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Bovine Herpesvirus 1 Glycoprotein B Does Not Productively Interact with Cell Surface Heparan Sulfate in a Pseudorabies Virion Background

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Attachment to cell surface heparan sulfate proteoglycans is the first step in infection by several alphaherpesviruses. This interaction is primarily mediated by virion glycoprotein C (gC). In herpes simplex virus, in the absence of the nonessential gC, heparan sulfate binding is effected by glycoprotein B. In contrast, gC-negative pseudorabies virus (PrV) infects target cells via a heparan sulfate-independent mechanism, indicating that PrV virion gB does not productively interact with heparan sulfate. To assay whether a heterologous alphaherpesvirus gB protein will confer productive heparan sulfate binding on gC-negative PrV, gC was deleted from an infectious PrV recombinant, PrV-9112C2, which expresses bovine herpesvirus 1 (BHV-1) gB instead of PrV gB. Our data show that gC-negative PrV–BHV-1 gB recombinant 9112C2-ΔgCβ was not inhibited in infection by soluble heparin, in contrast to the gC-positive parental strain. Similar results were obtained when wild-type BHV-1 was compared with a gC-negative BHV-1 mutant. Moreover, infection of cells proficient or deficient in heparan sulfate biosynthesis occurred with equal efficiency by PrV-9112C2-ΔgCβ, whereas heparan sulfate-positive cells showed an approximately fivefold higher plating efficiency than heparan sulfate-negative cells with the parental gC-positive virus. In summary, our data show that in a PrV gC-negative virion background, BHV-1 gB is not able to mediate infection by productive interaction with heparan sulfate, and they indicate the same lack of heparin interaction for BHV-1 gB in gC-negative BHV-1.

Initiation of infection by several herpesviruses involves binding to heparan sulfate-carrying proteoglycans on the surfaces of target cells (2, 18, 19, 24, 26, 28, 29). This primary interaction is mediated by the glycoprotein C (gC) homologs of herpes simplex virus (HSV) (5), bovine herpesvirus 1 (BHV-1) (13, 19), and pseudorabies virus (PrV) (9, 18, 22, 30). Since gC is nonessential for productive replication of these viruses, gC-negative virus mutants either rely on other virion components to substitute for the missing gC function or infect cells via a heparan sulfate-independent mechanism (14, 25). In HSV, the essential glycoprotein B (gB) has been shown to effect heparan sulfate binding in the absence of gC (4). Therefore, gC-negative HSV is still sensitive to inhibition of infection by soluble heparin, and consequently, it infects heparan sulfate-deficient cells significantly less efficiently than cells which are not impaired in proteoglycan biosynthesis (4). In contrast, gC-negative PrV infects cells by a heparan sulfate-independent mechanism, which is indicated by the resistance of gC-negative PrV to inhibition by exogenous heparin (18) as well as by its equal plating efficiency on cells irrespective of the presence of heparan sulfate proteoglycans (8). This demonstrates that in the absence of gC, no other virion component is able to productively interact with cell surface heparan sulfate, resulting in infection, despite the proposed heparin-binding activity of isolated PrV gB (22, 23). To analyze whether a heterologous herpesvirus gB is able to provide gC-negative PrV with heparan sulfate-binding competence, we analyzed a recombinant PrV which lacks PrV gB but stably expresses BHV-1 gB. BHV-1 gB has previously been shown to functionally complement a replication-defective gB-negative PrV by transcomplementation (21) as well as after recombination of the BHV-1 gB gene into the genome of gB-negative PrV (10). Relevant to the studies reported here is the demonstration that isolated BHV-1 gB exhibits efficient heparin-binding activity (1, 11, 12).

To test whether BHV-1 gB exerts heparin-binding functions in a PrV background, the UL44 gene, which encodes gC, was deleted in PrV–BHV-1 gB recombinant 9112C2 (10). To this end, in a cloned 4.1-kbp *Pst*I fragment of wild-type PrV strain Ka DNA (7), the major portion of the UL44 open reading frame, as well as upstream sequences which also contain the 3′ terminus of the nonessential UL43 gene (20), was deleted (17). Into the remaining *Bam*HI site at the 3′ end of the UL44 gene, a gG–β-galactosidase (gG-βGal) expression cassette was inserted (Fig. 1) to allow easy identification of viral mutants (15), resulting in plasmid pBR-TT321. After cotransfection of virion DNA of recombinant 9112C2 with pBR-TT321 into Vero cells, viral progeny was screened for the appearance of blue plaques under a Bluo-Gal (Life Technologies, Eggenstein, Germany) agarose overlay. Blue plaques were picked by aspiration and purified three times. One representative isolate, designated PrV-9112C2-ΔgCβ, was further analyzed.

To check for correct deletion of the gC gene and insertion of the gG-βGal expression cassette, virion DNAs of PrV-9112C2 (Fig. 2, lanes 1) and PrV-9112C2-ΔgCβ (Fig. 2, lanes 2) were cleaved with *Bam*HI and the resulting fragments were separated in a 0.7% agarose gel. Fig. 2A shows the ethidium bromide-stained gel. It is evident that *Bam*HI fragment 2 of PrV-9112C2-ΔgCβ is increased in size compared to that of PrV-9112C2 and nearly comigrates with *Bam*HI fragment 1 of PrV-9112C2. This increase can be accounted for by insertion of the gG-βGal cassette. Hybridization with *Bam*HI fragment 2 (Fig. 2B) verified the identities of the respective fragments. Hybridization with a UL44-specific probe resulted in a signal in PrV-9112C2 but an absence of reactivity in PrV-9112C2-ΔgCβ,

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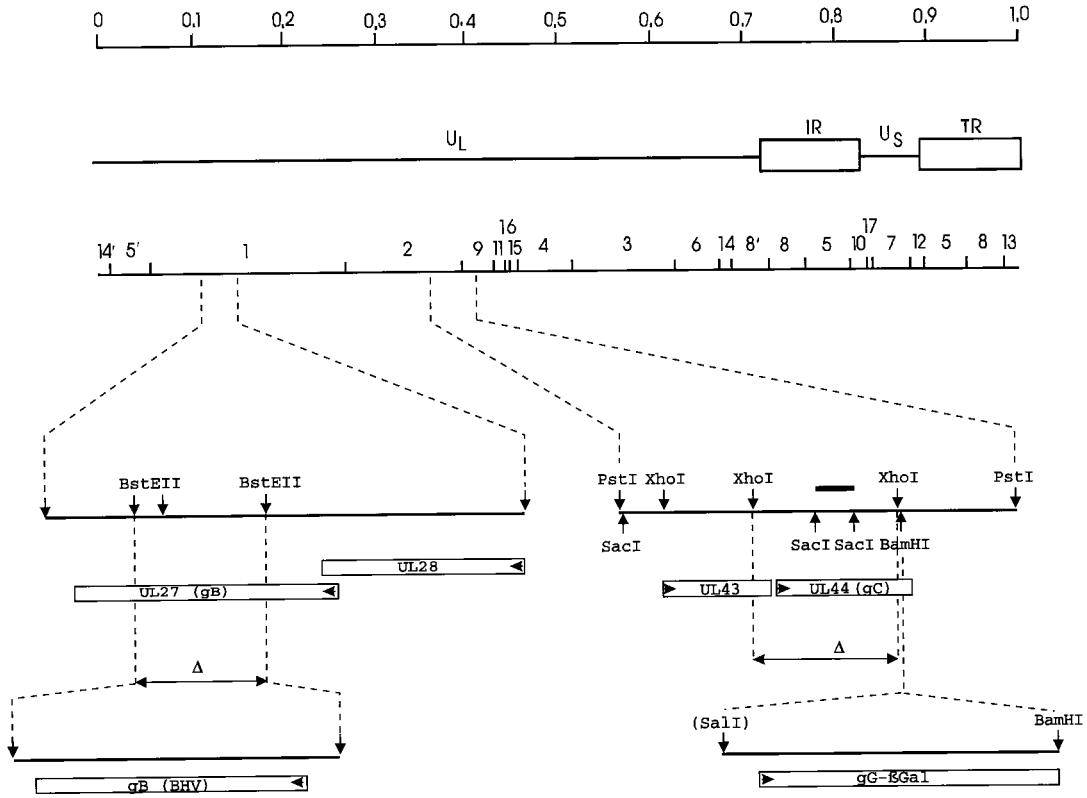

FIG. 1. Construction of PrV mutants. The PrV genome consists of a unique long (U_L) region and a unique short (U_S) region which is flanked by inverted repeats (IR, internal repeat; TR, terminal repeat) marked as boxes. After partial deletion of the PrV gB gene by *Bst*EII cleavage, the BHV-1 gB gene, including 5′ and 3′ transcriptional control elements, was inserted, resulting in PrV-9112C2 (10). Deletion of a 1.4-kbp *Xho*I fragment from the 4.1-kbp *Pst*I fragment encompassing the UL43 and UL44 genes resulted in deletion of most of the UL44 (gC) gene and also eliminated the 3′ terminus of the UL43 gene. Into the remaining *Bam*HI site a 3.5-kbp *Sal*I/*Bam*HI gG-βGal expression cassette was inserted, yielding PrV-9112C2-ΔgCβ. The *Sac*I fragment used as a UL44-specific hybridization probe (see Fig. 2) is indicated by a black bar.

demonstrating deletion of UL44 sequences (Fig. 2C). Insertion of the expression cassette is highlighted by hybridization with a β-Gal-specific probe (Fig. 2D).

To assay glycoprotein expression, virions were metabolically labelled with [^{35}S]methionine and [^{35}S]cysteine (Tran^{35}S-Label; ICN, Meckenheim, Germany); purified by centrifugation through a 40% sucrose cushion; immunoprecipitated with monoclonal antibodies against PrV gB (Fig. 3, lanes 1 to 4), BHV-1 gB (Fig. 3, lanes 5 to 8), PrV gC (Fig. 3, lanes 9 to 12), and PrV gD (Fig. 3, lanes 13 to 16); and analyzed in a reducing sodium dodecyl sulfate–10% polyacrylamide gel. Precipitation of wild-type PrV strain Ka virions showed the presence of PrV gB (Fig. 3, lane 1), PrV gC (Fig. 3, lane 9), and PrV gD (Fig. 3, lane 13) and the absence of BHV-1 gB (Fig. 3, lane 5). Analysis of gC-negative PrV demonstrated the presence of PrV gB and gD (Fig. 3, lanes 2 and 14) and the absence of BHV-1 gB and PrV gC (Fig. 3, lanes 6 and 10). In contrast, recombinant PrV-9112C2 did not specify PrV gB (Fig. 3, lane 3) but contained BHV-1 gB (Fig. 3, lane 7). PrV gC and gD were present as expected (Fig. 3, lanes 11 and 15). PrV-9112C2-ΔgCβ showed the presence of BHV-1 gB and PrV gD (Fig. 3, lanes 8 and 16) and a lack of PrV gB and gC (Fig. 3, lanes 4 and 12). Due to the reducing conditions in the gel, the disulfide-linked gB complexes of PrV and BHV-1 partially dissociated into the two subunits. Together these data verify the genomic profiles and glycoprotein expression of the virus strains.

Attachment of extracellular virions via cell surface heparan sulfate proteoglycans is efficiently inhibited in the presence of exogenous heparin (reviewed in reference 25), which is structurally similar to heparan sulfate (6). To test for heparin sensitivity, PrV-9112C2 and PrV-9112C2-ΔgCβ were titrated in the presence or absence of 50 μg of heparin per ml during a 1-h infection period on either African green monkey kidney (Vero) or bovine kidney (MDBK) cells. Thereafter, the medium was replaced by a semisolid methylcellulose medium devoid of heparin, and plaques were counted 3 days later. As shown in Table 1, plaque formation of PrV-9112C2 was inhibited more than 10-fold by exogenous heparin in both cell lines. In contrast, PrV-9112C2-ΔgCβ was not inhibited at all at this concentration. Increasing the heparin concentration to 500 μg per ml also did not impair the infectivity of PrV-9112C2-ΔgCβ (data not shown).

To test for heparin sensitivity in BHV-1, wild-type BHV-1 and a gC-negative deletion mutant (27) were also assayed. Data shown in Table 1 demonstrate that titers of wild-type BHV-1 decreased in the presence of 50 μg of heparin per ml, although not to the same extent as previously described (19) or as seen in gC-positive PrV. However, in the absence of gC, BHV-1 infectivity was no longer affected by heparin. As has been observed with PrV, elevating the heparin concentration to 500 μg per ml did not influence the results. This indicates that gC is the only BHV-1 glycoprotein that productively interacts with heparan sulfate and implies that BHV-1 gB in a BHV-1 background does not exert heparin-binding activity.

Since exogenous heparin might have pleiotropic effects not

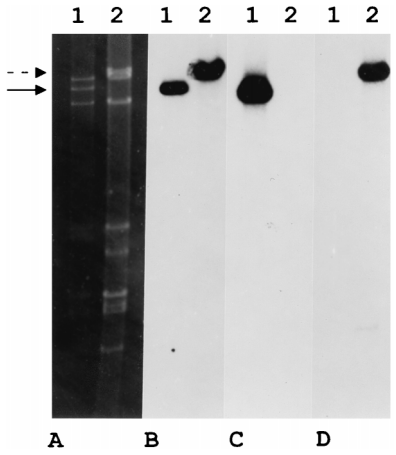

FIG. 2. Genomic characterization of PrV–BHV-1 gB recombinant viruses. Virion DNA of PrV–BHV-1 gB recombinant PrV-9112C2 (lanes 1) or gC-deleted derivative PrV-9112C2-ΔgCβ (lanes 2) was cleaved with *Bam*HI, and resulting fragments were separated in a 0.7% agarose gel. (A) Ethidium bromide stained gel. The positions of wild-type *Bam*HI fragment 2, encompassing the UL44 gene (solid arrow), and of the larger mutant fragment (broken arrow) are indicated. Results of hybridization with labelled *Bam*HI fragment 2 (B), a UL44-specific probe (C) (for location, see Fig. 1), and a β-Gal-specific probe (D) are shown.

necessarily related to a specific inhibition of infection, the infectivities of PrV-9112C2 and PrV-9112C2-ΔgCβ were assayed on gro2C cells, which are deficient in heparan sulfate biosynthesis but synthesize other glycosaminoglycans, such as chondroitin sulfate (3). These cells were derived from murine L cells and were shown to specifically lack heparan sulfate proteoglycans (3). Cells in six-well tissue culture dishes were infected with serial dilutions of either virus for 1 h at 37°C. Thereafter, the inoculum was removed and cells were overlaid with methylcellulose medium. Three days after infection, monolayers were fixed with 5% formalin and stained with crystal violet, and plaques were counted. As shown in Table 2, titers of both viruses on parental murine L cells were lower than those on Vero or MDBK cells (see Table 1). This has been observed before for various PrV mutants (8). However, whereas the plating efficiency of PrV-9112C2 was decreased approximately fivefold on heparan sulfate-deficient gro2C cells, PrV-9112C2-ΔgCβ exhibited similar titers on L and gro2C cells. Since BHV-1 infects murine cells only very inefficiently, similar experiments could not be performed with the corresponding gC-positive and gC-negative BHV-1.

Our data show that BHV-1 gB in a PrV background, despite its proficiency in binding heparin when assayed in isolated form (1, 11, 12), is unable to productively interact with heparan sulfate on cell surfaces to initiate infection. The results also indicate that in a gC-negative BHV-1 virion, BHV-1 gB fails to productively interact with heparan sulfate. Therefore, a heparin-binding capacity of glycoproteins in isolation does not necessarily imply a similar function in the virion. In this context, it is notable that PrV gB can functionally complement a gB-negative HSV mutant which is deficient in initiating infection (16). Since PrV virion gB does not exhibit heparan sulfate binding in the absence of gC (8), it remains to be analyzed

TABLE 1. Heparin inhibition of infectivity of PrV and BHV-1 mutants[a]

Virus (cell line)	Titers of virus[b]		Ratio of virus titers with heparin/ without heparin
	Without heparin	With heparin	
PrV-9112C2 (Vero)	2.7×10^6	2.1×10^5	0.08
PrV-9112C2-ΔgCβ (Vero)	1.9×10^5	2.1×10^5	1.1
PrV-9112C2 (MDBK)	7.4×10^6	6.4×10^5	0.09
PrV-9112C2-ΔgCβ (MDBK)	5.4×10^5	5.2×10^5	1
PrV-9112C2 (MDBK)	6.9×10^6	2.6×10^5	0.04
PrV-9112C2-ΔgCβ (MDBK)	1.6×10^6	1.8×10^6	1.1
BHV-1 (MDBK)	2.4×10^6	7.6×10^5	0.3
BHV-1 gC (MDBK)	1.2×10^5	1.1×10^5	0.9

[a] Three independent stocks of each PrV mutant were titrated on Vero or MDBK cells in the absence or presence of 50 μg of heparin per ml for 1 h at 37°C. In addition, BHV-1 and a gC-negative BHV-1 mutant (27) were titrated on MDBK cells. Thereafter, the inoculum was removed and cells were overlaid with a semisolid methylcellulose medium devoid of heparin. After 3 days, plaques were counted.

[b] Indicated as PFU per milliliter of virus suspension.

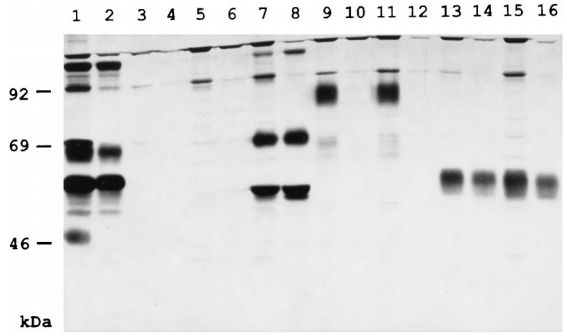

FIG. 3. Glycoprotein expression of PrV mutants. Virions of wild-type PrV strain Ka (lanes 1, 5, 9, and 13), an isogenic gC-negative β-Gal insertion mutant (lanes 2, 6, 10, and 14), PrV-9112C2 (lanes 3, 7, 11, and 15), and PrV-9112C2-ΔgCβ (lanes 4, 8, 12, and 16) were metabolically labelled with [^{35}S]methionine and [^{35}S]cysteine, purified by centrifugation through a 40% sucrose cushion, lysed, and precipitated with monoclonal antibodies directed against PrV gB (lanes 1 to 4), BHV-1 gB (lanes 5 to 8), PrV gC (lanes 9 to 12), and PrV gD (lanes 13 to 16). Precipitates were separated in a reducing sodium dodecyl sulfate–10% polyacrylamide gel and visualized by fluorography.

TABLE 2. Infection of L and gro2C cells with PrV-9112C2 and PrV-9112C2-ΔgCβ[a]

Expt no. and virus	Virus titers[b] in:		Ratio of virus titers in gro2C/L cells
	L cells	gro2C cells	
1			
9112C2	8×10^5	3×10^5	0.4
9112C2-ΔgCβ	1.8×10^5	2×10^5	1.1
2			
9112C2	7×10^5	1.5×10^5	0.2
9112C2-ΔgCβ	3.3×10^5	4×10^5	1.2
3			
9112C2	6.6×10^5	1.3×10^5	0.2
9112C2-ΔgCβ	1.1×10^5	1.2×10^5	1.1

[a] Murine L or heparan sulfate-deficient gro2C cells were infected with serial dilutions of PrV-9112C2 or PrV-9112C2-ΔgCβ. After 1 h at 37°C, the inoculum was removed and cells were overlaid with a semisolid methylcellulose medium. Three days after infection, plaques were counted and titers were determined. Results from three independent experiments with different virus stocks are shown.

[b] Indicated as PFU per milliliter of virus suspension.

whether a gB- and gC-deleted HSV (4) can also be complemented by PrV gB and whether the resulting transcomplemented virus can infect target cells by a heparan sulfate-independent mechanism.

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