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Citation for published version:

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Document Version:
Publisher's PDF, also known as Version of record

Published In:
J Virol

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The Class II Membrane Glycoprotein G of Bovine Respiratory Syncytial Virus, Expressed from a Synthetic Open Reading Frame, Is Incorporated into Virions of Recombinant Bovine Herpesvirus 1

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The Class II Membrane Glycoprotein G of Bovine Respiratory Syncytial Virus, Expressed from a Synthetic Open Reading Frame, Is Incorporated into Virions of Recombinant Bovine Herpesvirus 1

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Received 22 August 1997/Accepted 16 January 1998

The bovine herpesvirus 1 (BHV-1) recombinants BHV-1/eGori and BHV-1/eGsyn were isolated after insertion of expression cassettes which contained either a genomic RNA-derived cDNA fragment (BHV-1/eGori) or a modified, chemically synthesized open reading frame (ORF) (BHV-1/eGsyn), which both encode the attachment glycoprotein G of bovine respiratory syncytial virus (BRSV), a class II membrane glycoprotein. Northern blot analyses and nuclear runoff transcription experiments indicated that transcripts encompassing the authentic BRSV G ORF were unstable in the nucleus of BHV-1/eGori-infected cells. In contrast, high levels of BRSV G RNA were detected in BHV-1/eGsyn-infected cells. Immunoblots showed that the BHV-1/eGsyn-expressed BRSV G glycoprotein contains N- and O-linked carbohydrates and that it is incorporated into the membrane of infected cells and into the envelope of BHV-1/eGsyn virions. The latter was also demonstrated by neutralization of BHV-1/eGsyn infectivity by monoclonal antibodies or polyclonal anti-BRSV G antisera and complement. Our results show that expression of the BRSV G glycoprotein by BHV-1 was dependent on the modification of the BRSV G ORF and indicate that incorporation of class II membrane glycoproteins into BHV-1 virions does not necessarily require BHV-1-specific signals. This raises the possibility of targeting heterologous polypeptides to the viral envelope, which might enable the construction of BHV-1 recombinants with new biological properties and the development of improved BHV-1-based live and inactivated vector vaccines.

Bovine herpesvirus 1 (BHV-1), a member of the subfamily Alphaherpesvirinae with a double-stranded DNA genome of approximately 136 kbp, causes infectious rhinotracheitis and infectious pustular vulvovaginitis as the most common clinical symptoms in cattle (27, 34, 39). Vaccination with attenuated live viruses or inactivated virions is widely used to control the disease and to reduce the concomitant financial losses. As with other large DNA viruses, interest exists in the use of recombinant BHV-1 as an improved live vaccine against BHV-1 infection (1, 21, 42) or as a vector for bi- or multivalent vaccines against BHV-1 and additional bovine pathogens (17, 18). To date, incorporation of heterologous genes into the genome of BHV-1 has concentrated mainly on the expression of the procaryotic lacZ gene to identify essential and nonessential genes or as a reporter gene for analytical studies (3, 8, 12, 15, 20, 29, 37, 38, 45). Recently, BHV-1 has been used to express biologically active bovine interleukins (21, 32) and glycoproteins of pseudorabiesvirus (19, 31). However, expression of RNA virus-encoded proteins by BHV-1 has not been published so far. Remarkably, expression of genes from cytoplasm-replicating viruses by other herpesviruses of mammals has only rarely been reported (5, 43).

Attempts to express the fusion glycoprotein F and the attachment glycoprotein G of bovine respiratory syncytial virus (BRSV), a pneumovirus of the family Paramyxoviridae which is also prevalent worldwide and causes severe respiratory disease in young calves similar to the disease caused by human respiratory syncytial virus in children (4), were not successful (13, 33). Although the cDNA fragments encoding the respective glycoproteins were flanked by transcription control elements that are active in the genomic context of BHV-1 (21), no BRSV-specific transcripts were detected in cells infected with the BHV-1 recombinants (13) (see below). We therefore assumed that RNAs containing the authentic BRSV sequences were unstable in the nuclei of infected cells. To test this assumption, the BHV-1 glycoprotein D (gD) codon usage preferences (13, 40) were used to construct a modified open reading frame (ORF) encoding the BRSV G glycoprotein by chemically synthesized oligonucleotides.

In this report, we show that expression of the attachment glycoprotein G of BRSV (BRSV G glycoprotein), a type II membrane glycoprotein (36, 44), by BHV-1 was dependent on the modification of the base composition of the ORF encoding BRSV G glycoprotein, that virions produced by the recombinant contained the BRSV G glycoprotein, and that the presence of this protein in the viral envelope does not significantly interfere with the infectivity of BHV-1. Our findings suggest that RNA viruses which replicate in the cytoplasm can contain sequences or sequence elements that lead to instability of transcripts within the nucleus.
MATERIALS AND METHODS

Cell culture and viruses. BHV-1 strain Schönbock (BHV-1-Scho) was obtained from O. C. Straub (Federal Research Centre for Virus Diseases of Animals, Tübingen, Germany) and propagated on Madin-Darby bovine kidney cell clone Bu100 (MDBK-Bu100; kindly provided by W. Lawrence and L. Bello, University of Pennsylvania, Philadelphia, Pa.). The cells were grown in Dulbecco's minimum essential medium supplemented with 5% fetal calf serum (FCS), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.35 mg of 3-t-glutamine per ml. The g-negative mutant BHV-1/86–221 was propagated on the constitutively g-expressing cell line BU-Dorff as described previously (38).

Construction and cloning of the BRSV G ORF. Oligonucleotides were synthesized in a Biosearch 8700 instrument and purified by gel electrophoresis. Complementary oligonucleotides were mixed in equal molar amounts in 10 mM Tris-HCl (pH 7.5), boiled for 5 min, and slowly cooled to room temperature. All the cloning procedures were performed by established methods (35). The following double-stranded DNA fragments were generated (restriction enzyme cleavage sites used for cloning are shown in boldface type).

Fragment 1: dedemejer, Intervet International, Boxmeer, The Netherlands), which contains a cDNA fragment encoding the BRSV G glycoprotein, was cleaved with BglII. A 770-bp fragment, which encompasses codons 1 to 257 of the BRSV G ORF, was ligated into plasmids pROMe and pROMe (21) cleaved with BglII followed by insertion of a synthetic DNA fragment, obtained by hybridizing oligonucleotides 5'-GCGACATACCTCTCATATGAT-3' and 5'-CATGTTTATATGAGGTCG-3', as described above, into a single Ncol cleavage site to provide a stop codon. The resulting plasmids, in which codon 259 was changed from CAA (encoding glutamine) to ATG (encoding methionine), were named pROMeGori and pROMeGori, respectively.

To construct recombinant plasmid pROMeGori, plasmid pROMe was cleaved with BglII, blunt ended with Klenow polymerase, and used for the insertion of the synthetic G ORF, isolated from plasmid pBSRVGori (after cleavage with EcoRI and blunt endin with Klenow polymerase). Hybridization of cRNA, the same fragment was integrated into the BglII-cleaved and blunt-ended plasmid vector pSP73 (Promega, Heidelberg, Germany) to give pSP73syn.

For in vitro transcription of the BRSV G ORF, a blunt-ended ClaI fragment encompassing this ORF in plasmid pROMeGori, was inserted into EcoRV-cleaved pSP73. The resulting plasmid was designated pSP73syn. The correctness and orientation of the BRSV G ORFs were determined byideoxy sequencing, which showed that the 5' ends of the Gori and Gori ORFs were placed adjacent to the T7 or SP6 promoter, respectively.

In vitro transcription and translation. Plasmids pSP73syn and pSP73syn were linearized with BglII and HindIII, respectively, and cRNA was transcribed by T7 or SP6 RNA polymerase in the presence of the cap analog m7GpppG as specified by the manufacturer (Boehringer GmbH, Mannheim, Germany). In vitro translation of the cRNAs was performed in the presence of 60 μCi of [35S]methionine per reaction mixture as recommended by the supplier (Promega). Labelled proteins were visualized by fluorography after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) as described previously (16).

RNA isolation, Northern blot hybridization, and nuclear runoff analysis. Whole-cell RNA and cytoplasmic RNA was isolated as described previously (14, 35). Glyoxal-treated RNA (5 μg) was separated in 1% formaldehyde gels, transferred to nitrocellulose filters, and hybridized to 32P-labeled DNA by established procedures (14, 35). Nuclear runoff transcription assays were performed by the method of Greenberg and Ziff (11). Labeled RNA was hybridized to equimolar (1.5 pmol) amounts of plasmid DNA dotted onto nitrocellulose filters with a dot blot device (Schleicher & Schuell, Dassel, Germany). Bound radioactivity was visualized by autoradiography and quantitated by Cerenkov counting.

Antibodies and sera. BRSV G glycoprotein-specific monoclonal antibody (MAb) 20 and MAb 57 and the polyclonal serum directed against BHV-1 gD are described elsewhere (8, 9, 23). The anti-VacGori serum was raised in rabbits infected with a recombinant vaccinia virus expressing the Gori ORF under control of the p7.3 promoter. The rabbits were inoculated with 5 × 107 PFU and bled 4 weeks after a booster immunization with 5 × 108 PFU given 1 week after the initial infection. The BRSV-neutralizing titer was 1:80 in the presence of complement. Polyclonal anti-BHSV hyperimmune serum 2106 was raised in gnotobiotic calves after repeated inoculation with BRSV and had a BRSV-neutralizing titer of 1:10,000 in the absence of complement (10).

Western blotting and immunoprecipitation. For Western blotting (immunoblotting), cells or purified virions were lysed in sample buffer. For analysis of secreted proteins, infected cells were cultured with Dulbecco's minimal essential medium containing 1% FCS. Culture supernatants were harvested, cleared of debris by centrifugation in PBS, fixing with 3% paraformaldehyde in PBS and sequentially incubated with appropriate dilutions of MAbs or sera in a final volume of 100 μl, and incubated overnight at 37°C. The cultures were overlaid with semisolid medium 2 h later, and plaques were counted 2 to 3 days after infection. The percent neutralization-resistant infectivity was calculated from the results with controls incubated without antibody.

Inoculation of cells for indirect immunofluorescence. MDBK-Bu100 cell cultures were infected with approximately 100 PFU and overlaid with semisolid medium. After the development of plaques, the cultures were fixed with 3% paraformaldehyde in PBS and sequentially incubated with appropriate dilutions of MAbs or sera in PBS containing 4% dithiothreitol. All sera were preabsorbed with conjugated goat anti-species immunoglobulin (Dianova, Hamburg, Germany).

Fragment 1 was ligated into plasmid vector pUC18 after cleavage with AarII and BamHI. In the resulting plasmid, pU1, the AarII cleavage site of pUC18 was destroyed. Plasmid pU1 was cleaved with Ncol and BamHI and received fragment 2 to produce pU2, into which fragment 3 was ligated after digestion with AarII and BamHI to yield plr1. In parallel, pUC19 DNA was cleaved with HindIII and PstI, fragment 7 was inserted, and the resulting plasmid, pF7, was cleaved with SpeI and SpeI and received fragment 6 to generate pF6. Plasmid pF6 was incubated with PstI and BstII, and fragment 5 was inserted. The plasmid obtained, pF5, was treated with PstI and ClaI, and fragment 4 was integrated, resulting in plasmid pF4. Plasmid pF4 was cleaved with XbaI, blunt ended with Klenow polymerase, and ligated into this DNA, the HindIII cleavage site of fragment 1 of pF3 was ligated after blunt end of the HindIII site. The resulting plasmid, pBRSVGsyn, contained the reconstructed BRSV G ORF flanked by EcoRI cleavage sites.

DSS (HIV-1) plasmid constructions. Plasmid pRSV02 (kindly provided by Paul Sondermejer, InterNetwork International, Boxmeer, The Netherlands), which contains a
RESULTS

Rationale for synthesis of a modified BRSV G glycoprotein ORF. To express the BRSV G glycoprotein by recombinant BHV-1, recombination plasmid pROMeGori, which contains a cDNA fragment encompassing the BRSV G glycoprotein ORF under control of the murine cytomegalovirus (MCMV) early 1 promoter (Fig. 1), was cotransfected with purified BHV-1/80–221 DNA into MDBC cells and the progeny virus was serially diluted and plated on MDBC cells. Since the ORF encoding the essential gD is replaced by a lacZ expression cassette in BHV-1/80–221 (8), only viruses which have acquired the gD ORF by replacement of the lacZ cassette by the insert of the recombination plasmid should be able to replicate on non-complementing cells (21). Virus from plaques that did not stain blue under a Bluo-Gal-containing agarose overlay were again subjected to titer determination on MDBC cells, and isolates which produced only “white” plaques were plaque purified once more and characterized further (see Fig. 4).

Analysis of MDBC cells infected with the resulting recombinant BHV-1/eGori gave no indication of BRSV G glycoprotein expression by indirect immunofluorescence (see Fig. 7), and no stable transcripts were detected within cytoplasmic RNA (data not shown) or whole-cell RNA (see Fig. 5). BRSV G glycoprotein expression was also not detected in transient-expression experiments with plasmid pROMieGori (Fig. 1) in which the BRSV G glycoprotein ORF is under the control of the MCMV immediate-early 1 enhancer/promoter element (data not shown). In vitro translation of mRNA, transcribed in vitro from pSPGori, resulted in synthesis of a polypeptide whose apparent molecular mass of 31 kDa (Fig. 2, lane 2) was in good agreement with the calculated size of 29 kDa (24). This result indicated that mRNA from the BRSV Gori ORF contained within pROMeGori has the potential to direct protein synthesis, and we assumed that the failure to detect BRSV G glycoprotein-specific gene products was due to instability of BRSV G glycoprotein transcripts in the nuclei of BHV-1/eGori-infected cells. Therefore, a modified ORF encoding the BRSV G glycoprotein (Fig. 3) was chemically synthesized, isolated from plasmid pBRSVGsyn, and inserted into pROMe and pSP73. The resulting plasmids were named pROMeGsyn and pSPGsyn, respectively, and RNA transcribed from pSPGsyn directed the synthesis of a 31-kDa protein in a rabbit reticulocyte lysate (Fig. 2, lane 3) that comigrated with the polypeptide synthesized in vitro from the Gori ORF, supporting the conclusion.
that the 31-kDa protein represents the primary translation product encoded by the BRSV G glycoprotein ORF.

Integration of the BRSV-Gsyn ORF into the genome of BHV-1/80–221. Recombination plasmid pROMeGsyn was cotransfected with purified BHV-1/80-221 DNA into MDBK cells, and the recombinant virus, BHV-1/eGsyn, was isolated as described above for BHV-1/eGori. To demonstrate integration of the expression cassettes into the genomes of the respective viruses, MDBK cells were infected with BHV-1/eGori, BHV-1/eGsyn, and BHV-1/80–221, phenotypically complemented by propagation on gD-expressing cells (8). Whole-cell DNA was prepared 20 h postinfection (p.i.), cleaved with HindIII, transferred to nitrocellulose filters after size separation in 0.6% agarose gels, and hybridized to Gsyn-, Gori-, and lacZ-specific 32P-labeled DNA fragments. The sizes of the fragments that hybridized to the respective probes were as expected (data not shown). We concluded that BHV-1/eGori and BHV-1/eGsyn were generated by homologous recombination as envisaged.

Transcription of the BRSV G ori and BRSV G syn ORFs in recombinant BHV-1-infected cells. To test for transcription from the recombinant BRSV G genes, MDBK cells were infected with BHV-1/eGsyn (Fig. 4, lanes 1, 3, 5, and 7) and BHV-1/eGori (lanes 2, 4, 6, and 8). Cytoplasmic RNA was isolated at 6 h p.i., size separated by agarose gel electrophoresis, and transferred to nitrocellulose filters. A 32P-labeled DNA probe representing the BRSV Gsyn ORF detected an RNA of 1.3 kb after infection with BHV-1/eGsyn (lane 1) and, after extended exposure, a transcript of 1.8 kb (lane 5) whose synthesis is initiated approximately 500 bp upstream from the MCMV e1 promoter (13). Even after a longer exposure, Gori ORF-specific transcripts of the expected size were not detected (lanes 2 and 6). The faint smear in lane 6 might indicate the presence of fragmented Gori RNA. Hybridization of the filters with 32P-labeled DNA encompassing the BHV-1 gD ORF showed that comparable amounts of gD mRNA were detected in BHV-1/eGsyn-infected cells (lanes 3 and 7) and BHV-1/eGori-infected cells (lanes 4 and 8), demonstrating that
absence of stable transcripts from the BRSV G ori ORF was not due to degradation of the RNA from BHV-1/eGori-infected cells. Substantially the same results were obtained with RNAs isolated 16 h after infection with BHV-1/eGsyn and BHV-1/eGori in the absence or presence of phosphonoacetic acid (data not shown).

To support the assumption that transcripts from the BRSV G ori ORF are unstable in BHV-1/eGori-infected cells, elongation of initiated transcripts in BHV-1/eGori- and BHV-1/eGsyn-infected MDBK cells was analyzed by nuclear runoff transcription assays. 32P-labeled RNA, synthesized in nuclei isolated at 6 h p.i., was hybridized to DNA from the plasmid vector pSP73 or from plasmids pSPGori, pSPGsyn, or pSPgD dotted onto nitrocellulose membranes. Elongation of RNA from both BRSV G ORFs was detected (data not shown). The ratio of bound radioactivity between the respective BRSV G ORFs are unstable in BHV-1/eGori-infected cells, elongation of initiated transcripts in BHV-1/eGori and BHV-1/eGsyn-infected MDBK cells was analyzed by nuclear runoff transcription assays. 32P-labeled RNA, synthesized in nuclei isolated at 6 h p.i., was hybridized to DNA from the plasmid vector pSP73 or from plasmids pSPGori, pSPGsyn, or pSPgD dotted onto nitrocellulose membranes. Elongation of RNA from both BRSV G ORFs was detected (data not shown). The ratio of bound radioactivity between the respective BRSV G ORFs was 1.7 with RNA from BHV-1/eGori-infected cells and 2.1 with RNA from BHV-1/eGsyn-infected cells, demonstrating that the G ori ORF was transcribed in BHV-1/eGori-infected cells. These results are in accordance with the conclusion that BHSV-1-expressed BRSV Gori RNAs are unstable in the nuclei of infected cells.

Identification and characterization of BHV-1-expressed BRSV G glycoprotein. To test for the expression of the BRSV G glycoprotein in BHV-1/eGsyn-infected cells, BRSV G glycoprotein-specific MAb 20 and a polyclonal antiserum (anti-VacGsyn), raised in rabbits after infection with G syn-expressing recombinant vaccinia virus, were used to stain wild-type BHV-1 strain Schönböken (BHV-1/Scho), BHV-1/eGori, or BHV-1/eGsyn-induced plaques by indirect immunofluorescence. The cultures were fixed 2 days after infection and incubated with the BHV-1 gD-specific MAb 21/3/3, MAb 20, or the anti-VacGsyn serum. Antibodies bound on the cell surface were visualized by using DTAF-conjugated anti-species immunoglobulin G and fluorescence microscopy. As shown in Fig. 5, MAb 20 and the anti-VacGsyn serum bound only to cells in plaques generated by BHV-1/eGsyn whereas MAb 21/3/3 reacted with gD on the surface of cells infected with BHV-1/Scho, BHV-1/eGsyn, and BHV-1/eGori. The conclusion that the BRSV Gsyn ORF-encoding protein is expressed on the surface of BHV-1/eGsyn-infected cells was also demonstrated by flow cytometry with living cells and the same antibodies as above (data not shown).

To further characterize the BRSV Gsyn ORF-encoded gene product, proteins from infected cells (Fig. 6, lanes 1 to 4), purified virions (lanes 5 to 7), and the cell culture medium from BHV-1/eGsyn-infected cells (lane 8) were analyzed by immunoblotting because MAb 20 and the anti-VacGsyn serum did not work for immunoprecipitation. MAb 20 did not specifically bind to proteins from cells infected with BHV-1/Scho (Fig. 6, lane 1) and BHV-1/eGori (lane 2) or to proteins from purified BHV-1/Scho and BHV-1/eGori virions (lanes 5 and 6) but reacted strongly with proteins with apparent molecular masses of 38 and 43 kDa from BHV-1/eGsyn-infected cells (lane 3). In addition, several weaker-staining bands ranging in size from 70 to about 100 kDa were detected. To elucidate
FIG. 6. Identification of the BHV-1/eGsyn-expressed BRSV G glycoprotein. MDBK cells were infected with BHV-1/Schö (lanes 1 and 5), BHV-1/eGori (lanes 2 and 6), and BHV-1/eGsyn (lanes 3, 4, 7, and 8). Proteins from infected cells, harvested at 10 h.p.i. (lanes 1 to 4), from purified virions (lanes 5 to 7), and from cell culture medium (lane 8), were analyzed by immunoblotting with BRSV G glycoprotein-specific MAb 20. Proteins shown in lane 4 were from cells incubated with cycloheximide (100 μg/ml) for 2 h before lysis. Apparent molecular masses are indicated in kilodaltons.

FIG. 7. Evidence that BHV-1-expressed G glycoprotein contains N- and O-linked sugars. (a and b) Purified BHV-1/eGsyn virions were resuspended in PBS plus 0.5% NP-40 and digested overnight at 37°C with neuraminidase (lanes 2), neuraminidase and O-glycosidase (lanes 3), N-glycosidase F (lanes 4), and without enzyme (lanes 1). Digestion products were analyzed by immunoblotting with G glycoprotein-specific MAb 20 (a) or a polyclonal, BHV-1 G-specific rabbit antiserum (b). (c) Purified, [35S]methionine-labeled BHV-1/Schö virions were lysed and incubated with G-specific MAb 21/3/3. Immunoprecipitated proteins were incubated as described above, separated on SDS–10% polyacrylamide gels, and visualized by fluorography. The apparent molecular masses, indicated in kilodaltons on the left, were calculated from the migration of prestained (a and b) or 14C-methylated protein molecular mass standards (c) run on the respective gels.

DISCUSSION

Analysis of the expression from the authentic BRSV G glycoprotein ORF after infection of cells with BHV-1/eGsyn indicated that the transcripts are unstable in the nucleus. We assumed that this was due to the nucleotide sequence of the RNA, which might contain signals that do not interfere with the stability of the RNA in the cytoplasm of cells infected with BRSV or recombinant vaccinia virus expressing the BRSV G glycoprotein (24) but result in degradation of the transcripts in the nucleus. Therefore, the complete BRSV G glycoprotein ORF was reconstructed by using synthetic oligonucleotides. The codon usage of the modified ORF was adjusted to that of BHV-1 G, resulting in an increase of the G+C content from 42 to 62%, and the introduction of putative splice donor signals (30) was avoided. The synthetic ORF was inserted into the genome of BHV-1 under the control of the MCMV e1 promoter (2, 21), and analysis of the gene products in BHV-1/eGsyn-infected cells demonstrated transcription of mRNA.
which was translated into proteins recognized by BRSV G glycoprotein-specific MAb 20 and the anti-VacGsyn serum. Although we did not attempt to delineate sequence motifs leading to instability of the Gori transcripts in the nuclei, our results strongly suggest their existence, which might also explain the failure to express the authentic ORF encoding BRSV fusion protein F by BHV-1 (13). Introduction of a series of single restriction enzyme cleavage sites into the Gsyn ORF will be helpful in identifying sequence elements that prevent the generation of mRNA from the genomic BRSV G glycoprotein ORF, which may be beneficial for the development of systems for the expression of RNA virus genes in the nuclei of mammalian cells.

The BRSV G glycoprotein is a type II membrane glycoprotein with a 37-amino-acid N-terminal cytoplasmic domain followed by 29 hydrophobic amino acids representing the transmembrane region. The remaining 191 amino acids are mainly hydrophilic and constitute the extracellular domain (24). Among BHV-1/eGsyn-infected cell proteins, MAb 20, MAb 57, and the anti-VacGsyn serum detected proteins with apparent molecular masses of 38, 43, and 70 to 120 kDa. Inhibition of de novo protein synthesis by cycloheximide resulted in a decrease of the signals generated by the 38- and 43-kDa proteins and a concomitant increase in intensity and size of the large-protein band. Although the exact precursor-product relationship awaits clarification, we assume that the 38- and 43-kDa proteins represent precursor molecules of the mature BRSV G glycoprotein. This assumption is in accordance with results reported by Lerch et al. (24), who demonstrated that in cells infected with a BRSV G glycoprotein-expressing recombinant vaccinia virus, two proteins which migrated with an apparent molecular mass of 43 kDa and as a broad band between 68 and 97 kDa specifically reacted with an anti-BRSV serum in immunoblots. They further provided evidence that the 43-kDa form represents a precursor containing only N-linked carbohydrates whereas the 68- to 97-kDa molecules constitute the mature N- and O-glycosylated BRSV G glycoprotein.

Expression of the BRSV G glycoprotein in BHV-1/eGsyn-infected cells was analyzed only by immunoblotting because the BRSV G glycoprotein-specific MAb 20 and the anti-VacGsyn serum did not work for immunoprecipitation and MAb 57 precipitated only the mature glycoprotein after synthesis in the presence of [35S]methionine and [35S]cysteine (not shown). The observation that antibodies against the BRSV G glycoprotein bind in immunoblots but fail to immunoprecipitate the antigen is, although surprising, not without precedent and has also been reported by Lerch et al. (24). The reason for this apparent paradox, however, remains unclear.

Immunofluorescence and flow cytometry (data not shown) with MAb 20 and the anti-VacGsyn serum demonstrated that BHV-1/eGsyn-infected cells expressed the BRSV G glycoprotein on the cell surface, and we conclude that it is tightly anchored to the cell membrane since no secreted molecules were found in the medium from BHV-1/eGsyn-infected cultures. Thus, our results indicate Golgi processing and post-Golgi transport of the BHV-1/eGsyn-encoded BRSV G glycoprotein to the cell surface, comparable to the situation in BRSV- or recombinant vaccinia virus-infected cells (24).

Since alphaherpesviruses probably do not encode type II glycoproteins, we investigated whether the BRSV G glycoprotein that presumably lacks any herpesvirus-specific targeting signals is incorporated into BHV-1 particles. Western blot analysis with MAb 20 revealed incorporation of the BRSV G glycoprotein into purified virus particles, and this was confirmed by the susceptibility of BHV-1/eGsyn virions to neutralization by the anti-VacGsyn serum, the anti-BRSV immune serum, and MAb 57 in the presence of complement. That these antibodies did not neutralize in the absence of complement was not too surprising, since it is unlikely that the BRSV G glycoprotein exhibits a biological function for the infectivity of BHV-1. In addition, flow cytometry demonstrated that MAb 20 and the anti-VacGsyn serum bound to living cells infected with purified BHV-1/eGsyn virions in the presence of cycloheximide to prevent de novo protein synthesis (13). This further supports the conclusion that the orientation of the BRSV G glycoprotein within the viral envelope is correct.

To our knowledge, this is the first description of a class II membrane glycoprotein becoming incorporated into alphaherpesvirus virions. The presence of the BRSV G glycoprotein in the given background of BHV-1/eGsyn, exerted only a slight influence on the entry of virions into target cells in comparison to the situation for wild-type BHV-1 (data not shown). Although no repaired viruses were included in this study, we believe that it is reasonable to assume that BRSV G glycoprotein does not significantly interfere with the envelope structures required for the initial steps of the BHV-1 infection.

This study has practical implications for herpesvirus-based vector vaccine development: (i) it indicates that modification of RNA virus genes may be required for efficient expression, and (ii) it raises the possibility of targeting foreign antigens as class II membrane (glyco)proteins into the envelope of herpesvirus virions, which might be important when booster vaccinations with live recombinant viruses are inefficient due to...
existing immunity to the vector, a situation observed with recombiant vaccinia viruses (13, 26).

ACKNOWLEDGMENTS

We thank Julie Furze for providing MA85, Helmut Stephan and Elke Zorn for photography, J. Naval for corrections, and Bernd Köllnner for assistance with the flow cytometry.

Financial support for synthesis and expression of the Gc505 ORF by Intervet International BV is greatly appreciated. G.K. was supported by grant BIOT-CT93-0489 from the Commission of the European Communities.

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