primer pairs P1-P2 and P3-P4 (Fig. 1) and pBHV-5gE5'3' DNA as a template. These primer pairs incorporated HindIII and BamHI (P1-P2) and KpnI-EcoRI (P3-P4) sites at their 5' and 3' ends of the 2.25-kb and 1.76-kb PCR products, respectively. These PCR-generated fragments were cleaved with appropriate enzymes and cloned into the appropriate sites of pGEM3Z separately, resulting in pBHV-5gE5' 1-203 and pBHV-5gE3' 218-598, respectively. pBHV-5gE3' was then digested with EcoRI and KpnI, and the 1.76-kb fragment was gel purified and ligated to EcoRI- and KpnI-digested pBHV-5gE5' 1-203 DNA. In the resulting clone, pBHV-5gE Δ 204-218/temp, the coding region for these 15 amino acids was deleted and the reading frame after the deletion site was changed. To restore the reading frame, plasmid pBHV-5 gE Δ 204-218/temp was digested with BamHI and KpnI, blunt ended with mung bean nuclease, and religated. In the resulting plasmid, pBHV-5 gE Δ 204-218F, the extra sequence added by the PCR was removed with mung bean nuclease, which also restored the frame without adding extra amino acids. The sequence of the glycine-rich gE epitope deletion was confirmed by DNA sequencing.

Construction of gE5 epitope-deleted BHV-5. To introduce the engineered deletion into BHV-5, full-length BHV-5 gE-deleted, β -galactosidase-expressing virus (11) and linearized pBHV-5 gE Δ 204-218 DNAs were cotransfected into MDBK cells. Colorless plaques, under Blueo-gal (Gibco-BRL, Life Technologies) overlay, were plaque purified, and the expression of mutant BHV-5 gE was verified with rabbit polyclonal antibodies specific for the glycine-rich epitope coding region (11) or the carboxy-terminal 210 amino acids of BHV-1 gE (41). To verify that the gE sequence in gE epitope-deleted virus had the intended deletion and there was no other alteration, DNA sequencing of the entire gE coding region was performed.

Construction of gE5 epitope-rescued BHV-5. To generate the gE5 epitope-rescued BHV-5, plasmid pBHV-5 gE5'3' (11) was linearized and cotransfected with full-length gE5 epitope-deleted virus DNA. MDBK cells grown in 12-well

plates were infected with viruses from randomly picked plaques. Extraction of infected-cell DNA was performed by a slight modification of the method described earlier (9). Revertants were identified by amplifying a 133-bp fragment with revertant-specific primers P1 (5'-GCGTGTACTTCCTGTACGACC-3', located 116 bp upstream of the epitope coding region) and P2 (5'-CCTTCCCCCTCCCCGCCCTCG-3', located within the gE epitope coding region).

Preparation of radiolabeled mock- and virus-infected cell lysates and immunoprecipitation. For steady-state labeling, confluent MDBK cells were infected with 5 PFU of wild-type BHV-5 or recombinant gE epitope-deleted BHV-5. At 4 h postinfection, complete growth medium (Dulbecco's modified Eagle's medium with 5% fetal bovine serum) was replaced with serum-free and cysteine- and methionine-free medium (Sigma, St. Louis, Mo.). At 6 h postinfection, the medium was replaced with cysteine- and methionine-free medium, 1% serum, and 50 μ Ci of [35 S]methionine-cysteine per ml. Mock infections with virus-free medium were always included in the analysis. Lysate was collected at 16 h postinfection (10 h of labeling) in extraction buffer (40% [wt/vol] suspension) as described previously (11). Immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described previously (11).

For pulse-chase analysis, confluent MDBK cells were infected with wild-type and recombinant gE epitope-deleted BHV-5 viruses as above, and at 4 h postinfection the medium was replaced with cysteine- and methionine-free medium. At 6 h postinfection, the medium was replaced with cysteine- and methionine-free medium containing 1% serum and 100 μ Ci of [35 S]methionine-cysteine per ml. The infected cells were incubated at 37°C for 30 min. At the end of the labeling period, the cells were washed twice in warmed (37°C) serum-free medium and either harvested immediately (0-min chase) or incubated at 37°C in complete growth medium for an additional 15 to 240 min. At the end of each chase period, individual samples were processed as previously described (11, 41). Radioactively labeled infected-cell lysates were immunoprecipitated with gE-specific antibody and analyzed by SDS-PAGE as described previously (11).

Glycopeptidase F and EndoH digestion. The immunoprecipitated viral proteins were released from the Sepharose beads with immobilized protein A by boiling in 20 μ l of 0.5% SDS and 1% β -mercaptoethanol for 10 min. The supernatants were transferred to fresh tubes. For glycopeptidase F digestion, the supernatants were adjusted to 50 mM sodium phosphate (pH 7.5) and 1% NP-40 and incubated with 1,000 U of glycopeptidase F (New England Biolabs) in a total volume of 26 μ l for 1 h at 37°C. For endoglycosidase H (EndoH) digestion, the supernatants were adjusted to 50 mM sodium citrate (pH 5.5) and incubated with 100 U of EndoH (New England Biolabs) in a total volume of 22.2 μ l for 1 h at 37°C. The digested samples were subjected to SDS-PAGE, and labeled proteins were visualized via autoradiography.

Indirect immunofluorescence surface expression assay. MDBK cells grown on chamber slides were infected with 5 PFU of gE epitope-deleted or wild-type BHV-5. At 6 h postinfection, cells were fixed with 3.5% neutral buffered formaldehyde (30 min) and permeabilized with 0.5% Igepal CA 630 (Sigma). They were blocked with 5% horse serum in phosphate-buffered saline for 1 h at room temperature, incubated for 2 to 3 h at room temperature with anti-gE-specific rabbit antibody, washed, and stained with carboxyanine dye (Cy2)-conjugated donkey anti-rabbit immunoglobulin G (Jackson Immune Research, Inc.). Slides were coverslipped with Gel/mount (Biomedex Corp., Foster City, Calif.). Optical images were collected under a 100 $\times$ objective with a Bio-Rad MRC1024ES confocal laser scanning microscope with the 488-nm excitation and 522-nm emission module.

Animal experiments. Four-week-old New Zealand White rabbits weighing 500 to 600 g (Myrtles Rabbitry, Thomson Station, Tenn.) were used. Rabbits were maintained in laboratory isolation cages in our vivarium throughout the experiments. Food and water were freely available. All procedures were approved by the Kansas State University Animal Care and Use Committee.

A rabbit seizure model described previously (10, 29) was used to compare the neuropathogenic properties of gE epitope-deleted BHV-5, gE-deleted BHV-5, wild type, and gE epitope-reverted BHV-5 recombinant viruses. Unless otherwise mentioned, 2×10^7 PFU of virus were inoculated per nostril. Following infection, the rabbits were observed four times per day for the appearance of neurological symptoms. Nasal swab samples were obtained on day 3 postinfection for virus isolation. For the survival study, rabbits were euthanized at 14 days postinfection or when they developed severe neurological signs. For the virus isolation study, rabbits were euthanized at 10 days postinfection or when they showed severe neurological signs. Brains were sectioned and processed as described earlier (10).

To compare the neural spread of different viruses, rabbits were inoculated with each virus as described above. Rabbits were euthanized and perfused transcardially at 6, 8, 10, and 12 days postinfection for gE epitope-deleted viruses or at

10 and 12 days postinfection for the gE-deleted virus. Rabbits infected with the gE epitope-reverted or wild-type BHV-5 were euthanized at 10 days postinfection or when the animals showed severe neurological signs. The brain was processed for immunohistochemistry as described earlier (29).

To determine virus replication in the olfactory neuroepithelium, rabbits were inoculated with each virus as described above. Rabbits were euthanized on day 5 postinfection. Following euthanasia, the head was removed and the section of the skull containing the midmaxillary region to the middle of the orbit (including the bulb) was dissected and fixed en bloc in 10% buffered neutral formalin (2 to 3 days). Then whole-mount immunofluorescence-confocal microscopy with monospecific bovine anti-BHV-5 serum and neurofilament-specific antibody was performed as described earlier (12).

RESULTS

Construction and analysis of gE epitope-deleted and gE epitope-reverted BHV-5 viruses. A schematic showing the location of the glycine-rich gE epitope and the strategy used for the deletion of the epitope is shown in Fig. 1. Briefly, sequences flanking the epitope coding regions were generated by long PCR as described in Materials and Methods, cleaved with appropriate enzymes, and cloned. In the resulting plasmid, the epitope coding region was deleted in-frame without altering the rest of the gE coding region. Several recombinant BHV-5 viruses with their gE epitope coding region deleted were isolated by rescuing a gE-deleted virus constructed earlier (11) with a plasmid DNA containing the epitope-deleted gE version (Fig. 1). A gE epitope-deleted BHV-5 was analyzed by sequencing the entire gE open reading frame coding region and used for subsequent studies. The BHV-5 gE epitope revertant virus was generated by rescuing the gE epitope-deleted virus with plasmid DNA containing the entire gE gene and its flanking sequences.

Purified virion lysates from BHV-5 gE epitope-deleted and BHV-5 gE epitope reverted (wild-type) viruses were analyzed by immunoblotting with gE5 epitope-specific and rabbit polyclonal antibodies specific for the carboxy-terminal half of gE (11, 41). The results showed that when immunoblotted with the anti-gE5 epitope-specific antibody, the wild-type gE (94-kDa) band was detected in the infected-cell lysates (Fig. 2A), while no gE-specific band was recognized in the gE epitope-deleted virus-infected cell lysates (Fig. 2A). However, an identical blot immunoblotted with antibody generated against the carboxy-terminal 210 amino acids of the gE coding sequence (41) recognized a slightly smaller, 93-kDa, gE-specific band in the lane containing gE epitope-deleted virus-infected cell lysate (Fig. 2B). Sequencing results (data not shown) confirmed that gE5 epitope-deleted BHV-5 contained the intended in-frame deletion of the epitope coding region but encodes the rest of gE.

Synthesis of mutant gE and mutant gE/gI interaction in epitope-deleted BHV-5-infected cells. Together, the gE/gI homologues in alphaherpesviruses, including BHV-5, form a non-covalently linked hetero-oligomer complex, which is required for gE and gI maturation and function. Immunoprecipitation analysis and pulse-chase analysis of wild-type BHV-5- and BHV-5gE epitope-deleted virus-infected cell proteins with gE-specific antibody showed that in both cases gE/gI complex formation occurred during the 30-min pulse (Fig. 3). The dominant gE bands observed immediately after the pulse (0-min sample) were an 86-kDa (endoplasmic reticulum-processed wild-type gE precursor, see below) and an 85-kDa (endoplas-

FIG. 2. Immunoblotting analysis of BHV-5 epitope-deleted (BHV-5 gE epitopeΔ) and BHV-5 epitope-reverted (BHV-5 gE epitopeR) viruses. (A) Identification of gE protein in wild-type BHV-5, BHV-5gE epitopeΔ, and BHV-5gE epitopeR viruses by immunoblotting with BHV-5 gE epitope-specific (residues 204 to 218) rabbit polyclonal antibody. (B) Immunoblotting of an identical blot with a rabbit polyclonal antibody specific for the carboxy-terminal 210 amino acids of BHV-1 gE that cross-react with BHV-5 gE (11, 41).

mic reticulum-processed epitope-deleted gE precursor, see below). The dominant gI bands observed immediately after pulse were 46-kDa (endoplasmic reticulum processed gI precursor) for both BHV-5 wild-type and the gE epitope-deleted viruses (Fig. 3). In the case of wild-type BHV-5, a 62-kDa band (Golgi-processed mature gI, see below) was also dominant immediately after the pulse (0 min). By 30 min of chase, the 94-kDa (Golgi-processed wild-type gE, see below) and 93-kDa (Golgi-processed, epitope-deleted gE, see below) forms were dominant, and by 240 min of chase the 86-kDa (wild-type) and 85-kDa (gE epitope-deleted) gE precursors had diminished significantly (Fig. 3). The dominant gI band after 30 to 60 min through 240 min of chase was the 62-kDa mature Golgi-processed band (see below). The 46-kDa (endoplasmic reticulum-processed gI) diminished significantly after 30 to 60 min of chase, while a 45-kDa band (gI^C) representing a proteolytically cleaved band of the mature 62-kDa gI became apparent (Fig. 3).

Analysis of gE/gI processing in wild-type and gE epitope-deleted BHV-5-infected cell lysates by glycosidase F and EndoH treatment. The gE and gI precursors are predicted to acquire EndoH-sensitive, N-linked glycosylation in the endoplasmic reticulum, while the mature form of gE and gI (Golgi modified) are predicted to contain EndoH-resistant, N-linked oligosaccharides. As a control, both the endoplasmic reticulum- and the Golgi apparatus-modified N-linked oligosaccharides can be removed by glycosidase F.

To determine the EndoH resistance of the gE expressed by gE epitope-deleted BHV-5, steady-state (10 h) labeled and wild-type BHV-5 and BHV-5gE epitope-deleted virus-infected cell extracts pulsed and chased for 0, 15, and 30 min were immunoprecipitated with gE-specific antibody and treated with EndoH as described in Materials and Methods. For a

FIG. 3. Autoradiograph showing pulse-chase analysis of gE in BHV-5 and BHV-5 gE epitopeΔ virus-infected cell lysates. Infected MDBK cells were pulse-labeled for 30 min in 100 μ Ci of [³⁵S]cysteine-methionine per ml beginning at 6 h postinfection. Cell monolayers were then washed twice with serum-free DMEM and incubated in complete growth medium without labeled cysteine and methionine. Detergent extracts were prepared from identical samples harvested at times ranging from 0 to 240 min following the labeling period. Extracts were then subjected to immunoprecipitation with gE-specific rabbit antiserum (11, 41), and the precipitated proteins were separated by SDS-PAGE (10% gel). Mock, BHV-5, and BHV-5gE epitopeΔ (left-most three lanes) represent cell lysates after steady-state labeling for 10 h beginning at 6 h postinfection. Note that in the case of gE epitope-deleted BHV-5, both the mature (93 kDa) and precursor forms (85 kDa) of gE are slightly smaller (approximately 1 kDa), which corresponds to the predicted molecular sizes of the deleted epitope coding region. The 62-, 46-, and 45-kDa proteins representing mature gI, gI precursor (pgI), and a proteolytic cleavage fragment of mature gI (gI^C), respectively, are indicated.

control, 3 μ g of RNase B (New England Biolab), a high-mannose glycoprotein was treated with EndoH. The results of the EndoH experiments showed that the 94- and 93-kDa mature gE bands for wild-type and gE epitope-deleted viruses, respectively, are resistant to EndoH digestion (Fig. 4) while RNase B (data not shown) and the endoplasmic reticulum-processed 86-kDa and 85-kDa gE bands in the 0-, 15-, and 30-min pulse-chase samples were sensitive (cleaved by EndoH to slightly faster migrating bands 0.5 to 1 kDa smaller in mass) (Fig. 4). The results of these experiments also showed that the 62-kDa (Golgi-processed mature gI) and 45-kDa gI^C (resulting from proteolytic cleavage of the mature 62-kDa gI) bands coprecipitated with gE from steady-state samples were resistant to EndoH, but the 46-kDa gI precursor coprecipitated in 0-, 15-, and 30-min pulse-chase samples, presumably processed in the endoplasmic reticulum, is EndoH sensitive, as evidenced by its conversion to a faster-migrating 44-kDa band (Fig. 4).

The results of glycosidase F digestion (data not shown) revealed that the mature gE bands of wild-type and gE epitope-deleted viruses were equally sensitive to the enzyme. Taken together, these results indicate that the mutant gE synthesized by the gE epitope-deleted BHV-5 was complexed with the gI in the endoplasmic reticulum, transported to Golgi, and processed to its mature form within the Golgi apparatus. There were no discernible differences in glycosylation and processing between the wild-type and epitope-deleted gE or between the wild-type and epitope-deleted gI.

FIG. 4. EndoH digestion of gE and gI in BHV-5gE epitope Δ and wild-type BHV-5. Infected MDBK cells were steady-state labeled or pulse-labeled for 30 min and chased for 0, 15, or 30 min as in Fig. 3. Lysates of labeled cells were immunoprecipitated with gE-specific polyclonal rabbit serum and adjusted to 0.5% SDS. Samples were boiled for 10 min and digested with EndoH (+) as described in Materials and Methods. SDS-PAGE of the samples was performed as described for Fig. 3. For a control, untreated samples (–) are shown. Note that the 46-kDa precursor gI was sensitive to EndoH after 0, 15, and 30 min of chase (yielding a faster-migrating 44.5-kDa band), while the 45-kDa gI^C is EndoH resistant in steady-state samples.

Plaque size of BHV-5 gE epitope-deleted virus in MDBK cells. The plaque sizes of gE epitope-deleted BHV-5, gE-deleted BHV-5 (11), gE epitope-reverted BHV-5, and wild-type BHV-5 viruses on MDBK cells were compared at 48 h postinfection as described earlier (11). The recombinant gE epitope-deleted BHV-5 produced wild-type-sized plaques, while gE-deleted BHV-5 produced much smaller plaques (data not shown). These results indicated that deletion of the epitope

coding region had no apparent effect on cell-to-cell spread in MDBK cells.

Surface expression of mutant gE relative to wild-type gE. To validate the results of the pulse-chase and EndoH experiments, we determined the surface expression of epitope-deleted gE. MDBK cells infected with wild-type and gE epitope-deleted BHV-5 were stained with gE-specific antibody at 6 h postinfection, and gE-specific staining was present on both the wild-type and gE-epitope-deleted virus-infected cell surfaces (Fig. 5). Therefore, deletion of the epitope coding sequences did not have a discernible effect on the surface expression of gE.

Pathogenicity of gE5 epitope-deleted virus in rabbits. In the initial survival experiment, 8 of 10 rabbits infected with BHV-5 gE epitope-deleted virus (2×10^7 PFU/nostril) showed no detectable neurological signs through 10 days postinfection. Two rabbits showed mild neurological signs, characterized by head twitching and hyperexcitation, which did not progress to seizures. The rabbits were euthanized at 14 days postinfection, and their brains were not processed further (Table 1). This experiment was repeated in an additional 10 rabbits, with 5×10^7 PFU of BHV-5gE epitope-deleted virus/nostril (second experiment). Once again, mild neurological signs were observed in 3 of 10 rabbits. These rabbits were euthanized at 14 days postinfection, and their brains were not processed further (Table 1). In a third experiment, six rabbits were inoculated with BHV-5gE epitope-deleted virus and four rabbits were inoculated with gE epitope-reverted BHV-5 (2×10^7 PFU). They were euthanized on day 10 postinfection or when they showed severe neurological sign (for the revertant), and their brains were processed for virus isolation. Virus was isolated from the olfactory bulb (10-fold less than the revertant), anterior cortex (100-fold less than the revertant), and posterior cortex (10-fold less than the revertant), which were considerably lower than the amounts isolated from rabbits infected with

FIG. 5. Surface expression of gE in cells infected with wild-type and BHV-5 gE epitope Δ viruses. MDBK cells were infected with wild-type and BHV-5 gE epitope Δ at 5 PFU per cell. At 8 h postinfection, the cells were fixed, permeabilized, reacted with anti-gE polyclonal antibody, and stained with Cy2-conjugated donkey anti-rabbit immunoglobulin G. In the case of the Cy2 control (data not shown), the slides were incubated with PBS prior to staining with Cy2. The anti-gE antibody used for surface labeling is specific for the carboxy-terminal 210 amino acids of BHV-1 gE (41). This antibody is not suitable for surface labeling without permeabilization. Samples were examined with a Bio-Rad MRC1024Es confocal laser scanning microscope. Confocal images were collected with a 100 $\times$ objective with a 488-nm laser line for excitation and 522-nm emission filter, with the phase contrast mode of the Bio-Rad LaserSharp imaging program. Arrows point to surface labeling. Bar, 20 μ m.

TABLE 1. Summary of clinical signs and virus isolation

BHV-5	No. of animals	Neurological signs ^a	Avg PFU/nose at 3 dpi	Virus isolation ^b		
				Olfactory bulb	Anterior cortex	Posterior cortex
gE epitopeΔ (expt 1)	10	Mild (2)	ND	ND	ND	ND
gE epitopeΔ (expt 2)	10	Mild (3)	3.8×10^3	ND	ND	ND
gE epitopeΔ (expt 3) ^f	6	Mild (1)	0.8×10^3	+ ^d (3)	+ ^c (5)	+ ^e (5)
gE epitopeR (expt 3)	4	Seizure (3)	1.2×10^3	++ ^d (2)	+++ (4)	+++ (4)

^a Numbers in parentheses indicate the number of animals showing clinical signs or of brain segments positive for virus isolation.

^b +, 1 to 190 PFU/g. ++, 200 to 1990 PFU/g. +++, >2,000 PFU/g. ND, not determined.

^c Lower end of the range.

^d Mid range.

^e Upper end of the range.

^f Rabbits in expt. 3 were euthanized at 10 dpi or when they showed severe neurological signs.

gE epitope-reverted BHV-5 (Table 1). gE epitope-deleted BHV-5 grew efficiently in the naso-olfactory mucosa, and the amount of virus shed was comparable to that shed by rabbits infected with gE epitope-reverted BHV-5 (Table 1).

To determine the spread of BHV-5 gE epitope-deleted BHV-5 in the central nervous system following intranasal infection, 12 rabbits were infected as above, and three rabbits each were euthanized on days 6, 8, 10, and 12 postinfection, and perfused transcardially, and the brain was processed for immunohistochemistry as described earlier (29). The experiment was repeated once with eight rabbits, and two rabbits each were euthanized on days 6, 8, 10, and 12 days postinfection. To compare the spread of gE epitope-reverted, wild-type, and gE-deleted BHV-5 in the central nervous system, six rabbits were similarly infected with the viruses. Rabbits infected with gE epitope-reverted or wild-type BHV-5 were euthanized at 10 days postinfection or when they showed severe neurological signs. For gE-deleted BHV-5, three rabbits each were euthanized at 10 and 12 days postinfection. The results are shown in Fig. 6 and 7 and summarized in Table 2.

In the rabbits infected with gE epitope-deleted BHV-5, virus-specific antigen was first detected in the olfactory bulb at 8 days postinfection (data not shown). At 10 and 12 days postinfection, a small number of neurons in the anterior olfactory nucleus (100 to 200/field at a magnification of 5×), piriform cortex (100 to 200/field at 5×), and amygdala (25 to 50/field) were also stained for viral antigen (Fig. 6). The number of neurons in rabbits infected with the gE epitope-deleted BHV-5 was significantly lower (at least 10- to 20-fold) in most of these areas (Fig. 6) than the number in rabbits infected with the gE epitope-reverted BHV-5 (more than 2,000 to 3,000 labeled neurons in the anterior olfactory nucleus, piriform cortex, and amygdala). In rabbits infected with the gE epitope-deleted BHV-5, we never observed infected neurons in the hippocampus, dentate gyrus, cingulate cortex, dorsal raphe, locus coeruleus, and lateral dorsal tegmentum (Fig. 7 and Table 2). In contrast, rabbits infected with gE5 epitope-reverted BHV-5 contained immunostained neurons in the olfactory bulb at 4 to 6 days postinfection (data not shown), and a large number of immunostained neurons were found in the anterior olfactory nucleus, piriform cortex, amygdala, and cingulate cortex at 8 to 10 days postinfection (Fig. 6 and 7 and Table 2). Additionally, the locus coeruleus, lateral dorsal tegmentum, and dorsal ra-

phe of rabbits infected with gE 5 epitope-reverted and wild-type BHV-5 had infected neurons (Table 2).

BHV-5 gE epitope-deleted virus infects the olfactory receptor neurons and is transported to the bulb but has significantly reduced neural spread and neurovirulence. To determine whether the deletion of the epitope affects the ability of the virus to infect olfactory receptor neurons and/or to be transported from the olfactory receptor neurons to the olfactory bulb, four rabbits were infected with gE epitope-deleted or wild-type BHV-5 and two rabbits were infected with gE-deleted BHV-5 (11). Immunofluorescence-confocal microscopy of the olfactory epithelium was performed at 5 days postinfection with anti-BHV-5 and anti-neurofilament-specific (neurofilament 200- and 160-kDa proteins) antibodies (12). As shown in Fig. 8, the olfactory receptor cells (bipolar neurons) within the olfactory epithelium were infected by all three virus types. Optical images of connective tissues underneath the respiratory layer revealed viral proteins in the olfactory nerve fibers of rabbits infected with wild-type BHV-5, gE-deleted BHV-5, and gE epitope-deleted BHV-5. Taken together, the results suggest that gE epitope-deleted BHV-5 can enter the brain through the olfactory pathway; it then replicates and spreads relatively inefficiently compared to wild-type BHV-5.

DISCUSSION

Previously we reported that the ectodomain of BHV-5gE contains a glycine-rich epitope coding region showing divergence from the corresponding gE region of BHV-1 (11). The current study was conducted to determine the role of this BHV-5 gE-specific epitope in BHV-5 neuroinvasion and neurovirulence. Our results show that deletion of the epitope did not affect the plaque size of the virus on MDBK cells and gE maturation or its interaction with gI, but it attenuated BHV-5 neurovirulence in rabbits.

The pulse-chase analysis and EndoH digestion results show that the epitope-deleted gE was processed in the Golgi like wild-type gE, and the mutant gE also reached the cell surface. Immunoblotting analysis of purified virions demonstrated that the epitope-deleted gE was incorporated into the virus envelope. Collectively, these results demonstrated that epitope-deleted gE was not retained in the endoplasmic reticulum or

FIG. 6. Localization of viral antigen in brain sections. Animals were inoculated intranasally with BHV-5gE epitope Δ , BHV-5 gE-deleted (gE Δ), BHV-5 gE epitope R, or BHV-5 wild-type (WT) viruses as described in Materials and Methods. The animals were euthanized on days 6, 8, 10, and 12 postinfection for BHV-5 gE epitope Δ , 10 and 12 days postinfection for BHV-5gE Δ , or when they showed neurological signs for BHV-5 gE epitopeR and wild-type BHV-5 (8 to 10 days postinfection). Their brains were processed for immunohistochemical analysis as described in Materials and Methods. Sections for BHV-5 gE epitope Δ and BHV-5gE Δ are from day 12 postinfection. BHV-5gE epitopeR and wild-type virus-infected rabbit brain sections are from day 10 postinfection. Representative sections of the anterior olfactory nucleus (AON), piriform cortex (PIR), and amygdala (AMYG) are shown. Bar in anterior olfactory nucleus and piriform cortex, 1,000 μ m; bar in amygdala, 500 μ m.

Golgi complex in cells infected with the gE epitope-deleted virus.

In PRV and herpes simplex virus, the extracellular domain of gE is important for cell-to-cell spread *in vitro*. However, the cytoplasmic domains of gE and gI are also essential for this process (13, 37, 38). In MDBK cells, the gE epitope-deleted virus produced wild-type-size plaques and did not have any discernible cell-to-cell spread defect. Brains from rabbits in-

fectected with gE epitope-deleted BHV-5 contained 10- to 20-fold fewer infected neurons and yielded 10- to 100-fold less virus compared to rabbits infected with gE epitope-reverted BHV-5. For BHV-5, gE is important for the spread of virus from a presynaptic to a postsynaptic neuron and for neurovirulence *in vivo* (11). Even though the gE epitope-deleted virus is slightly more virulent than the gE-deleted virus, its ability to infect and replicate within the second and third-order olfactory tract neu-

FIG. 7. Localization of viral antigen in representative sections showing the hippocampus (HIPPO), lateral dorsal tegmentum (LDT), and cingulate cortex (CG). In this assay, wild-type and gE BHV-5 epitopeR spread to the hippocampus, lateral dorsal tegmentum, and cingulate cortex. However, no labeling was found in these areas for gE epitopeΔ and gEΔ BHV-5. Arrows point to infected neurons. Bar in hippocampus, 1,000 μ m; bar in lateral dorsal tegmentum and cingulate cortex, 500 μ m.

rons was significantly reduced. In herpes simplex virus and varicella-zoster virus, the ectodomains of gE and gI are important for F_c binding, which is a virulence determinant because it helps the virus evade the immune system (4, 5, 7, 20, 21, 23, 25, 30). BHV-1 and BHV-5 gE do not have F_c binding activity when tested against bovine and rabbit sera (41; S. I. Chowdhury, unpublished data). Therefore, the loss of virulence is not associated with F_c binding activity.

In alphaherpesviruses, all known gE endocytosis motifs are located within the cytoplasmic tail (34, 36–38). In PRV and vari-

cella-zoster virus, YXXL motifs are important for gE endocytosis and trans-Golgi localization (34, 38). The BHV-5 gE cytoplasmic tail also contains two YXXL motifs (11); their role in endocytosis, recycling, trans-Golgi localization of BHV-5 gE, and gE-mediated neurovirulence is currently being tested. Since we did not manipulate the cytoplasmic tail of the epitope-deleted gE and the sequencing did not show any alteration in the cytoplasmic tail sequence, it is unlikely that the deletion of the epitope coding sequences within the gE ectodomain affected gE endocytosis or other functions mediated by the gE cytoplasmic tail.

TABLE 2. Summary of viral spread in the brain after intranasal inoculation

Virus	Presence and location of viral antigen ^a								
	OB	AON	PIR	HIPPO	AMYG	CG	LC	DR	LDT
BHV-5	++	++++ ^b	++++ ^b	+++	++++ ^b	+++	+	++	+
BHV-5gEepiR	++	++++ ^b	++++ ^b	+++	++++ ^b	+++	+	++	+
BHV-5gEepiΔ	+	++ ^b	++ ^b	—	++ ^c	—	—	—	—
BHV-5gEΔ	+ ^c	+ ^b	+ ^c	—	—	—	—	—	—

^a Indicated as no labeling (—) or 1 to 25 (+), 30 to 150 (++), 160 to 500 (+++), or 550 to 3,000 (++++ labeled neurons per field at a magnification of ×5. OB, olfactory bulb; AON, anterior olfactory nucleus; PIR, piriform cortex; HIPPO, hippocampus; AMYG, amygdala; CG, cingulate cortex; LC, locus coeruleus; DR, dorsal raphe; LDT, lateral dorsal tegmentum. Scores for the BHV-5 gE epitopeR, wild-type, and BHV-5 gEΔ are averages for six rabbits, and for each segment, five sections were counted (30 slides for each section). Scores for BHV-5 gE epitopeΔ are averages for 10 rabbits, and for each brain segment, five sections (total, 50 sections) were analyzed.

^b Higher end of the range.

^c Lower end of the range.

In alphaherpesviruses, gE and gI hetero-oligomers function to increase the efficiency of cell-to-cell transmission between fibroblasts, epithelial cells, or neurons and neurovirulence (15–17, 40). In a pulse-chase assay, we noticed that the Golgi-processed gI of the wild-type virus was dominant over the

Golgi-processed gI of the gE epitope-deleted virus during 30 min of pulse-labeling, but this effect was not discernible after various times of chase and in steady-state-labeled samples. It is possible that initially, there is a slight delay in gE-gI interactions which might have resulted in slower maturation of gI at

FIG. 8. Localization of virus-specific antigens by immunofluorescence-confocal microscopy in the processes of olfactory receptor neurons of rabbits infected with BHV-5 gE epitopeR, BHV-5 gE epitopeΔ, and BHV-5 gEΔ viruses. Viral antigen (red fluorescence) as detected by BHV-5-specific bovine polyclonal antibody is seen in the processes of olfactory receptor neuronal cells (marked by arrows). The processes are labeled with neurofilament-specific antibodies (green fluorescence). Merged images show colocalization of viral and neurofilament antigen. Bar, 30 μm.

an early phase. This could also potentially affect the neurovirulence of the mutant virus. However, in BHV-5, gE alone is sufficient to promote neuron-to-neuron spread and neurovirulence, because a gI-deleted BHV-5 has wild-type neural spread and retains significant neurovirulence in rabbits (1a). Therefore, it is unlikely that a slight delay in the gI maturation at the early phase of the gE-gI interaction is responsible for the considerable reduction in the neural spread and neurovirulence of the gE epitope-deleted BHV-5.

In the BHV-5 gE ectodomain, there are 10 cysteine residues which are also conserved in the BHV-1 gE sequence (11). These cysteine residues are clustered in two areas called the C1 (76 to 100) and C2 (270 to 326) regions. The C1 but not the C2 region is required for the gE-gI interaction in BHV-1 (39). The glycine-rich gE epitope is located 103 amino acids downstream and 52 amino acids upstream of the C1 and C2 regions, respectively. Since deletion of the epitope coding region had no apparent effect on gE-gI interaction other than causing a slight delay in the early phase of interaction, as noted above, the conformation of the epitope-deleted gE in the C1 region is largely unaffected. The disulfide-bonded structure of alphaherpesvirus gE is not known. Therefore, we cannot exclude the possibility that the deletion affected gE conformation downstream of the C1 region.

PRV mutants expressing only the gE ectodomain retain the ability to spread anterogradely in a rat eye model (37, 38), which indicates that the gE ectodomain is important in trans-synaptic spread of the virus between neurons. In herpes simplex virus type 1, cell lines expressing the gE-gI ectodomain without the cytoplasmic tail interfered with cell-to-cell spread of the virus (13), which reinforces the idea that the gE ectodomain binds a receptor present in the cell junction (15). Another mechanism by which gE could affect neuron-to-neuron spread of herpesviruses involves preferential sorting of viral glycoproteins or vesicles containing viral glycoproteins to the axon terminal (19). Cytoplasmic tail of the gE sequences in alphaherpesviruses contain potential sorting motifs (1). In polarized epithelial cells, the sorting of nascent virions to lateral surfaces of polarized epithelial cells is mediated by the gE cytoplasmic tail sequences (26). In PRV, the cytoplasmic tail domain is also responsible for gE-mediated neurovirulence (37, 38). We did not manipulate the cytoplasmic tail, and there was no alteration in the cytoplasmic tail sequence of the mutant virus. Therefore, the spread defect and reduced neurovirulence of the gE epitope-deleted virus are due to the effect of the deletion within the ectodomain.

According to Dingwell et al. (13, 17), gE promotes neuron-to-neuron spread of herpes simplex virus type 1 by binding to a neuronal protein concentrated at neuronal synapses. It is possible that the glycine-rich sequence of BHV-5 gE ectodomain binds to a neuronal protein which acts as a viral receptor and which are concentrated at neuronal synapses. Thus, by binding to such a protein, BHV-5 gE might promote neuron-to-neuron transmission and neurovirulence. Alternatively, as discussed earlier, the deletion of the glycine-rich region could also have an effect on gE conformation in and around the glycine-rich region. In either case, binding to such a neuronal protein would be affected resulting in defective neuronal spread and reduced neurovirulence.

Analysis of the gE sequences of equine herpesvirus type 1,

herpes simplex virus type 1, PRV, and varicella-zoster virus (accession numbers NC_001491, NC_001806, AY368490, and NC_001348, respectively) did not reveal a conserved glycine-rich motif in the corresponding gE regions. Therefore, it might be the gE region rather than the glycine-rich gE sequence is important for the binding with a putative neuronal factor. We suggest that the BHV-5 glycine-rich gE epitope (gE5 epitope) coding region promotes anterograde neuronal transport from the first-order neurons to the second-order neurons in the bulb and neurovirulence of BHV-5 within the central nervous system of rabbits.

We reported earlier that BHV-5 gE alone did not transfer the BHV-5 neurovirulence property to BHV-1 (11). In that construct, the BHV-1 Us9 gene was inadvertently deleted. Recently, we determined that Us9 is essential for BHV-5 anterograde transport from the first-order olfactory receptor neuron to the bulb and that the Us9-deleted BHV-5 cannot invade the central nervous system (12). Future experiments in our laboratory will test whether insertion of the gE5 epitope alone into the BHV-1 gE protein will transfer the neurovirulence property to BHV-1 containing its own or a BHV-5 Us9.

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