JOURNAL OF VIROLOGY, Sept. 1989, p. 4055–4059
0022-538X/89/094055-05$02.00/0

Vol. 63, No. 9

Replacement of the Pseudorabies Virus Glycoprotein gIII Gene with Its Postulated Homolog, the Glycoprotein gC Gene of Herpes Simplex Virus Type 1

M. E. WHEALY, A. K. ROBBINS, AND L. W. ENQUIST*

Central Research and Development Department, Experimental Station, P.O. Box 80328, E. I. du Pont de Nemours & Company, Wilmington, Delaware 19880-0328

Received 17 February 1989/Accepted 5 May 1989

gIII, the major envelope glycoprotein of pseudorabies virus (PRV), shares approximately 20% amino acid similarity with glycoprotein gC of herpes simplex virus type 1 (HSV-1) and HSV-2. We describe here our first experiments on the potential conservation of function between these two genes and gene products. We constructed PRV recombinants in which the gIII gene and regulatory sequences have been replaced with the entire HSV-1 gC gene and its regulatory sequences. The gC promoter functions in the PRV genome, and authentic HSV-1 gC protein is produced, albeit at a low level, in infected cells. The gC protein is present at the cell surface but cannot be detected in the PRV envelope.

All herpesviruses encode a set of structural membrane glycoproteins. These glycoproteins, found predominantly in the virus envelope and on the surfaces of infected cells, play fundamental roles in the infectious process (19). DNA sequence analysis of glycoprotein genes cloned from a variety of diverse herpesviruses has revealed significant DNA or protein homology among certain glycoprotein genes. It is of interest to understand whether such shared information reflects, for example, a general functional requirement for assembly and activity of herpesvirus particles, motifs for specific aspects of herpesvirus pathogenicity, or simply vestiges of an original common ancestor with little or no functional significance.

Pseudorabies virus (PRV) is a neurotropic swine herpesvirus encoding at least seven glycoproteins (1, 4). Several of the PRV glycoproteins share homology with glycoproteins of herpes simplex virus type 1 (HSV-1). For example, PRV glycoprotein gII shares significant DNA sequence homology with HSV-1 glycoprotein gB, and the proteins also exhibit limited immunological cross-reactivity (12). The PRV glycoproteins gIII (13), gp50 (11), and gH (E. A. Petrovskis, P. W. H. Duffus, D. R. Thomsen, A. L. Meyer, and L. E. Post, 13th International Herpesvirus Workshop, p. 217, 1988) have essentially no DNA homology to HSV but exhibit significant similarity at the protein level with HSV gC, gD, and gH, respectively. In this study we present initial work designed to address the functional relationship of PRV gIII and HSV-1 gC.

A significant problem is that to date, little evidence other than the distant amino acid homology exists to suggest that PRV gIII and HSV gC have any functions in common. Certainly, no evidence for immunological cross-reactivity exists. Absence of gIII does not affect PRV virulence for chicks (10), while inactivation of gC significantly reduces HSV virulence for mice (9). While both genes are not absolutely required for virus growth in tissue culture (5, 14, 19), PRV gIII is known to be important for efficient adsorption to or entry into cells (16) and for efficient release of virus from infected cells (21). HSV-1 gC, on the other hand, apparently plays little role in virus adsorption or virus release (19). It is well established that HSV gC binds the C3b component of complement (2), but no such activity for PRV gIII has been reported. Given the paucity of specific functions, we designed complementation experiments to determine if HSV-1 gC and PRV gIII retained any of the perhaps more general functions, such as ability to be incorporated into heterologous virion envelopes or to take part in glycoprotein complexes. If gC could not be incorporated into virion envelopes, it might still be able to participate in and interfere with certain aspects of PRV glycoprotein assembly and localization, perhaps altering PRV growth (e.g., reduced final titer or rate of virus release).

We began our study by determining if the HSV-1 gC gene could function in the PRV genome. To do this, we inserted the entire gC coding sequences and promoter region into a plasmid (pALM10) that contained a 1.4-kilobase (kb) deletion of the gIII promoter and 87% of the gIII coding sequences but retained sequences upstream and downstream of gIII (including the gIII polyadenylation site). A 3.7-kb *Sal*I fragment of HSV-1 containing the regulatory and coding sequences of gC was inserted in pALM10 in both orientations. The insertion was at the second *Xho*I site from the left shown in PRV10 (Fig. 1). These plasmids (pALM63 and pALM64) were cotransfected with PRV-Becker (PRV-Be) viral DNA into PK15 cells. We anticipated that homologous recombination would result in the replacement of the gIII gene with the HSV-1 gC gene and its promoter. These recombinants could be easily recognized by their gIII null phenotype by using the black-plaque assay as previously described (6, 14, 17). The resulting viruses were designated PRV63 and PRV64 (portions shown in Fig. 1A). All work with recombinant viruses was done in accordance with the National Institutes of Health guidelines for recombinant DNA research and were performed at biosafety level 2 in a biosafety level 3 facility.

To demonstrate that PRV63 and PRV64 contained the correct insertions, nucleocapsid DNA was digested with *Sph*I, and the resulting fragments were resolved on a 1% agarose gel and blotted onto nitrocellulose for Southern blot analysis as previously described (14, 18). The probe used in the hybridization was a 4.3-kb *Pst*I fragment containing the gIII coding region and flanking sequences. Two *Sph*I sites,

* Corresponding author.

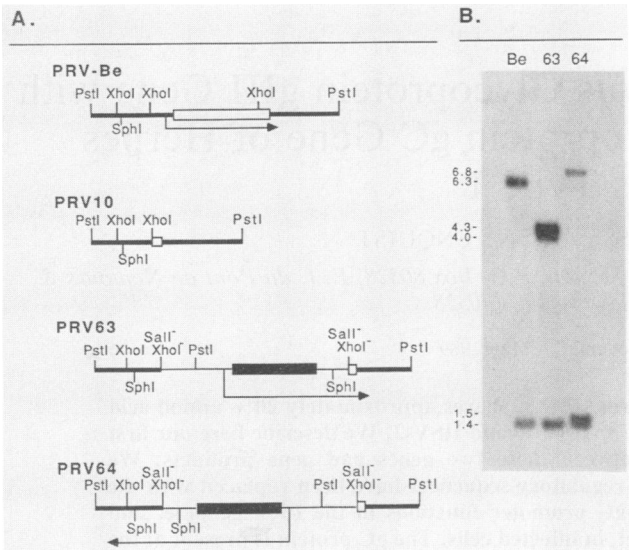

FIG. 1. Construction and analysis of PRV recombinants. (A) *Pst*I fragments containing the coding regions for gIII in PRV-Be and HSV-1 gC in PRV63 and PRV64. Relevant restriction endonuclease sites are indicated. The gIII and gC coding sequences are indicated by open and closed boxes, respectively. The thick black line denotes PRV sequences, and the thin black line indicates HSV-1 sequences. The direction and extent of expected transcripts are indicated by arrows below the boxes. The drawings are not to scale. (B) Southern blot analysis of nucleocapsid DNA extracted from PRV-Be (Be), PRV63 (63), and PRV64 (64). The sizes of the fragments were estimated by using molecular size standards and are indicated (in kilobases) to the left of the blot.

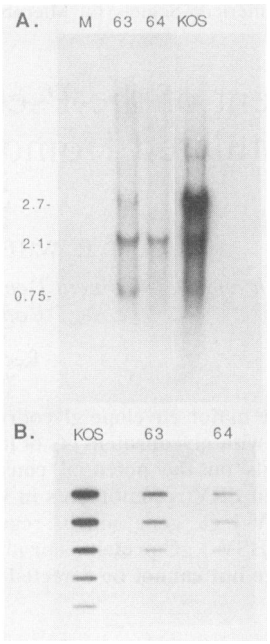

FIG. 2. Northern and slot blot analyses of total cytoplasmic RNA extracted at 16 h postinfection. (A) Northern blot analysis of uninfected and PRV63- and PRV64-infected PK15 cells and of HSV-1 KOS-infected Vero cells. The observed RNA species are indicated to the left of the Northern blot. (B) Slot blot analysis. Samples (10 μg) of RNAs extracted from PRV63, PRV64, and HSV-1 KOS-infected Vero cells were serially diluted twofold, denatured, and transferred to nitrocellulose in a slot blot apparatus. Lanes: M, mock infection; KOS, HSV-1 KOS; 63, PRV63; and 64, PRV64.

critical for Southern blot analysis (Fig. 1B), lie just outside the indicated *Pst*I fragment in the PRV genome: one is located 900 base pairs to the left of the upstream *Pst*I site, and the other is located 2.5 kb to the right of the downstream *Pst*I site. The data from the analysis are shown in Fig. 1B. When the gC-containing fragment was inserted in the same orientation as gIII, the expected fragments of 4.3, 4.0, and 1.4 kb were seen (Fig. 1B, lane 63). In the opposite orientation, bands at 6.8, 1.5, and 1.4 kb were observed (Fig. 1B, lane 64). We conclude that the replacements of the gIII genes with the HSV-1 gC genes constructed in vitro were transferred correctly to the PRV by homologous recombination.

We used these two recombinant viruses to examine the following: (i) whether the gC gene will function in the context of a PRV infection; (ii) whether gC, if expressed, will be exported and localized properly on the surfaces of infected cells and in the virus envelope; and (iii) whether gC, if expressed, will affect PRV growth.

The regulated expression of HSV-1 gC has been studied extensively (3, 7, 8). It is a typical late gene, in that it requires DNA replication for expression. PRV gIII is also a late gene. When viral DNA synthesis was blocked by phosphonoacetic acid, no gIII transcription was observed (unpublished observations). Homa and colleagues have shown that the 15-base-pair sequence, GGGTATAAATTC CGG, located between bases −34 and −20 of the gC promoter is essential for expression of gC as a strict γ2 gene. The gIII promoter has not been analyzed in the same detail, but a similar motif (GCGTTTTTAAAACCGCG) is found around the putative gIII promoter (13). Homa and colleagues showed in transfection experiments that the gC 15-base-pair sequence contains signals responsive to transactivation by the HSV viral immediate-early gene products. The 15-base-pair promoter region of gC is intact in both PRV63 and PRV64, but sequences at the 3′ end of the gC mRNA have been altered. PRV63 lacks the gC polyadenylation signal, but the gIII polyadenylation signal remains intact. In PRV64, the gC polyadenylation signal is lacking and is replaced with uncharacterized DNA upstream of the gIII gene. Consequently, it was not clear where PRV64 gC mRNA would be terminated and polyadenylated, but it should be larger than the normal gC message.

To analyze the steady-state levels of gC-specific mRNA produced in a PRV63 or PRV64 infection, total cytoplasmic RNA was extracted at 16 h postinfection from PK15 either mock infected or infected with PRV63 or PRV64. As a positive control, total cytoplasmic RNA was extracted at 16 h postinfection from Vero cells infected with HSV-1 KOS. A 5-μg portion of each RNA was denatured, fractionated on an agarose-formaldehyde gel, and transferred to nitrocellulose for Northern (RNA) blot analysis (20). The probe used was a 3.7-kb *Sal*I fragment containing all of the gC coding sequences (Fig. 2A). It is clear from this analysis that the major gC-specific messages produced in a PRV63 infection were indistinguishable from those produced in an HSV-1 KOS infection. In PRV63, the full-length gC message of 2.7 kb was present along with two smaller transcripts of approximately 2.1 and 0.75 kb, whereas in PRV64-infected cells, only the 2.1-kb transcript and a small amount of the 0.75-kb transcript were detected. We assume that in PRV64, gC is translated from a message larger than 2.7 kb but is present at reduced levels. These results are consistent with expression from both recombinants that initiate from an HSV promoter, but use different PRV terminators. This analysis also dem-

onstrates that the quantity of gC message is reduced significantly compared with the quantity in an HSV-1 infection. Quantitation of RNA by slot blot analysis and subsequent densitometry revealed that PRV63 and PRV64 express about 8- to 12-fold less gC-specific RNA than HSV-1 KOS does (Fig. 2B).

Taken together, the Northern blot and slot blot analyses demonstrate that the gC gene can be transcribed when stably maintained in the PRV genome with an endogenous HSV promoter (presumably the gC promoter). This expression, however, is reduced compared with gC expression in an HSV-1 infection. These results could be explained by the lack of HSV regulatory gene products needed for activation of gC transcription, by interference by PRV regulatory gene products, or by the lack of authentic gC 3′ ends in the transcripts.

We next determined if gC produced by PRV63 and PRV64 in PK15 cells was synthesized, exported, and localized correctly. Fractionation and immunoprecipitation of infected cells, virions, and media have been described previously (15). Briefly, PK15 cells or Vero cells (for HSV-1 KOS) were infected at a multiplicity of infection of 10 with PRV-Be, PRV63, PRV64, or HSV-1 KOS. Infected cells were labeled continuously with [^{3}H]glucosamine (50 μCi/ml) until 16 h postinfection. Each plate was fractionated into infected cells, released virions, and media free of released virions. Figure 3A shows the results of these immunoprecipitations from infected cells. Lane gIII under Be shows the steady-state labeling pattern for gIII; that is, a characteristic, heterogeneous, 92-kilodalton mature protein and a 74-kilodalton precursor protein. Lane gC under Be demonstrates that there was no cross-reactivity between the gC antiserum and gIII. Conversely, the lanes under KOS demonstrate that there was no cross-reactivity between the gIII antibody and gC produced by HSV-1 KOS. Lanes 63 and 64 show that PRV63 and PRV64 produce gC that is reactive with the gC polyvalent serum and not with gIII serum. Both the characteristic precursor and product forms of gC were observed. Consistent with in vitro translation results (data not shown), PRV63 produced more gC than PRV64 did. The amount of gC immunoprecipitated for PRV63 and PRV64 (Fig. 3A) resulted from four times the amount of infected-cell extract compared with that used to analyze PRV-Be or KOS.

The medium analysis (Fig. 3B) revealed an unexpected but reproducible result. A significant portion of the gC antigen produced not only by PRV63 and PRV64 but also in HSV-1 KOS infections was found in the media. This phenotype was also observed in HEL cells (data not shown), indicating that it is not a cell-specific phenomenon. We have constructed gIII-gC hybrid proteins and have demonstrated that this phenotype requires only the carboxy-terminal 39 amino acids of gC (manuscript in preparation). While it was difficult to detect a strong signal for gC in infected cells unless four times the standard amount of extract was immunoprecipitated (Fig. 3A), gC in the media of PRV63 and PRV64 infections was relatively easy to detect. The data in Fig. 3B were obtained by immunoprecipitating equal amounts of media from all four infections.

Total virion profiles (not immunoprecipitated) (Fig. 3C, lanes Be, 63, 64, and KOS) indicated that the infections had progressed to the same extent, because approximately equal quantities of virion proteins were recovered. In an attempt to increase sensitivity of detection of gC protein in the PRV virions, four times more purified PRV63 and PRV64 virions than PRV-Be virions were used in the immunoprecipitation analysis. Even with this increased quantity of sample, it was

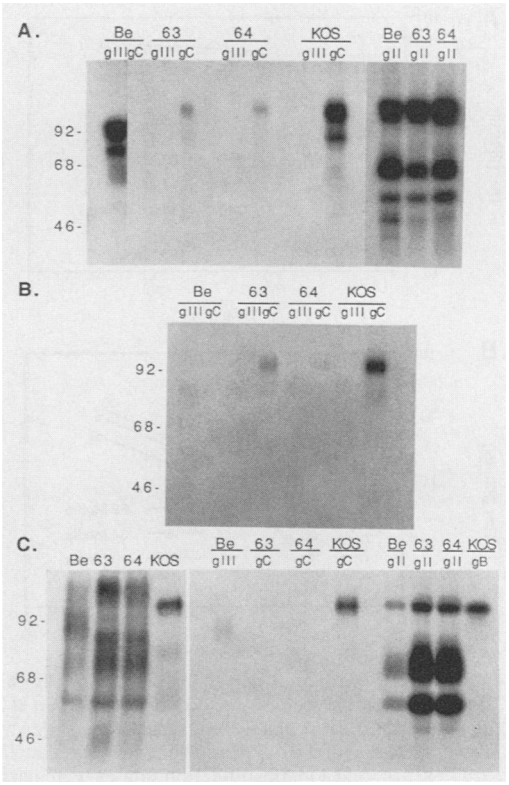

FIG. 3. Localization of [^{3}H]glucosamine-labeled gIII and gC. The samples were fractionated into cell (A), medium (B), and virion (C) fractions, and samples of each were used for immunoprecipitation. Proteins were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel, and fluorography was used for visualization of the ^{3}H-labeled polypeptides. The virus strain used for infection is indicated across the top of each panel as follows: Be, PRV-Be; 63, PRV63; 64, PRV64; KOS, HSV-1 KOS. Antisera used for immunoprecipitation were as follows. Goat polyvalent 282 serum (gIII) was prepared against a denatured *Escherichia coli* Cro-gIII fusion protein and was reactive with native and denatured gIII antigen. Goat polyvalent 284 serum (gII) was prepared against immunoaffinity-purified gII and was reactive with native and denatured gII antigen (284 serum was used as a control for infection and integrity of fractions). Rabbit polyvalent gC serum (gC) was prepared and kindly provided by G. Kikuchi (the gC antiserum was reactive with denatured and native gC). Rabbit polyvalent gB serum (gB) was prepared and kindly provided by R. L. Burke.

not possible to detect gC in the envelopes of PRV63 and PRV64. The more intense gII profiles for PRV63 and PRV64, but not for PRV-Be, demonstrate that four times the amount of purified virions were actually immunoprecipitated.

While gC was not localized in PRV virions, it did become localized at the cell surface. Both PRV63- and PRV64-infected cells expressed gC on the cell surface, as determined by positive reaction of PRV63 and PRV64 plaques with gC antibody in the black-plaque test (data not shown).

PRV gIII is known to aid in the efficient release of plaque-forming virus from PK15 cells, a phenotype that is easily monitored by measuring virus in the supernatant of infected cells (21). In a typical PRV-Be infection, plaque-forming virus begins to accumulate in the media about 7 h postinfection, but for infections with gIII null mutants, virus release is delayed more than 3 h (15). We compared production and release of plaque-forming virus in single-step growth experiments to determine if gC could complement

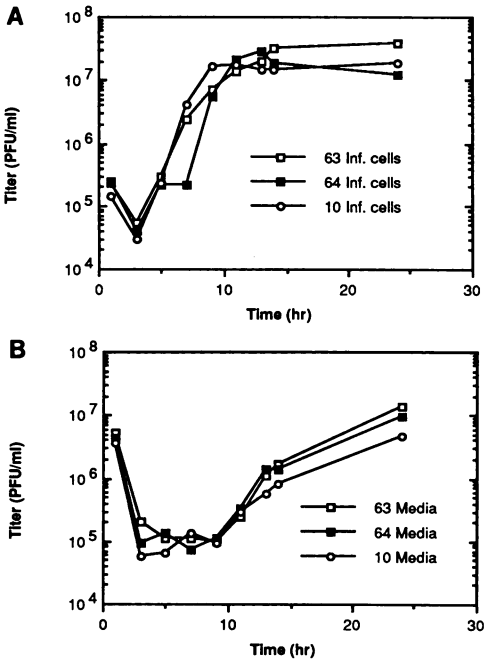

FIG. 4. Single-step growth analysis of PRV10, PRV63, and PRV64. The experiments were done as described by Ryan et al. (15) and Whealy et al. (21). PK15 cells were infected with PRV strains at a multiplicity of infection of 5 PFU per cell and incubated at 37°C. At 1, 3, 5, 7, 9, 11, 13, 14, and 24 h postinfection, plates were harvested, and the plaque-forming-virus titers of the infected-cell (A) and media (B) fractions were determined. Media fractions were cleared of cells and debris by low-speed centrifugation prior to titer determinations. Infected cell fractions were washed free of released virions before titer determinations. 63, PRV63; 64, PRV64; and 10, PRV10; Inf., infected.

the release defect of a virus carrying a defined gIII deletion. Any interference in extent and kinetics of intracellular plaque-forming virus production was also monitored. PRV10, a virus carrying a 1.4-kb gIII deletion lacking the gIII promoter and most of its coding sequences (14), was compared with PRV63 and PRV64, which have the identical gIII deletion (Fig. 4). It is clear that expression of gC does not improve or retard growth of either virus. Significantly, the release defect is not complemented; increase of plaque-forming virus in the media for all three infections can be detected only after 10 h.

These experiments represent our initial attempts to determine if there is conservation of function between PRV gIII and its postulated homolog, HSV-1 gC. Even though the number of assayable functions was limited, several conclusions emerged from our complementation experiments. We have demonstrated that the gC glycoprotein of HSV-1 KOS could be expressed from a PRV vector. Since gC was expressed from the cloned HSV-1 fragment in either orientation, expression was most likely from the HSV gC promoter. gC expression was low but consistent with the reduced level of gC mRNA produced. Surprisingly, a significant fraction of gC produced by PRV63, PRV64, and HSV-1 KOS was found in the media of infected cells. The glucosamine-labeled gC proteins expressed by PRV63 and PRV64 were indistinguishable from authentic HSV-1 gC in electrophoretic mobility, suggesting that posttranslational modifications were essentially identical. Moreover, the gC proteins could be localized to the cell surface as demonstrated by antibody staining (data not shown). No gC protein was detected in PRV63 or PRV64 virions under our immunoprecipitation conditions or in more sensitive Western immunoblot experiments (data not shown).

These results indicated that basic export and localization signals of gC can function in a heterologous infection; however, it is interesting that gC is not found in the PRV envelope. Because of low gC expression by these recombinant viruses, we cannot unequivocally state that specific localization signals for incorporation into PRV virions were defective. There may be a concentration threshold for incorporation into virions. On the other hand, it may be that gC has evolved sufficiently from PRV gIII that it lacks specific signals or the proper conformation to participate in interactions with other factors necessary for efficient inclusion in the PRV virus envelope. This would imply that localization of glycoproteins to herpesvirus virion envelopes is a highly specific process. Further work is necessary to establish this point.

Single-step growth experiments indicated that gC could not complement the virus release defect of a gIII null mutant. From these experiments, we found no evidence that HSV-1 gC participated in or interfered with PRV virion assembly and virus production. Given these observations and the reservation that gC was expressed at a low level, our current suggestion is that PRV gIII and HSV-1 gC have diverged sufficiently that few, if any, complementing functions remain.

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