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Glycoprotein gB (gII) of Pseudorabies Virus Can Functionally Substitute for Glycoprotein gB in Herpes Simplex Virus Type 1

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Glycoproteins homologous to gB of herpes simplex virus (HSV) constitute the most highly conserved family of herpesvirus glycoproteins. All gB homologs analyzed so far have been shown to play essential roles in penetration and direct viral cell-to-cell spread. In studies aimed at assessing whether the high sequence homology is also indicative of functional homology, we analyzed the ability of the gB-homologous glycoprotein (former designation gII) of pseudorabies virus (PrV) to complement a gB$^-$ HSV type 1 (HSV-1) mutant and vice versa. The results show that a PrV gB-expressing cell line phenotypically complemented the lethal defect in gB$^-$ HSV-1 whereas reciprocal complementation of a gB$^-$ PrV mutant by HSV-1 gB was not observed.

The glycoprotein gB homologs constitute the most highly conserved group among herpesvirus glycoproteins. They have been identified in every herpesvirus analyzed so far, and identity at the amino acid level can be high, e.g., 63% between pseudorabies virus (PrV) gB (originally designated gII [26]) and bovine herpesvirus 1 (BHV-1) gB (originally designated gI [32]). (At the 1993 International Herpesvirus Workshop, researchers working on alphaherpesviruses agreed upon a common nomenclature for alphaherpesvirus glycoproteins. Hence, the name gB will be used for the related alphaherpesvirus glycoproteins that are the subject of this communication). The high degree of sequence conservation might be indicative of common functions of the gB homologs in the herpesvirus life cycle, and it has indeed been shown that gB-homologous glycoproteins play essential roles in virus penetration into host cells and direct viral cell-to-cell spread from infected to adjacent noninfected cells (5, 11, 20, 22, 24). To examine whether these proteins can execute their function in a heterologous herpesvirus background, we concentrate on analyzing the capabilities of different gB proteins to complement defects in the homologous glycoprotein in several alphaherpesviruses.

Our approach involved a PrV mutant, 4112, lacking gB because of a deletion removing approximately 60% of the gB coding sequence and concomitant insertion of a β-galactosidase reporter gene construct. This mutant is defective in both penetration and direct viral cell-to-cell spread and can, therefore, be propagated only on cell lines providing PrV gB in *trans* (24, 25). Further studies demonstrated that not only cells expressing PrV gB but also cell lines expressing the homologous BHV-1 gB were able to support productive replication of gB$^-$ PrV, indicating that BHV-1 gB was able to compensate for the defect in gB-deficient PrV (25). Final proof for the ability of BHV-1 gB to fully complement in vitro the defect associated with lack of PrV gB in a PrV background was obtained after isolation of a viable PrV gB$^-$ PrV recombinant that carries and expresses the BHV-1 gB gene (14).

PrV gB and BHV-1 gB share several properties. They are encoded by two closely related viruses (1, 4) and are highly homologous (63% amino acid identity), and both are proteolytically processed into subunits that remain linked via disulfide bonds (8, 15, 28, 31). In contrast, gB of herpes simplex virus type 1 (HSV-1) exhibits only 50% identity at the amino acid level to PrV gB (23, 26) and is not proteolytically processed (2, 6, 12, 27). It was therefore of interest to analyze whether PrV gB and HSV-1 gB would be capable of *trans*-complementing defects in the respective gB-homologous protein in the different virus backgrounds. Results from studies on a pseudodiploid herpes simplex virus that expressed BHV-1 gB as well as HSV-1 gB suggested that HSV-1 gB function could be at least partially executed by BHV-1 gB after complement-independent neutralization with a monoclonal antibody (MAb) directed against HSV-1 gB (19). These results are difficult to interpret, however, because unaltered HSV-1 gB was still present in virions and functionally or structurally important domains other than the one targeted by the MAb remained unaltered.

To avoid this problem and to facilitate our studies, we constructed a gB-negative HSV-1 mutant by insertion of an expression cassette containing the *Escherichia coli* β-galactosidase gene under the control of the PrV glycoprotein gG(gX) promoter (17) into the *Sal*I site of the gB gene in HSV-1 strain KOS. In transient expression assays, it had previously been observed that HSV-1 was able to transactivate efficiently the early gG(gX) promoter of PrV (16). The resulting mutant, which is dependent on *trans*-complementing cells for productive replication (see Fig. 1 and 2), was designated KO1252-gBβ. Radioimmunoprecipitations with several gB-specific MAbs did not detect any gB-specific protein in noncomplementing cells infected with this mutant (16). To assay for complementation, the gB$^-$ PrV mutant 4112 (25) and the gB$^-$ HSV-1 mutant KO1252-gBβ were tested for ability to form plaques and to replicate on Vero cells expressing the homologous or heterologous form of gB. The N7 cell line carries the PrV gB gene and was previously shown to complement the defect of gB$^-$ PrV (25). The T9 cell line was isolated by transfecting Vero cells with plasmid pMN12 and selecting for resistance to Geneticin. Plasmid pMN12 carries the HSV-1(KOS) gB gene (from a *Bal*I site ca. 150 bp upstream of the coding sequence to a *Kpn*I site downstream) inserted with linkers between the *Bam*HI and *Eco*RI sites of plasmid pRJ40 (13). Although pMN12 contains the human metallothionein promoter upstream of the gB gene, no constitutive expression of gB was detected, and expression could not be induced by

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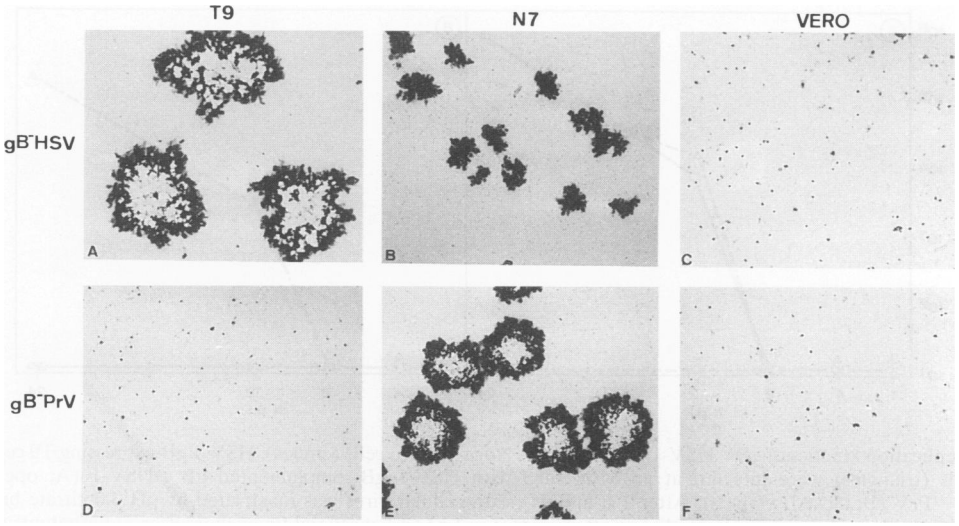

FIG. 1. Plaque assays of phenotypically complemented gB⁻ HSV-1 and gB⁻ PrV. The gB⁻ HSV-1 mutant KO1252-gBβ (A to C) or the gB⁻ PrV mutant 4112 (D to F) after homologous complementation was used for infection of either HSV-1 gB-expressing T9 cells (A and D), PrV gB-expressing N7 cells (B and E), or normal Vero cells (C and F) under plaque assay conditions. Three days after infection, monolayers were fixed and stained with X-Gal. In panels A, B, and E, comparable amounts of virus were used for infection. For panels C, D, and F, a 20-fold higher amount of virus was used to more clearly demonstrate single blue-staining infected cells.

heavy metal ions but could be induced by HSV-1 infection. Moreover, the cell line complemented the defects of gB⁻ HSV-1 mutants, including both KO1252-gBβ and KO82, a linker-inserted gB-null mutant (kindly provided by S. Person, Pittsburgh, Pa.) that was included for comparison in all our studies.

To analyze whether PrV gB was able to complement the defect in cell-to-cell spread associated with lack of gB in HSV-1, gB⁻ HSV-1 mutants that had been produced on cells expressing HSV-1 gB were plated on T9 cells (Fig. 1A), N7 cells (Fig. 1B), or normal Vero cells (Fig. 1C) under plaque assay conditions with a methylcellulose overlay. Three to seven days after infection, the monolayers were fixed and stained for β-galactosidase expression with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), resulting in a dark blue stain (for KO1252-gBβ; Fig. 1), or fixed and stained with crystal violet (for KO82; data not shown). As expected, KO1252-gBβ formed blue plaques on HSV-1 gB-expressing T9 cells (Fig. 1A). It was, however, unable to form plaques on normal Vero cells (Fig. 1C). Only single infected cells were observed, indicating that infection of primary target cells by the complemented gB⁻ HSV-1 occurred but spread of infection to neighboring cells did not ensue because of lack of the essential gB. Interestingly, blue plaques also formed on PrV gB-expressing N7 cells (Fig. 1B), although they were smaller than those on T9 cells. Wild-type HSV-1 plaques were of similar size on all three cell lines (data not shown). Numbers of plaques of gB⁻ HSV-1 were similar on T9 cells ($2.5 \times 10^5 \pm 0.3 \times 10^5$) and N7 cells ($2.2 \times 10^5 \pm 0.2 \times 10^5$), which shows that plating efficiencies were comparable on both cell lines. This result indicated that PrV gB was able to complement the missing cell-to-cell spread function in gB⁻ HSV-1, albeit less efficiently than the homologous HSV-1 gB. Identical results were obtained with the gB-null mutant KO82 (data not shown).

When phenotypically complemented gB⁻ PrV was similarly analyzed, plaque formation was observed only on PrV gB-expressing N7 cells (Fig. 1E), whereas on T9 cells (Fig. 1D), as well as on normal Vero cells (Fig. 1F), only single infected primary target cells were observed. Therefore, whereas PrV gB was able to complement the missing cell-to-cell spread function in gB⁻ HSV-1, reciprocal complementation of gB⁻ PrV spread by HSV-1 gB did not take place.

To analyze whether the heterologous forms of gB were able to complement the replication defects of the HSV-1 and PrV mutants, kinetics of infectious virus production by synchronously infected cells were determined. T9, N7, and normal Vero cells were infected with either gB⁻ HSV-1 (Fig. 2A) or gB⁻ PrV (Fig. 2B) at a multiplicity of infection (MOI) of 5 for 1 h at 37°C. Input virus had been complemented by the homologous form of gB. To inactivate residual extracellular input virus, monolayers were treated for 1 min with citrate buffer, pH 3.0. Immediately thereafter, and at 4, 8, 12, and 24 h postinfection, supernatants were harvested and titrated on either T9 cells (gB⁻ HSV-1 infections; Fig. 2A) or N7 cells (gB⁻ PrV infections; Fig. 2B). To assay for intracellular infectious virus, monolayers were scraped into medium, freeze-thawed twice, and titrated. Viral titers were calculated by adding the amount of extra- and intracellular infectious virus. Results shown in Fig. 2 represent averages of two independent experiments. It can be seen that after infection of N7 cells by complemented gB⁻ HSV-1, infectious virus progeny appeared with similar kinetics as well as similar final titers as on T9 cells. No differences in the relative amounts of released and cell-bound infectious virions were observed (data not shown). On normal Vero cells, no infectious virus was produced either extracellularly or intracellularly, although cells exhibited a pronounced cytopathic effect similar to that seen on T9 and N7 cells (limit of detection, 10 PFU/ml). The infectious virus produced by both N7 and T9 cells was phenotypically complemented but still mutant, on the basis of findings that the virus titers on Vero cells were approximately 10,000-fold less than those on T9 cells. This shows that, after infection by gB⁻ HSV-1, both T9 and N7 cells were able to produce infectious virus progeny, whereas normal Vero cells were unable to do so. In contrast, only on N7 cells could infectious gB⁻ PrV progeny be detected whereas infectious virions were not formed in

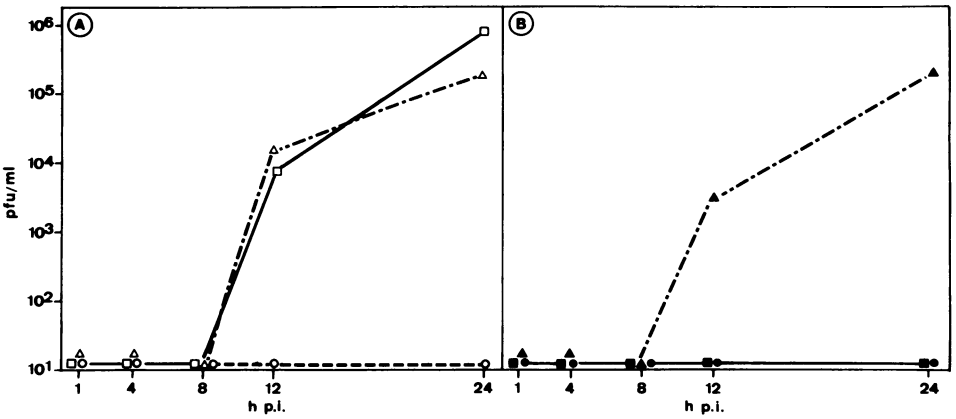

FIG. 2. One-step replication kinetics of gB⁻ HSV-1 and gB⁻ PrV. Normal Vero cells (circles), HSV-1 gB-expressing T9 cells (squares), or PrV gB-expressing N7 cells (triangles) were infected at an MOI of 5 with HSV-1 gB-complemented gB⁻ HSV-1 (A; open symbols) or PrV gB-complemented gB⁻ PrV (B; closed symbols). After 1 h at 37°C, extracellular virus was inactivated by pH 3.0 citrate buffer treatment, and monolayers were overlaid with fresh medium. Immediately afterwards (t = 1 h) and at several times thereafter, supernatants and cell pellets were harvested and titrated on either T9 (A) or N7 (B) cells. Values indicated represent averages obtained from two different experiments after titers of intra- and extracellular virus were combined.

either Vero or T9 cells. These results demonstrate that PrV gB was able to substitute functionally for HSV-1 gB in HSV-1 particles, restoring infectivity to them, whereas in the reciprocal situation, complementation was not observed.

To assay directly for incorporation of PrV gB into gB⁻ HSV-1 virions, complement-dependent neutralization tests were performed. As shown in Table 1, gB⁻ HSV-1 complemented with HSV-1 gB behaved like wild-type HSV-1 regarding sensitivity to neutralization by HSV-specific MAbs and resistance against neutralization by PrV-specific MAbs. In contrast, gB⁻ HSV-1 complemented with PrV gB acquired resistance against neutralization by HSV-specific anti-gB antibodies and sensitivity to neutralization by PrV-specific anti-gB antibodies. This unequivocally demonstrates that PrV gB was incorporated into gB⁻ HSV-1 virions.

The observed lack of complementation of gB⁻ PrV after propagation on T9 cells could be due to failure of PrV to transactivate the resident HSV-1 gB gene. To test for transactivation, T9 (Fig. 3A), N7 (Fig. 3B), or normal Vero (Fig. 3C) cells were infected at an MOI of 2 with either gB⁻ HSV-1 (Fig. 3, lanes 1 to 3) or gB⁻ PrV (Fig. 3, lanes 4 to 6), and proteins were labelled from 2 to 24 h postinfection with [^{35}S]methionine. Immunoprecipitations of infected cell lysates were performed with anti-gB(HSV-1) MAb II-105-1 (lanes 1 and 4 [21]), anti-gD(HSV-1) MAb I-99-1 (lanes 3 [21]), anti-gB(PrV) MAb 5/14 (lanes 2 and 5 [15]), and anti-gD(PrV) (formerly designated gp50) MAb MCA50-1 (lanes 6 [29]). As shown in Fig. 3, HSV-1 gB was precipitated in similar amounts from T9 cells after infection with either PrV or HSV-1. Multiple gB-specific bands were probably due to oligomerization (6) as well as to limited proteolytic degradation during extraction from the Vero-derived cells, which is commonly observed (33). As expected, the 155-kDa PrV gB complex and the 120-kDa glycosylated precursor were precipitated from N7 cells infected with either PrV or HSV-1. In reducing gels, correct proteolytic processing of PrV gB was observed (data not shown). From normal Vero cells, neither HSV-1 gB nor PrV gB could be precipitated after infection with either virus. Precipitations with the respective anti-gD MAbs served as controls.

TABLE 1. Neutralization of phenotypically complemented gB⁻ HSV-1[a]

MAb	Neutralization (%)			
	PrV	HSV-1	gB⁻ HSV-1 (T9)	gB⁻ HSV-1 (N7)
Anti-gB (PrV)	99	2	7	96
Anti-gD (PrV)	99	4	5	8
Anti-gB (HSV-1)	5	99	99	3
Anti-gD (HSV-1)	3	99	90	89

[a] gB⁻ HSV-1 was propagated either on HSV-1 gB-expressing T9 cells (gB⁻ HSV-1 [T9]) or on PrV gB-expressing N7 cells (gB⁻ HSV-1 [N7]). For comparison, wild-type PrV and wild-type HSV-1 grown on normal Vero cells were included. Approximately 500 PFU of each virus was incubated with an appropriate antibody dilution adjusted to yield between 90 and 99% neutralization in the homologous system after addition of 5% normal rabbit serum as a source for complement. Neutralization was assessed as percent plaque reduction compared with controls that had been reacted with a non-herpesvirus-specific MAb. MAbs directed against PrV gB (MAb 5/14), PrV gD (MAb MCA50-1), HSV-1 gB (MAb IE12), and HSV-1 gD (MAb IE1) were used.

Previously, we demonstrated that BHV-1 gB could substitute for PrV gB in a PrV background (14, 25). We show here that PrV gB is able to complement both defects associated with absence of gB in HSV-1, i.e., lack of infectivity and failure to execute direct cell-to-cell spread. Thus, despite the lower sequence homology, PrV gB can substitute for HSV-1 gB in an HSV-1 background. This also indicates that a proteolytically processed gB homolog is able to execute functions similar to those of the uncleaved gB in HSV-1, at least in cell culture.

The lack of complementation of the defect in gB⁻ PrV by HSV-1 gB is not due to absence of HSV-1 gB expression or peculiarities of the T9 cell line. With two other independently obtained gB-expressing cell lines, a similar lack of complementation was observed (16). Preliminary immunoelectron-microscopical analyses failed to detect HSV-1 gB in the gB⁻ PrV virion envelope (30). It is also conceivable that the absence of proteolytic cleavage in HSV-1 gB might interfere with its function in a PrV background. With a pseudodiploid HSV-1 that in addition to HSV-1 gB expresses a BHV-1 gB into which a deletion of the proteolytic cleavage site had been engineered (3), it has previously been proposed that proteolytic processing was not necessary for BHV-1 gB to complement a function of HSV-1 gB that had been neutralized by a MAb. However, this

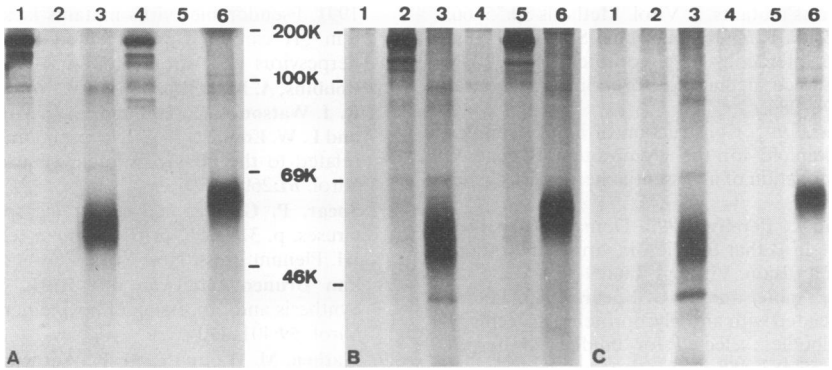

FIG. 3. Transactivation of the resident gB gene in T9 cells after infection with PrV. HSV-1 gB-expressing T9 (A), PrV gB-expressing N7 (B), or normal Vero (C) cells were infected at an MOI of 2 with either gB$^-$ HSV-1 (lanes 1 to 3) or gB$^-$ PrV (lanes 4 to 6) and labelled with [^{35}S]methionine from 2 until 24 h postinfection. Thereafter, cells were lysed and proteins were immunoprecipitated with MAbs directed against HSV-1 gB (II-105-1; lanes 1 and 4), PrV gB (5/14; lanes 2 and 5), HSV-1 gD (I-99-1; lanes 3), or PrV gD (MCA50-1; lanes 6). Precipitates were separated in a sodium dodecyl sulfate–10% polyacrylamide gel under nonreducing conditions. Positions of molecular size markers are indicated.

pseudodiploid situation in which both gB homologs are still present in virions and infected cells is clearly different from our assay system using viruses containing either homologous or heterologous gB. Therefore, it remains to be seen whether addition of a proteolytic cleavage site to HSV-1 gB will alter its complementation capabilities. Interestingly, the gB homolog of varicella-zoster virus which resembles PrV gB in proteolytic processing appears to be incapable of complementing a gB defect in HSV-1 (7).

The results presented here provide the basis for further studies on functional comparison between the gB homologs of PrV and HSV. It has, for example, been shown that HSV-1 gB exhibits heparin-binding properties and may therefore, especially in the absence of the major heparin-binding protein, gC, represent an important mediator of primary viral attachment (9, 10). In contrast, PrV gB does not appear to bind to heparin in the absence of the heparin-binding protein PrV gC (previously designated gIII), and therefore, gC$^-$ PrV has to attach by a heparin-independent pathway (18). Experiments are in progress to determine whether in gC-deficient HSV-1 PrV gB can also substitute for HSV-1 gB function. In addition, PrV gB has been shown to interact with PrV gC (18). Whether PrV gB is also able to interact with HSV-1 gC in an HSV-1 background is under investigation.

Further studies on functional complementation between homologous proteins of different herpesviruses should help to more clearly understand the basis for glycoprotein function and provide a framework for analysis of molecular interactions essential for initiation of herpesvirus infection.

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