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Synthesis, Processing, and Oligomerization of Bovine Herpesvirus 1 gE and gI Membrane Proteins

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This study reports the identification and initial characterization of the precursors, modified forms, and oligomers of bovine herpesvirus 1 (BHV-1) gI and gE proteins with polyvalent rabbit serum specific for gI or gE. Our experiments used the Colorado strain of BHV-1 and mutant viruses with insertions of the *Escherichia coli lacZ* gene into the predicted gE and gI reading frames. We also translated the gE and gI open reading frames in vitro and expressed them in uninfected cells using eukaryotic expression vectors. Precursor-product relationships were established by pulse-chase analysis and endoglycosidase H and glycopeptidase F digestions. Like the homologous glycoproteins of herpes simplex virus type 1, pseudorabies virus, and varicella-zoster virus, BHV-1 gI and gE are modified by N-linked glycosylation and associate with each other soon after synthesis, forming a noncovalent complex in infected and transfected cells. An analysis of mutant and wild-type-virus-infected cells and transfected COS cells expressing gE or gI alone suggested that gE-gI complex formation is necessary for efficient processing of the gE precursor to its mature form. One new finding was that unlike the other alphaherpesvirus gI homologs, a fraction of pulse-labeled gI synthesized in BHV-1-infected cells apparently is cleaved into two relatively stable fragments 2 to 4 h after the pulse. Finally, we incubated BHV-1-infected cell extracts with nonimmune mouse, rabbit, horse, pig, and calf sera and found no evidence that gE or gI functioned as Fc receptors as reported for the herpes simplex virus type 1 and varicella-zoster virus homologs.

The genes encoding membrane proteins gE and gI are conserved among the alphaherpesviruses that have been sequenced. In all instances studied to date, expression of gE and gI is required for full pathogenic potential in animals but is not required for growth in tissue culture (11). In infected cells, these proteins are localized to the plasma membrane, the virion envelope, and all internal membranes (except for mitochondria). The conservation of gE and gI genes among diverse alphaherpesviruses as well as their maintenance in physically separated populations of wild-type viruses is given as evidence of their importance in virus survival. Nevertheless, we are only beginning to understand the biology and functions of gE and gI.

Biological functions ascribed to gE and gI include cell-cell spread, binding of antibody immunoglobulin G (IgG) (Fc receptor), and virulence. Previous work indicated that gE and gI functioned in cell-cell spread of herpes simplex virus type 1 (HSV-1) and pseudorabies virus (PRV) in tissue culture and in animal infections (11). In 1992, Zsak et al. (36) noted that PRV mutants lacking the gE homolog produced small plaques relative to wild-type virus under conditions in which extracellular progeny virus was neutralized. Jacobs et al. (14) found that a PRV mutant lacking codons 125 and 126 of the PRV gE gene formed small plaques on some but not all cell types. HSV-1 mutants that do not express gI or gE also produced small plaques in the presence of neutralizing antibodies (6). HSV and varicella-zoster virus (VZV) gE and gI form a noncovalent complex in infected cells that binds the Fc region of IgG (15, 16, 24). Complex formation, but not IgG binding, has been observed for the gI and gE homologs of PRV (32, 36). Finally, gE and gI mutants of HSV-1 and PRV exhibited significantly reduced virulence and a diminished or altered ability to spread to and within some, but not all, aspects of the central nervous system in animals (4, 11–14, 26).

The gE homologs of several alphaherpesviruses (HSV-1, VZV, EHV-1, and PRV) are posttranslationally modified by N- and O-linked glycosylation, phosphorylation, and sulfation (7, 11, 24, 33, 35). The gI homologs that have been analyzed are also glycosylated and phosphorylated. The functions of none of these modifications are known. Grose and colleagues (24, 33, 35) presented evidence that cellular casein kinases I and II phosphorylate the cytoplasmic tail of the VZV gE homolog. Similarly, the gE homologs may be sulfated on carbohydrate or tyrosine residues in the extracellular domain. Edson (7) has demonstrated that the gE homolog of VZV is sulfated on both carbohydrate and tyrosine. He identified a potential consensus site of tyrosine sulfation in the gE homolog of VZV as well as in the gE homologs of HSV-1, HSV-2, and PRV. This suggests that tyrosine sulfation may be a general posttranslational modification of the gE homologs of herpesviruses that establish latent infections in sensory or autonomic ganglia.

The open reading frames (ORF) coding for gI and gE are located adjacent to one another in the short unique region of the bovine herpesvirus 1 (BHV-1) genome and have been designated U_S ORF-6 and U_S ORF-7, respectively (19). The predicted gI primary translation product of 380 amino acids (19) contains one potential N-linked oligosaccharide attachment site. The predicted gE primary translation product of 575 amino acids (19, 28) contains two potential N-linked oligosaccharide attachment sites and two possible O-linked oligosaccharide attachment sites (28). Both predicted gE and gI amino acid sequences contain stretches of hydrophobic amino acids at the N terminus (putative signal sequence) and near the C

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terminus (transmembrane sequence), which is typical of class I integral membrane proteins. All alphaherpesviruses encode gE and gI homologs and, despite the disparate genomes and pathogenic niches of these viruses, the homologs appear to have common sequence motifs and biological functions. Thus, comparative analysis of homologs is likely to provide valuable insight into structure and function. Accordingly, in this report, we examine the synthesis, processing, and oligomerization of the gE and gI proteins of BHV-1.

MATERIALS AND METHODS

Virus and cell culture. The Colorado-1 strain of BHV-1 was obtained from the American Type Culture Collection. Virus was propagated in Madin-Darby bovine kidney (MDBK) cells as previously described (18).

Construction of BHV-1 mutants K25 and K36. Details of the construction of the K25 and K36 BHV-1 mutants will be published later. A brief description follows. A cloned copy of the 8.4-kb *Hin*dIII-K fragment of BHV-1, which lies entirely within the short unique region of the genome, was subjected to partial digestion at random sites with *Hae*III. Full-length linear molecules were isolated and ligated to a β-galactosidase expression unit. Recircularized plasmid molecules were transformed into *Escherichia coli*, and the positions of inserts within the K-fragment of individual clones were determined by restriction enzyme analysis. Clones containing inserts in the gI coding region (designated K36) and the gE coding region (designated K25) were cotransfected along with intact BHV-1 DNA into bovine cells, and viable recombinant viruses containing the inserts were recognized by their ability to form blue plaques.

Production of gE- and gI-specific rabbit polyclonal antisera. gE and gI proteins were produced in *E. coli* as follows. DNA fragments from pBH144 (encoding the complete ORF of BHV-1 gI) and from pBH145B (encoding the complete ORF of BHV-1 gE) were subcloned into the appropriate pEx31 expression vectors (30). This vector system uses the bacteriophage lambda P_L promoter regulated by the temperature-sensitive *c*I857 repressor. BHV-1 gI amino acid residues 86 to 380 and BHV-1 gE amino acid residues 366 to 575 were expressed and purified essentially as described by Strebel et al. (30). Briefly, the cells were disrupted with lysozyme and then sonicated and sequentially extracted with increasing concentrations of urea. After dialysis in phosphate-buffered saline (PBS), the soluble fraction was used for injection into rabbits. The rabbits (New Zealand White) were primed with 250 μg of antigen in complete Freund's adjuvant and were given booster injections at intervals of 3 to 4 weeks with the same amount of protein in incomplete Freund's adjuvant. Sera were titrated by Western blotting (immunoblotting) and immunoprecipitation.

Construction of pBH144 and pBH145B. To construct pBH144, the 1.55-kb *Sma*I-*Nsp*V fragment containing the entire 1.14-kb gI ORF was isolated from the *Hin*dIII-K (18) fragment of BHV-1 (Colorado-1 strain). The *Nsp*V end was blunted by filling (Klenow fragment), and the fragment was cloned into the *Sma*I site of pUC18 with the 5′ end of gI near the *Eco*RI site and the 3′ end near the *Hin*dIII site of pUC18. The *Sma*I site was regenerated in the process. To construct pBH145B, we took advantage of the fact that *Nsp*V-cut fragments and *Acc*I-cut pUC18 yield similar cohesive 2-base overhangs that can be ligated together. The 1.13-kb *Nsp*V-*Hin*dIII fragment containing the 5′ end of gE was isolated from the *Hin*dIII-K fragment of BHV-1, and the 0.72-kb *Hin*dIII-*Nsp*V fragment containing the 3′ end of gE was isolated from the *Hin*dIII-F (18) fragment of BHV-1. Both fragments were combined with *Acc*I-cut pUC18, and the entire mixture was treated with T4 ligase. Plasmid clones containing the 1.13-kb *Nsp*V-*Hin*dIII and 0.72-kb *Hin*dIII-*Nsp*V fragments ligated together at their *Hin*dIII ends and inserted into the *Acc*I site of pUC18 were isolated and characterized by restriction enzyme analysis. Clone 145B contains the 1.73-kb gE ORF oriented with the 5′ end near the *Hin*dIII site of pUC18 and the 3′ end near the *Eco*RI site of pUC18.

In vitro transcription-translation. Cell-free translation was done with a Promega transcription-translation coupled reticulocyte lysate. A typical reaction mixture contained 0.5 μg of template DNA, encoding the complete ORF of BHV-1 gI and BHV-1 gE, fused to the T7 promoter in pGEM-11Z (Promega) and 20 μCi of [^{35}S]methionine (1,000 Ci/mmol; New England Nuclear Corp.).

Preparation of steady-state, [^{35}S]cysteine-labeled extracts. Confluent MDBK cell monolayers in 60- or 100-mm tissue culture dishes were infected with wild-type BHV-1 at a multiplicity of infection of 5 PFU per cell or with mutant viruses K25 and K36 at a multiplicity of infection of 2 PFU per cell. Mock infections with virus-free media were always included in the analysis. At 4 h postinfection, complete growth medium (Dulbecco modified Eagle medium [DMEM], 5% fetal bovine serum) was replaced with serum-free, cysteine-free medium (Selectamine kit; GIBCO), and infected cells were incubated for an additional 2 h at 37°C. At 6 h postinfection, the serum-free, cysteine-free medium was replaced with medium containing 1/20 the usual concentration of cysteine, 0.5% serum, and 50 μCi of [^{35}S]cysteine (Amersham) per ml. Infected cells were then incubated at 37°C for an additional 10 h (until 16 h postinfection). At the end of the labeling period, the cells were washed once in ice-cold, serum-free medium and twice in ice-cold Tris-buffered saline (0.15 M NaCl, 0.01 M Tris [pH 7.4]). Washed cell monolayers were then incubated in ice-cold extraction buffer (0.15 M NaCl, 0.01 M Tris [pH 8.0], 0.01 M EDTA, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 1.0 mM phenylmethylsulfonyl fluoride, 0.1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone [TLCK], 0.1 mM L-1-tosylamide-2-phenylethyl-chloromethyl ketone [TPCK], and 2 μg of aprotinin per ml) at approximately 10^7 cells per ml for 1 h at 4°C. The cells were then scraped into the extraction buffer, and the suspension was centrifuged at 13,000 × *g* for 15 min at 4°C. The resulting supernatant (cell extract) was recovered and stored at −70°C.

Pulse-chase analysis. Confluent MDBK cell monolayers in 60-mm tissue culture dishes were infected with wild-type BHV-1 at a multiplicity of infection of 5 PFU per cell. At 4 h postinfection, complete growth medium (DMEM, 5% fetal bovine serum) was replaced with serum-free, cysteine-free medium (Selectamine kit; GIBCO), and infected cells were incubated for an additional 2 h at 37°C. At 6 h postinfection, the serum-free, cysteine-free medium was replaced with medium containing 1/20 the usual concentration of cysteine, 0.5% serum and 100 μCi of [^{35}S]cysteine (Amersham) per ml, and the cells were incubated at 37°C for 30 min. At the end of the labeling period, the cells were washed twice in prewarmed (37°C) serum-free medium and were 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 of the chase periods, individual samples were harvested by being washed twice in ice-cold Tris-buffered saline (0.15 M NaCl, 0.01 M Tris [pH 7.4]) and by being incubated in 0.5 ml of ice-cold extraction buffer (0.15 M NaCl, 0.01 M Tris [pH 8.0], 0.01 M EDTA, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 1.0 mM phenylmethylsulfonyl fluoride, 0.1 mM TLCK, 0.1 mM TPCK, and 2 μg of aprotinin per ml) for 1 h at 4°C. The cells were then scraped into the extraction buffer, and the suspension was centrifuged at 13,000 × *g* for 15 min at 4°C. The resulting supernatant (cell extract) was recovered and stored at −70°C.

Transient transfection assays. COS cells were transiently transfected with expression plasmids encoding the complete ORF of BHV-1 gI or gE. We used the DEAE-dextran precipitation method with slight modifications (29). Briefly, COS cells at 50% confluence in 6-cm dishes were transfected in modified Eagle medium–*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid containing 1 mg of DEAE-dextran per ml and 10 μg of purified DNA per ml. After 4 h at 37°C, the transfection mixture was removed and the cells were treated with 15% glycerol in DMEM for 2 min. The cells were then returned to DMEM–10% calf serum and 100 μM chloroquine diphosphate for 60 h to allow protein expression. At 60 h posttransfection, the cells were labeled for 4.5 h with [^{35}S]cysteine, and the native extract was immunoprecipitated as described elsewhere (32).

Immunoprecipitations. Labeled cell extracts (50 to 100 μl) were mixed with 4 μl of gE- or gI-specific polyclonal rabbit antiserum and 40 μl of a 50% (vol/vol) suspension of protein A-Sepharose (Pharmacia) in extraction buffer, and the mixture was incubated for 90 min at 4°C with frequent mixing. Immune complexes were collected by centrifugation and were washed twice in extraction buffer, once in extraction buffer–0.1% sodium dodecyl sulfate (SDS) and once in 50 mM Tris (pH 8)–0.1% Nonidet P-40. The washed complexes were then resuspended in 20 μl of 2× sample buffer, heated to 100°C, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Labeled proteins were visualized via autoradiography.

Polyacrylamide gel analysis of labeled proteins. SDS-PAGE analysis of immunoprecipitated proteins was performed essentially as described previously (18).

Glycopeptidase F and endoglycosidase H digestions. A 200-μl volume of [^{35}S]cysteine-labeled BHV-1-infected cell extract was adjusted to 0.4% SDS and boiled for 10 min. The resulting extract was preabsorbed with 50 μl of protein A-Sepharose beads which had been preincubated with 10 μl of normal rabbit serum and again with 50 μl of protein A-Sepharose beads without bound antibody. This preabsorbed extract was then subjected to two sequential immunoprecipitations. First, the extract was incubated for 90 min at 4°C with 15 μl of washed protein A-Sepharose beads which had been preincubated with 10 μl of gI-specific antiserum. The protein A-Sepharose beads were then pelleted, and the extract was transferred to a separate tube containing 15 μl of washed protein A-Sepharose beads preincubated with 10 μl of gE-specific antiserum and incubated for an additional 90 min at 4°C. The two resulting protein A-Sepharose-antibody-antigen complexes were then washed twice in extraction buffer and once in extraction buffer containing 0.1% SDS and were heated at 100°C for 5 min in 25 μl of 0.2% SDS–0.1 M 2-mercaptoethanol. After pelleting of the protein A-Sepharose beads, the supernatants (containing the immunoprecipitated gI and gE) were recovered and transferred to new tubes. For glycopeptidase F digestions, 5 μl of the immunoprecipitated material was incubated in 40 mM sodium phosphate (pH 7)–2% Nonidet P-40 with 1 U of glycopeptidase F in a total volume of 50 μl for 16 h at 30°C. For endoglycosidase H digestions, 5 μl of the immunoprecipitated material was incubated in 50 mM sodium citrate (pH 5.5) with 0.005 U of endoglycosidase H in a total volume of 50 μl for 16 h at 30°C. The digested samples were subjected to SDS-PAGE, and labeled proteins were visualized via autoradiography.

RESULTS

Identification of the gE and gI polypeptides synthesized in BHV-1-infected cells. The antisera raised against gE and gI fusion proteins specifically immunoprecipitated proteins pro-

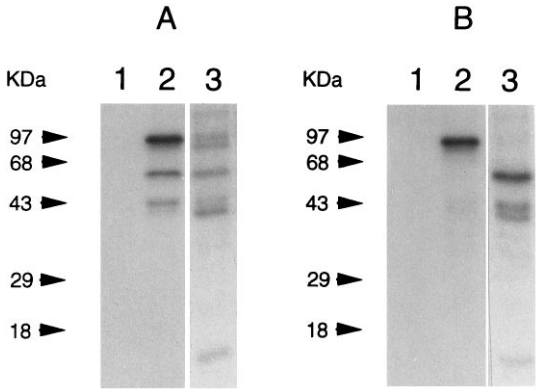

FIG. 1. Immunoprecipitation and SDS-PAGE analysis of gE and gI polypeptides from BHV-1-infected cells. Mock-infected and virus-infected MDBK cells were labeled with 50 μCi of [^{35}S]cysteine for 10 h beginning at 6 h postinfection. Detergent extracts of infected cells were prepared and analyzed by immunoprecipitation with gE- or gI-specific antiserum and then by SDS-PAGE and autoradiography of the dried gels. (A) Immunoprecipitations performed on untreated extracts. (B) prior to immunoprecipitation, extracts were adjusted to 0.4% SDS and heated for 10 min at 100°C. Lanes: 1, mock-infected cell extract immunoprecipitated with gE-specific antiserum; 2, BHV-1-infected cell extract immunoprecipitated with gE-specific antiserum; 3, BHV-1-infected cell extract immunoprecipitated with gI-specific antiserum. The positions of molecular size markers are indicated to the left of each panel.

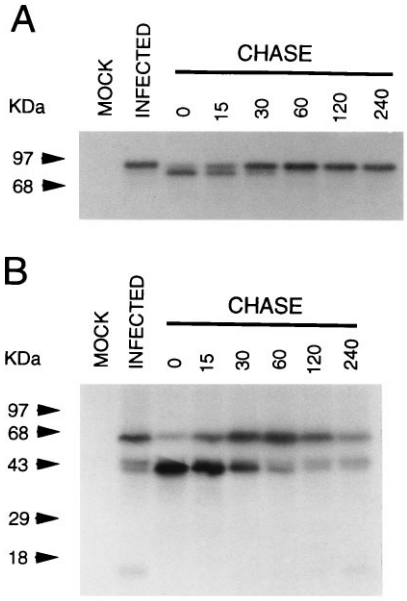

FIG. 2. Pulse-chase analysis of BHV-1 gE and gI polypeptides. BHV-1-infected MDBK cells were pulse-labeled for 30 min in 100 μCi of [^{35}S]cysteine per ml beginning at 6 h postinfection. Cell monolayers were then washed twice with serum-free DMEM and were incubated in complete growth medium without labeled cysteine. Detergent extracts were prepared from identical samples harvested at times ranging from 0 to 240 min after the labeling period, adjusted to 0.4% SDS, heated, and analyzed by immunoprecipitation with gE- or gI-specific antiserum and then by SDS-PAGE and autoradiography of the dried gels. (A) Samples immunoprecipitated with gE-specific antiserum. (B) Samples immunoprecipitated with gI-specific antiserum. MOCK, mock-infected cells labeled continuously for 10 h with [^{35}S]cysteine; INFECTED, BHV-1-infected cells labeled continuously for 10 h with [^{35}S]cysteine; CHASE, pulse-labeled, virus-infected cells harvested at the indicated times (in minutes) following the labeling period. The positions of molecular size markers are indicated to the left of each panel.

duced in BHV-1-infected cells (Fig. 1). Under nondenaturing conditions, the gE-specific antiserum precipitated five labeled polypeptides with molecular masses of 92, 84, 61.5, 45, and 39.5 kDa (Fig. 1A, lane 2), while the gI-specific antiserum precipitated six polypeptides (Fig. 1A, lane 3), five of which comigrated in SDS-PAGE with those precipitated by the gE-specific antiserum. The additional gI-specific polypeptide had an apparent molecular mass of 16 kDa. No detectable polypeptides were precipitated from identically labeled, mock-infected cell extracts (Fig. 1A, lane 1). Verification that the polypeptides were indeed encoded by the gE or gI gene came from analysis of BHV-1 gE and gI mutants (see below).

Previous work has shown that gE homologs coimmunoprecipitate with the respective gI homologs in HSV-1 (15)-, PRV (32, 37)-, and VZV (25, 34)-infected cells. Thus, it seemed likely that the comigrating proteins immunoprecipitated by both BHV-1-monospecific antisera reflected the same phenomenon. To examine this, we immunoprecipitated infected-cell extracts after denaturation with 0.4% SDS and boiling for 10 min. As shown in Fig. 1B, each antiserum reacted with a subset of the same five or six polypeptides seen prior to treating the extracts with SDS and heat. The gE-specific antiserum precipitated polypeptides of 92 and 84 kDa (Fig. 1B, lane 2) while the gI-specific antiserum precipitated polypeptides of 61.5 kDa, 45 kDa, 39.5 kDa and 16 kDa (Fig. 1B, lane 3). We conclude that, like the other reported gE-gI homologs, BHV-1 gE and gI form a noncovalent oligomer in infected cells.

Kinetics of BHV-1 gE and gI synthesis and processing in infected cells. We determined the precursor-product relationships between the multiple polypeptide species detected with each of our monospecific antisera by pulse-chase labeling experiments. BHV-1-infected cells were exposed to 100 μCi of [^{35}S]cysteine per ml for a period of 30 min (pulse) beginning at 6 h postinfection as described in Materials and Methods. Following the pulse label, excess unlabeled cysteine was added and samples were taken at 0, 15, and 30 min and at 1, 2, and 4 h. gE- and gI-specific polypeptides were immunoprecipitated under denaturing conditions and were analyzed on SDS-PAGE gels. Figure 2A shows the result obtained with the gE-specific antiserum. The dominant gE band observed immediately following the pulse (0-min sample) was the 84-kDa species. The 92-kDa species appeared soon after, and by 15 min of chase, the 84- and 92-kDa species were nearly equal. By 2 h of chase, the 84-kDa band was no longer detectable. These results indicate that the 84-kDa species is the precursor synthesized in the endoplasmic reticulum (ER) and the 92-kDa species results from posttranslational modification of the 84-kDa species as it moves through the ER and Golgi cisternae.

An identical experiment with the gI-specific antiserum is shown in Fig. 2B. The dominant gI band immediately following the pulse was the 39.5-kDa species. The 61.5-kDa species detected at zero time increased in intensity by 60 min of chase as the 39.5-kDa form diminished. We conclude that the 39.5-kDa form is the ER precursor which is converted into the 61.5-kDa species during export. Unlike the pattern observed for the 92-kDa protein of gE, the modified gI 61.5-kDa species diminished in intensity between 1 and 4 h of chase. During this same time period, two new bands of 45- and 16-kDa were detected and increased slightly in intensity. This may indicate that the 61.5-kDa form of gI is processed into two smaller fragments.

Kinetics of gE-gI oligomer formation. As shown above, steady-state labeled, infected-cell extracts contained complexes of gE and gI that can be coimmunoprecipitated with either gE- or gI-specific antiserum. The following experiments were done to determine when oligomerization occurred after synthesis. We used the pulse-chase format and made extracts under nondenaturing conditions prior to immunoprecipitation. As shown in Fig. 3, complexes are detected at the zero time

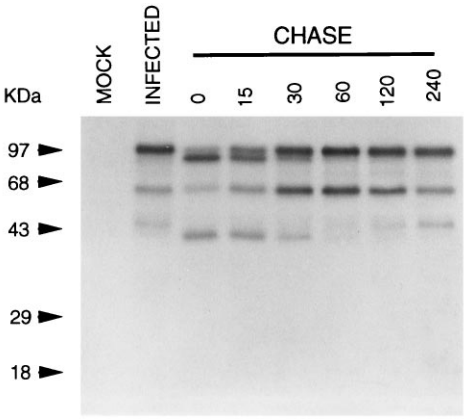

FIG. 3. Pulse-chase analysis of the gE-gI complex in BHV-1-infected cells. BHV-1-infected cells were pulse-labeled for 30 min in 100 μCi of [^{35}S]cysteine per ml beginning at 6 h postinfection. Cell monolayers were then washed twice with serum-free dMEM and were incubated in complete growth medium without labeled cysteine. Detergent extracts were prepared from identical samples harvested at times ranging from 0 to 240 min following the labeling period. Extracts were then analyzed by immunoprecipitation with gE-specific antiserum and then by SDS-PAGE and autoradiography of the dried gels. MOCK, mock-infected cells labeled continuously for 10 h with [^{35}S]cysteine; INFECTED, BHV-1-infected cells labeled continuously for 10 h with [^{35}S]cysteine; CHASE, pulse-labeled, virus-infected cells harvested at the indicated times (in minutes) following the labeling period. The positions of molecular size markers are indicated to the left of each panel.

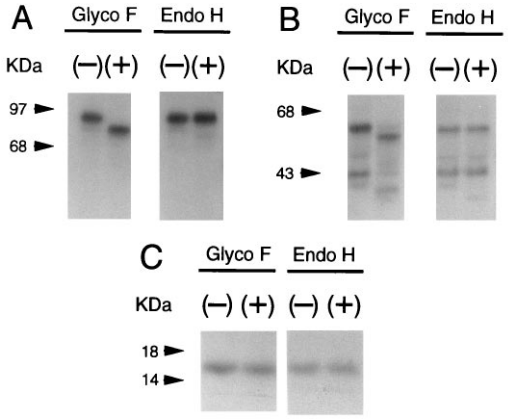

FIG. 4. Glycopeptidase (Glyco) F and endoglycosidase (Endo) H digestion of BHV-1 gE and gI. An aliquot of [^{35}S]cysteine-labeled, BHV-1-infected cell extract was adjusted to 0.4% SDS and was heated to dissociate gE-gI complexes. gE and gI were then sequentially immunoprecipitated and digested with glycopeptidase F or endoglycosidase H. Control and enzyme-treated samples were analyzed by SDS-PAGE and autoradiography of the dried gels. (A) Identical samples of immunoprecipitated gE incubated without (−) or with (+) the indicated enzymes. (B) Identical samples of immunoprecipitated gI incubated without (−) or with (+) the indicated enzymes. (C) A longer autoradiographic exposure of the SDS-PAGE gel shown in panel B showing only the 16-kDa gI polypeptide. The positions of molecular size markers are indicated to the left of each panel.

point, indicating that complex formation occurred during the 30-min pulse. The coimmunoprecipitate from the 30-min pulse-labeling was enriched for the precursor forms of gE and gI (84- and 39.5-kDa forms), suggesting that complex formation occurred in the ER.

We noted that the complex precipitated with the gE antiserum contained no detectable gI precursor (39.5 kDa) after a 2-h chase (Fig. 3). The pulse-labeled gI precursor is certainly present at this time point and is stable even after 4 h of chase (Fig. 2B). Thus, it is likely that the gI precursor matures to the 61.5-kDa mature form as part of the gE-gI complex. The 39.5-kDa gI precursor population present at 2 and 4 h after the 30-min pulse must have failed to complex with gE. We also found that the 45-kDa but not the 16-kDa proteolytic fragment of gI was precipitated with anti-gE serum. This finding suggests that the association of gI with gE involves a region(s) contained within the 45-kDa fragment but not the 16-kDa fragment.

Glycopeptidase F and endoglycosidase H treatment of BHV-1 gE and gI. The kinetic analysis described above provided both temporal and molecular mass indications of precursor-product relationships. Further verification of these assignments was obtained by endoglycosidase digestion experiments that measure which species of gE and gI contain N-linked glycosylation modifications predicted from DNA sequence analysis (19, 28). The gE and gI precursors are predicted to acquire endoglycosidase H-sensitive, N-linked glycosylation in the ER, while the mature forms of gE and gI are predicted to contain endoglycosidase H-resistant, N-linked oligosaccharides. As a control, both the ER and Golgi apparatus-modified N-linked oligosaccharides can be removed by glycopeptidase F. Sensitivity is detected by a reduction in apparent molecular mass after SDS-PAGE. As described in Materials and Methods, gI and gE were sequentially immunoprecipitated from an aliquot of steady-state (10 h), [^{35}S]cysteine-labeled, BHV-1-infected cell extract which had been heated in the presence of SDS to disrupt gE-gI oligomers (Fig. 4). Under these labeling conditions, the mature species of both gE and gI contain more label than do the precursors.

The first finding from this experiment is that the previously identified forms of gE (84 and 92 kDa; Fig. 4A) as well as three of the four previously identified forms of gI (39.5, 45, and 61.5 kDa; Fig. 4B) contained N-linked oligosaccharides, as evidenced by their conversion to faster-migrating forms following glycopeptidase F treatment (Fig. 4A and B). However, the 16-kDa form of gI was resistant to glycopeptidase F, indicating that it contained no N-linked oligosaccharides (Fig. 4C).

Identical samples of immunoprecipitated gE and gI were also treated with endoglycosidase H (Fig. 4). The predicted precursor forms of both gE and gI (84 and 39.5 kDa, respectively) were sensitive to endoglycosidase H and were converted to faster-migrating forms. The predicted mature species of gE (92 kDa) and gI (61.5 kDa) were resistant to endoglycosidase H, indicating that all of their N-linked oligosaccharides had been processed to complex-type structures. Similarly, the 45-kDa species of gI is also resistant. Taken together, the kinetic analysis and the endoglycopeptidase data confirm the precursor-product relationship of the intracellular gE and gI protein species established by pulse-chase experiments.

Characterization of BHV-1 mutants with *lacZ* insertions in the gE and gI coding sequences. Insertion of a β-galactosidase expression cassette into the gE and gI coding sequences was accomplished as described in Materials and Methods. Two viable viruses, designated K25 (insertion in gE) and K36 (insertion in gI), were isolated and characterized. Both mutants form small plaques on MDBK cell monolayers (data not shown). These mutants were used to verify the specificity of our gE and gI antiserum and to gain insight into the synthesis and processing of gE and gI in infected cells. Steady-state, [^{35}S]cysteine-labeled extracts were prepared after infection with wild-type BHV-1, K25, and K36. The extracts were denatured to disrupt gE-gI oligomers, and labeled proteins were immunoprecipitated with gE- and gI-specific antisera.

The results obtained with mutant K25 are shown in Fig. 5A. No gE-specific protein was found after immunoprecipitation

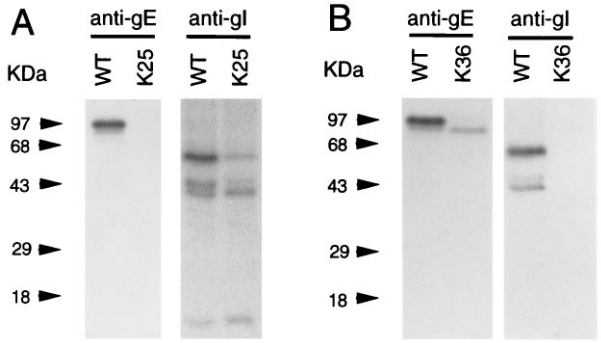

FIG. 5. Immunoprecipitation and SDS-PAGE analysis of gE and gI polypeptides from mutant virus-infected cells. Cells infected with wild-type (WT) virus and with BHV-1 mutants K25 (gE negative) and K36 (gI negative) were labeled with 50 μCi of [^{35}S]cysteine per ml for 10 h beginning at 6 h postinfection. Detergent extracts of infected cells were then adjusted to 0.4% SDS and were heated for 10 min at 100°C prior to immunoprecipitation. Immunoprecipitated polypeptides were separated via SDS-PAGE and were visualized by autoradiography of the dried gels. (A) Wild-type and K25-infected cell extracts immunoprecipitated with the indicated antisera. (B) wild-type (WT) and K36-infected cell extracts immunoprecipitated with the indicated antisera. The positions of molecular size markers are indicated to the left of each panel.

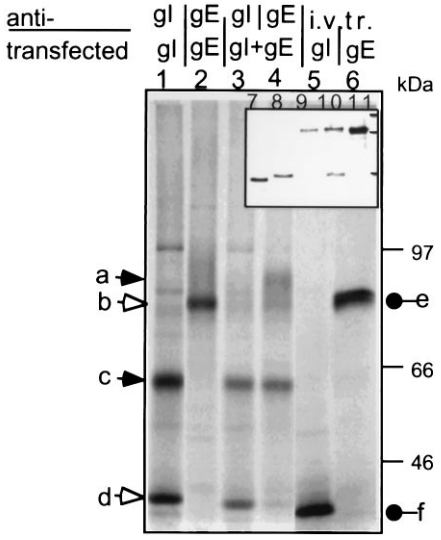

FIG. 6. Immunoprecipitation of gI and gE from transfected and infected cells with antipeptide antisera. (Large gel) COS cells were transiently transfected either separately or in a cotransfection with the expression plasmids encoding gE or gI. At 48 h posttransfection, the cells were labeled with [^{35}S]cysteine for 4.5 h and were immunoprecipitated under nondenaturing conditions with the antisera indicated at the top of the figure. Lanes: 1, gI; 2, gE; 3 and 4, cotransfections of both gE and gI plasmids; 5 and 6, labeled proteins produced after in vitro translation (i.v. tr.) of RNA from a gI expression plasmid and a gE expression plasmid, respectively. On the left, black arrows point to the mature forms of gE (a) and gI (c), while white arrows point to precursor forms of gE (b) and gI (d). The in vitro-translated primary protein products of gE (e) and gI (f) are noted on the right. (Small inset) The in vitro translation products of gI (lane 7) and gE (lane 11) compared with immunoprecipitated gI (lanes 8 and 10) and gE (lanes 9 and 10) from BHV-1-infected cells after 40 min of labeling with [^{35}S]cysteine after the prior addition of detergents (lanes 8 and 9) or without detergents (lane 10). Marker protein migration is indicated by dashes on the right side of the inset: first marker, 117 kDa; second marker, 82 kDa; third marker, 47 kDa.

with the gE-specific antiserum, while the gI-specific antiserum brought down all four forms of gI observed in wild-type infected cells (16, 39.5, 45, and 61.5 kDa). However, in contrast to wild-type virus infections in which the 61.5-kDa mature form was predominant, K25-infected cells contained significantly more 39.5-kDa gI precursor. In addition, in K25-infected cells, the 16-kDa form of gI was more abundant than the 45-kDa form, in contrast to the results from cells infected with wild-type virus.

The results obtained with mutant K36 are shown in Fig. 5B. No gI-specific polypeptides reacted with the gI-specific antiserum, while the gE-specific antiserum precipitated an appreciable amount of the 84-kDa gE precursor. However, in contrast to an infection with wild-type virus, the 92-kDa mature form of gE was barely detectable after infection by K36.

In vitro transcription-translation and transient expression of the BHV-1 gE and gI genes. We examined the synthesis and modification of gE and gI using in vitro translation and transient transfection as described in Materials and Methods. This analysis enabled us to corroborate further the precursor-product relationship of the various intracellular forms of gE and gI. The complete ORF of BHV-1 gI and gE was subcloned into the pcDNAI/Amp eukaryotic expression vector (Invitrogen). These plasmids were used in a coupled, in vitro transcription-translation reaction to produce the primary translation products of gE and gI, which were compared with immunoprecipitated gE and gI from infected cell extracts (see the small inset in Fig. 6). The predicted primary gI in vitro translation product is 39.9 kDa, and the observed protein migrated at approximately 38 kDa (Fig. 6, inset, lane 7). The gI precursor detected after 40 min of labeling of infected cells migrated slightly more slowly than the in vitro-translated species, most likely reflecting addition of a single high-mannose, N-linked glycosylation modification (Fig. 6, inset, lane 8). The primary gE in vitro translation product migrated at 84 kDa, not at 61 kDa, as predicted from the amino acid sequence (Fig. 6, inset, lane 11). The gE precursor detected after 40 min of labeling migrated similarly at 84 kDa (Fig. 6, inset, lane 9), despite the putative addition of two N-linked glycosylation modifications. This anomalous migration was also seen when gE and gI oligomers were studied (Fig. 6, inset, lane 10). The 84-kDa gE and the 39.5-kDa gI precursors and no other species are precipitated by gE antiserum.

In the transfection experiments, plasmids encoding gI and gE were added to cells either individually or together, and after 60 h, the cells were labeled for 4.5 h with [^{35}S]cysteine. The labeled cells were harvested, and extracts were prepared under nondenaturing conditions to observe oligomer formation after immunoprecipitation with either gE or gI antiserum (the first four lanes of Fig. 6). The first lane shows the results after transfection of the gI-expressing plasmid. We observed two primary gI-specific species: the 39.5-kDa precursor (Fig. 6, d) migrating slightly more slowly than the in vitro-translated gI protein (f), and the 61.5-kDa species (c) corresponding to the Golgi apparatus-modified, gI mature form. The second lane shows the results after transfection of the gE-expressing plasmid. We observed a strong band at 84 kDa (b) corresponding to the predicted gE ER precursor (and comigrating with the in vitro-translated species [e]) and a faint, diffuse signal corresponding to the Golgi apparatus-modified, mature gE species (a). The following two lanes of Fig. 6 show the results of cotransfection of the gE and gI plasmids and immunoprecipitation with anti-gI serum (lane 3) and anti-gE serum (lane 4). Although no clear coimmunoprecipitation of any gE species can be seen in lane 3 with the anti-gI serum, the gE band was clearly visible upon longer exposure. In contrast, the mature forms of gI and gE were both readily immunoprecipitated with anti-gE serum. It is noteworthy that the amount of gE precursor is less, and more mature gE was made during the cotransfection with the gI expression plasmid than when the gE plas-

mid was transfected alone (Fig. 6, lane 2). An obvious band corresponding to the 45-kDa gI species seen in infected cells (Fig. 1, lane 2) was not observed in lane 4 of Fig. 6. It is possible that this difference reflects an activity present in infected cells that is absent in the transient expression experiment.

DISCUSSION

The short unique region of the BHV-1 genome encodes four membrane glycoproteins homologous to HSV-1 gG, gD, gI, and gE (19). Until our study, only the gD polypeptide of this group had been unambiguously identified in BHV-1-infected cells. In the present report, we identified and provided an initial characterization of the precursors, modified forms, and oligomers of BHV-1 gI and gE proteins using polyvalent rabbit serum specific for gE or gI.

BHV-1 encodes at least nine membrane glycoproteins (see reference 20 for references) that share many properties with the homologous glycoproteins of other alphaherpesviruses. Specifically, (i) BHV-1 gB functions in viral attachment binding first to cell surface heparan sulfate and then with much higher affinity to an unknown, non-heparan sulfate cellular receptor (20, 21). gB also functions in penetration of the host cell membrane (22). (ii) gC is dispensable for growth in tissue culture (22, 23), is involved in attachment of virions to host cells via binding to a cellular heparan sulfate (21, 27), and binds bovine but not porcine or human complement factor C3b (10). (iii) gD is essential for virion infectivity (8), interferes with BHV-1 infection when expressed in stably transformed bovine cells (5, 8, 31) or when cells are incubated in the presence of a soluble form of the glycoprotein (21), and is involved in viral attachment to the host cell (5, 8, 22). (iv) gG is dispensable for growth in tissue culture (our unpublished observation). (v) gH appears to be essential for virion infectivity (3). Finally, (vi) gE (17) and gI are dispensable for growth in tissue culture (this report). Viruses lacking either of these glycoproteins exhibit a small plaque phenotype (reference 11 and our unpublished observations), and a virus lacking gE exhibits reduced virulence (17).

In infected and transfected cells, the gI precursor is likely to be the 39.5-kDa species which is most likely modified by a single endoglycosidase H-sensitive, N-linked, high-mannose oligosaccharide. After passage through the Golgi cisternae, further modifications are added that increase the apparent molecular mass to 61.5 kDa, which is characteristic of the mature form of gI. Unlike the other alphaherpesvirus gI homologs, a fraction of pulse-labeled gI synthesized in BHV-1-infected cells apparently is cleaved into two relatively stable fragments, 2 to 4 h after the pulse. The larger fragment, likely consisting of most of the ectodomain of gI, remains complexed with gE (see below), while the smaller fragment, perhaps consisting of the transmembrane and cytoplasmic domains, is not detectably associated with gE. We include protease inhibitors in the extract buffers so that proteolysis after the cells are lysed is unlikely. Further studies are required to determine if this processing occurs on the surface of infected cells or in lysosomes or if the cleaved gI ectodomain is found in the virion envelope.

The gE protein is first detected as an 84-kDa form that most likely contains two endoglycosidase H-sensitive, N-linked oligosaccharides. Modification by Golgi enzymes further increases the apparent molecular mass to approximately 92 kDa. The observed molecular mass of the gE precursor and the in vitro translation product is considerably larger than that predicted from the primary sequence. The gE protein sequence is highly acidic ($pK_a = 4.5$), which may contribute to its anomalous migration in SDS-PAGE; however, further work is necessary to understand this phenomenon.

As predicted from their deduced amino acid sequences, both BHV-1 glycoproteins contain N-linked oligosaccharides, as evidenced by their sensitivity to glycopeptidase F. However, both proteins contain additional modifications, perhaps O glycosylation, since glycopeptidase F treatment does not yield a single deglycosylated form of either gE or gI. Computer analysis with a program called NetOglyc (9) suggested that nine sites (serine or threonine residues) in BHV-1 gE and eight sites in BHV-1 gI were likely to be O glycosylated (sites in the putative signal sequence, transmembrane region, or cytoplasmic domain of both glycoproteins were omitted). It should be noted that two of the nine sites identified in the BHV-1 gE amino acid sequence closely matched the two putative O-glycosylation sites reported by Rebordosa et al. (28). In addition, VZV gE and gI have both been shown to contain O-linked as well as N-linked oligosaccharide moieties (25, 34).

Like the homologous glycoproteins of HSV-1 (15), PRV (32, 37), and VZV (25, 34), BHV-1 gI and gE associate with each other soon after synthesis, forming a noncovalent complex in infected and transfected cells. An analysis of mutant and wild-type-virus-infected cells and transfected COS cells expressing gE or gI alone or together suggested that gE-gI complex formation is necessary for efficient processing of the gE precursor to its mature form. The gE precursor accumulates after infection with K36 (defective for gI expression), and the gI precursor accumulates after infection with K25 (defective for gE expression). This observation is similar to previous observations with the PRV gE and gI homologs (32). Similar results were obtained in the transfection experiment with cells that are nonpermissive for natural BHV infection. Thus, we conclude that protein maturation of BHV-1 gE and gI is independent of cell type and virus infection. It must be stressed that the defect in export and modification of gE in the absence of gI is a kinetic defect and not absolute; indeed, gE can be detected easily on the surface of BHV-1 gI mutant-infected cells (data not shown).

Our observation that a fraction of the 61.5-kDa endoglycosidase H-resistant form of BHV-1 gI appears to be proteolytically processed, yielding 45- and 16-kDa forms, was unexpected, since it had not been reported for other gI homologs. Since the smaller 16-kDa form was resistant to glycopeptidase F treatment, while the 45-kDa form was sensitive, and the single predicted N-linked glycosylation site in the gI amino acid sequence is near the amino terminus of the predicted polypeptide, we believe that the smaller cleavage product represents the carboxyl-terminal portion of the glycoprotein. The apparent size of the smaller fragment (16 kDa), as determined by SDS-PAGE analysis, suggests that the cleavage of gI occurs in the ectodomain of the glycoprotein. The 16-kDa fragment would thus consist of a small portion of the ectodomain along with the entire cytoplasmic and transmembrane regions of gI. This conclusion is also supported by the mutant virus data, which showed that although cleavage of the 61.5-kDa form of gI had occurred in K25-infected cells, the 45-kDa fragment was underrepresented compared with cells infected with wild-type virus, in agreement with the conclusion that this fragment lacks a transmembrane anchor. Since the 45- but not the 16-kDa form of gI can be coprecipitated with gE, perhaps if any association between the gE and gI cytoplasmic or transmembrane regions occurs, it is either transient or unstable under the conditions used in this study (or both). In this regard, Basu et al. (1) showed that a 106-amino-acid region of the HSV-1 gE ectodomain (amino acids 183 to 288) was sufficient to mediate complex formation with gI (presumably through interaction

with a portion of the gI ectodomain). Finally, infection by BHV-1 K25 (gE minus) shows that in the absence of gE, gI is more readily cleaved into the 45- and 16-kDa forms (Fig. 5a). The significance of the cleavage of BHV-1 gI for its function or for that of the gE-gI complex remains to be investigated.

HSV-1 gE as well as the gE-gI complex has been shown to bind the Fc domain of IgG (2, 16). We attempted to detect similar IgG binding by BHV-1 gE or the gE-gI complex. To do so, we incubated BHV-1-infected cell extracts with nonimmune mouse, rabbit, horse, pig, and calf sera under conditions similar to those used by Johnson et al. (16) to demonstrate IgG binding by the HSV-1 gE-gI complex. We precipitated the resulting immune complexes with protein A-Sepharose. SDS-PAGE analysis of these immune precipitates revealed several labeled polypeptides; however, none of these comigrated with any known gE or gI polypeptides (data not shown). Thus, we have no evidence that the BHV-1 gE-gI complex is an Fc receptor. This result is consistent with and strengthens the currently held view that BHV-1-infected cells do not possess Fc receptor-like activity.

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