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Early Interactions of Pseudorabies Virus with Host Cells: Functions of Glycoprotein gIII

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Adsorption of mutants of pseudorabies virus (PrV) lacking glycoprotein gIII is slower and less efficient than is that of wild-type virus (C. Schreurs, T. C. Mettenleiter, F. Zuckermann, N. Sugg, and T. Ben-Porat, J. Virol. 62:2251–2257, 1988). To ascertain the functions of gIII in the early interactions of PrV with its host cells, we compared the effect on wild-type virus and gIII$^-$ mutants of antibodies specific for various PrV proteins. Although adsorption of wild-type virus was inhibited by polyvalent antisera against PrV as well as by sera against gIII and gp50 (but not sera against gII), adsorption of the gIII$^-$ mutants was not inhibited by any of these antisera. These results suggest that, in contrast to adsorption of wild-type PrV, the initial interactions of the gIII$^-$ mutants with their host cells are not mediated by specific viral proteins. Furthermore, competition experiments showed that wild-type Prv and the gIII$^-$ mutants do not compete for attachment to the same cellular components. These findings show that the initial attachment of PrV to its host cells can occur by a least two different modes—one mediated by glycoprotein gIII and the other unspecific. gIII$^-$ mutants not only did not adsorb as readily to cells as did wild-type virus but also did not penetrate cells as rapidly as did wild-type virus after having adsorbed. Antibodies against gIII did not inhibit the penetration of adsorbed virus (wild type or gIII$^-$), whereas antibodies against gII and gp50 did. It is unlikely, therefore, that gIII functions directly in virus penetration. Our results support the premises that efficient adsorption of PrV to host cell components is mediated either directly or indirectly by gIII (or a complex of viral proteins for which the presence of gIII is functionally essential) and that this pathway of adsorption promotes the interactions of other viral membrane proteins with the appropriate cellular proteins, leading to the rapid penetration of the virus into the cells. The slower penetration of the gIII$^-$ mutants than of wild-type PrV appears to be related to the slower and less efficient alternative mode of adsorption of PrV that occurs in the absence of glycoprotein gIII.

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, 12–15, 17–20, 23; L. Post, personal communication); four of these glycoproteins have been shown to be nonessential for growth in cell cultures (2, 6, 8, 13, 18, 19, 21).

We have been interested in ascertaining the functions of these nonessential glycoproteins and have focused (among others) on glycoprotein gIII, one of the major nonessential glycoproteins of PrV. Glycoprotein gIII is a homolog of glycoprotein gC of herpes simplex virus (17). Cells infected with mutants of PrV deficient in gIII produce virus populations that have a lower titer of infectious virus than do virus populations produced by cells infected with wild-type virus (6, 19, 22). 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$^-$ mutants and reflects the important role that gIII plays in the stable adsorption of the virus to its host cell (19). However, since gIII$^-$ virus is infectious, adsorption of PrV can occur not only by a gIII-mediated process but also by another, slower process (19).

The effect of glycoprotein gIII on virus adsorption could be either direct or indirect. Thus, glycoprotein gIII could either mediate adsorption directly (i.e., be directly responsible for the adsorption process) or enhance adsorption by interacting with or modifying the configuration of other glycoproteins which are essential for the adsorption process. The alternative, gIII-independent mode of adsorption could thus be mediated by proteins that normally do not play a role in adsorption, could be mediated by the unmodified viral proteins which, when modified by gIII, lead to a more efficient adsorption, or could be unspecific (i.e., not mediated by specific viral proteins).

The experiments presented in this paper were designed to distinguish among these possibilities and to probe the pathways of PrV adsorption and penetration into host cells.

MATERIALS AND METHODS

Virus strains and cell cultures. PrV(Ka) is a strain that has been carried in our laboratory for more than 25 years. The isolation and characterization of the gIII$^-$ mutants used have been described previously (10, 11). Some of the results obtained with the PrV(Ka) gIII$^-$ mutants discussed in this paper were corroborated by results obtained with gIII$^-$ mutants of the Becker strain (kindly supplied by L. Enquist). Rabbit kidney (RK) and Madin-Darby bovine kidney (MDBK) cells were cultivated in Eagle synthetic medium plus 5% dialyzed serum (EDS). Virus was titrated by plaque assay in RK or MDBK monolayer cultures. Most experiments were performed with both RK and MDBK cells; some quantitative but not qualitative differences between the results obtained in experiments with these two cell types were sometimes observed.

Media and solutions. The following media and solutions were used: EDS; EDS plus 5-fluorouracil (20 μg/ml) and thymidine (5 μg/ml); Tris-buffered saline containing 1% crystalline bovine albumin (TBSA); and NaCl (0.15 M)-sodium citrate (0.015 M)-sodium dodecyl sulfate (1%).

Radiochemicals. [^{3}H]thymidine (specific activity, 45 Ci/mmol) was purchased from New England Nuclear Corp.

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Labeling and purification of virus. Virus was purified as described previously (1). In brief, cell monolayers were incubated for 24 or 48 h in EDS–5-fluorouracil–thymidine (a procedure that inhibits cellular DNA synthesis without affecting virus growth [5]). The cells were then infected (multiplicity of infection, 5 PFU per cell) and incubated in EDS containing [^{3}H]thymidine (20 μCi/ml) for 24 h. The medium was collected and clarified by centrifugation at 4,000 × *g* for 10 min. The supernatant containing the extracellular virus was centrifuged on a TBSA–30% sucrose cushion at 15,000 rpm for 1 h in an SW27 rotor (Beckman Instruments, Inc.). The virus pellet was suspended gently in 1 ml of TBSA and centrifuged on a sucrose gradient as described previously (1). 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 virus particles in the preparation was determined by counting particles relative to a known number of latex particles in an electron microscope. The number of particles was also calculated from the amount of DNA in the preparations of purified virions. In the original virus stocks, the ratio of particles to PFU was approximately 20:1; after virus purification it ranged between 100:1 and 500:1, depending on the virus preparations.

Virus neutralization. One-milliliter samples of virus (approximately 150 PFU/ml) were incubated for 1 h at 37°C with the appropriate antisera and then added to RK or MDBK cells grown in 50-mm petri dishes. After a 1-h adsorption period, the cells were overlaid with agarose, and plaques were counted 4 days later. The percent reduction in titer caused by treatment of the virus with antisera was determined.

Virus adsorption. Monolayers of cells were pretreated with TBSA to minimize unspecific adsorption of the virus. Purified [^{3}H]thymidine-labeled virus in TBSA, prepared as described above, was added to RK monolayers. One hour later the monolayers were washed extensively, the cells were scraped into NaCl-sodium citrate-sodium dodecyl sulfate, and the amount of radioactivity that was associated with the cell monolayers was determined. The kinetics of adsorption of infectious virus were ascertained as described previously (19).

Virus penetration. Monolayers of MDBK or RK cells were precooled to 2°C and infected with 1 ml of an ice-cold suspension of 150 PFU of virus. After 1 h at 2°C, the monolayers were washed extensively and cold EDS containing antiserum was added. After an additional 30 min at 2°C, the cultures were shifted up to 37°C, further incubated for 30 min, and overlaid with agarose. The reduction in titer caused by the antiserum treatment was determined.

Antisera. The following antisera were used. Polyvalent anti-PrV pig serum was obtained from pigs that had recovered from infection with wild-type PrV(Ka); it was used at a final concentration of 1:300. Polyvalent anti-gIII$^-$ mutant pig serum was obtained from pigs that had recovered from infection with PrV(Ka) gIII$^-$; it was used at a final concentration of 1:100. Anti-gII polyvalent sera and anti-gIII polyvalent sera were goat sera prepared against denatured *Escherichia coli cro*-gII or *cro*-gIII fusion proteins (generous gifts from L. Enquist); each was used at a final concentration of 1:40. Anti-gII monoclonal antibody (MAb) mixture was a mixture of equal amounts of M2, M3, M5, and M8 MAbs reactive with gII (4); it was used at a final concentration of 1:40. Anti-gIII MAb mixture was a mixture of equal amounts of M1, M4, M6, and M7 MAbs against gIII (4); it was used at a final concentration of 1:40. Anti-gp50 MAb was a generous gift from C. Marchioli; it was used at a final concentration of 1:30.

TABLE 1. Effect of various antisera and MAbs on the adsorption of wild-type PrV[a]

Antiserum	cpm of virus adsorbed to cell monolayers (10^2)	% Inhibition of adsorption
Control (preimmune serum)	270	0
Polyvalent pig anti-PrV (wild type)	53	80
Polyvalent pig anti-PrV (gIII$^-$ mutant)	61	78
Anti-gII polyvalent	255	6
Anti-gII MAb mixture	232	14
Anti-gIII polyvalent	32	88
Anti-gIII MAb mixture	63	77
Anti-gp50 MAb	78	71

[a] Purified [^{3}H]thymidine-labeled wild-type PrV was prepared as described in Materials and Methods, suspended in TBSA (6.7×10^5 cpm/ml), and incubated for 1 h at 37°C with the indicated antisera (at the concentrations given in Materials and Methods). One milliliter of virus was then added to each RK cell monolayer (which had been pretreated for 30 min with TBSA to reduce unspecific binding) and incubated for 60 min at 37°C. The monolayers were washed extensively, and the number of counts that remained associated with the cells was determined. The results are the averages for triplicate cultures; the variation between similarly treated cultures did not exceed 15%.

RESULTS

Effect of various antibodies on virus adsorption. To clarify the role of gIII in the processes leading to the initiation of the infective process, we compared the effects of various antibodies on the early stages of infection with wild-type virus and gIII$^-$ mutants. As a first step, we ascertained which of the antibodies at our disposal interfered with the adsorption of wild-type PrV(Ka).

In these experiments, antisera at concentrations that would reduce virus infectivity by approximately 80 to 95% were used, and their ability to interfere with the stable association of radiolabeled virus with cells was determined (Table 1). Polyvalent anti-PrV sera from pigs that had been inoculated with either wild-type virus or a gIII$^-$ mutant inhibited adsorption equally well. The inhibition of virus adsorption by either serum was sufficient to account for most of the neutralizing activity. However, these antisera were also quite effective in inhibiting virus penetration (see below). It has been established that antibodies against gIII inhibit virus adsorption (4; see also Table 1). The finding that antisera with similar neutralizing powers and obtained either from animals infected with a gIII$^-$ mutant or from animals infected with wild-type virus inhibit adsorption equally well indicates that antibodies against surface proteins other than gIII must also be quite effective in preventing adsorption. Indeed, although none of the antibodies against gII significantly affected adsorption, an MAb against gp50 did. We previously reported (19) that the same MAb against gp50 affects adsorption of PrV only slightly. In the experiments reported here, higher concentrations of the MAb were used, with a consequent significant increase in its effect on adsorption.

To determine whether the antibodies that inhibited adsorption of wild-type virus would also affect adsorption of gIII$^-$ mutants, we performed experiments similar to those summarized in Table 1 with the gIII$^-$ mutants. Surprisingly, we found that none of the antibodies, including polyvalent

TABLE 2. Inhibition of adsorption of wild-type and gIII$^-$ viruses by PrV-specific gIII$^-$ antiserum[a]

Expt	cpm of the following virus/monolayer (10^3):					
	Wild type			gIII$^-$ mutant		
	Input	Adsorbed		Input	Adsorbed	
		Without antiserum	With antiserum		Without antiserum	With antiserum
1	1,205.0	62.8 (5.2)	7.0 (0.6)	1,120.3	18.6 (1.7)	19.8 (1.8)
2	230.3	10.2 (4.4)	2.0 (0.9)	240.7	2.4 (1.0)	2.6 (1.1)

[a] The experiment was performed as described in Table 1, footnote *a*. Numbers in parentheses indicate the percentage of input virus adsorbed.

sera against wild-type PrV, interfered with adsorption of the gIII$^-$ mutants. The results of two representative experiments in which we compared the ability of polyvalent sera obtained from pigs that had recovered from infection with a gIII$^-$ mutant to inhibit adsorption of wild-type PrV and gIII$^-$ mutants are summarized in Table 2. The results obtained with these antisera are presented in preference to those obtained with antisera against wild-type virus because the former antisera do not contain antibodies against gIII which might favor their interaction with wild-type virus over their interaction with the gIII$^-$ mutants. However, as mentioned above, similar results were also obtained with sera obtained from pigs that had been infected with wild-type virus.

Under the experimental conditions used here, approximately 5% of the purified wild-type virus and less than 2% of the gIII$^-$ mutants adsorbed stably to the cells. Although this percentage varied somewhat between experiments, it was independent of the multiplicity of infection; exposure of the cells to fivefold more or fivefold less virus gave similar percentages of adsorption (data not shown), indicating that in these experiments the virus was present in nonsaturating concentrations. When the virus was preincubated with the antiserum, the amount of wild-type virus that adsorbed to the cells was reduced considerably (by more than 80%); adsorption of the gIII$^-$ mutants was, however, not significantly affected. This finding indicates that adsorption of the gIII$^-$ mutants occurs by a mechanism that is different from that of wild-type virus; it does not appear to be mediated by viral proteins.

Lack of competition between wild-type virus and gIII$^-$ virus for cellular receptors. If the mode of adsorption of the gIII$^-$ mutants differed from that of wild-type virus, the gIII$^-$ mutants might not recognize the same cellular receptors as does wild-type virus. To ascertain whether this was indeed the case, we determined the degree to which unlabeled wild-type virus could interfere with the adsorption of ^{3}H-labeled wild-type virus or ^{3}H-labeled gIII$^-$ mutants (Table 3).

Although the cells were infected with a similar number of wild-type and gIII$^-$ physical particles, as expected only 25% as much gIII$^-$ virus as wild-type virus adsorbed to the cells. Unlabeled wild-type virus competed with labeled wild-type virus, but it competed much less effectively with labeled gIII$^-$ mutants. Thus, while a 10-fold excess of unlabeled virus inhibited adsorption of wild-type virus by 55%, it did not inhibit adsorption of the gIII$^-$ mutants. In the converse experiment, unlabeled gIII$^-$ virus also competed much more effectively with labeled gIII$^-$ virus than with labeled wild-type virus (data not shown). These results show that wild-type and gIII$^-$ virions do not compete for attachment to the same cellular components.

TABLE 3. Competition for adsorption between wild-type virus and gIII$^-$ mutant[a]

No. of particles of unlabeled competing wild-type virus/cell	cpm of the following virus/monolayer (10^4):	
	Wild type	gIII$^-$ mutant
0	1.67	0.46
5,000	0.75 (55)	0.49 (0)
50,000	0.29 (83)	0.29 (37)

[a] Monolayers of RK cells (4×10^6 per plate) were pretreated with TBSA for 30 min and infected with 5×10^5 cpm (approximately 500 particles per cell) of either ^{3}H-labeled wild-type virus or ^{3}H-labeled gIII$^-$ mutants as well as with the indicated number of unlabeled competing wild-type virus particles. The infected cell monolayers were incubated at 37°C for 1 h and washed four times with EDS, and the number of counts that remained associated with them was determined. The results represent the averages for triplicate cultures; the variation between individual cultures did not exceed 15%. Numbers in parentheses indicate the percent reduction in ^{3}H-labeled virus adsorbed.

Neutralization of wild-type virus and gIII$^-$ mutants by various anti-PrV antibodies. Since, in contrast to adsorption of wild-type virus, adsorption of gIII$^-$ mutants was not affected by anti-PrV sera, it was of interest to compare the neutralization of these viruses by various antisera (Table 4). All the antibodies that we tested neutralized the infectivity of wild-type virus, with the exception of a mixture of MAbs against gII. Although the mixture of MAbs against gII used here did not neutralize PrV(Ka), a polyvalent antiserum against a gII fusion protein produced in *E. coli* did, indicating that glycoprotein gII can be the target of neutralizing antibodies. Indeed, whereas our MAbs against gII were not neutralizing, other neutralizing antibodies against gII have been isolated (C. Schreurs, personal communication). It is clear, therefore, that in addition to antibodies against gIII (4) and gp50 (3a, 20), antibodies against gII can also have complement-independent neutralizing activity.

The gIII$^-$ mutants were neutralized by all the antibodies that also neutralized wild-type virus, with the exception of those against gIII. Furthermore, the same mixture of MAbs against gII that did not neutralize wild-type virus consistently had some neutralizing activity against the gIII$^-$ mutants; the gIII$^-$ mutants appeared to be more sensitive than wild-type virus to neutralization by the other antisera as well. The increased sensitivity of the gIII$^-$ mutants to both convalescent-phase serum obtained from pigs infected with either wild-type virus or gIII$^-$ mutants and the polyvalent serum against gII was confirmed by ascertaining the kinetics of neutralization of gIII$^-$ mutants and wild-type virus by these sera (unpublished results).

TABLE 4. Neutralization of wild-type virus and gIII$^-$ mutants by various antisera or MAbs[a]

Antiserum	% Reduction in titer of:	
	Wild-type virus	gIII$^-$ mutants
Control (preimmune serum)	0	0
Polyvalent pig anti-PrV (wild type)	95	100
Polyvalent pig anti-PrV (gIII$^-$ mutant)	96	100
Anti-gII polyvalent	84	99
Anti-gII MAb mixture	0	50
Anti-gIII polyvalent	84	0
Anti-gIII MAb mixture	91	0
Anti-gp50 MAb	80	90

[a] The procedures used to measure virus neutralization as well as the serum concentrations used are described in Materials and Methods.

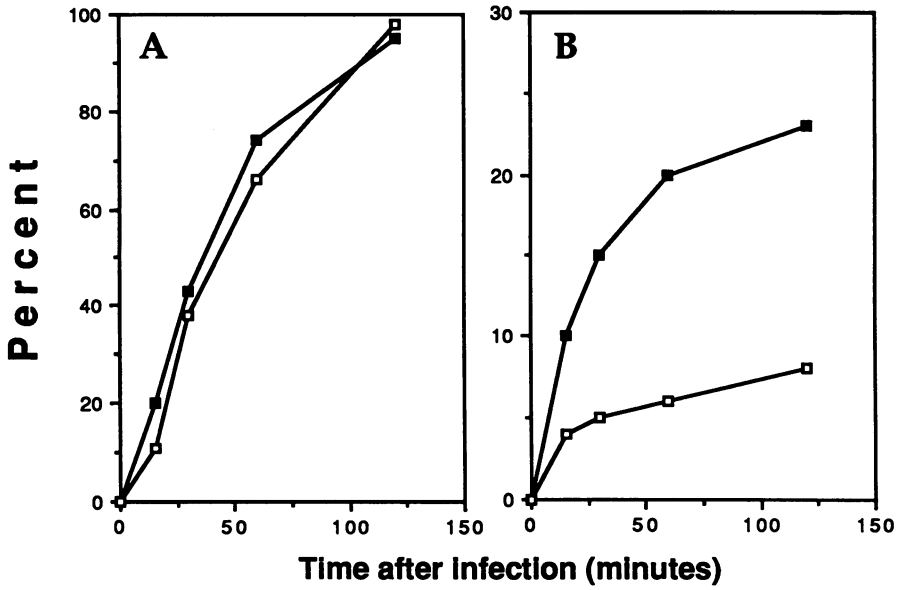

FIG. 1. Adsorption and sensitivity to anti-PrV antiserum of wild-type virus (A) and a gIII$^-$ mutant of PrV(Ka) (B). The virus preparations were diluted in EDS to approximately 150 PFU/ml (under the assay conditions used), and 1 ml of each was added to cultures of MDBK cells grown in 50-mm petri dishes. At the indicated times, the cultures were washed extensively (four times) to remove unadsorbed virus and further incubated for 1 h with 1 ml of EDS (closed symbols) or EDS containing convalescent-phase pig anti-PrV antiserum (open symbols). The cultures were again washed (once) and then overlaid with agarose. The number of plaques was counted after 4 days of incubation. The number of plaques that developed on plates from which the inoculum had not been removed and which were overlaid with agarose 2 h after inoculation was considered 100%.

Effect of various anti-PrV antibodies on virus penetration. Since gIII$^-$ mutants were more readily neutralized by various antisera than was wild-type virus (Table 4) but their adsorption was not affected by these antisera (Table 2), it seemed likely that the antisera would interfere more effectively with the penetration into cells of the gIII$^-$ mutants than of wild-type virus.

Figure 1 shows the results of an experiment in which the sensitivities of adsorbed wild-type PrV(Ka) and a gIII$^-$ deletion mutant of PrV(Ka) to convalescent-phase serum were tested at various times after inoculation of cultures of MDBK cells. As expected, the gIII$^-$ mutant did not adsorb as rapidly or as efficiently to the cells as did wild-type virus; by 2 h after infection, approximately 95% of the wild-type virus but only approximately 25% of the gIII$^-$ mutant had adsorbed to the cells (as determined by a comparison of the number of plaques that developed on infected monolayers from which unadsorbed virus had been removed by extensive washing prior to overlay with agarose with the number that developed on similarly infected monolayers that had not been washed). Treatment of the monolayers to which virus had adsorbed with neutralizing antibodies at various times after the addition of the virus did not affect plaque formation by wild-type virus; even at 15 min after infection (when only a small percentage of the total virus had adsorbed) the addition of antiserum did not affect the number of plaques that developed. Thus, after adsorption, wild-type virus quickly loses its sensitivity to neutralization by antiserum, probably because it has been internalized by the cell. Adsorption and penetration into the cells of wild-type virus appear, therefore, to be temporally closely linked at 37°C. The adsorbed gIII$^-$ mutant, on the other hand, behaved quite differently. By 2 h postinfection, a large proportion of the adsorbed virus was still sensitive to anti-PrV antiserum; the number of plaques that appeared on monolayers that had been washed extensively but that had not been treated with antiserum was significantly larger than the number that appeared on monolayers that had been washed extensively and had been treated with antiserum. Results similar to those shown in Fig. 1 were also obtained when two other independently isolated gIII$^-$ mutants were tested. We conclude that gIII not only plays an important role in the attachment (adsorption) of the virus to the cells but that it also contributes to the penetration of the virus into the cells.

The slower adsorption and penetration of the gIII$^-$ mutants than of wild-type virus were correlated with a lag in the initiation of the infective process. This conclusion was documented by monitoring the kinetics of accumulation of viral RNA (data not shown); it could also be deduced on the basis of the kinetics of viral DNA synthesis in the infected cells (Fig. 2).

The slower penetration of gIII$^-$ virus than of wild-type virus into the cells could be ascribed either to a direct role of gIII in penetration or to the alternative mode of attachment of the gIII$^-$ mutants to the cells. To gain more information about the role that gIII may play in penetration, we attempted to dissociate the process of adsorption from that of penetration and to determine whether various antibodies would affect the penetration of the gIII$^-$ mutants and wild-type virus differently. In these experiments, cell monolayers were precooled and incubated with virus at 2°C for 1 h. Under these conditions, adsorption will occur but penetration will be delayed. Unadsorbed virus was removed by extensive washing, and the monolayers were subsequently treated for 30 min at 2°C with different antisera. The cultures were then shifted up to 37°C and overlaid with agarose. The number of plaques that developed was determined (Table 5).

Both wild-type virus and the gIII$^-$ mutants that had adsorbed to the cells at 2°C remained susceptible to neutralization by all the antisera and MAbs tested, with the exception of those against gIII. The fact that antibodies against gIII did not affect the penetration of wild-type virus indicates

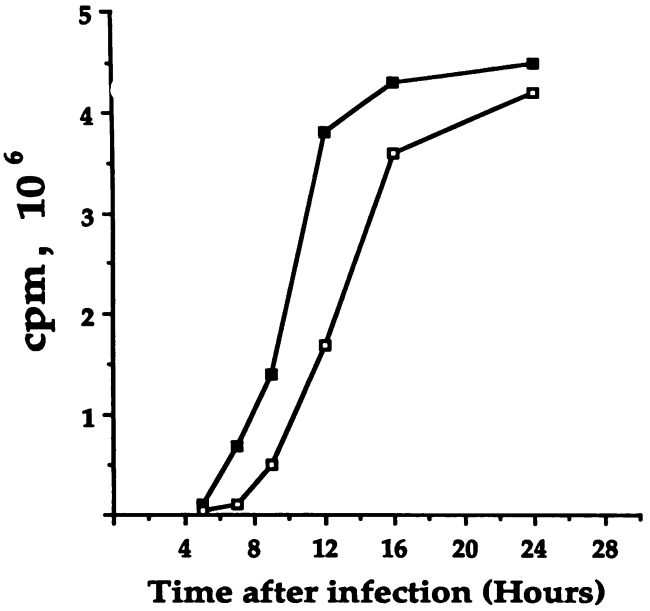

FIG. 2. Viral DNA synthesis in cells infected with wild-type virus and a gIII$^-$ mutant of PrV(Ka). RK cells (4×10^6 per sample) were incubated for 24 h in EDS–5-fluorouracil–thymidine to inhibit cellular DNA synthesis (5). They were then infected with either wild-type PrV (5 PFU per cell) (■) or a gIII$^-$ mutant (20 PFU per cell) (□). After a 2-h adsorption period, the inoculm was removed and the cells were overlaid with EDS containing [^{3}H]thymidine (20 μCi/ml). At intervals, the cells were harvested and the amount of [^{3}H]thymidine incorporated into viral DNA was determined.

that gIII is probably not directly involved in the penetration process. Antibodies against gII and gp50, on the other hand, both inhibited the penetration of the virus quite effectively. Interference of the antibodies with virus penetration was more marked for the gIII$^-$ mutants than for wild-type virus. Indeed, greater interference of the antibodies with the penetration of the gIII$^-$ mutants than with that of wild-type virus was not unexpected, since the antibodies were more effective in neutralizing gIII$^-$ mutants than in neutralizing wild-type virus (Table 4) without having any effect on the adsorption of the gIII$^-$ mutants (Table 2).

DISCUSSION

The experiments presented in this paper were designed to elucidate the function of glycoprotein gIII in the early interactions of PrV with its host cells. Although the results obtained with only one type of gIII$^-$ mutant are presented, some of the same experiments were also performed with four different independently isolated types of gIII$^-$ mutants [two of the PrV(Ka) strain and two of the Becker strain], with identical results. It is clear, therefore, that the difference in the behavior of wild-type virus and of the gIII$^-$ mutants can be attributed to the defect in gIII and not to another adventitious mutation.

TABLE 5. Neutralization of adsorbed virus (inhibition of penetration) by various antisera or MAbs[a]

Antiserum	% Reduction in titer of:	
	Wild-type virus	gIII$^-$ mutants
Control (preimmune serum)	0	0
Polyvalent pig anti-PrV (wild type)	75	100
Polyvalent pig anti-PrV (gIII$^-$ mutant)	95	100
Anti-gII polyvalent	85	100
Anti-gIII polyvalent	0	0
Anti-gIII MAb mixture	0	0
Anti-gp50 MAb	60	95

[a] The experiment was performed as described in Materials and Methods. Virus (approximately 150 PFU per plate) was allowed to adsorb to the cells at 2°C. The cell monolayers were washed extensively and treated with the antibodies. They were then shifted up to 37°C and incubated for 30 min, washed once, and overlaid with agarose. Plaques were counted 4 days later.

The salient findings of the experiments presented in this paper may be summarized as follows. (i) Adsorption of wild-type virus is inhibited by all the antisera and MAbs that we have tested, with the exception of those against gII. (ii) Adsorption of gIII$^-$ mutants is not affected by any of the antisera or MAbs that we have tested. (iii) Wild-type virus and gIII$^-$ mutants do not appear to compete for the same cellular receptors during the initial attachment of the virus to the cells. (iv) Mutants defective in gIII not only do not adsorb to cells as efficiently as does wild-type virus but also do not penetrate as readily into the cells. (v) Penetration of both wild-type virus and gIII$^-$ mutants is affected by antibodies against gII and gp50 but not by those against gIII.

Adsorption of gIII$^-$ mutants is slower and less efficient than is that of wild-type virus (19). It is therefore clear that gIII plays some role in adsorption. The original intent in performing the experiments described in this paper was to determine whether the role that gIII plays in adsorption is direct or indirect. The results presented here do not provide conclusive evidence for either of these possibilities. However, the observation that adsorption of gIII$^-$ mutants was not affected by polyvalent anti-PrV sera indicates that gIII$^-$ mutants probably bind to the cells nonspecifically, i.e., attachment of these virions to the cells is not mediated by specific viral proteins. Furthermore, the finding that an excess of wild-type virus interferred to a much lower extent with the adsorption of gIII$^-$ mutants than with that of wild-type virus (and vice versa) indicates that both wild-type virus and gIII$^-$ mutants initially attach to different cellular components. Thus, gIII appears to be essential to the normal efficient process that leads to the recognition and adsorption of the virus to some surface component of the cell; in its absence, the virus attaches to alternative cellular components, a process that is less efficient and does not appear to depend on any specific viral protein.

Recently, it was reported that the initial interaction of herpes simplex virus with cells is by binding of the virus to heparan sulfate (23). We have similarly found that wild-type but not gIII$^-$ mutants of PrV binds to a heparinlike substance on the cell surface. Furthermore, glycoprotein gIII is the viral protein that binds most readily to heparin (manuscript in preparation). These findings reinforce the conclusions drawn from the experiments presented in this paper that gIII mediates the binding of wild-type virus to certain cellular components while binding of the gIII$^-$ mutants to the cells involves different cellular components.

Although PrV-specific antisera interfere with the adsorption of wild-type virus but not of the gIII$^-$ mutants, the gIII$^-$ mutants are nevertheless neutralized more readily by these antisera than is wild-type virus. The supersensitivity of the gIII$^-$ mutants to the antisera may be due, at least in part, to the slower mode of penetration of the gIII$^-$ mutants and to the consequent longer interval of vulnerability of the gIII$^-$ mutants to the sera. The presence of gIII may, however, also affect the interaction of other proteins with antibodies either by steric hindrance or by mediation of conformational changes of the membrane proteins; in the absence of gIII,

epitopes of some viral proteins that are normally hidden may be revealed. Whatever the case may be, the salient findings emerging from our results are that, while in contrast to adsorption of wild-type virus, adsorption of gIII$^-$ virus is not affected by any of the antisera against PrV, penetration of mutant virus is affected by all the antisera that also inhibit penetration of wild-type virus. Thus, while adsorption of the gIII$^-$ mutants appears to be independent of any specific viral protein, penetration of the gIII$^-$ mutants appears to be dependent on the same proteins that also affect penetration of wild-type virus.

It is interesting to note that gIII$^-$ mutants not only do not adsorb as readily to the cells as does wild-type virus but also are not internalized as rapidly as is wild type virus. A similar observation has also been made by T. Mettenleiter (personal communication). The delay in the penetration of the gIII$^-$ mutants into the cells could, in principle, reflect a direct role of gIII in penetration. It could, however, also be related to the alternative mode of adsorption the gIII$^-$ mutants use. The fact that several different antibodies against gIII do not affect penetration of adsorbed wild-type virus (while antibodies against gII and gp50 do) argues against the notion that gIII plays a direct role in penetration. Furthermore, since the penetration of gIII$^-$ virus appears to be dependent on the same proteins that also affect the penetration of wild-type virus, it appears possible that once they have adsorbed to the cells, the gIII$^-$ mutants use the same pathways of entry into the cells as does wild-type virus. The delay in penetration of the gIII$^-$ mutants into the cells may thus be linked to the alternative mode of adsorption the mutants use.

The following sequence of events leading to the initiation of infection is consistent with our findings and is an attractive working model on which to base further investigations. The first step of the interaction of the virus with the host cell is attachment of the virus to a cellular component. Glycoprotein gIII (or a complex of viral proteins for which the presence of gIII is functionally essential) plays an important role in this process. The gIII-mediated adsorption of the virus to the cell promotes the interaction of other viral membrane proteins with the appropriate cellular proteins, thereby triggering the processes that eventually lead to the penetration of the virus into the cells. Because antibodies against gII and gp50 interfere with virus penetration, these two viral glycoproteins may well be involved in penetration. After gIII-mediated adsorption, penetration of the virus occurs rapidly, and the two processes cannot be uncoupled at 37°C. Penetration of the adsorbed gIII$^-$ mutants is, however, much slower. Since penetration of the gIII$^-$ mutants is inhibited by the same antibodies as is penetration of wild-type virus, the gIII$^-$ mutants may use the same pathway of penetration into the cells as does wild-type virus. However, because of the altered mode of adsorption of the gIII$^-$ mutants, the interactions of the viral membrane proteins with the appropriate cellular protein(s) which trigger virus penetration may be delayed, thereby delaying the penetration of the virus.

Our results show clearly that glycoprotein gIII is an essential part of the viral components that attach to the cell, i.e., it is the viral receptor-binding protein or is part of a viral receptor-binding protein complex. Our data are also compatible with a role of glycoproteins gII and gp50 in virus penetration. The possibility that glycoproteins gII and gp50 play a role in penetration is reinforced by the fact that gB and gD of herpes simplex virus (which are the homologs of gII and gp50 of PrV, respectively [12, 16]) have been shown to be essential for the internalization of this virus (3, 7, 12, 16). It remains to be seen whether gC of herpes simplex virus (the homolog of gIII of PrV [17]) also plays a role in the attachment of herpes simplex virus to the cells, as does gIII of PrV, i.e., whether it is the protein or part of a protein complex that mediates the attachment of herpes simplex virus to the cells.

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