Glycoprotein Bb, the N-Terminal Subunit of Bovine Herpesvirus 1 gB, Can Bind to Heparan Sulfate on the Surfaces of Madin-Darby Bovine Kidney Cells†

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The present study confirms our previous finding made by using heparin affinity chromatography that bovine herpesvirus 1 gB can bind to heparin-like structures. In order to locate the functional domain for heparin binding, we expressed the extracellular portion of gB (gBt) and the large subunit of gB (gBb) in Madin Darby bovine kidney (MDBK) cells under the control of the bovine heat shock protein 70A gene promoter. The recombinant gBt and gBb were both efficiently secreted from the transfected cells. They were shown to have structural and antigenic properties similar to those of authentic gB. Like authentic gB, both gBt and gBb were able to bind heparin-Sepharose as well as heparan sulfates on MDBK cells. Thus, we suggest that at least one heparin-binding domain is localized in gBb, the N-terminal portion of gB, which agrees with the presence of clusters of prolines and basic residues, thought to be essential for heparin binding.

Bovine herpesvirus 1 (BHV-1), a member of the *Alphaherpesviridae* subfamily, is an economically important pathogen in cattle (9). The genome of BHV-1 encodes more than 33 structural proteins, among which there are at least nine membrane-associated glycoproteins (1, 6, 15, 16, 18, 25, 29–31, 37). The three major glycoproteins, gB, gC, and gD, have been shown to play a role in the virus entry process (20). gC is the dominant heparin-binding glycoprotein involved in the initial step in virus adsorption to cells, whereas gB and gD are important for subsequent interactions (21, 35). Previous studies have shown that BHV-1 gC but not gB could bind heparin (33). However, during our characterization of cell-binding properties of the three major BHV-1 glycoproteins, we found that the binding of both gB and gC to MDBK cells could be inhibited by exogenous heparin, suggesting that BHV-1 gB is also involved in heparin binding (19). This is consistent with observations for herpes simplex virus 1 (HSV-1) and a recent report for BHV-1 (3, 12). In order to gain a better understanding of the function of gB in BHV-1 entry, we carried out further studies on the heparin-binding property of gB.

BHV-1 gB consists of 933 amino acids with features characteristic of type I integral membrane proteins, including a putative signal peptide sequence located between amino acid residues 50 and 67 and a transmembrane domain between residues 759 and 828 that is followed by a 104-amino-acid cytoplasmic tail (42, 47). Like the gB homologs of most other herpesviruses, including pseudorabies virus (PRV) (22), varicella-zoster virus (32), human cytomegalovirus (2), and equine herpesvirus (27, 45), mature BHV-1 gB exists predominantly as a covalently linked heterodimeric complex derived from a common primary translation product by proteolytic cleavage, while a small fraction remains in an uncleaved form (22, 27, 44). This cleavage event does not occur in HSV gB. As one of the

essential viral glycoproteins, BHV-1 gB plays an important role in virus entry, including initial virus attachment and subsequent penetration involving membrane fusion (5, 7, 8, 20, 42).

We previously demonstrated that purified gB as well as gC and gD could effectively block infection with both wild-type and gC-negative BHV-1 when added during virus adsorption, suggesting that these three major glycoproteins may participate in the BHV-1 attachment process (20). In a subsequent study, we demonstrated that all three major glycoproteins have saturable cellular receptors which are heparan sulfate (HS) or non-HS components (19). We also demonstrated that gB has two binding sites to bind to HS and an unknown non-HS component on MDBK cells. An initial gB-heparin binding event is required for the secondary binding to the non-HS cellular receptor. Our observation that gB binds to heparin is in agreement with a recent report by Byrne et al. (3) but is contradictory to a previous report by Okazaki et al. (33). In both previous studies, heparin chromatography, which has also been widely used to identify the heparin-binding proteins in other herpesviruses, was used (4, 12, 28, 38). In order to confirm that BHV-1 gB does bind to heparin, we used the same approach in the present study.

To assess the binding of the viral protein to heparin, BHV-1-infected MDBK cells were labeled with 50 μ Ci of [³⁵S]methionine-cysteine (ICN) per ml at 37°C for 6 to 10 h and then lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl [pH 7.5], 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.02% NaN₃). Cell lysates (0.5 ml) were incubated with 0.5 ml of heparin-Sepharose (Pharmacia), which was saturated in RIPA buffer. After 1 h at 4°C, the supernatant was collected as the unbound fraction and the heparin-Sepharose was extensively washed with RIPA buffer. The bound proteins were dissociated from the heparin-Sepharose by incubation with a mixture of RIPA buffer, 2% SDS, and 2 mg of heparin per ml at 60°C for 5 min. All the samples including original lysates and unbound and bound fractions were subjected to immunoprecipitation. As shown in Fig. 1A, all three glycoproteins were present in the original cell lysates. After incubation of infected

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FIG. 1. Binding of BHV-1 glycoproteins to heparin-Sepharose. ^{35}S -labeled BHV-1-infected MDBK cell lysates were incubated with heparin-Sepharose. The samples of original lysates before heparin-Sepharose incubation (A), unbound cell lysates after incubation with heparin-Sepharose (B), and bound proteins dissociated from heparin-Sepharose (C) were subjected to immunoprecipitation with pooled monoclonal antibodies against gB, gC, or gD. Molecular mass standards (kilodaltons) are indicated on the right.

cell lysates with heparin-Sepharose, the amounts of both gC and gB were greatly reduced in the unbound fraction but the amount of gD was not (Fig. 1B). In addition, gC and gB, but not gD, could be recovered from the bound proteins released from the heparin-Sepharose (Fig. 1C). These data confirm that gC and gB have the ability to bind heparin.

In order to demonstrate that under our experimental conditions heparin binding of gB and gC was indeed a specific event, we tried to block this binding by using soluble heparin, as well as chondroitin sulfate and dermatan sulfate (Sigma), two other glycosaminoglycans found on cell surfaces. We found that at a concentration of 100 $\mu\text{g}/\text{ml}$, soluble heparin could block nearly all of the gC binding and 50% of the gB binding to heparin-Sepharose (Fig. 2). However, even at a concentration of 1,000 $\mu\text{g}/\text{ml}$, chondroitin sulfate and dermatan sulfate could not inhibit the binding of gB or gC to heparin-Sepharose. In this experiment, soluble heparin showed a differential capacity to inhibit the binding of gB and gC to heparin-Sepharose. The highest heparin concentration used completely blocked the binding of gC to heparin-Sepharose but blocked only 75% of the binding of gB (Fig. 2C). It is of interest that soluble heparin inhibited the binding of gC to heparin-Sepharose more efficiently than the binding of gB (Fig. 2). This difference provides evidence that BHV-1 gB and gC have different avidities in heparin binding. In HSV, gB and gC have been found to bind to different structures of heparin (10, 11, 34). It was suggested that HSV gB interacts with more highly sulfated regions of HS, which may cause gC-negative HSV mutants to be more resistant to heparin washing than wild-type viruses, in which gC-heparin binding is dominant (11). In our study, gB and gC may bind differently to immobilized heparin, resulting in the different sensitivities to heparin inhibition. In contrast, soluble heparin, which was used as the inhibitory reagent, has a lower molecular weight than Sepharose-immobilized heparin. It was previously found that heparin with shorter saccharide chains may be less efficient in interacting with viral heparin-binding proteins (23). Therefore, the immobilized heparin might have higher avidity than the soluble heparin in gB binding, thereby resulting in the incomplete inhibition of the binding of gB to heparin-Sepharose by soluble

heparin (Fig. 2). The difference in binding avidity of gB for soluble heparin and immobilized heparin may also explain the previous failure to recognize gB as a heparin-binding protein (33), because soluble heparin may not be able to recover the bound gB from heparin-Sepharose under certain experimental conditions. Byrne et al. also found that under certain circumstances, gB bound to the heparin matrix could not be eluted by heparin solution (3). In order to dissociate bound gB from the Sepharose, optimized conditions different from those previously reported (3, 4, 12, 28, 33, 38) were used in the present study.

To further study the heparin-binding property of gB, we expressed the extracellular portion (gBt) and the N-terminal subunit (gBb) of gB in mammalian cells. BHV-1 gB was synthesized as a 933-amino-acid precursor, which was subsequently cleaved into two subunits, i.e., the amino-terminal fragment, gBb, and the carboxyl-terminal fragment, gBc (40, 47). The location of the cleavage site has been confirmed by amino-terminal sequencing of gBc showing that alanine at position 506 is the amino-terminal residue (42). On the basis of the location of the transmembrane anchor and the cleavage

FIG. 2. Heparin inhibition of binding of gB and gC to heparin-Sepharose. ^{35}S -labeled BHV-1-infected MDBK cell lysates were first incubated with different amounts of heparin, chondroitin sulfate (C.S.), or dermatan sulfate (D.S.) at 4°C for 1 h and then further incubated with heparin-Sepharose for another hour. After extensive washing, bound proteins were dissociated and subjected to immunoprecipitation to detect gB (A) and gC (B). The precipitated bands of heparin incubation groups were scanned by a Bio-Rad video densitometer (model 620), and the amounts in each band are indicated as a percentage of the control which has no heparin as an inhibitive reagent (C). Concentration units in panels A and B are micrograms per milliliter.

FIG. 3. Immunoprecipitation of gBt and gBb from transfected cell lines. Cells grown in T-25 flasks were heat shocked at 43°C for 6 h (+HS) or left untreated (–HS) and labeled with [³⁵S]Met-Cys. Cells and culture medium were collected separately and each precipitated with gB-specific monoclonal antibodies. The samples were separated on SDS–8.5% polyacrylamide gels under non-reducing (A) or reducing (B) conditions. Lanes BHV1, gB precipitated from the wild-type BHV-1-infected MDBK cells; lanes C, cellular fraction; lanes M, medium; HS, heat shock. Molecular mass standards (kilodaltons) (KD) are indicated on the right.

site, the gBt and gBb genes were constructed so that they contained the sequences coding for residues 1 to 763 and residues 1 to 505, respectively. The plasmid p3KHSPG4HU (17), which contains a truncated BHV-1 gD gene under the control of the bovine heat shock protein 70A gene promoter and the aminoglycoside phosphotransferase gene under the control of the simian virus 40 early promoter, was used as the parental plasmid for constructing gBt and gBb transfer vectors. The newly generated plasmids p3KHSPGBt and p3KHSPGBb have the gBt and gBb genes, respectively, in place of the truncated gD gene in the parental plasmid. By using the Lipofectin transfection method, two stable G418-resistant cell lines, gBt-03 and gBb-03, were established to express gBt and gBb, respectively. Both gB-expressing cell lines exhibited morphology and growth properties indistinguishable from those of parental MDBK cells. The expression of gB products can be induced by incubating the cell lines at 43°C, i.e., heat shock treatment. The heat-enhanced gBt expression is shown in Fig. 3A, lanes M. Under the conditions tested, both forms of expressed gB were readily detected in the culture medium (Fig. 3A, lanes C). Under nonreducing conditions, gBt existed as a single band with a molecular mass of about 115 kDa (Fig. 3A); under reducing conditions, the 74-kDa and 38-kDa bands were dominant, although the 115-kDa band was still visible (Fig. 3B). The 74-kDa band had the same mobility as the gBb subunit present in the BHV-1-infected cell lysate, whereas the 38-kDa band was significantly smaller than the authentic gBc. The difference in apparent molecular mass between the truncated and authentic gBc was estimated to be 17 kDa, which approximates the estimated molecular mass of the truncated portion of gB. Therefore, the reduced molecular mass of gBt could be attributed to the truncation of its transmembrane and cytoplasmic domains. With respect to gBb, the product expressed by

cell line gBb-03 had a mobility similar to that of authentic gBb (Fig. 3B). On the basis of these studies, we anticipated that the expressed gBt and gBb from the bovine heat shock expression system were structurally similar to authentic gB. The maturation rates of the recombinant gBs were also compared with that of the authentic gB by pulse-chase experiments and were found to be similar. Our results indicated that all three forms of gB have similar glycosylation (data not shown). In order to compare the antigenicity of the recombinant gBs with that of the authentic gB, a panel of gB-specific monoclonal antibodies and laboratory animals were used. We found that both gB products reacted with all eight monoclonal antibodies tested with a degree of reactivity similar to that exhibited by authentic gB in the enzyme-linked immunosorbent assay (ELISA) (Table 1). Similar results were observed with immunoprecipitation assays (data not shown). Among these monoclonal antibodies, some recognize only properly configured or glycosylated epitopes (40, 41). Therefore, our results suggested that both gBt and gBb retain not only linear but also conformation- and glycosylation-dependent epitopes. We also compared the immunogenicity of the expressed gB products with authentic gB by assessing their ability to elicit immune responses in mice. We found that all three forms of gB, authentic gB, gBt, and gBb, induced comparable titers of total anti-BHV-1 antibody and of anti-BHV-1 neutralizing antibody (data not shown).

Since the recombinant gBt and gBb were structurally and antigenically similar to the authentic gB, they were used as reagents for further studies to identify the region of gB that contains the heparin-binding activity. The expressing cells were first subjected to heat shock for 4 h at 43°C and then starved and labeled as above. After labeling, samples of the supernatant were harvested and used for a heparin affinity chromatography study. Both gBt and gBb were found to bind to heparin-Sepharose (Fig. 4C). These results are consistent with those for BHV-1-infected cell lysates (Fig. 4B and C), indicating that authentic gB, gBt, and gBb could all bind heparin. The heparin-binding profiles of gC and gD served as controls and were consistent with previous results (Fig. 1).

To demonstrate that gBt and gBb could bind HS on permissive MDBK cells, we incubated MDBK cells with affinity-purified, iodine-labeled gBt or gBb, as previously reported (19). We found that soluble heparin blocked the binding of glycoprotein to cells in a dose-dependent manner (Fig. 5A), indicating that binding is mediated by HS. This result was further supported by experiments in which cells depleted of HS by heparinase treatment were not able to bind labeled proteins

TABLE 1. Reactivity of monoclonal antibodies with gB, gBt, and gBb^a

MAb	Epitope	ELISA titer ^b		
		gB	gBt	gBb
3F3	II	2,560	2,560	2,560
1E11	III	1,280	1,280	1,280
1F8	IVa	1,280	1,280	640
3C7	IVb	1,280	2,560	1,280
3G11	IVb	640	1,280	640
5G11	IVc	1,280	1,280	640
6G11	IVc	640	1,280	640
1F10	V	1,280	640	640

^a gB monoclonal antibodies (MAbs) and epitopes were described previously (39, 43).

^b Titers are the reciprocals of the highest dilution of gB products which show positive ELISA values (an optical density at 405 nm of at least 0.1). A 1:10 dilution corresponds to 0.04 µg of protein per well (17).

FIG. 4. Binding of radiolabeled gBt and gBb to heparin-Sepharose. ^{35}S -labeled BHV-1-infected MDBK cell lysates and media from gBt-03 or gBb-03 were incubated with heparin-Sepharose. The samples of original lysates or media before heparin-Sepharose incubation (A), unbound cell lysates or media after incubation with heparin-Sepharose (B), and bound proteins dissociated from heparin-Sepharose (C) were subjected to immunoprecipitation with pooled monoclonal antibodies against gB, gC, or gD. In lanes BHV, immunoprecipitations were done with combined monoclonal antibody pools (gB-gC-gD) or with the indicated individual pools (C). Molecular mass standards (kilodaltons) are indicated on the right.

(Fig. 5B). Although both gBt and gBb can specifically bind HS on the surfaces of MDBK cells, they may have different binding capacities, since at low heparin concentrations (0.1 to 10 $\mu\text{g}/\text{ml}$), gBb expressed higher levels of binding to cells than gBt, and it also showed slightly higher binding levels in the heparin-

nase treatment experiment (Fig. 5). These data suggest that the gBb subunit has the heparin-binding activity.

Previous studies of HSV and PRV have shown that the interaction between gC and cellular HS plays the dominant role in initial viral attachment (13, 26, 46, 48). However, it has been found that gC-negative HSV-1 has the same heparin-dependent binding pattern as the wild-type virus, whereas for gC-negative PRV, a proteoglycan-independent attachment was reported (14, 28), indicating that different mechanisms could exist for the early events in entry of different herpesviruses. In regard to BHV-1, our previous data showed that heparin is involved in the attachment of gC-negative BHV-1 (19), which suggested that another viral component(s) can bind HS. HSV gB has been found to bind to heparin-Sepharose columns (12), whereas gB of PRV can bind heparin only in the presence of gC (28). In contrast, gC was previously reported to be the only heparin-binding protein in BHV-1 (33), which is contradictory to a recent report and our studies (3, 19; also this paper). We suspect that differences in the experimental conditions between the study by Okazaki et al. and our studies are responsible for the different conclusion.

In this communication, we describe the production and characterization of gBt and gBb. Both gB products have proper posttranslational modifications, including intrachain disulfide bond formation, proteolytic cleavage, and glycosylation, indicating that they possess structural and antigenic properties similar to those of authentic gB. Both gBt and gBb were able to bind heparin-Sepharose (Fig. 4); in addition, the iodinated proteins could bind to MDBK cells in a heparin-sensitive manner (Fig. 5). The different capacities of the two gBs for binding to cellular HS may be caused by a conformational change that may result in better exposure of the heparin-binding domain(s) on gBb. It has been suggested that clusters of basic amino acids, including lysine and arginine, are involved in the binding of polyanionic heparin-like structures by viral glycoproteins

FIG. 5. Effects of heparin and heparinase treatment on binding of iodinated gBt and gBb to MDBK cells. For the heparin inhibition assay (A), confluent MDBK cells were incubated with heparin at the indicated concentrations at 4°C for 1 h, which was followed by the addition of ^{125}I -labeled gBt or gBb and incubation at 4°C for 4 h. Cell-associated radioactivity was measured with a gamma counter. For heparinase treatment (B), cells were first treated with heparinase at the indicated concentrations at 37°C for 1 h. Then the cells were incubated with ^{125}I -labeled gBt or gBb for 4 h at 4°C , which was then followed by washing and counting. The counts per minute bound in the presence of 100 μg of the unlabeled protein per ml was defined as the background count, which has been subtracted from all sample counts. The amount bound in the absence of the inhibitive factor was defined as 100%. The curves were drawn from triplicate sample counts, and standard deviations are indicated.

TABLE 2. Basic amino acid clusters in BHV-1 gB^a

Position	Sequence
212–221.....	LV DKKW R CL S
225–234.....	Y LR S GR K V V A
343–352.....	A T G RR L KE P V
368–377.....	W V P K R K N V C S

^a Sequence from T. Zamb (47). Boldface indicates clusters of lysine and arginine.

(21, 36). In HSV gB, a short lysine- and proline-rich region located near the N-terminal portion of the polypeptide was thought to be the heparin-binding domain (36). The basic residue clusters, B-B-X-B and B-B-B-X-X-B, found in other heparin-binding proteins have been recognized as heparin-binding motifs (24) in which the basic amino acids (B) and the hydrophobic amino acids (X) are specifically arranged. Although in BHV-1 gB, there is no obvious lysine-rich region like that in HSV, we found some basic residue clusters (Table 2) and a proline-rich region in the N-terminal portion of gB. These regions may be the potential heparin-binding domain(s). As shown in Fig. 2A and Fig. 4, the precursor form of gB was also found to bind to heparin-Sepharose, indicating that the binding of gB to heparin does not require intracellular modifications such as glycosylation and proteolytic cleavage. Therefore, the primary sequence but not the posttranslational modification appears to be important for the heparin binding of gB. Comparison of the binding of gBt and gBb to MDBK cells revealed that the heparin-binding activity was mostly correlated with gBb (Fig. 5). To prove that gBb is the functional heparin-binding domain, we also expressed the truncated gBc part, residues 506 to 763 of gB, and examined its heparin-binding ability. We found that this truncated C-terminal part of gB cannot bind heparin-Sepharose (data not shown). On the basis of these observations, we conclude that the potential heparin-binding domain(s) of gB is located in the N-terminal gBb region.

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