The gIII Glycoprotein of Pseudorabies Virus Is Involved in Two Distinct Steps of Virus Attachment

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The entry of herpesviruses into cells involves two distinct stages: attachment or adsorption to the cell surface followed by internalization. The virus envelope glycoproteins have been implicated in both stages. Pseudorabies virus attaches to cells by an early interaction that involves the viral glycoprotein gIII and a cellular heparinlike substance. We examined the role of gIII in the attachment process by analysis of a set of viruses carrying defined gIII mutations. The initial attachment of gIII mutants with an internal deletion of 134 amino acids (PrV2) to MDBK cells was indistinguishable from that of wild-type virus. The adsorption of these mutants was, however, much more sensitive than that of wild-type virus to competing heparin. Furthermore, while attachment of wild-type virus to MDBK cells led to a rapid loss of sensitivity to heparin, this was not the case with PrV2, which could be displaced from the cell surface by heparin after it had attached to the cells. We conclude that glycoprotein gIII is involved in two distinct steps of virus attachment and that the second of these steps but not the first is defective in PrV2.

Pseudorabies virus (PrV), a herpesvirus of swine, encodes at least eight different glycoproteins. Seven of the genes encoding these glycoproteins have been mapped and sequenced (9-11, 14-19, 23). Four have been shown to be nonessential for growth in cell culture (2, 9, 15, 20, 24, 25). We have been interested in ascertaining the functions of these nonessential glycoproteins and have focused on glycoprotein gIII, the homolog of glycoprotein gC of herpes simplex virus (19).

Cells infected with mutants of PrV that do not express gIII produce virus populations that have a lower titer of infectious virus than do cells infected with wild-type virus (7, 22, 25). The lower yield of infectious virus produced by gIII⁻ mutant-infected cells is attributable, at least in part, to the reduced infectivity of the gIII⁻ mutant and reflects the important role gIII plays in the stable adsorption of the virus to its host cell (22). It has been reported recently that the gC glycoprotein of herpes simplex virus, the homolog of glycoprotein gIII of PrV, also plays a principal role in virus adsorption (3, 5). While the primary pathway of adsorption of wild-type PrV to cells is through an early interaction that involves the viral glycoprotein gIII and a cellular heparinlike substance, an alternative mode of adsorption exists, which is used by the virus in the absence of either the viral gIII protein or the cellular heparinlike receptor (28). A similar situation also appears to apply for herpes simplex virus (3, 5).

The early events leading to the initiation of the infectious process of most enveloped viruses, including the herpesviruses, can be divided into two distinct stages: attachment or adsorption to the cell surface followed by internalization, which is accompanied or followed by removal of the virus envelope. It is likely that each of these stages involves several distinct steps. In this report, we address the attachment of PrV to cells and examine the role of glycoprotein gIII in this process. In particular, we used defined gIII

mutations to determine the domains of gIII involved in attachment. Using this approach, we demonstrated that the attachment process includes at least two different steps and that gIII is involved in both.

MATERIALS AND METHODS

Media and solutions. Media and solutions used in this study include the following: Eagle's synthetic medium plus 5% dialyzed bovine serum (EDS); EDS plus 5-fluorouracil (20 µg/ml) and thymidine (5 µg/ml) (EDS-FU); Tris-buffered saline (TBS) containing 1% crystalline bovine albumin (TBSA); and TBS containing 2% sodium dodecyl sulfate (SDS).

Radiochemicals and chemicals. [³H]thymidine (specific activity, 45 Ci/mmol) was purchased from New England Nuclear Corp. Heparin was purchased from Sigma Chemical Co.

Cells and virus mutants. Madin-Darby bovine kidney (MDBK), pig kidney, or rabbit kidney cells were cultivated in EDS. Virus was titrated by plaque assay in MDBK monolayer cultures.

The mutants used have been described previously in detail (19, 25). To facilitate the understanding of the experiments in this report, we briefly summarize their structures and phenotypes in Fig. 1. The wild-type gIII gene encodes a protein of 479 amino acids. PrV10, a mutant of PrV(Be), and PrV(Ka)gIII⁻ have a deletion of the gIII promoter as well as most (more than 87%) of the gIII-coding sequence (Fig. 1); they produce no immunoreactive gIII protein (20, 21). PrV4, a mutant of PrV(Be), produces a truncated form of gIII that is predicted to terminate at codon 157. The gIII protein expressed by PrV4 is secreted into a medium and is not incorporated into virion envelopes. PrV2, a mutant of PrV(Be), and the mutant PrV(Ka)2 contain a gIII gene with an in-frame internal deletion of 402 bp from the central part of the gene (deletion of codons 157 to 292). A mature gIII glycoprotein of 68 kDa apparent molecular mass is synthesized by cells infected with these mutants, and this glyco-

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FIG. 1. Diagram of the PrV gIII protein and description of mutant viruses. The 479-amino-acid (aa) gIII protein is indicated at the top. The amino terminus (NH₂) and carboxy terminus (COOH) are indicated. Numbers refer to specific amino acids or codons, as indicated below. The PrV(Ka) gIII gene has not been completely sequenced, but we assume that the locations of signal sequence and transmembrane and glycosylation sites coincide with those established for PrV(Be) (19).

protein is found in the virion envelopes. PrV30, a mutant of PrV(Be), contains a linker insertion at codon 460 resulting in a frameshift that replaces the last 19 amino acids of wild-type gIII with 34 amino acids from a different reading frame (25). Most of the PrV30 gIII protein is secreted into the medium because it lacks a functional membrane anchor. Unlike PrV4 and PrV10, PrV30 virions contain reduced, but detectable, quantities of gIII in the envelope (20). Thus, the mutants PrV4 and PrV10 do not contain gIII in their envelope, while PrV2 virions have essentially wild-type quantities of a 68-kDa form of the gIII protein. Immunoprecipitation analysis (Fig. 2) suggested that PrV30 virions contain less than 10%

FIG. 2. Immunoprecipitation of [³H]glucosamine-labeled gIII- and gII-specific proteins from purified virions. PK15 cells were infected with PrV(Be) and PrV30 in the presence of [³H]glucosamine as described in Materials and Methods. Extracellular virus particles were purified and analyzed with gIII- and gII-specific antibodies. Lanes 1, 2, and 3, PrV(Be); lanes 4, 5, and 6, PrV30. Lanes 1 and 4, gIII-specific M1 monoclonal antibody; lanes 2 and 5, 282 polyvalent anti-gIII serum; lanes 3 and 6, gII-specific M3 monoclonal antibody. Immune complexes were collected by adsorption to Pansorbin (*Staphylococcus aureus*) and fractionated by electrophoresis on a 10% polyacrylamide-SDS gel. ³H-labeled polypeptides were detected by fluorography. Molecular mass markers are indicated (kilodaltons).

of the level of wild-type gIII in PrV(Be) virions.

Labeling of virus for immunoprecipitations. PK cells were infected at a multiplicity of infection of 10 with either PrV(Be) or PrV30 and were labeled continuously throughout 16 h of infection at 37°C by growth in medium containing 50 μCi of [³H]glucosamine per ml.

Monoclonal antibodies and immunoprecipitation. The procedures used for immunoprecipitation and the monoclonal antibodies against glycoprotein gIII have been described previously (4).

Labeling and purification of virus. To obtain [³H]thymidine-labeled virus, we incubated cell monolayers for 24 or 48 h in EDS-FU (a procedure that inhibits cellular DNA synthesis without affecting virus growth [6]). The cells were then infected (multiplicity of infection, 5 PFU per cell) and incubated in EDS containing [³H]thymidine (20 μCi/ml) for 24 h.

To purify the virions, the medium was collected and clarified by centrifugation at 4,000 × *g* for 10 min, and the supernatant containing the extracellular virus was centrifuged on a TBSA–30% sucrose cushion at 15,000 rpm for 1 h in a Beckman SW29 rotor. The virus pellet was resuspended gently in 1 ml of TBSA and centrifuged on a sucrose gradient, as described previously (1, 4). Fractions were collected, and the virus peak was localized. The virus was diluted with TBSA and sedimented on a TBSA–30% sucrose cushion by centrifugation at 15,000 rpm for 1 h in an SW27 rotor. The number of PFU/10⁶ cpm varied between approximately 2 × 10⁸ PFU/10⁶ cpm for wild-type virus and 1 × 10⁶ PFU/10⁶ cpm for gIII null mutants (the infectivity of gIII[−] mutants is reduced). The number of viral particles (as determined by the amount of DNA in the preparations) per 10⁶ cpm was approximately the same for the wild type and all the mutants (2 × 10¹⁰ particles per 10⁶ cpm).

Adsorption of labeled virus. Purified [³H]thymidine-labeled virus in TBSA, prepared as described above, was incubated with monolayers of MDBK cells which had been preincu-

TABLE 1. Inhibition of plaque formation by monoclonal antibodies against gIII

Virus	Plaque formation (% of control) ^a				Adsorption ^b with or without antibody mixture:	
	M1	M7	M16	Mixture	-	+
PrV(Ka)	10	6	7	4	89.0	3.7
PrV(Ka)2	100	100	100	100	70.0	68.3
PrV(Ka)gIII ⁻	100	100	100	100	2.8	2.6
PrV(Be)	100	100	100	3	65.3	2.9
PrV2	100	100	100	100	59.0	63.2
PrV4	ND ^c	ND	ND	100	ND	ND
PrV10	ND	ND	ND	100	3.7	4.1
PrV30	ND	ND	ND	3	ND	ND

^a Approximately 5×10^2 PFU of each of the virus stocks was incubated for 1 h in Eagle's medium with or without the indicated individual monoclonal antibody (final dilution, 1:20) or an equal mixture of the three (final dilution, 1:20). The virus was then added to MDBK cells and allowed to adsorb for 1 h. The monolayers were washed to remove unadsorbed virus and overlaid with agarose. The percentage of plaques that developed after treatment with the monoclonal antibodies was determined.

^b Approximately 2×10^5 cpm of [³H]thymidine-labeled virus in 0.2 ml of TBSA was either incubated with the monoclonal antibody mixture or left untreated. The virions were then diluted fivefold in TBSA, added to MDBK monolayers, and allowed to adsorb for 1 h at 37°C. The monolayers were washed extensively to remove unadsorbed virus, and the number of counts (cpm $\times 10^2$) associated with the monolayers was determined.

^c ND, not determined.

bated in TBSA for 15 min to minimize nonspecific binding. The monolayers were further incubated for 1 h at 37°C and then washed extensively to remove unadsorbed virus. The cells were scraped into SSC (0.15 M NaCl plus 0.015 M sodium citrate)-SDS, and the amount of radioactivity that was associated with the cell monolayers was determined.

RESULTS

Neutralization of gIII mutants by monoclonal antibodies against gIII. Previously, it was shown that monoclonal antibodies raised against gIII of PrV(Ka) immunoprecipitate this protein from wild-type PrV(Ka) or PrV(Be) but not from gIII null mutants of either virus (21, 22). Of the four mutants (PrV2, PrV10, PrV4, and PrV30) (see Fig. 1 for structures of mutants), only PrV30 expresses a gIII protein capable of binding these monoclonal antibodies (19, 25). Thus, it appeared that these monoclonal antibodies interact with the central domain of the gIII molecule defined by the deletion in PrV2. Alternatively, the lack of reactivity of the monoclonal antibodies with the PrV2 glycoprotein may be the result of a conformational change resulting from the deletion of amino acids 157 to 292. To extend these observations, we determined whether the gIII mutants were neutralized by these monoclonal antibodies and whether these monoclonal antibodies would inhibit adsorption of these viruses.

Of the individual monoclonal antibodies against gIII tested, all neutralized wild-type PrV(Ka), but none was capable of neutralizing PrV(Be) (Table 1). However, a mixture of three of the monoclonal antibodies was effective in neutralizing PrV(Be) and PrV30. This mixture did not neutralize PrV4, PrV10, or PrV2. The mutants of the PrV(Ka) strain, PrV(Ka)gIII⁻ and PrV(Ka)2 [two mutants derived from the PrV(Ka) strain that carry mutations similar to those of PrV10 and PrV2, respectively], also were not neutralized by these antibodies (Table 1).

The pooled monoclonal antibodies against gIII also inhib-

ited the binding to MDBK cells of radiolabeled wild-type PrV(Be) and PrV(Ka) but not PrV2 and PrV10 or PrV(Ka)gIII⁻ and PrV(Ka)2 (Table 1). These results corroborate previous observations indicating that gIII was essential for adsorption (22). Furthermore, they extend these findings to show that a mutant containing only trace amounts of gIII in its envelope (PrV30) is still neutralized by gIII-specific antibodies.

Because the gIII monoclonal antibodies inhibit wild-type and PrV30 virus adsorption (4) (Table 1), and because the PrV2 mutants, which lack the central structural elements of gIII protein, are not neutralized or immunoprecipitated by these antibodies (21), we ascertained whether the central segment of gIII has a role in virus attachment to cells by comparing the adsorption characteristics of each gIII mutant.

Adsorption of different mutants defective in gIII to MDBK cells. Figure 3 shows the adsorption to MDBK cells of wild-type PrV(Be) and PrV(Ka) and of the various mutants of these virus strains defective in gIII. PrV30 and PrV2, as well as PrV(Ka)2 (Fig. 1), adsorbed as well as wild-type virus to MDBK cells. The gIII null mutants PrV4, PrV10, and PrV(Ka)gIII⁻ (Fig. 1) adsorbed less well than wild-type virus. A large proportion of these mutants could be removed from the monolayers by washing, as indicated by the fact that they yielded a higher titer (i.e., a larger number eventually did adsorb and did form plaques) if the monolayers were overlaid with agarose without prior washing. These findings confirm the previous conclusion that the gIII null mutants adsorb a slower rate than wild-type virus (22).

The noteworthy observation emerging from this experiment was that PrV2 and PrV(Ka)2 adsorbed to the MDBK cells as well as wild-type virus. Thus, despite the fact that PrV2 lacks the domain (or has an altered domain) of gIII necessary for interaction with the neutralizing antibodies, the ability of PrV2 to adsorb to MDBK cells did not appear to be detectably impaired. The other finding to emerge from these experiments is that the reduced amount of gIII present in the PrV30 virions is sufficient for efficient adsorption.

In the experiment summarized in Fig. 3, we determined the adsorption efficiencies of the different mutants by comparing the number of plaques that developed when the monolayers were washed after the adsorption period with those that developed when the inoculum was not removed before the plates were overlaid with agarose. This method is predicated on the assumption that virus that has not adsorbed during the adsorption period will eventually adsorb to the cells if left on the monolayers. However, this method will not reveal what proportion of the infectious virions in the inoculum indeed did adsorb. To circumvent this difficulty, another approach was used.

To compare the relative adsorptions of the wild type and the mutants, we made mixtures of a mutant and wild-type virus or of two mutants. Part of the mixture was allowed to adsorb to cell monolayers. The cells were washed to remove unadsorbed virus, and the DNA associated with the cells was analyzed and compared with the DNA obtained from the virus in the inoculum used to infect the cells. The ratio of the two mutants in the initial inoculum and in the virus population that had adsorbed to the cells could be determined by the distinctive hybridization patterns of the restriction enzyme digests of the two. Because of the different sizes of the deletions in the gIII gene in the mutants, the genomes of the mutants and of the wild type can be distinguished from each other.

The results of a representative experiment (Fig. 4) show

FIG. 3. Adsorption of various gIII mutants to MDBK cells. The virus preparations were diluted in medium to give approximately 200 PFU/ml, and 1 ml of each was added to cultures of MDBK cells. At the indicated times, some of the cultures were washed extensively and overlaid with agarose, while others were overlaid with agarose without removing the inoculation fluid. Plaques were counted after 4 days of incubation. The 100% value (approximately 200 PFU) is the number of plaques that developed in nonwashed cultures. (A) PrV(Be) and gIII mutants derived from PrV(Be). (B) PrV(Ka) and gIII mutants derived from PrV(Ka).

that the ratio of PrV(Be) to PrV2 DNA was approximately the same in the original virus inoculum and in the cell-associated virus. On the other hand, the ratio of PrV10 to PrV2 DNA in cell-associated virus was much lower than that in the inoculum used to infect the cells. Scanning of appropriately exposed autoradiograms and quantitation of the areas under the peaks confirmed that PrV(Be) and PrV2

adsorbed equally well to MDBK cells but that PrV10 adsorbed at least 10-fold less well than PrV(Be). Thus, while PrV10 adsorbs poorly to MDBK cells, the adsorption of PrV2 and wild-type virus to MDBK cells is similar.

Effect of heparin on adsorption of mutants to MDBK cells. The initial interaction of wild-type PrV with its host cell is mediated by glycoprotein gIII and a cellular heparinlike receptor. This interaction is blocked effectively by heparin (13). gIII⁻ mutants adsorb by an alternative mode that is independent of the heparinlike cellular receptor (27). To ascertain to what extent the different mutants adsorb to their host cells either via the viral gIII-heparinlike receptor interaction or via the alternative mode of adsorption, we ascertained the effect of heparin during the adsorption period on plaque formation of the wild type and of the gIII mutants. (The effect of heparin on plaque formation is a measure of its effect on adsorption [13]). The results of a representative experiment, in which the effects of different concentrations of heparin on the mutants were ascertained, are summarized in Fig. 5. Plaque formation of wild-type PrV(Be) and PrV(Ka) or of mutant PrV30 was similarly sensitive to heparin. As expected from previous findings (13), the presence of heparin during the adsorption period had much less effect on plaque formation of gIII null mutants [PrV4 and PrV10 or PrV(Ka)gIII⁻] than it did on that of wild-type virus. Concentrations that reduced plaque formation of the wild type by more than 99% reduced plaque formation of these mutants by less than 50%. Surprisingly, PrV2 and PrV(Ka)2 were considerably more sensitive to heparin than was wild type. While plaque formation by wild-type virus was inhibited by approximately 80% with 0.3 μ g of heparin per ml, the same degree of inhibition of PrV2 or PrV(Ka)2 was achieved with as little as 0.003 μ g/ml (i.e., a 100-fold-lower concentration). Because two viruses with the same deletion in the gIII gene [PrV2, PrV(Ka)2] behaved in an identical manner, the hypersensitivity of these mutants to heparin must be directly related to the mutation in the gIII

FIG. 4. Relative adsorption of PrV(Be), PrV2, and PrV10 to MDBK cells. Mixtures of PrV(Be) and PrV2 or PrV2 and PrV10 (approximately 10^7 PFU each) were made. One milliliter of each mixture was added to monolayers of MDBK cells and allowed to adsorb for 1 h. The monolayers were then washed to remove unadsorbed virions, the cells were harvested, and the DNA was extracted. The virus in the original mixtures, as well as in each individual virus preparation, was also purified, and the DNA was extracted. The DNA preparations were digested with *Pst*I and probed with the nick-translated 4.3-kb *Pst*I fragment (the gene encoding gIII is included in this fragment). Lanes: 1, PrV(Be); 2, PrV2; 3, original mixture of PrV(Be) and PrV2; 4, virions that became associated with cells infected with the mixture of PrV(Be) and PrV2; 5, PrV2; 6, PrV10; 7, original mixture of PrV2 and PrV10; 8, virions that became associated with cells infected with the mixture of PrV2 and PrV10.

FIG. 5. Effect of the presence of heparin during adsorption on plaque formation of wild type or of gIII mutants of PrV. Monolayers of MDBK cells were inoculated with 1 ml of TBASA containing the indicated concentration of heparin for 10 min at 37°C. Approximately 500 PFU of either wild type or the gIII mutants was then added, and the monolayers were incubated for 1 h. They were then washed extensively to remove the heparin and the unadsorbed virus and overlaid with agarose. Plaques were counted 4 days later. (A) PrV(Be) and gIII mutants of PrV(Be). (B) PrV(Ka) and gIII mutants of PrV(Ka).

gene. We conclude that, compared with wild-type virus, adsorption of the PrV2 mutants is hypersensitive to heparin.

Similar results were also obtained when the effect of heparin on adsorption of radiolabeled virus was determined (Table 2). Concentrations of heparin (0.3 $\mu\text{g/ml}$) that reduced the amount of radiolabeled cell-associated PrV2 and PrV(Ka)2 by approximately 97% reduced adsorption of wild-type virus by only approximately 60%.

The greater sensitivity of PrV2 to heparin reflects a defect in a step required for the stable interaction of the virus with its host cells. The higher sensitivity of PrV2 mutants to heparin could be due to a higher affinity of PrV2 to exogenously added heparin (in contrast to its affinity for the heparinlike receptor on the cell surface, which appears to be the same). On the other hand, it might reflect the fact that stable adsorption is a multistep process. Thus, the first interaction of the virus with the cells might be via a low-affinity binding of gIII with the cell surface heparinlike receptor molecule, this interaction being reversible by competing heparin. A second step of adsorption resulting in a more stable interaction between the virus and a cell membrane component

might then take place. If the interaction of PrV2 with the heparinlike cellular component is normal but the second step of adsorption is delayed, the presence of competing heparin would be more effective in interfering with the stable adsorption of PrV2 than with that of wild-type virus.

First, to distinguish between these possibilities, we looked for differences in the affinity of heparin for the gIII proteins of PrV(Be) and PrV2. [^3H]glucosamine-labeled glycoprotein extracts obtained from the purified virions were incubated with increasing amounts of heparin acrylic beads, and the amount of gIII that had bound to the acrylic beads was estimated after polyacrylamide gel electrophoresis as described previously (13). Similarly, heparin acrylic beads were incubated with increasing amounts of [^3H]glucosamine-labeled extracts obtained from PrV(Be) and PrV2 purified virions. No difference in the binding of the gIII (or gII) of PrV(Be) and PrV(B2) could be detected in any of these experiments (data not shown). Thus, PrV2 does not appear to have a higher affinity for heparin than does wild-type virus.

Conclusive evidence for the two-step binding hypothesis was obtained by determining whether PrV2 bound to cell membrane became resistant to exogenously added heparin more slowly than did wild-type virus. To attempt to slow the processes involved in adsorption, as well as to prevent virus penetration, monolayers of MDBK cells were inoculated with virions at 2°C. At various times thereafter, the monolayers were washed to remove unadsorbed virus and heparin was added to one set of infected monolayers; another set served as a control. The rationale for this experiment was that if adsorption is a two-step process and PrV2 is defective in the second of these steps, PrV2 that had adsorbed under the conditions used might be displaced by the competing heparin, while adsorbed wild-type virus might not.

The results obtained are summarized in Fig. 6. As expected, wild-type PrV and PrV2 adsorbed to the cells similarly, i.e., the amount of virus inoculum that could not be removed from the cells by washing at various times after

TABLE 2. Effect of heparin on adsorption of radiolabeled PrV(Be) and PrV2^a

Concn of heparin ($\mu\text{g/ml}$)	10 ⁴ cpm/monolayer ^b		
	PrV(Be)	PrV2	PrV(Ka)2
None	1.90	2.30	1.73
0.3	0.80 (58)	0.07 (98)	0.05 (97)
0.6	0.12 (94)	0.05 (98)	0.03 (98)
1.0	0.04 (98)	0.05 (98)	0.05 (97)

^a Approximately 2×10^5 cpm of [^3H]thymidine-labeled virus was added to monolayer cultures of MDBK cells which had been incubated with TBASA and the indicated concentration of heparin. After a 1-h adsorption, the monolayers were washed and the amount of radioactivity associated with them was determined.

^b The numbers in parentheses indicate the percent inhibition by heparin.

FIG. 6. Displacement of adsorbed PrV(Be) (A) and PrV2 (B) from the cell surface by heparin. Monolayers of MDBK cells were cooled to 2°C and inoculated with cold (2°C) virus (approximately 500 PFU per cell). At the indicated times, the monolayers were washed. Some were incubated with TBSA, and some were incubated with TBSA containing heparin (10 µg/ml). The cultures were further incubated for 1 h at 2°C, washed, shifted up to 37°C, and overlaid with agarose, and plaques were counted 4 days later. ●, no heparin; ○, heparin treated.

inoculation was approximately the same. However, while the wild-type virus that had remained associated with the monolayers after they had been washed extensively was not affected by the addition of heparin, a similar treatment of cultures to which PrV2 had adsorbed reduced significantly the number of plaques obtained. This was particularly apparent when heparin was added at early times after inoculation. Thus, after a 15-min and a 60-min adsorption period, 85 and 50%, respectively, of the adsorbed PrV2 virus but none of the wild-type virus could be displaced from the monolayers by heparin.

These experiments demonstrate that after wild-type virus adsorbs to the cells, it rapidly becomes resistant to the competing effect of exogenously added heparin, even when adsorption occurs at 2°C. In contrast, after PrV2 adsorption, cell-associated virions can still be displaced from the cells by the addition of heparin to the culture. These findings indicate that adsorption of PrV to its host cell involves at least two steps. During the first step, the virus adsorbs to a heparinlike surface moiety, a process that is mediated by glycoprotein gIII. This process occurs similarly when wild type and PrV2 mutants are adsorbed to MDBK cells. Once this first step has taken place, the virus cannot be washed off the monolayer with medium but can be displaced from the cell surface by exogenously added heparin. The second step involves another interaction of virus proteins with the cell surface. After this step has occurred, the virus cannot be displaced from the cell surface by heparin. This second step occurs rapidly after attachment of wild type to the cellular heparinlike receptor; it is, however, delayed after attachment of a mutant such as PrV2, which carries a shortened version of glycoprotein gIII.

DISCUSSION

Glycoprotein gIII is multifunctional, playing roles in virus adsorption and virus release, as well as indirectly in virus penetration (8, 12, 13, 22, 25, 26). It is also a major determinant in eliciting both neutralizing antibodies (2) and cell-mediated immune responses (27). It appears therefore

that gIII, although nonessential for growth in vitro (2, 20), plays a central role in the biology of PrV.

We have been interested in identifying the domains of gIII that control the different facets of its functions. The experiments we presented in this report compare the biological properties of wild-type PrV, gIII null mutants, gIII mutants that express a shortened protein, and mutants that have reduced amounts of gIII in the virus envelope. These experiments were prompted by the observation that the central region of gIII plays a critical role in the neutralization of PrV by gIII-specific antibodies.

Interaction of wild-type and mutant gIII proteins with anti-gIII monoclonal antibodies. The gIII monoclonal antibodies we used were raised against the gIII protein of PrV(Ka). Each of these monoclonal antibodies is capable of neutralizing PrV(Ka) and inhibiting the adsorption of this virus to the cell. These monoclonal antibodies are probably not directed against continuous epitopes because they only react with native, not denatured, gIII (unpublished data). While the gIII protein of PrV(Be) reacts with each of these monoclonal antibodies and is immunoprecipitated by them (21), individually, none of these antibodies is capable of neutralizing PrV(Be) or of inhibiting its adsorption to host cells. A mixture of three of the monoclonal antibodies against gIII, each of which is directed against a different epitope (unpublished data), will, however, neutralize PrV(Be) and inhibit its adsorption. The reason for the cooperative effect of the three monoclonal antibodies is unclear. It is possible that after reacting with all three of the antibodies, the conformation of gIII is modified, thereby causing the inhibition of adsorption. If this is correct, after reacting with only one of the antibodies, the conformation of the gIII protein of PrV(Be) [but not that of PrV(Ka)] must remain competent for virus adsorption.

The fact that the monoclonal antibodies do not bind to the gIII protein of PrV2 might indicate that these antibodies recognize an epitope in the middle third of the gIII protein, i.e., the part of the molecule that is missing in the PrV2 gIII gene. However, the absence of this epitope might also affect

the configuration of the protein, rendering it unable to interact with the monoclonal antibodies. Since the monoclonal antibodies inhibit adsorption, and since PrV2 adsorbs well to at least some cell types (Fig. 3), it appears that the domain responsible for reactivity of gIII with the neutralizing antibodies and those responsible for binding to the cell surface receptors are not identical. Therefore, after binding to wild-type gIII, the antibodies must either sterically obstruct the adsorption site on the wild-type gIII protein or modify its conformation, thereby rendering the adsorption site inaccessible.

Adsorption of PrV to MDBK cells is a two-step process. Stable adsorption of a virus to its host cells is generally defined as binding of the virus to the cell so that it cannot be dislodged from the cell surface by extensive washing. By this criterion, PrV2 adsorbs to MDBK cells as well as wild-type virus. However, the binding of PrV2 differs from that of wild-type virus in that after initial binding to the cells, the latter becomes rapidly resistant to exogenously added heparin while PrV2 does not. Thus, when adsorption is performed at 2°C, PrV2 adsorbs as well as wild type to MDBK cells but remains sensitive to heparin for a considerably longer time. Since at 2°C PrV adsorbs to but does not penetrate the cells (8, 28), the hypersensitivity of PrV2 to heparin cannot be ascribed to a delay in its penetration into the cells. Furthermore, it is unlikely that the increased sensitivity of bound PrV2 is due to the fact that the gIII of PrV2 does not bind to the heparinlike receptor as firmly as does the gIII of PrV. If this were the case, the same proportion of adsorbed PrV2 virus would be sensitive to heparin at all times after inoculation of the cells. This is clearly not so; the percentage of adsorbed PrV2 that becomes resistant to treatment with heparin increases with time.

These findings indicate that at least two interactions of the virus with the cells occur during the primary adsorption process. The initial interaction between gIII and the heparinlike cellular receptor is low in affinity, and after it has occurred, the virus can be displaced from the cells by exogenously added heparin. The second interaction results in a more stable binding of the virus to the cells; after this interaction, the virus can no longer be displaced from the cell surface by heparin. The second step occurs slowly when PrV2 adsorbs to cells and rapidly when wild-type PrV adsorbs to the cells. Adsorption and resistance to exogenously added heparin are thus temporally coupled in wild-type PrV and are uncoupled in PrV2.

The second interaction of the virus with the cell surface which leads to the heparin-resistant adsorption of the virus is due to either an additional, high affinity, interaction of the virus with the heparinlike receptor or an interaction of the virus with another cellular receptor. Whatever the case may be, it is clear that the middle third of gIII, missing from PrV2 [and PrV(Ka)2], is either directly or indirectly involved in mediating the second step. (It is noteworthy that five of the eight N-linked glycosylation sites in gIII are removed by this deletion.) Thus, either the configuration of the PrV2 gIII glycoproteins is such that the second step in adsorption is delayed or weakened or the domain of gIII that is missing in PrV2 is necessary to mediate the second step in adsorption. Alternatively, the defect in gIII could affect the second step in adsorption indirectly by interfering with or failing to promote the interaction of a cell component with another viral protein (not gIII), a protein that is normally involved in mediating the second step of adsorption. At present, we

have no evidence that would allow us to favor either of these alternatives.

Our experiments clearly show that PrV adsorption consists of at least two distinguishable steps and that gIII is not only responsible for the first interaction of the virus with the heparinlike receptor but also affects a second binding step in adsorption. Even low levels of gIII in virions are sufficient to promote both steps in adsorption. PrV30 expresses a gIII protein with a defective membrane anchor domain. As a result, a significant quantity of gIII is secreted into the medium and the PrV30 virions contain significantly less gIII in the virus envelope than does wild-type virus. We estimate that PrV30 virions have less than 10% of the normal levels of gIII (Fig. 2). Nevertheless, PrV30 is only slightly defective in growth compared with gIII null mutants (25). The experiments in this report demonstrate that PrV30 initiates infection via the residual gIII present on its envelope; it is neutralized by gIII-specific antibodies and completes both steps in adsorption as well as wild-type virus.

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REFERENCES

1. Ben-Porat, T., J. M. Demarchi, and A. S. Kaplan. 1974. Characterization of defective interfering viral particles present in a population of pseudorabies virions. *Virology* 61:29-37.
2. Ben-Porat, T., J. M. DeMarchi, B. Lomniczi, and A. S. Kaplan. 1986. Role of glycoproteins of pseudorabies virus in eliciting neutralizing antibodies. *Virology* 154:325-334.
3. Campadelli-Fiume, G., D. Strippe, A. Boscaro, E. Avitabile, L. Foa-Tomasi, D. Barker, and B. Roizman. 1990. Glycoprotein C-dependent attachment of herpes simplex virus to susceptible cells leading to productive infection. *Virology* 178:213-222.
4. Hampl, H., T. Ben-Porat, L. Ehrlicher, K.-O. Habermehl, and A. S. Kaplan. 1984. Characterization of the envelope proteins of pseudorabies virus. *J. Virol.* 52:583-590.
5. Herold, B. C., D. WuDunn, N. Soltys, and P. G. Spear. 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. *J. Virol.* 65:1090-1098.
6. Kaplan, A. S., and T. Ben-Porat. 1961. The action of 5-fluorouracil on the nucleic acid metabolism of pseudorabies virus-infected and non-infected rabbit kidney cells. *Virology* 13:78-92.
7. Keeler, C. K., M. E. Whealy, and L. W. Enquist. 1986. Construction of an infectious PrV recombinant expressing a glycoprotein gIII beta-galactosidase fusion protein. *Gene* 50:215-224.
8. Mettenleiter, T. C. 1989. Glycoprotein gIII deletion mutants of pseudorabies virus are impaired in virus entry. *Virology* 171:623-625.
9. Mettenleiter, T. C., N. Lukacs, and H. J. Rziha. 1985. Pseudorabies virus avirulent strains fail to express a major glycoprotein. *J. Virol.* 56:307-311.
10. Mettenleiter, T. C., N. Lukacs, and H.-J. Rziha. 1985. Mapping of the structural gene of pseudorabies virus glycoprotein A and identification of two non-glycosylated precursor polypeptides. *J. Virol.* 53:52-57.
11. Mettenleiter, T. C., N. Lukacs, H.-J. Thiel, C. Schreurs, and H.-J. Rziha. 1986. Location of the structural gene of pseudorabies virus glycoprotein complex gII. *Virology* 152:66-75.
12. Mettenleiter, T. C., C. Schreurs, F. Zuckermann, T. Ben-Porat, and A. S. Kaplan. 1988. Role of glycoprotein gIII of pseudorabies virus in virulence. *J. Virol.* 62:2712-2717.
13. Mettenleiter, T. C., L. Zsak, F. Zuckermann, N. Sugg, H. Kern, and T. Ben-Porat. 1990. Interaction of glycoprotein gIII with a cellular heparinlike substance mediates adsorption of pseudorabies virus. *J. Virol.* 64:278-286.

14. Petrovskis, E. A., J. G. Timmins, M. A. Armentrout, C. C. Marchioli, R. J. Yancey, Jr., and L. E. Post. 1986. DNA sequence of the gene for pseudorabies virus gp50, a glycoprotein without N-linked glycosylation. *J. Virol.* **59**:216–223.
15. Petrovskis, E. A., J. G. Timmins, T. M. Gierman, and L. E. Post. 1986. Deletions in vaccine strains of pseudorabies virus and their effect on synthesis of glycoprotein gp63. *J. Virol.* **60**:1166–1169.
16. Petrovskis, E. A., J. G. Timmins, and L. E. Post. 1986. Use of gt11 to isolate genes for two pseudorabies virus glycoproteins with homology to herpes simplex virus and varicella-zoster virus glycoproteins. *J. Virol.* **60**:185–193.
17. Rea, T. J., J. G. Timmins, G. W. Long, and L. E. Post. 1985. Mapping and sequence of the gene for the pseudorabies virus glycoprotein which accumulates in the medium of infected cells. *J. Virol.* **54**:21–29.
18. Robbins, A. K., D. J. Dorney, M. W. Wathen, M. E. Whealy, C. Gold, R. J. Watson, L. E. Holland, S. D. Weed, M. Levine, J. C. Glorioso, and L. W. Enquist. 1987. The pseudorabies virus gII gene is closely related to the gB glycoprotein gene of herpes simplex virus. *J. Virol.* **61**:2691–2701.
19. Robbins, A. K., R. J. Watson, M. E. Whealy, W. W. Hays, and L. W. Enquist. 1986. Characterization of a pseudorabies virus glycoprotein gene with homology to herpes simplex virus type 1 and type 2 glycoprotein C. *J. Virol.* **58**:339–347.
20. Robbins, A. K., M. E. Whealy, R. J. Watson, and L. W. Enquist. 1986. The pseudorabies virus gene encoding glycoprotein gIII is not essential for growth in tissue culture. *J. Virol.* **59**:635–645.
21. Ryan, J. P., M. E. Whealy, A. K. Robbins, and L. W. Enquist. 1987. Analysis of pseudorabies virus glycoprotein gIII localization and modification by using novel infectious viral mutants carrying unique *EcoRI* sites. *J. Virol.* **61**:2962–2972.
22. Schreurs, C., T. C. Mettenleiter, F. Zuckermann, N. Sugg, and T. Ben-Porat. 1988. Glycoprotein gIII of pseudorabies virus is multifunctional. *J. Virol.* **62**:2251–2257.
23. Wathen, M. W., and L. M. K. Wathen. 1984. Isolation, characterization, and physical mapping of a pseudorabies virus mutant containing antigenically altered gp50. *J. Virol.* **51**:57–62.
24. Wathen, M. W., and L. M. K. Wathen. 1986. Characterization and mapping of a nonessential pseudorabies virus glycoprotein. *J. Virol.* **58**:173–178.
25. Whealy, M. E., A. K. Robbins, and L. W. Enquist. 1988. Pseudorabies virus glycoprotein gIII is required for efficient growth in tissue culture. *J. Virol.* **62**:2512–2515.
26. Zsak, L., T. C. Mettenleiter, N. Sugg, and T. Ben-Porat. 1989. Release of pseudorabies virus from infected cells is controlled by several viral functions and is modulated by cellular components. *J. Virol.* **63**:5475–5477.
27. Zuckermann, F., L. Zsak, T. C. Mettenleiter, and T. Ben-Porat. 1990. Pseudorabies virus glycoprotein gIII is a major target antigen for murine and swine virus-specific cytotoxic T lymphocytes. *J. Virol.* **64**:802–812.
28. Zuckermann, F., L. Zsak, L. Reilly, N. Sugg, and T. Ben-Porat. 1989. Early interactions of pseudorabies virus with host cells: functions of glycoprotein gIII. *J. Virol.* **63**:3323–3329.