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Bovine Herpesvirus 1 UL49.5 Homolog Gene Encodes a Novel Viral Envelope Protein That Forms a Disulfide-Linked Complex with a Second Virion Structural Protein†

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We previously reported that the genome of bovine herpesvirus 1 (BHV-1) contains an open reading frame (ORF) homologous to the herpes simplex virus UL49.5 ORF, and as with the herpes simplex virus UL49.5 ORF, the deduced amino acid sequence of the BHV-1 UL49.5 homolog (UL49.5h) contains features characteristic of an integral membrane protein, implying that it may constitute a functional gene encoding a novel viral envelope protein. This communication reports on the identification of the BHV-1 UL49.5h gene product. By employing an antibody against a synthetic BHV-1 UL49.5h peptide and an UL49.5h gene deletion mutant, the primary product of BHV-1 UL49.5h gene was identified as a polypeptide with a size of approximately 9 kDa; in both infected cells and isolated virions, the UL49.5h products were found to exist in three forms: monomer, disulfide-linked homodimer, and disulfide-linked heterodimer containing a second viral protein with a size of about 39 kDa. *O*-Glycosidase digestion and [^{3}H]glucosamine labelling experiments showed that the UL49.5h protein is not glycosylated. Although the deduced amino acid sequence contains putative sites for myristylation and phosphorylation, we were unable to detect either modification. Surface labelling and trypsin digestion protection experiments showed that the BHV-1 UL49.5h protein was present on the surface of infected cells and on the surface of mature virions. Nonionic detergent partition of isolated virions revealed that the UL49.5h protein is more tightly associated with the virion tegument-nucleocapsid structure than envelope protein gD. The results from this study demonstrate that the BHV-1 UL49.5h gene encodes a nonglycosylated virion envelope protein which may associate with virion internal structures by forming a complex with the 39-kDa virion structural protein.

Bovine herpesvirus 1 (BHV-1) is a member of the *Alphaherpesvirinae* subfamily. It is an important cattle pathogen responsible for a wide variety of clinical diseases, including conjunctivitis, respiratory disease, reproductive tract lesions, abortion, and encephalitis as well as fatal systemic infection. In addition, BHV-1 has been recognized as the most important viral factor that predisposes cattle to succumb to respiratory opportunistic bacterial infection. Such a combined viral-bacterial infection constitutes the major etiology of shipping fever pneumonia, one of the most costly diseases in the cattle industry (10, 38).

A BHV-1 virion contains at least 33 structural proteins, among which 13 have been suggested to be associated with the virion envelope (5, 22). Three major BHV-1 envelope glycoproteins have now been identified and characterized. They are glycoproteins gB, gC, and gD (formery called gI, gIII, and gIV, respectively) (9, 23, 32). In addition, BHV-1 genes homologous to those for herpes simplex virus (HSV) gG, gI, gE, and gH have been mapped and sequenced (15, 21). For human HSV, the prototype virus of the *Alphaherpesvirinae* subfamily, 14 membrane proteins have been characterized so far, namely, gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, and the gene products of UL20, UL24, and UL34 (1, 2, 6, 11, 12, 19, 20, 24, 25, 29, 30). It is conceivable that BHV-1 may encode membrane proteins similar to those of HSV. The membrane proteins of alphaherpesviruses play important roles in virus replication, particularly in the virus entry process. For example, it is now known that envelope proteins gB, gC, and gD are involved in virus attachment, and gB, gD, gH, and gK are also involved in virus penetration (reference 12; see reference 31 for a review). Furthermore, for pseudorabies virus, another member of the alphaherpesviruses, the envelope proteins gI and gE constitute important determinants in neurotropism (7, 8, 14, 36).

More recently, it was recognized that the HSV genome contains an additional putative membrane protein gene, namely, UL49.5 (3, 28). Subsequently, the genes homologous to HSV UL49.5 were also identified in a number of other herpesviruses, including BHV-1 (18), equine herpesvirus (4, 27), varicella-zoster virus (4) and Marek's disease virus (37). Therefore, the UL49.5 homolog genes appear to be highly conserved among different herpesviruses. However, at present, the actual identity and function of the protein products coded for by these UL49.5 homolog genes are still unknown. The present study was designed to identify and characterize the BHV-1 UL49.5h gene product. By employing an antiserum against a synthetic peptide of the predicted UL49.5h gene product, we were able to demonstrate that the BHV-1 UL49.5h gene encodes a novel virion envelope protein.

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MATERIALS AND METHODS

Virus and cells. The Cooper strain of BHV-1 was obtained from the National Veterinary Services Laboratories, Ames, Iowa. The BHV-1 mutant vDUCZ, which contains a UL49.5h and dUTPase gene deletion, was produced in this laboratory (18). Viruses were propagated in Madin-Darby bovine kidney (MDBK) cells grown in minimum essential medium (GIBCO/BRL Laboratories,

Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS; GIBCO/BRL).

Production of UL49.5h antiserum. An oligopeptide corresponding to amino acid residues 21 to 41 of the predicted BHV-1 UL49.5h protein sequence was synthesized and conjugated to keyhole limpet hemocyanin with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. New Zealand White rabbits were immunized intramuscularly with 200 μg of the peptide-keyhole limpet hemocyanin conjugate in complete Freund's adjuvant. The rabbits were given boosters of 200 μg of the conjugate in incomplete Freund's adjuvant 4 weeks later, and this was repeated four times at 2-week intervals. One week after the final immunization, serum was collected and stored at −70°C until used.

Purification of virions. BHV-1 virions were purified from the supernatant of virus-infected MDBK cell culture by an Na-K tartrate gradient, as described before (16). Briefly, cell supernatant containing virus was clarified by low-speed centrifugation at 1,000 × *g*. Virus was harvested by pelleting through 30% sucrose in phosphate-buffered saline (PBS) at 100,000 × *g* for 60 min. The virus pellet was resuspended in 0.05 M Tris-HCl–0.15 M NaCl–10 mM EDTA, pH 8.0, applied to a 20 to 50% Na-K tartrate discontinuous gradient, and centrifuged at 100,000 × *g* for 90 min. After centrifugation, the virus band was collected and diluted in PBS, and this step was followed by an additional centrifugation at 100,000 × *g* for 60 min. The virus pellet was resuspended in PBS and stored at −70° until used.

Metabolic labelling and immunoprecipitation. Subconfluent MDBK cells grown in T-25 flasks were infected with virus at a multiplicity of infection of 5. Radiolabelling with [^{3}H]glucosamine (100 μCi/ml in glucose-deficient minimum essential medium supplemented with 10% dialyzed FBS; Amersham), [^{3}H]myristic acid (25 μCi/ml in minimum essential medium supplemented with 10% dialyzed FBS; Amersham), or Tran^{35}S-label (50 μCi/ml in a methionine-cysteine-deficient minimum essential medium supplemented with 10% FBS; ICN) was carried out for 16 h beginning 1 h postinfection; radiolabelling with [^{32}P]phosphoric acid (100 μCi/ml in a phosphate-deficient medium supplemented with 10% dialyzed FBS; ICN) was carried out at 16 h postinfection for 1 h. Immunoprecipitation, following labelling, was carried out as previously reported (16) with modifications. The radiolabelled cells from each T-25 flask were lysed in 300 μl of RIPA buffer composed of 0.15 M NaCl, 10 mM Tris-HCl, 1% deoxycholic acid, 1% Nonidet P-40 (NP-40), 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, and 0.5 mg of aprotinin per ml (pH 8.0) at 4°C for 30 min. The cell lysate was clarified by centrifugation, the supernatant was collected and incubated with antibodies at 4°C for 2 h, and then 150 μl of packed protein A-Sepharose beads which had been preabsorbed with a normal MDBK cell lysate was added. After an additional 2-h incubation at 4°C, the samples were washed three times with RIPA buffer and once with 10 mM Tris-HCl (pH 7.2). After the final wash, the beads were resuspended in 150 μl of 2× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer either with or without 2% 2-mercaptoethanol and boiled for 5 min. The samples were separated by SDS-PAGE. The rabbit anti-UL49h serum (17) and the rabbit anti-UL49.5h serum were used at a dilution of 1:100; a pool of gD monoclonal antibodies (MAbs) (34) was used at a dilution of 1:1,000.

Western blot analysis. Western blot (immunoblot) procedures were carried out as previously described (13). Cell lysate was prepared at 16 h postinfection by directly lysing infected cells in SDS-PAGE sample buffer.

***O*-Glycosidase treatment.** The proteins were labelled with [^{35}S]methionine and immunoprecipitated as described above. The Sepharose-protein A beads containing the precipitated proteins were reconstituted in 10 mM Tris-HCl containing 0.5% NP-40 and 0.5 M NaCl (pH 7.6) and incubated with 150 mU of neuraminidase (Boehringer Mannheim, Laval, Quebec, Canada) per ml at 37°C for 2 h. After incubation, the samples were further reconstituted in 50 μl of 10 mM Tris-maleate (pH 6.8) and incubated with 2 mU of *O*-glycosidase (Boehringer Mannheim) at 37°C for 16 h. Control samples without the enzymatic treatment were processed in parallel. Samples were separated by SDS-PAGE under reducing conditions.

Biotinylation of infected cells and virions. MDBK cells grown in a T-150 flask were infected with virus at a multiplicity of infection of approximately 5. At 16 h postinfection, the cells were collected by scraping and washed once with PBS containing 100 mM EDTA and once with normal PBS without EDTA. The cells were then resuspended in 600 μl of PBS. Sulfo-NHS-biotin (Pierce, Rockford, Ill.) was freshly prepared in dimethyl sulfoxide at a concentration of 50 mg/ml. For biotinylation, the cells were incubated with sulfo-NHS-biotin at a final concentration of 1 mg/ml either in the presence or absence of 1% NP-40 at 4°C for 60 min. After incubation, samples were quenched with 100 mM glycine for 10 min at 4°C. For purified virions, the biotinylation reaction was carried out with 100 μl of virions at a concentration of 1 mg/ml. After biotinylation, the samples were precipitated with antibodies by the same procedure as the immunoprecipitation, separated by SDS-PAGE, and blotted onto nitrocellulose paper. Specific protein bands were detected by reactions with avidin-conjugated horseradish peroxidase at a dilution of 1:3,000 (Bio-Rad).

Trypsin digestion of isolated virions. Purified virions at a concentration of 1 mg/ml were incubated with 0.01 mg of trypsin (*N*-tosyl phenylalanine chloromethyl ketone [TPCK]-treated trypsin; 10,500 U/mg; Sigma) per ml either in the presence or absence of 1% NP-40 at 37°C for 5 min. Immediately after the incubation, samples were supplemented with phenylmethylsulfonyl fluoride to a final concentration of 1 mM and an equal volume of 2× SDS-PAGE sample buffer and boiled for 5 min. Samples were separated by SDS-PAGE, and this step was followed by Western blotting with antibodies.

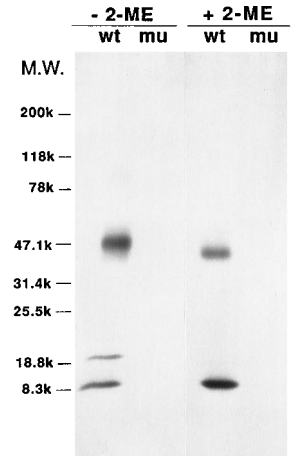

FIG. 1. Immunoprecipitation of the BHV-1 UL49h gene product from virus-infected cells. MDBK cells were infected with wild-type BHV-1 (wt) and a UL49.5h deletion mutant, vDUCZ (mu), and labelled with 50 μCi of Tran^{35}S-label per ml of medium for 16 h. After the labelling, the cells were lysed and precipitated with the anti-UL49.5h peptide antiserum. The precipitated samples were separated by SDS–6-to-20% gradient PAGE under nonreducing (− 2-ME) and reducing (+ 2-ME) conditions. M.W., molecular weight markers; 2-ME, 2-mercaptoethanol.

Nonionic detergent partition of isolated virions. Purified virions (100 μl) at a concentration of 1 mg/ml were incubated with either 1% NP-40 or 1% NP-40 plus 1 M NaCl in PBS at 4°C for 30 min. After incubation, the samples were layered on top of 50 μl of 30% sucrose in a 250-μl centrifuge tube and centrifuged at 178,000 × *g* for 30 min in an Airfuge (Beckman). After centrifugation, the supernatant and the pellet were collected separately. Samples were separated by SDS-PAGE under reducing conditions, and this step was followed by Western blotting with a mixture of BHV-1 gD-specific MAbs, UL49h antiserum (17), and UL49.5h antiserum. When immunoprecipitation was involved, ^{35}S-labelled virions purified from the supernatant of one T-150 flask of infected MDBK cells were divided into two equal portions. Two-thirds of the labelled virions were treated with NP-40 and then by centrifugation, as described above. The remaining one-third of the labelled virions were used as a control. The control sample, the supernatant, and the pellet of the NP-40-treated sample were precipitated separately with a mixture of BHV-1 gD-specific MAbs and UL49h- and UL49.5h-specific rabbit antisera. The resultant precipitated samples were separated by SDS-PAGE under reducing conditions.

RESULTS

Identification of UL49.5h polypeptide in virus-infected cells. As a first step to identify the BHV-1 UL49.5h gene products, we synthesized an oligopeptide corresponding to residues 21 to 41 of the deduced UL49.5h amino acid sequence (17). The peptide was conjugated to keyhole limpet hemocyanin and used to immunize rabbits. As a result, hyperimmune serum against the synthetic peptide was produced.

Immunoprecipitation with anti-peptide serum followed by SDS-PAGE analysis identified several distinct products from wild-type BHV-1-infected cells but not from UL49.5h gene deletion mutant-infected cells (Fig. 1). Under nonreducing conditions, the precipitated products were separated into three distinct bands with calculated relative molecular masses of about 47, 19, and 9 kDa, respectively; under reducing conditions, only two bands at sizes of approximately 39 and 9 kDa were present. In order to better assess the relationship among these different species, immunoprecipitated samples were subjected to different partial reducing conditions and then analyzed by SDS-PAGE. As shown in Fig. 2, under various partial reducing conditions, there was an inverse correlation between the density of the 47-kDa band and the density of the 39- and 9-kDa bands. It should be noted that the lane which contained

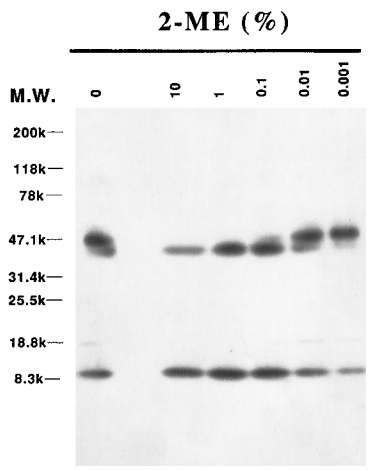

FIG. 2. SDS-PAGE analysis of UL49.5h protein under different partial reducing conditions. ^{35}S-labelled UL49.5h precipitate (see the legend to Fig. 1) was divided into five aliquots. To each aliquot, 2-mercaptoethanol (2-ME) was added at specific concentrations as indicated. After being boiled for 1 min, the samples were separated by SDS–6-to-20% gradient PAGE. M.W., molecular weight markers.

no 2-mercaptoethanol also contained a band at a size of about 39 kDa. This result was most likely caused by the diffusion of the high concentration of reducing agent present in the adjacent lane; the unique shape of the band is consistent with this explanation. The results from the immunoprecipitation suggest that the primary product of the UL49.5h gene is a polypeptide with a size of about 9 kDa. The 19-kDa species and 47-kDa species that were detected only under nonreducing conditions may represent a disulfide-linked UL49.5h homodimer and a disulfide-linked heterodimer consisting of the 9-kDa UL49.5h polypeptide and a second heterologous protein with a size of about 39 kDa, respectively.

In order to further ascertain that the 39-kDa protein was indeed a coprecipitated product rather than a different form of UL49.5h oligomer which could not be dissociated by the denatured gel, Western blot analysis was carried out. In contrast to immunoprecipitation, Western blotting failed to detect the 39-kDa band (Fig. 3), indicating that the 39-kDa species was not recognized by the antiserum and therefore must be a heterologous cellular or viral component which coprecipitates with the UL49.5h product via disulfide linkage.

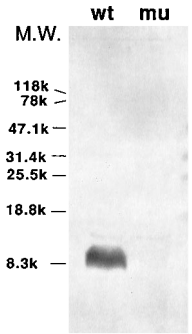

FIG. 3. Western blot analysis of UL49.5h protein from virus-infected cells. Subconfluent MDBK cells grown in six-well plates were infected with wild-type BHV-1 (wt) and mutant vDUCZ (mu). At 16 h postinfection, cells were collected, directly lysed in SDS-PAGE sample buffer, and separated by SDS–15% PAGE, which was followed by standard Western blot procedures. The anti-UL49.5h peptide serum was used as the primary antibody at a dilution of 1:500. M.W., molecular weight markers.

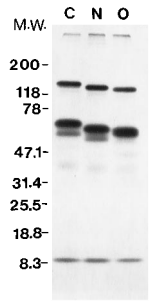

FIG. 4. SDS-PAGE analysis of the susceptibility of UL49.5h protein to *O*-glycosidase. MDBK cells were infected with wild-type BHV-1 and labelled with [^{35}S]methionine-cysteine for 16 h. Cell lysate was precipitated with a mixture of UL49.5h antiserum and gD MAbs. The immunoprecipitated sample was divided into three aliquots. Two of the aliquots were treated with either neuraminidase only (N) or neuraminidase plus *O*-glycosidase (O). The third aliquot served as a control (C), which was processed in parallel with the samples that received enzymatic treatment. Samples were separated by SDS–6-to-20% PAGE under reducing conditions. M.W., molecular weight markers.

UL49.5h gene product lacks glycosylation, myristylation, and phosphorylation. On the basis of the amino acid sequence analysis, the UL49.5h protein lacks the consensus sequence for N-linked glycosylation, but it contains a putative myristylation site at residue 46 and a putative phosphorylation site at residue 92. The lack of an N-linked glycosylation site prompted us to examine whether the UL49.5h protein contained any O-linked oligosaccharide modification by using *O*-glycosidase digestion. ^{35}S-labelled, immunoprecipitated samples were first treated with neuraminidase to remove the terminal sialic acid residues of O-linked oligosaccharides and then were digested with *O*-glycosidase, and this step was followed by analysis by SDS-PAGE. To control for the efficacy of the enzymatic digestion, gD, a viral protein that contains O-linked oligosaccharides (33), was coprecipitated and treated together with UL49.5h protein. As shown in Fig. 4, the combined neuraminidase and *O*-glycosidase treatment resulted in an apparent reduction in the molecular mass of gD, as expected; in contrast, the same enzymatic treatment did not affect the migration rate of the 9-kDa UL49.5h product. The lack of reduction of the molecular mass of the UL49.5h protein after the *O*-glycosidase treatment suggests that UL49.5h protein does not contain O-linked oligosaccharide. It is noted that gD exhibits several different bands. The band with an apparent molecular mass of 71 kDa represents a gD monomer and the band above the gD monomer is likely a gD dimer. The identities of the bands with faster migration rates than those of the gD monomer in the control and the neuraminidase-treated samples are not clear. They probably represent an incompletely reduced form of gD, migrating at a faster rate than that of its completely reduced counterpart.

As an alternative approach to detect UL49.5h glycosylation, we also labelled UL49.5h protein with [^{3}H]glucosamine in conjunction with immunoprecipitation by anti-peptide antibody. No specific product was detected (data not shown). This result further substantiates that the BHV-1 UL49.5h protein is not glycosylated.

Because of the presence of the myristylation and phosphorylation sites in the UL49.5h amino acid sequence, attempts to examine the respective modifications were also made by [^{3}H]myristic acid and $^{32}P_i$ labelling. No UL49.5h product could be detected by either label (data not shown), suggesting that the UL49.5h product lacks these modifications.

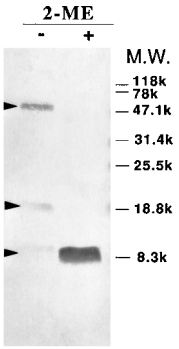

FIG. 5. Detection of the UL49.5h product in isolated virions. Samples (25 μg) of purified virions were separated by SDS–15% PAGE under nonreducing (−) and reducing (+) conditions, and then Western blotting was carried out with UL49.5h antiserum at a dilution of 1:500. M.W., molecular weight markers; 2-ME, 2-mercaptoethanol.

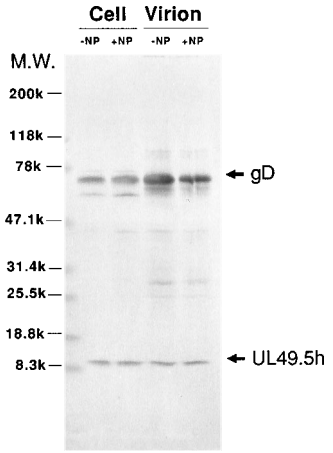

FIG. 6. Surface biotinylation of infected cells and isolated virions. Wild-type BHV-1-infected MDBK cells (Cell) collected at 16 h postinfection and isolated virions (Virion) were incubated with 1 mg of sulfo-NHS-biotin per ml of PBS in the presence (+NP) or absence (−NP) of 1% NP-40 at 4°C for 60 min, and this step was followed by incubation with 100 mM glycine for an additional 10 min at 4°C. After biotinylation, the samples were precipitated with a mixture of anti-UL49.5h serum and gD MAbs; immunoprecipitates were separated by SDS–6-to-20% gradient PAGE and blotted to nitrocellulose paper. Specific biotinylated protein bands were detected with an avidin-conjugated horseradish peroxidase. NP, NP-40; M.W., molecular weight markers.

UL49.5h gene product is a virion structural component. Having identified the UL49.5h gene product in virus-infected cells, we next examined whether the UL49.5h protein was also a virion structural protein. BHV-1 virions were purified from virus-infected cell culture supernatant via an Na-K tartrate gradient and analyzed by Western blotting with the UL49.5h-specific antiserum. As shown in Fig. 5, the UL49.5h products were indeed present in the isolated virions. In addition, the UL49.5h products present in the virions exhibited an electrophoretic pattern similar to that observed with virus-infected cell lysate. Thus, under nonreducing conditions, there were three major bands with apparent molecular masses of 47, 19, and 9 kDa, respectively, and under reducing conditions, there was a single 9-kDa band (it should be noted that Western blotting did not detect the 39-kDa heterologous protein as described above). Among the three species detected under the nonreducing conditions, the 47-kDa heterodimers appeared to be the most abundant. The presence of the 47-kDa heterodimer in isolated virions also indicated that the 39-kDa protein, with which the UL49.5h protein forms a heterodimer, is a virion protein.

Presence of UL49.5h protein on the plasma membrane and virion envelope. According to amino acid sequence analysis, the UL49.5h gene product is predicted to be an integral membrane protein. Nevertheless, our initial effort to determine its membrane localization by an indirect immunofluorescence assay failed to yield an unequivocal result. In addition, we found that the UL49.5h peptide-specific antiserum reacted very poorly with the intact virion on the basis of an enzyme-linked immunosorbent assay. We speculated that the particular antigenic epitope of the UL49.5h molecules present on the cell or virion surface might not be accessible to the anti-peptide antibody. Accordingly, two alternative approaches which do not rely on the reactivity of the antibody with membrane-associated molecules were taken to ascertain UL49.5h membrane localization. The first approach involves surface labelling by biotinylation. Infected cells and isolated virions were labelled with biotin either in the presence or in the absence of NP-40; UL49.5h protein along with viral protein gD were precipitated with their respective antibodies. The immunoprecipitated samples were separated by SDS-PAGE and transferred to nitrocellulose paper. The specific protein bands were detected with avidin-conjugated horseradish peroxidase. As shown in Fig. 6, both gD and UL49.5h protein were detected either in the presence or absence of NP-40. The fact that UL49.5h protein could be labelled without lysing the cells with NP-40 indicates that it is present on cell and virion surfaces. It is also noticed that there is a faint band with a size of about 39 kDa in both virion samples and in the cellular sample treated with NP-40. According to its electrophoresis mobility, this band may represent the 39-kDa protein that coprecipitates with UL49.5h protein.

The second approach examines the susceptibility of the UL49.5h protein present on intact virions to trypsin digestion. Isolated virions were treated with trypsin either in the presence or absence of NP-40; following separation by SDS-PAGE, bands were detected by Western blotting. As controls, virion envelope protein gD and virion tegument protein UL49h (17) were determined in parallel with the UL49.5h protein. To reduce the complexity of the protein bands, the individual samples were divided into two sets which were separated in parallel on the same gel. After transfer, one set of the samples was probed with gD antibody and the other was probed with a mixture of UL49h and UL49.5h antibodies. The results are shown in Fig. 7. In the absence of NP-40, UL49.5h protein and gD but not UL49h protein (32 kDa) were susceptible to the trypsin digestion, whereas in the presence of NP-40, all three proteins were digested. These results again suggest that the UL49.5h product is a membrane protein.

Nonionic detergent partitioning of UL49.5h gene product in isolated virions. Nonionic detergent partitioning is frequently used to determine the compartmentalization of virion structural components; envelope proteins can be readily released by nonionic detergent treatment, whereas tegument proteins may be released by a combined treatment with nonionic detergent and a high concentration of salt. In view of the fact that UL49.5h protein can form complexes with a heterologous 39-kDa viral component (Fig. 1 and 5), it was of interest to assess the detergent partition profile of this protein and to provide some information about the nature of the 39-kDa protein. Purified virions were treated with NP-40 either in the presence or absence of 1 M NaCl, and this step was followed by centrifugation; supernatant and the pellet were collected separately and analyzed by Western blotting. As references, virion enve-

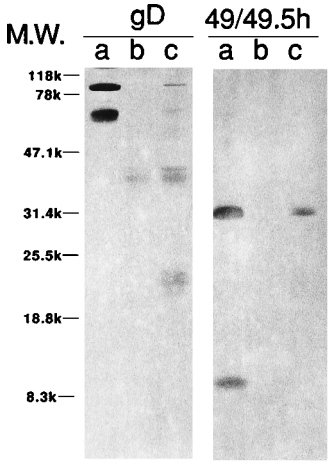

FIG. 7. Western blot analysis of the susceptibility of UL49.5h protein on isolated virions to trypsin digestion. Purified virions at a concentration of 1 mg/ml were incubated with 0.01 mg of TPCK-treated trypsin per ml of PBS in the presence (b) or absence (c) of 1% NP-40 at 37°C for 5 min. Immediately after the incubation, phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM; samples were boiled in SDS-PAGE sample buffer for 5 min. Each of the trypsin-treated samples and a control sample, i.e., the virions without trypsin treatment (a), were divided into two portions; the two sets of the identical samples were separated in parallel by SDS–6-to-20% PAGE. After separation, samples were blotted onto nitrocellulose paper. One set of the samples was probed with gD MAbs (gD), and the other was probed with a mixture of UL49h antiserum and UL49.5h antiserum (49/49.5h). M.W., molecular weight markers.

lope protein gD and tegument protein UL49h were detected along with UL49.5h protein by using a mixture of their respective antibodies. The control sample (Fig. 8 [C]) showed three major bands, namely, gD, UL49h protein, and UL49.5h protein, in descending order. Detergent treatment only resulted in complete release of gD, partial release of UL49.5h protein, and no discernible release of UL49h; detergent plus NaCl treatment resulted in complete release of gD and UL49.5h protein and partial release of UL49h protein (Fig. 8). The observation that the detergent treatment induced complete release of gD but only partial release of UL49.5h protein suggests that UL49.5h protein is more tightly attached to the viral internal structure than gD. To have a more direct appreciation of the 39-kDa protein in the virion, detergent partition

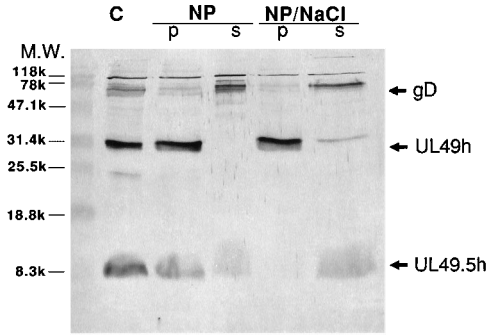

FIG. 8. Nonionic detergent partition of UL49.5h protein in isolated virions. Purified wild-type BHV-1 virions were treated with 1% NP-40 (NP) and 1% NP-40 plus 1 M NaCl (NP/NaCl) at 4°C for 30 min, and this step was followed by centrifugation at 178,000 × *g* for 30 min. After centrifugation, supernatant (s) and pellet (p) were collected separately and separated by SDS–15% PAGE under reducing conditions, and this step was followed by Western blotting. Virions without detergent treatment were included as a control (C). gD-, UL49h-, and UL49.5h-specific bands were detected with a mixture of gD MAbs, UL49h antiserum, and UL49.5h antiserum. M.W., molecular weight markers.

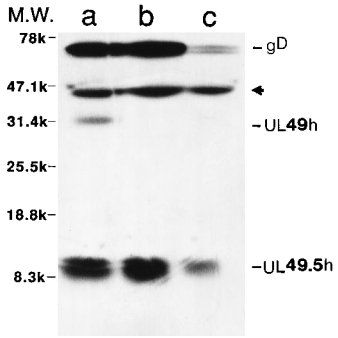

FIG. 9. Detection of the 39-kDa coprecipitate in isolated virions by immunoprecipitation. MDBK cells in a T-150 flask were infected with wild-type BHV-1 and labelled with Tran^{35}S-label. Virus was purified from the culture supernatant. Two-thirds of the virions were treated with 1% NP-40 as described in the legend to Fig. 6; the supernatant and the pellet were collected separately. The supernatant and the pellet, along with the remaining one-third of untreated virions, were precipitated separately with a mixture of BHV-1 gD MAbs and UL49h antiserum and UL49.5h antiserum. Samples were separated by SDS–6-to-20% gradient PAGE under reducing conditions. Lane a, untreated virions; lane b, supernatant; lane c, pellet. M.W., molecular weight markers. The arrow indicates the position of the 39-kDa coprecipitate.

experiments were repeated with ^{35}S-labelled purified virions, and these experiments were followed by immunoprecipitation. In this experiment, only the detergent treatment was performed. As shown in Fig. 9, the 39-kDa protein (indicated by the unspecified arrow) was indeed present in virions, and furthermore, it exhibited a partition profile similar to that of UL49.5h protein. It may be noted that in this particular experiment, the detection of UL49h protein was not optimal. However, after prolonged exposure of the X-ray film, the UL49h band was visible in lane c of Fig. 9, the lane that contained the pellet fraction.

DISCUSSION

Genes homologous to the HSV type 1 (HSV-1) UL49.5 gene have been identified in the genomes of a number of herpesviruses, including BHV-1 (3), equine herpesvirus (4, 27), varicella-zoster virus (4), and Marek's disease virus (37). However, at present, little is known about their protein products. Barker and Roizman previously showed that the cloning of the HSV-1 UL49.5 open reading frame sequence containing a fragment encoding a foreign antigen epitope into the thymidine kinase locus of the HSV-1 genome resulted in the expression of a 12-kDa protein (3). This experiment provided an initial indication that the HSV-1 UL49.5 open reading frame could be translated into a protein. In this report, by employing an antibody against a synthetic peptide of the BHV-1 UL49.5h gene and a UL49.5h gene deletion mutant, we have identified and characterized the protein product of the BHV-1 UL49.5h gene.

According to the deduced amino acid sequence, the BHV-1 UL49.5h gene product consists of 96 amino acids and has features of a typical type I integral membrane protein, with a putative signal peptide located between residues 1 and 21 and a transmembrane domain between residues 55 and 75 (17). Immunoprecipitation and Western blot analysis with an anti-UL49.5h peptide serum detected a primary product with a size of approximately 9 kDa in the wild-type BHV-1-infected cells but not in the UL49.5h gene deletion mutant-infected cells. The fact that the 9-kDa polypeptide was absent in the UL49.5h gene deletion mutant-infected cells confirms that it is a specific gene product of the BHV-1 UL49.5h gene. The molecular

mass of the UL49.5h protein estimated under the experiment conditions used is also consistent with the value predicted on the basis of the amino acid sequence, i.e., 10.02 kDa for the entire sequence or 8 kDa after the removal of the putative signal sequence.

Although an initial effort using an indirect immunofluorescence assay was unable to unequivocally localize the UL49.5h products to the cellular membrane, the results from both surface labelling and trypsin digestion experiments strongly suggest that UL49.5h protein is a membrane protein. Thus, we found that, like membrane glycoprotein gD, UL49.5h molecules present on both infected cells and isolated virions were accessible to surface labelling (Fig. 6) and the UL49.5h molecules present on virions were susceptible to trypsin digestion (Fig. 7). A possible explanation for the failure to detect the membrane localization by indirect immunofluorescence could be that when the molecule is present on the membrane surface, the particular peptide antigenic epitope might be unaccessible to the rabbit antiserum employed.

While most of the herpesvirus envelope proteins are glycoproteins, we found that BHV-1 UL49.5h product is not glycosylated. The evidence for this is as follows. First, UL49.5h protein does not have an N-linked glycosylation signal consensus sequence. Second, *O*-glycosidase digestion did not affect the migration rate of the UL49.5h product on SDS-PAGE gels. Since the UL49.5h product is a relatively small protein, any addition of oligosaccharide would be expected to have discernible effects on the overall molecular mass of this protein and thus its electrophoretic mobility. The lack of effect of *O*-glycosidase digestion on UL49.5h mobility suggests that UL49.5h protein contains no O-linked oligosaccharides. Finally, [^{3}H]glucosamine was unable to label the UL49.5h product.

An interesting feature of UL49.5h protein is that it forms both disulfide-linked homodimers and disulfide-linked heterodimers. We found that when immunoprecipitated samples were analyzed under nonreducing conditions by SDS-PAGE, the UL49.5 gene product migrated as three distinct bands with calculated relative molecular masses of approximately 47, 19, and 9 kDa, respectively; however, when it was separated under reducing conditions, two bands with sizes of 39 and 9 kDa were detected. These patterns are best interpreted as follows: (i) the 47-kDa product was a disulfide-linked heterodimer consisting of the 9-kDa UL49.5h product and the 39-kDa heterologous protein, and (ii) the 19-kDa product was a disulfide-linked homodimer of the 9-kDa UL49.5h product. In support of this contention, we found that under different partial reducing conditions, there was an inverse correlation between the amounts of 47- and 19-kDa products and amounts of the 39- and 9-kDa products; furthermore, according to Western blotting, anti-UL49.5h antibody did not react with the 39-kDa protein, indicating that the latter is not antigenically related to UL49.5h protein. In both infected cells and isolated virions, the 47-kDa heterodimers constitute the predominant species, which may imply that the heterodimer could represent the functional structure of UL49.5h protein.

The 39-kDa protein with which the UL49.5h protein forms a disulfide-linked complex is present not only in virus-infected cells but also in mature virions. The nature of this viral component is not clear. However, it is tempting to speculate that the 39-kDa protein is an internal virion protein. While there is no definitive evidence for this yet, some observations tend to support this notion. Immunoprecipitation of virus-infected cells by [^{3}H]glucosamine labelling failed to detect the 39-kDa protein (data not shown), indicating that it is not glycosylated, which is quite unusual for herpesvirus envelope proteins. The second indication stems from the nonionic detergent partition experiments. It was found that the 39-kDa protein coprecipitates with the UL49.5h protein and that both of them differed from the envelope protein gD in that the former appears to be more tightly associated with tegument-nucleocapsid structures. Since UL49.5h protein is an envelope protein, the observation that it is somehow associated with the tegument-nucleocapsid structures may indicate that it is linked with an internal virion structural component. In this regard, the 39-kDa protein appears to be a good candidate as the internal virion structural component with which UL49.5h protein forms a complex. It should be pointed out that, as shown in Fig. 6, a band with a size of about 39 kDa was detected in the virions even in the absence of detergent treatment; this band could be the 39-kDa protein with which UL49.5h protein forms a heterodimer. If so, this observation may suggest that the 39-kDa protein is also exposed on the virion surface. A definitive answer as to the actual nature of the 39-kDa protein may have to wait until further studies.

The functions of UL49.5 homologs are still unknown. It has been shown for BHV-1 and HSV-1 that the UL49.5 homologs are not essential for virus growth (17, 26). Recent evidence suggests that herpesvirus nonessential envelope proteins may function in restricted cell or tissue types. For example, nonessential proteins gI and gE of pseudorabies virus have been shown to be required for the dissemination of the virus among neuron cells (7), and HSV membrane protein coded for by gene UL20 has been shown to be required for virus assembly in Vero cells but not in others (35). In light of the observation that UL49.5 homologs are highly conserved among different herpesviruses, it is not unreasonable to expect that they may play important functions in virus replication. In this regard, the identification of the BHV-1 UL49.5h gene product establishes a basis for further elucidation of UL49.5h function(s).

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