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Pseudorabies Virus Glycoprotein gIII Is Required for Efficient Virus Growth in Tissue Culture

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Glycoprotein gIII of pseudorabies virus is a major antigen found in the envelopes of virus particles as well as in and on the surfaces of infected cells. It is not an essential gene product for virus growth in tissue culture. In this report, we provide evidence that, although it is not essential, the gIII protein is required for efficient virus growth and that gIII mutants are quickly outgrown by wild-type virus in mixed infections.

Pseudorabies virus (PRV) is a swine herpesvirus whose envelope contains at least five glycoproteins. Glycoprotein gIII of PRV is a major component of the virus envelope and is found on the surfaces of infected cells. While the function(s) of gIII is not well established, it has been implicated in virus entry into cells (3). gIII also shares significant amino acid similarity with herpes simplex virus type 1 glycoprotein gC (7), a complement C3b-binding protein. To date, no evidence for C3b binding to PRV gIII has been presented.

Previously we demonstrated that expression of the gIII gene was not essential for growth of PRV in tissue culture (8) by constructing two gIII deletion mutations and determining that the resulting mutant viruses were infectious in culture. We noted, however, that mutations affecting the gIII gene were not without effect; virus carrying a gIII deletion grew less well than wild-type virus. In this study, we examined this phenotype further by using two distinct classes of gIII-defective viruses, those with mutant gIII in their virion envelopes and those with no detectable envelope-associated gIII. We analyzed single-step growth in PK15 cells and compared the final titers of the viruses, the rate of appearance of infectious virus in cells, and the time at which plaque-forming virus was released into the medium. In general, compared with wild-type virus, gIII mutants grow to reproducibly lower titers and they are slower to produce plaque-forming virus in the culture medium. However, each mutant has a distinct growth phenotype; for example, those lacking gIII in the virus envelope are more severely affected than those carrying a mutated gIII in the envelope. Significantly, gIII mutants are quickly outgrown by wild-type virus in mixed infection.

A map of the gIII gene of PRV-Becker (PRV-Be, our wild-type virus) and five mutant gIII genes is shown in Fig. 1. The mutant genes were constructed and crossed onto infectious PRV-Be as described previously (8, 9). PRV29 and PRV30 will be described in detail in a subsequent publication (A. K. Robbins et al., manuscript in preparation).

The wild-type gIII gene encodes a protein of 479 amino acids containing eight potential N-linked glycosylation sites. The protein probably spans the lipid bilayer once because of a cluster of hydrophobic residues at positions 447 to 470. The proteins produced by the mutant viruses, with the exception of those made by PRV29 and PRV30, have been described in detail (8, 9). PRV10 is a deletion of the gIII promoter and more than 87% of the gIII-coding sequences. It produces no detectable gIII mRNA and no immunoreactive gIII protein. PRV4 produces a truncated, glycosylated fragment of gIII that is predicted to terminate at codon 157. PRV30 contains a frameshift-linker insertion at codon 460 that replaces the last 19 amino acids of gIII with 34 new amino acids from a different reading frame. The mutant gIII glycoproteins expressed by PRV4 and PRV30 are secreted into the medium because they lack the putative transmembrane and anchoring functions near the carboxy terminus. These proteins are incorporated poorly, if at all, into virion envelopes, probably because they lack proper targeting signals or membrane-anchoring functions. PRV2 and PRV29 produce mutant gIII glycoproteins that are readily detected in virion envelopes. PRV2 contains a gIII gene with an in-frame internal deletion of 402 base pairs that removes five of the eight potential N-linked glycosylation sites. PRV29 contains a gIII gene with an *Eco*RI linker at the *Bam*HI site (codon 460), which results in a glycoprotein with an insertion of four amino acids in the putative transmembrane region. PRV29 produces a gIII glycoprotein almost indistinguishable from that of the wild type, except that a small quantity of protein is found in the medium.

Table 1 summarizes the localization of wild-type and mutant gIII glycoproteins after infection of PK15 cells. Briefly, PK15 cells were infected with wild-type PRV-Be or mutant virus at a multiplicity of infection (MOI) of 10 and labeled with [^{3}H]glucosamine until 16 h postinfection. At this time, the cells were harvested and fractionated into medium and cell fractions. The medium fractions were further purified by centrifugation to separate virions. These techniques were described previously (9). Fractionated samples were prepared for immunoprecipitation with gIII-specific antibodies. The immunoprecipitates were resolved on a 7 to 17% sodium dodecyl sulfate-polyacrylamide gradient gel, and the ^{3}H-labeled polypeptides were detected by fluorography.

The magnitude of the growth defect of each gIII mutant depended on the mutation and the method of analysis. The defect generally could be seen as slower apparent release of plaque-forming virus into the medium and an overall lower titer when single-step growth studies were done (Fig. 2 and Table 2). It is important that cell-associated infectious virus accumulates with similar kinetics for all viruses; however, the release of infectious virus was altered for gIII mutants. In particular, for PRV-Be, the number of infectious particles released into the medium began to increase after 5 to 7 h. The gIII mutants showed delayed infectious particle release after 7 to 9 h. At 24 h postinfection, only PRV-Be had significantly more released virus than cell-associated virus.

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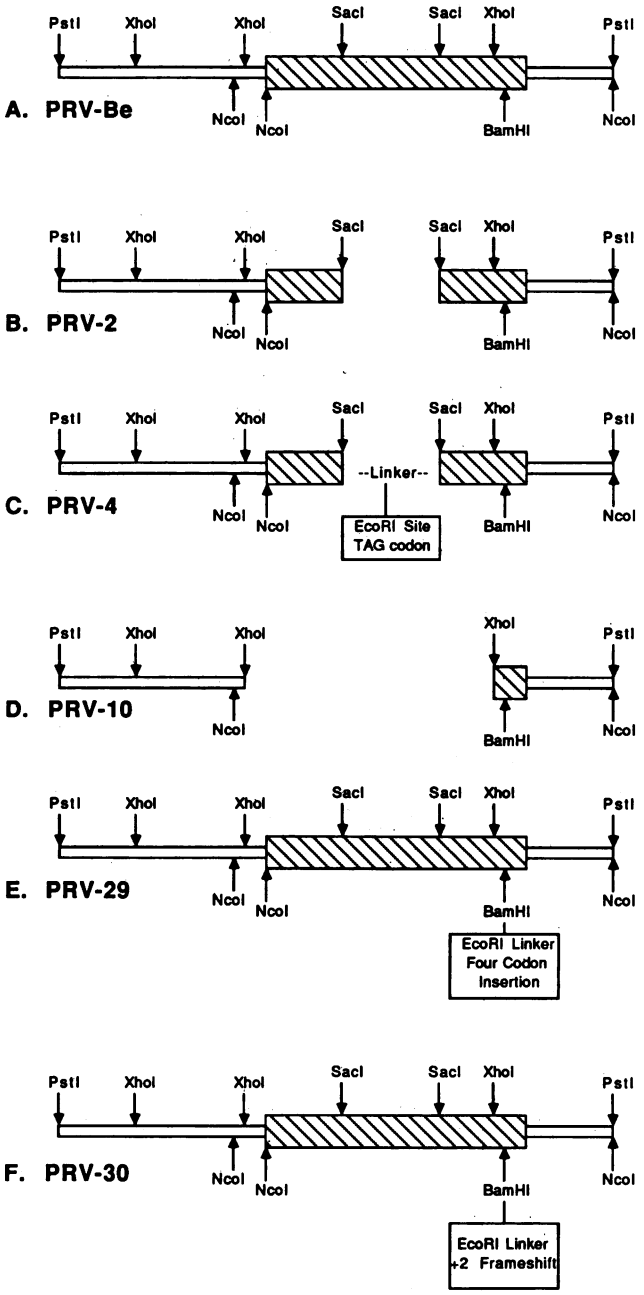

FIG. 1. Map of the *Pst*I fragment from PRV-Be and five PRV gIII mutants. ▨, gIII gene. Transcription is from left to right. (A) A 4.3-kilobase fragment containing the gIII gene of PRV-Be is expanded and detailed, with relevant restriction enzyme cleavage sites noted. (B) PRV2 contains a 402-base-pair deletion of the internal *Sac*I fragment in the gIII gene. A single *Sac*I site is regenerated. (C) PRV4 contains the same 402-base-pair *Sac*I deletion with a synthetic *Eco*RI linker containing an in-frame TAG stop codon inserted at the *Sac*I site. The TAG stop codon is codon 157 of the gIII gene. (D) PRV10 contains a deletion of the indicated *Xho*I fragment. This deletion removes 87% of the gIII-coding sequences, including 230 base pairs of the upstream putative transcriptional control sequences. (E) PRV29 contains a synthetic *Eco*RI linker inserted at the unique *Bam*HI site in the gIII gene. The insertion results in the addition of four extra amino acids in the putative transmembrane region of the gIII protein. (F) PRV30 contains a synthetic *Eco*RI linker inserted at the unique *Bam*HI site in the gIII gene. The insertion results in a +2 frameshift.

TABLE 1. Localization of wild-type and mutant gIII glycoproteins after infection of PK15 cells[a]

Virus	Amt of glycoprotein gIII detected in:		
	Infected cells	Virions	Medium
PRV-Be	+++	+++	−
PRV2	+++	+++	−
PRV4	++	−	++
PRV10	−	−	−
PRV29	+++	++	+−
PRV30	++	+−	++

[a] The relative amount of glycoprotein within the measured fraction compared with that in PRV-Be is indicated as follows: +++, wild-type levels; ++, less than wild type, but easily detectable; +−, barely detectable; −, not detectable. The data for PRV-Be, PRV2, PRV4, and PRV10 are found in references 8 and 9. Data for PRV29 and PRV30 are unpublished (Robbins et al., in preparation).

Of the five gIII mutants, PRV4 and PRV10 were most severely affected.

The growth defect was seen more dramatically in mixed infection with wild-type virus (Table 3). The rationale for the mixed infection experiments follows. The relatively subtle defect in single-step growth should result in slower cell-to-cell spread in a low-multiplicity infection. This phenotype should therefore present itself as a disadvantage in competition with wild-type virus. We therefore made artificial stock mixtures of wild-type virus and one of two gIII mutants (PRV2 or PRV10) by low-multiplicity infection and then compared the final ratio of wild-type virus to gIII mutant plaques with the initial ratio. The ratios of the initial mixtures were determined by plating on PK15 cells and screening in a black-plaque assay with the gIII-specific M1 monoclonal antibody (2, 6). PRV-Be plaques stain black, and both PRV2 and PRV10 plaques remain white (6). To determine the ratios in the initial mixtures, 100 to 1,000 plaques were screened; 500 to 10,000 plaques were screened to determine the ratio in the final stocks. The initial mixtures were used to infect PK15 cells at an MOI of 0.001. The MOI was determined by the total titer of each mixture, which was approximately 10^8 PFU/ml. The plates were harvested after 40 h, when total cytopathic effect was observed. The resulting virus was plated on PK15 cells, and plaques were screened in a black-plaque assay with M1 antibody. The results (Table 3) demonstrate that both gIII mutants were outgrown by wild-type virus and that PRV10, a gIII-null mutant, was most severely affected. PRV2, a gIII mutant with a truncated gIII protein in the envelope, was only marginally defective in single-step growth but was still overgrown by wild-type virus.

TABLE 2. Single-step growth of PRV-Be and five gIII mutants[a]

Virus	Final titers (PFU/ml)	Burst size (PFU/cell)
PRV-Be	1.2×10^9	1,000
PRV2	1.5×10^8	500
PRV4	6.6×10^7	50
PRV10	1.9×10^8	100
PRV29	1.1×10^9	1,000
PRV30	2.9×10^8	300

[a] PK15 cells were infected with each virus at an MOI of 5. After 24 h, the plates were harvested, medium and cells were pooled and sonicated briefly to release virus from cells and debris, and total plaque-forming virus was determined as described previously (8).

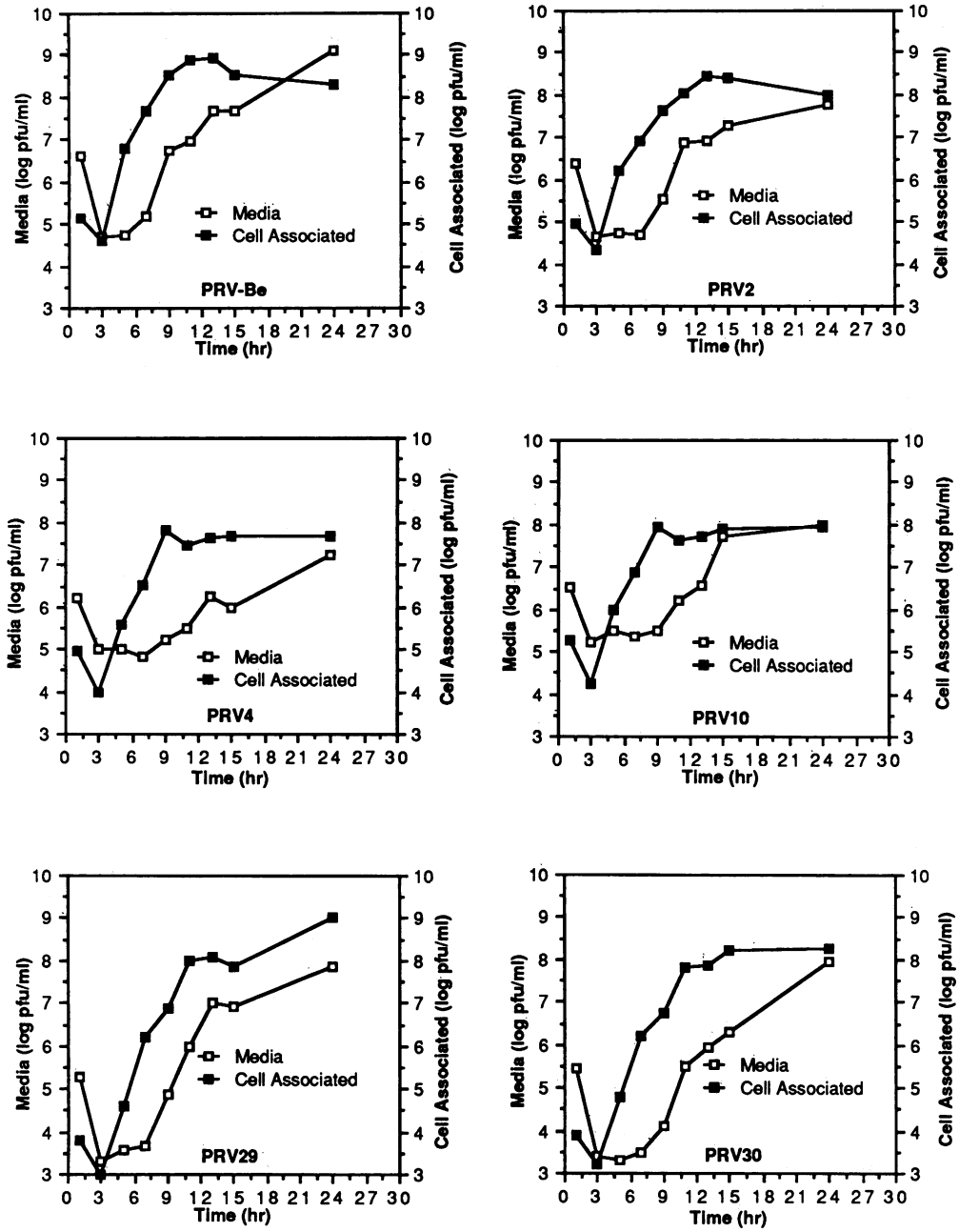

FIG. 2. Single-step growth curves of PRV-Be and gIII mutant viruses. PK15 cells were infected with PRV strains at an MOI of 5 and were incubated at 37°C. At 1, 3, 5, 7, 9, 11, 15, and 24 h postinfection, plates were harvested and the virus titers of the cell and medium fractions were determined separately and plotted. The infecting virus strain is indicated at the bottom center of each panel. Data for PRV-Be, PRV2, and PRV4 were taken from reference 7 and replotted.

We have concluded the following from these experiments. First, PRV strains with defective gIII genes usually produce lower titers than the parental virus in PK15 cells. The magnitude of this effect is relatively small (from 2- to 20-fold) and varies with the mutation, the method of making virus stocks, and the cell line. It is noteworthy that mutants retaining gIII in their envelopes (e.g., PRV2 and PRV29) are less defective than those totally lacking envelope gIII (PRV4 and PRV10). Second, although the growth defect of gIII mutants is small, it is significant and reproducible. The effects, however, are most evident in mixed infections with wild-type virus. During multiple cycles of growth, the wild type rapidly overgrows the mutants. The defect is most severe for PRV10, a gIII-null mutation. Third, from single-step growth curve data, we have noted that gIII mutants appear to be partially defective in the release of infectious virus from the infected cell (Fig. 2) (4, 9). Mutants not only exhibit a lag in the appearance of infectious particles in the medium but also show an increase in cell-associated virus.

The rather subtle growth phenotype of gIII mutants can have major effects in certain experiments in which a wild-type gIII gene is present. For example, we have observed (data not shown) that in crosses in which gIII mutations on plasmids are crossed with wild-type virus, the apparent

TABLE 3. Plaque ratio in infection mixtures of PRV-Be and two gIII-defective mutants

Infection mixture[a]	Ratio of PRV to gIII mutant plaques in[b]:		Increase (fold)
	Initial mixture	Final stock	
PRV-Be + PRV2	0.5	2	4
	0.07	0.25	3.6
	0.0005	0.013	26
PRV-Be + PRV10	4	846	212
	0.25	38	152
	0.023	12	522

[a] PRV2 contains a truncated gIII protein; PRV10 contains no gIII protein.
[b] The initial mixtures were plated on PK15 cells, and the final stocks were harvested after 40 h. Ratios were determined as described in the text.

recombination frequency which gives rise to a gIII mutant virus is quite low (typically 0.1%). The reciprocal cross, in which a wild-type gIII gene on a plasmid is crossed to a gIII mutant virus, gives a very high apparent recombination frequency (typically 5 to 10%). It is clear that these frequencies are combinations of the actual recombination frequency and the growth advantage which the wild-type virus has over gIII mutants.

It is well established that several herpesvirus glycoprotein genes are not essential for propagation in tissue culture. However, little work has been done on the effects of these mutations on efficiency of virus production. In this report, we have shown that while PRV glycoprotein gIII is not required for growth in tissue culture, it is required for efficient virus propagation. Viruses expressing wild-type gIII have a significant selective advantage during laboratory growth. A number of questions remain concerning the role of gIII in the virus life cycle and the growth defect phenotype of certain gIII mutants. For example, T. Ben-Porat (personal communication) has found that gIII mutants show reduced adsorption to cells. The rather dramatic overgrowth of wild-type virus in mixed low MOI infections with gIII mutants may reflect both the slower release and subsequently poorer adsorption of gIII mutants. Furthermore, the physical stability of gIII mutant virus particles must be examined. The lower titer of plaque-forming virus released from cells may well reflect some instability of gIII-defective virions. We do know that gIII mutants are not fragile; stocks of gIII mutants are as stable as wild-type stocks during several cycles of freeze-thaw (unpublished observations).

Little is known about the role of gIII in virulence and virus growth in animals. Preliminary studies suggest that PRV gIII mutants remain virulent in chicks (Ben-Porat, personal communication). It is interesting that the avirulent strain Bartha expresses an altered gIII protein and contains several other lesions, including a deletion of glycoprotein gI (1; unpublished observations). Ben-Porat and colleagues have noted that glycoproteins in the unique short region of PRV play a role in virus release from certain cells (2, 6). Glycoprotein gI is particularly critical. They have also noted that combinations of glycoprotein defects yield viruses that are less virulent than the single-deletion mutants (6; personal communication). Not all glycoprotein defects are deleterious. Mettenleiter and colleagues also have demonstrated that deletions of glycoprotein gI provide a strong selective advantage in certain cell lines, including chicken embryo fibroblasts (5). It is clear that much remains to be learned concerning the role of individual glycoproteins and how they function in concert with the other virus-encoded glycoproteins in growth and virulence.

One practical concept that emerges from this study is a note of caution for live PRV vaccines that are defective for gIII (or any defect that imparts a growth defect in the presence of the wild-type function). Obviously, nonreverting gIII mutations should be used given the strong selection for $gIII^+$. However, this is not a perfect solution, because if there is opportunity for mixed infection with $gIII^+$ virus in animals or in the production facility, recombination events replacing most gIII mutations may be highly represented in virus yields.

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