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Production and characterization of bovine herpesvirus 1 glycoprotein B ectodomain derivatives in an hsp70A gene promoter-based expression system*

Brief Report

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Summary. Different derivatives of bovine herpesvirus 1 (BHV-1) glycoprotein B (gB) ectodomain were expressed in a novel heat-shock expression system. The putative ectodomain, gBt, and the N-terminal subunit, gBb, were of the expected molecular weight and were secreted. Their production were heat-inducible and the purified proteins were able to elicit antibody responses in mice of a comparable level as induced by authentic gB. The truncated C-terminal subunit, gBct, was retained in the endoplasmic reticulum. Our studies suggest that the gBb subunit may play a major role in constituting the overall configuration of gB and is required for the intracellular transport of gB.

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Bovine herpesvirus-1 (BHV-1) glycoprotein B (gB) is one of the essential viral glycoproteins [27, 28]. Previous studies have shown that gB plays an important role in virus entry including initial virus attachment and subsequent penetration involving membrane fusion [4, 5, 7, 13, 25]. It also represents a dominant viral antigen that induces protective immunity in the natural host [1, 2, 9, 14, 22]. Because of its importance in viral immunology and infectivity, this glycoprotein was expressed in mammalian cells in order to gain a further understanding regarding its biological function.

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BHV-1 gB is a type I integral membrane protein. The membrane anchor region is located between residues 759 to 828, which is followed by a 104 amino acid cytoplasmic tail [15, 27, 29]. A cleavage site is located in the middle of the gB molecule, between residue 505 and 506, which divides the molecule into two subunits. Therefore, mature BHV-1 gB exists predominantly as a covalently linked heterodimeric complex, derived by proteolytic cleavage from a common primary translation product, while a small fraction remains in an uncleaved form [21, 23, 25]. Because expression of full-length gB has been shown to cause membrane fusion and to be toxic to transfected cells [5, 7], we expressed the gB ectodomain without its membrane anchor region. The truncated gB derivatives were expressed in MDBK cells under control of a heat-inducible, bovine heat-shock protein 70A (hsp70A) gene promoter [11]. The recombinant gB products were characterized with respect to their intracellular processing and their antigenic properties.

To produce gB expressing cell lines, transfer vectors containing the hsp70A gene promoter followed by a neomycin resistance gene and the respective gB genes were constructed and used to transfect MDBK cells. In the construction of transfer vectors, plasmid p3KHSPG4HU [11], which contains a truncated BHV-1 gD gene under the control of the bovine hsp70A gene promoter and the aminoglycoside phosphotransferase gene under the control of the SV40 early promoter, was used as the parental plasmid. In order to express the gB ectodomain, gBt, we constructed a gB gene that lacks the sequence coding for the putative membrane anchor and cytoplasmic domains. For gBb, a subunit representing the amino terminal fragment of the proteolytic cleavage products, the gene was constructed to encode a protein which terminates at the proteolytic cleavage site. Two transfer vectors, pHSgBt and pHSgBb, were first constructed to contain BHV-1 gB amino acids 1-763 (gBt) and 1–505 (gBb) respectively. The plasmids were used to transfect MDBK cells with the help of Lipofectin (Gibco/BRL, Grand Island, NY) [11]. The resultant G418-resistant cells were screened for gB expression by a gB-specific monoclonal antibody-based immune dot blot assay. After single cell cloning, two cell lines, one expressing gBt and the other gBb, were subsequently established, and named gBt03 and gBb03, respectively. Both gB expressing cell lines exhibited morphology and growth properties indistinguishable from parental MDBK cells, and the expression of gB products was stable even after passages in the absence of G418 (data not shown).

For characterization of the molecular properties of gBt and gBb produced by the cell lines, an immunoprecipitation assay was performed [25], Fig. 1. We compared heat-activation of protein production in gBt03 and gBb03 cells, and found that both cell lines could produce more gB products following heat-shock treatment (+HS) than prior to heat-shock (−HS), indicating that gB production is under the control of the hsp70A promoter. Under non-reducing conditions, gBt existed as a single band with a molecular weight of about 115 kDa (result not shown); when reduced, the 74 kDa and 38 kDa bands were dominant, although the 115 kDa band was still visible (Fig. 1). The 74 kDa band had the same mobility as the gBb subunit present in the BHV-1-infected cell lysate, whereas

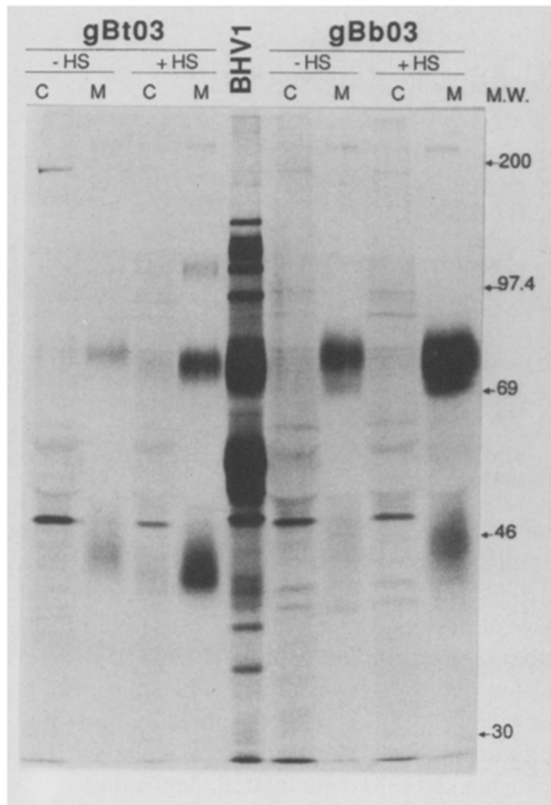

Fig. 1. Immunoprecipitation of gBt and gBb from transfected cell lines. Subconfluent MDBK cells grown in T-25 flasks were heat shocked at 43 °C for 4 h and labeled with ^{35}[S]-methionine. Cells and culture medium were collected separately and each precipitated with gB-specific monoclonal antibodies. The samples were separated on 8.5% SDS-PAGE gels under reducing conditions. Also included is gB precipitated from the wt BHV-1 infected MDBK cells (*BHV1*). *C* Cellular fraction; *M* medium; *HS* heat shock; *M.W.* molecular weight standards (kDa)

the 38 kDa band was significantly smaller than the authentic gBc (55 kDa). The difference in apparent molecular weight between the truncated and authentic gBc was estimated to be 17 kDa, which approximates the estimated molecular weight deleted from the authentic gB. Therefore, the reduced molecular weight of gBt could be attributed to the truncation of its transmembrane and cytoplasmic domains. In gBb03 cells, the expressed product had a mobility similar to authentic gBb, where the trace amount of 46 kDa product could be degraded protein. Under the conditions tested, gBt and gBb were readily detected in the culture medium, but little in the cell lysate (Fig. 1), indicating they were secreted from the cells.

Since we intended to produce the recombinant gB products in large amounts and to purify them from the media, we characterized the production of gB in the recombinant cell lines. The cells were subjected to daily heat-shock treatment by incubation at 43 °C for 4 h, whereafter the cells were returned to 37 °C for 20 h. This was repeated daily with a medium change prior to the next heat-shock cycle. The collected medium was quantitated for the presence of gB by enzyme-linked immunosorbent assay (ELISA) (Fig. 2). Briefly, microtiter plates were coated with media produced by the cell lines, along with affinity-purified BHV-1 gB standard. After incubation with gB-specific monoclonal antibodies, the plates were washed and incubated with mouse IgG-specific horseradish peroxidase-

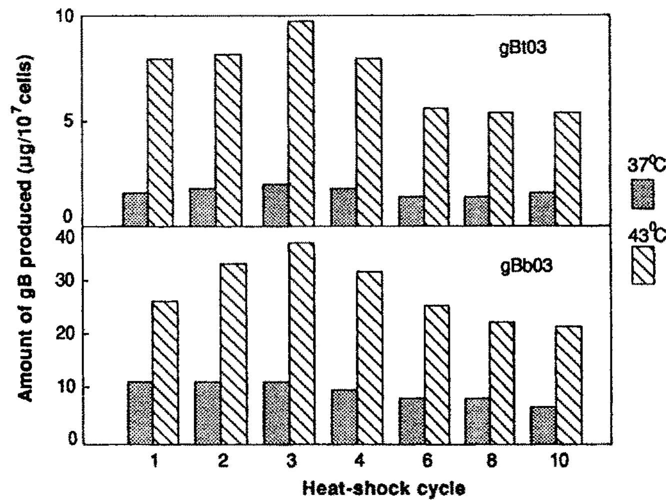

Fig. 2. Production of gBt and gBb by transfected cell lines in response to heat-shock treatment. Cell lines gBt03 and gBb03 were cultured in MEM with 10% FBS in T-150 flasks until confluent, culture media were then replaced with serum-free MEM, and cells were subjected to heat shock treatment. Each cycle of heat shock treatment consisted of incubating cells at 43 °C for 4 h, followed by incubation at 37 °C for 20 h. After each heat shock, culture medium was collected, and fresh serum-free MEM was added. The amount of gB present in the culture medium collected after each heat shock was determined by a quantitative ELISA using affinity-purified authentic gB as standard. The results represent averages of duplicate samples. Control, non-heat shocked cells were treated identically

conjugated goat antibody (BioRad, Mississauga, Ontario). This was followed by colour development with 2,2′-amino-di-(3-ethylbenzthiozoline sulfonate) (ABTS) (Boehringer-Mannheim, Laval, Quebec) and reading at 405 nm. The concentrations of gB products in the test samples were calculated based on the authentic gB standard. We found that in the absence of heat-shock, both cell lines exhibited a basal level of gB expression; after each heat shock, there was approximately a 2–5 fold increase in gBt and gBb production (Fig. 2). Both cell lines were found to be able to sustain their response to multiple heat shock treatments; even after the tenth cycle of heat-shock treatment there was still at least a two-fold induction of gB expression. In this particular experiment, after the tenth cycle, the accumulated gBt and gBb production per million cells was 7.5 µg and 28 µg, respectively. Since the two cell lines grew equally well, we speculated that the lower yield of gBt may be caused by a lower copy number of the gene present in the transfected cells.

An advantage of using the gB expressing cell lines is that the recombinant proteins are primarily secreted into the serum-free medium, and are therefore easier to purify. After collecting the media and passing them through an affinity column [20], the gB products are relatively pure. The purified gBs were analyzed for their reactivities with gB-specific monoclonal antibodies by an indirect ELISA and immunoprecipitation. Our results indicate that both gB products

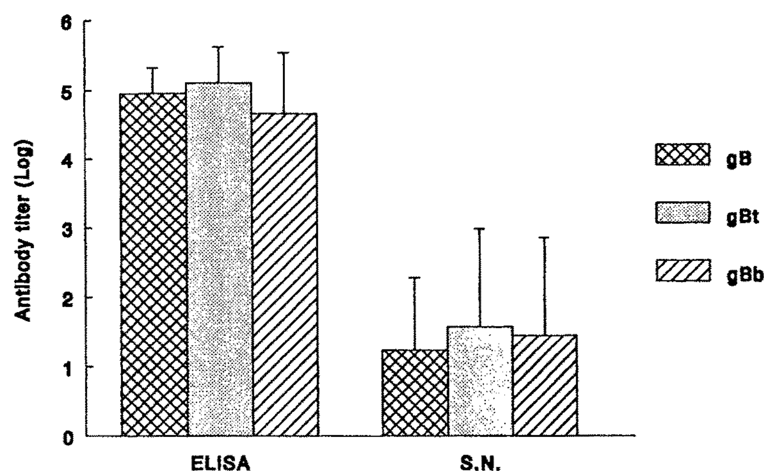

Fig. 3. Serum ELISA and neutralizing antibody titers of mice immunized with gB, gBt and gBb. Three groups of 10 mice each were primed with 10 µg of affinity-purified gB in complete Freund's adjuvant and boosted two weeks later with 10 µg of the same antigen in incomplete Freund's adjuvant. Serum antibody titers were determined by ELISA and virus neutralization assays (SN)

react with all gB-specific monoclonal antibodies tested, including some that only recognize conformation or glycosylation-dependent epitopes [19, 20] (data not shown). To further confirm that the recombinant gBs have authentic antigenicity, groups of CD-1 mouse were immunized with gBt, gBb or authentic gB. Mice were subcutaneously injected with 10 µg of affinity-purified protein in complete Freund's adjuvant, and boosted two weeks later with 10 µg of protein in incomplete Freund's adjuvant. One week after the boost, serum samples were collected and tested for gB-specific antibody titers and BHV-1 neutralization titers [13, 26]. As shown in Fig. 3, the three forms of gB induced comparable immune responses.

Previously, it has been shown that truncation in the cytoplasmic region of viral glycoproteins can change the protein's exocytosis pathway [3, 17]. We also focused on the processing and export of the recombinant gB products. By expressing gB in the mammalian cells, it is possible to study its structure and function without constructing viral mutants [16–18]. Results from immunoprecipitation suggested that gBt was able to form heterodimers similar to its authentic counterpart, and that the gBb had a similar molecular weight to authenitic gBb, indicating that both gB products produced by the cell lines were properly processed with respect to post-translational modification. In order to further characterize the maturation and transport processes of gBt and gBb, we performed pulse-chase experiments and compared the gB molecules produced by the expressing cell lines with authentic gB in BHV-1-infected cells. In these experiments, pulse-labeling was performed at 7 h post-infection for authentic gB and at 30 min after heat shock for gBt and gBb. Cells were starved for methionine

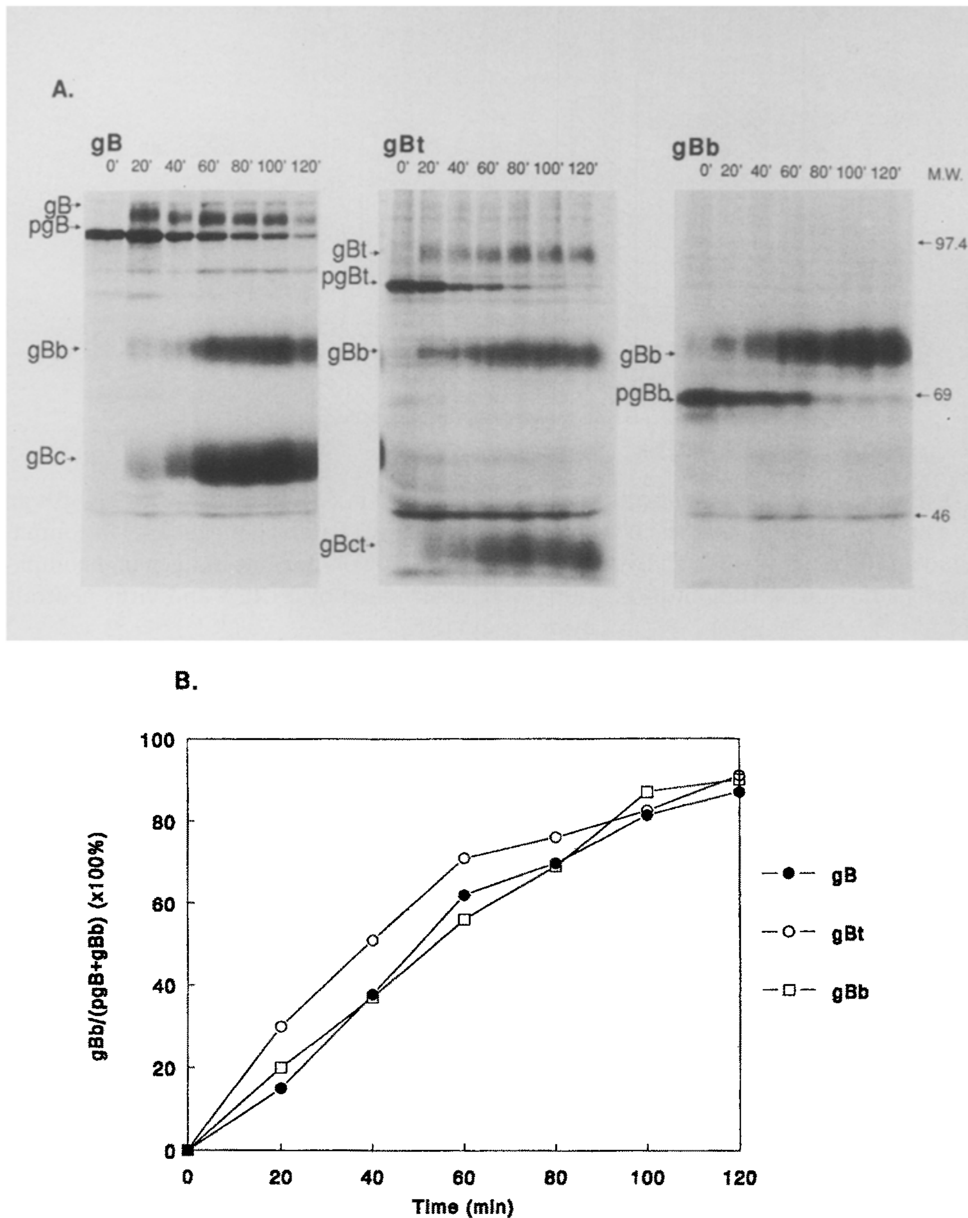

Fig. 4. Pulse-chase analysis of intracellular maturation of gB. MDBK cells infected with BHV-1 at 7 h postinfection and gB-expressing cell lines were pulse-labeled with ^{35}[S]-methionine for 30 min and chased with unlabeled methionine. At indicated time points, both cells and culture medium were collected, precipitated with gB-specific monoclonal antibodies and separated on SDS-8.5% polyacrylamide gels under reducing conditions (**A**). pgB, pgBt and pgBb are precursors and gB, gBt and gBb are mature products. The density of individual bands was scanned by a Bio-Rad video densitometer (model 620). The percent of gB converted to the mature form at a given time point was calculated on the basis of gBb, a subunit which is present in all three forms of the gB products. **B** shows the conversion curves of authentic gB, gBt and gBb by plotting the percent of the mature form of each of the gB products against chase time

in methionine-free medium for 30 min, and labeled by the addition of 200 μCi of [^{35}S]-methionine per ml of medium at 37 °C for 30 min. After labeling, supernatants were removed and cells were further incubated in MEM containing 2 mM methionine. In view of the fact that most of the gBb and gBt produced by the cell lines was secreted into the medium, while authentic gB was retained in cells, the samples from cellular and medium fractions were combined for detection of gB. At time 0, a dominant, 117 kDa band was detected in the BHV-1 infected cells, which represents high mannose oligosaccharide-containing gB precursor (Fig. 4 A, pgB) [21, 23]. This gB precursor was subsequently chased into a 130 kDa fully glycosylated, but uncleaved gB, as well as two cleaved products gBb and gBc. A similar pulse-chase pattern was observed with gBt. With respect to gBb expressed by cell line gBb03, the precursor existed as a 65 kDa band, which was chased into the 74 kDa mature gBb. As non-glycosylated gBb was reported to be 56 kDa [21], the observed 65 kDa gBb precursor is likely to contain the high-mannose oligosaccharides as does the authentic gB precursor. According to densitometry analysis, where the gBb bands present on all three forms of gB were used to calculate the conversion rates, gBt, gBb and authentic gB in BHV 1-infected cells showed similar rates of conversion from precursors to nature forms (Fig. 4B).

After we expressed the gB ectodomain, gBt, and its N-terminal subunit, gBb, we also expressed the truncated gBc portion containing residues 506 to 763, in the same expression system (Fig. 5A). In order to construct the transfer vector, a BHV-1 gC signal fragment was added to the N-terminal portion of gBct [6]. To our suprise, this product could not be successfully transported out of the cells (Fig. 5B). The C-terminal subunit of gBt is a fully glycosylated product, which has a molecular weight of 38 kDa (c, Fig. 5B and C). The recombinant gBct has an apparent molecular weight of 36 kDa, and is retained intracellularly (Fig. 5B). In order to compare the polypeptide backbone of gBct to that of gBt, we performed deglycosylation as previously reported [24]. It was found that the C-terminal subunit of gBt (c) could be reduced to a 34 kDa product by N-glycosidase F (PNGase, F, Boehringer-Mannheim) digestion (c2, Fig. 5C), which corresponds to the molecular weight of the unglycosylated polypeptide from residue 506 to 763. This subunit is also partially sensitive to endoglycosidase H (endo H, Boehringer-Mannheim) digestion (Fig. 5C). These results indicate that the C-terminal suunit of gBt contains complex oligosaccharides as well as high-mannose oligosaccharides which are sensitive to PNGase F and endo H respectively. The product c1 in PNGase F treatment probably is an incompletely deglycosylated product. This result is consistent with our previous observation [21] that gBc prossesses N-linked oligosaccharides of the high-mannose type. Both endo H and PNGase F modified the recombinant gBct to migrate at the c2 position, suggesting that it only contains high-mannose oligosaccharides, but no complex oligosaccharides. Our results indicate that the recombinant gBct is the same polypeptide as the C-terminal subunit of gBt. However, this product is not fully glycosylated by enzymes in the Golgi, because it can not be transported properly.

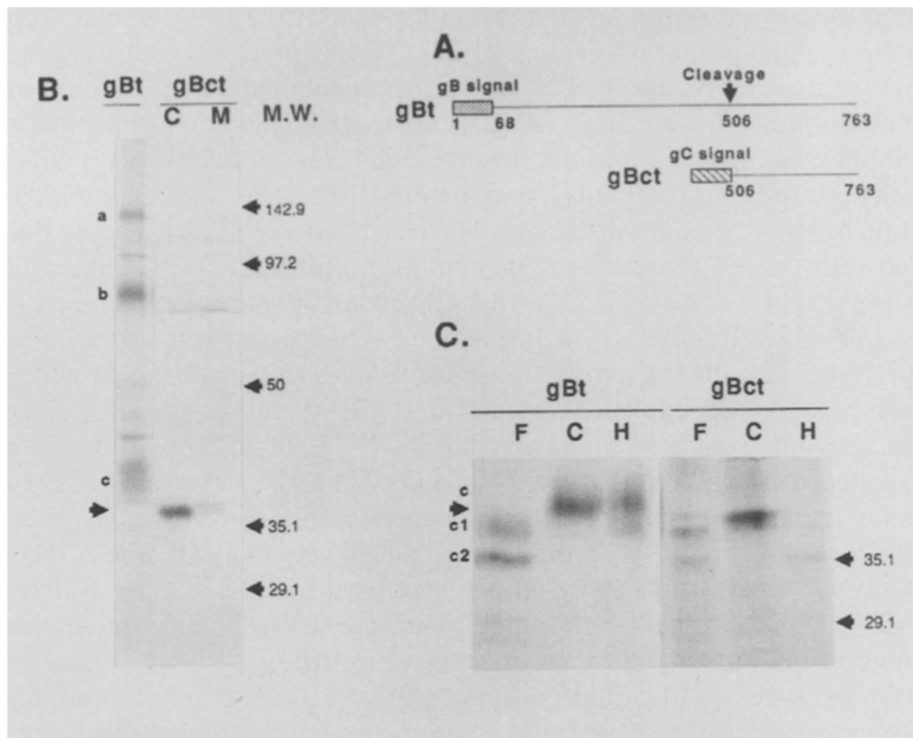

Fig. 5. Expression and deglycosylation of the C-terminal subunit of gBt. Schematic diagram of gBct expression which has a BHV-1 gC signal at the N-terminal position is shown in **A**. Samples from cells (*C* in **B**) or media (*M* in **B**) were immunoprecipitated with gBc-specific polyclonal antibody (20) and separated on SDS-8.5% polyacrylamide gels under reducing conditions with a gBt sample as a control (**B**). *a*, *b*, *c* correspond to uncleaved mature gBt and its cleaved products repectively. Endo H (*H*) and PNGase F (*F*) treatment of gBt and gBct are shown in **C**. The capital *C* on top indicates untreated control. *c1* and *c2* indicate two forms of endoglycosidase digested products from c (**C**). *M.W.* Molecular weight standard (kDa)

The processing and transport of viral glycoproteins have been studied extensively in other viruses especially for vesicular stomatitis virus G protein and paramyxovirus hemagglutinin-neuraminidase protein [3]. Viral glycoproteins are synthesized on the ribosomes located in the rough endoplasmic reticulum, then they pass through cellular organelles to obtain proper posttranslational modifications. In eukaryotic cells, proteins enter the exocytic pathway at the rough endoplasmic reticulum membrane and travel through the Golgi to the cell surface or to other cellular organelles. This process requires the protein to be in the correct conformation. Mutations which block the initial folding and perturb oligomerization can stop the transport to the cell surface [3]. In our study, both gBt and gBb underwent intracellular processing similar to authentic gB (Fig. 4), indicating that their overall conformation might be similar to authentic gB. Since we found that gBt forms dimer like authentic gB, but gBb does not (manuscript in prep.), the oligomer formation of recombinant gBs may not be critical for their

transport from the ER to the Golgi, therefore, another potential conformational signal may be responsible for transport. As the N-terminal subunit of gBt, gBb is successfully transported, this raises the question as to which part can provide such a transport signal. Obviously, gBb is the common component in both recombinant products. This was further supported by the expression of gBct, the C-terminal subunit of gBt. This subunit was found to be retained in the ER as a precursor form with high-mannose oligosaccharides, suggesting that the N-terminal subunit of gB, gBb, is required for the transport of the gB ectodomain from the ER to the Golgi where appropriate glycosylation and proteolysis occur. Previous study in HSV gB also suggested that for the efficient transport through the ER and the Golgi, a fragment which contains residues 441–475 is required, whereas the molecular dimerization is not [17]. From our results, it is possible to draw a three-dimensional picture for recombinant gBt where the gBb subunit must be exposed to provide functional conformation in the intracellular environment, whereas the C-terminal part, gBct, is not. This hypothesis is also supported by the antigenic comparison between the two recombinant gBs and the authentic gB in that they all raise comparable levels of antibody response in laboratory animals. The observation that gBb, which lacks the small subunit, was as efficacious as authentic gB in inducing an immune response is consistent with observation from a study involving monoclonal antibody mapping [8] and further supports the conclusion that the dominant immunogenic epitopes of gB are located within the gBb subunit. This subunit may also be responsible for inducing protective immunity in cattle [9, 10, 12], suggesting that gBb is the most exposed and immunogenic part in BHV-1 gB.

The hsp70A gene promoter-based expression system differs from most other commonly used expression systems since it does not require the addition of exogenous inducing agents. The induction of foreign gene expression can be simply carried out by raising the temperature. In this study, we extended the characterization of this system involving the expression of a highly complex viral glycoprotein, BHV-1 gB. The results show that induction of foreign gene expression by heat treatment in MDBK cells had no adverse effect on various properties of the expressed product such as intracellular processing and antigenicity; high levels of foreign gene expression could be readily achieved by multiple heat shock cycles of the cells. The results of this study demonstrate that both gBt and gBb can be expressed at high levels. The resultant gB products appear to be fully processed, secreted, and to retain antigenic properties similar to their authentic counterpart. This observation provides the basis for future studies using recombinant gBs as subunit vaccine candidates.

In conclusion, the observations made in this study have demonstrated that in BHV-1 gB, gBb is required for the transport of the gB ectodomain from the ER to the Golgi. This study provides as with further information about intracellular processing of BHV-1 gB, and substantiates the utility of the novel, hsp70 gene-promoter based expression system. Furthermore, the availability of gBt and gBb in relatively high quantities should facilitate future studies of this important viral component and its potential use as a BHV-1 vaccine.

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