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Interaction of Glycoprotein gIII with a Cellular Heparinlike Substance Mediates Adsorption of Pseudorabies Virus

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Glycoprotein gIII is one of the major envelope glycoproteins of pseudorabies virus (PrV) (Suid herpesvirus 1). Although it is dispensable for viral growth, it has been shown to play a prominent role in the attachment of the virus to target cells, since gIII$^-$ deletion mutants are severely impaired in adsorption (C. Schreurs, T. C. Mettenleiter, F. Zuckermann, N. Sugg, and T. Ben-Porat, J. Virol. 62:2251–2257, 1988). We show here that during the process of adsorption of PrV, the viral glycoprotein gIII interacts with a cellular heparinlike receptor. This conclusion is based on the following findings. (i) Heparin inhibits plaque formation of PrV by preventing the adsorption of wild-type virions to target cells. However, heparin does not interfere with the plaque formation of PrV mutants that lack glycoprotein gIII. (ii) Wild-type virions readily adsorb to matrix-bound heparin, whereas gIII$^-$ mutants do not. (iii) Pretreatment of cells with heparinase reduces considerably the ability of wild-type PrV to adsorb to these cells and to form plaques but does not negatively affect gIII$^-$ mutants. (iv) Glycoprotein gIII binds to heparin and appears to do so in conjunction with glycoprotein gII. Although heparin significantly reduces the adsorption of wild-type virus to all cell types tested, quantitative differences in the degree of inhibition of virus adsorption by heparin to different cell types were observed. Different cell types also retain their abilities to adsorb wild-type PrV to a different extent after treatment with heparinase and differ somewhat in their relative abilities to adsorb gIII$^-$ mutants. Our results show that while the primary pathway of adsorption of wild-type PrV to cells occurs via the interaction of viral glycoprotein gIII with a cellular heparinlike receptor, an alternative mode of adsorption, which is not dependent on either component, exists. Furthermore, the relative abilities of different cell types to adsorb PrV by the gIII-dependent or the alternative mode vary to some extent.

The processes leading to the initiation of infection of cells with herpesviruses are complex, but we know that the initial interaction of the virus with the cells can be divided into at least two stages. In the first stage, the virus binds to receptors on the cell surface; in the second stage, internalization of the virus occurs.

It has been suggested that the cellular receptor to which herpes simplex virus (HSV) adsorbs is a glycoprotein (17, 28) and that two cationic molecules, neomycin (10) and polylysine (11), inhibit HSV adsorption by binding to this receptor. WuDunn and Spear (27) have recently shown that the initial interaction of HSV with the cell surface is mediated by a heparan sulfate-containing entity. The HSV protein that is responsible for the recognition of heparan sulfate remains unknown, but adsorption of the virus to the cells is inhibited by antibodies against at least two different glycoproteins. Internalization of HSV (stage II) involves fusion of the viral and cellular membranes (18, 21). Several glycoproteins are thought to be required for the internalization into the cells of HSV (2, 4, 6, 7, 12, 20).

Adsorption and penetration of pseudorabies virus (PrV) are also clearly distinct processes that can be dissociated from each other at low temperatures (13, 29), and each is inhibited by monoclonal antibodies against different glycoproteins. Thus, several monoclonal antibodies against glycoprotein gII inhibit virus penetration but do not affect adsorption. Conversely, antibodies against glycoprotein gIII inhibit adsorption but not virus penetration (29).

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Adsorption of PrV to its host cell is impaired in mutants that lack glycoprotein gIII (23). Because of the reduced ability of the gIII$^-$ mutants to adsorb to their host cells and to form plaques, cells infected with such mutants yield lower titers of infectious virus (23, 26). Still, gIII$^-$ mutants are infectious; therefore, in addition to the gIII-mediated mode of adsorption, another, gIII-independent mode of adsorption which occurs at low frequency must exist. This alternative mode of adsorption appears to be independent of specific viral proteins—it is not affected by polyvalent neutralizing antisera (23). Furthermore, the alternative mode of adsorption used by the gIII$^-$ mutants is not mediated by the same cellular receptor to which wild-type virus adsorbs; wild-type and gIII$^-$ virions do not compete with each other for adsorption sites (23).

During the course of studies designed to elucidate the factors affecting adsorption of wild-type PrV and gIII$^-$ mutants of this virus, we tested the effects on virus adsorption of several polycationic and polyanionic substances, including heparin, which has been shown to inhibit the growth of herpesviruses (19, 24, 25, 27). Our results show that the initial binding of PrV is to a cell surface component that includes a heparinlike moiety. Furthermore, we show that the viral protein that mediates this interaction is glycoprotein gIII.

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 of PrV(Ka) have been described previously (14–16). The gIII$^-$ mutant of the Becker strain (B10) was generously provided by L. Enquist. Madin-Darby bovine kidney (MDBK), pig kidney (PK), or rabbit kidney (RK) cells were cultivated in Eagle synthetic medium plus 5% dialyzed bovine serum (EDS). Virus was titrated by plaque assay in RK, MDBK, chicken embryo fibroblast (CEF), or PK monolayer cultures.

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 (TBS) (0.136 M NaCl–2.6 mM KCl–0.01 M Tris hydrochloride [pH 7.5]–20 mM $MgCl_2$–1.8 mM $CaCl_2$ [pH 7.0]); TBS containing 1% crystalline bovine albumin (TBSA); TBS containing 1% sodium dodecyl sulfate (TBS-SDS); phosphate-buffered saline (PBS) (0.15 M NaCl–2.6 mM KCl–8 mM $MgCl_2HPO_4$–1 mM KH_2PO_4–20 mM $MgCl_2$–1.8 mM $CaCl_2$ [pH 7.0]); and PBS containing 1% crystalline bovine albumin.

Radiochemicals and chemicals. [^{3}H]thymidine (specific activity, 45 Ci/mmol) was purchased from Dupont, NEN Research Products. [^{35}S]cysteine (specific activity, >600 Ci/mmol) was purchased from Amersham Corp. Heparin, heparinase, and heparin acrylic beads were purchased from Sigma Chemical Co. Heparin CL6B Sepharose was purchased from Pharmacia, Inc.

Labeling and purification of virus. To obtain [^{3}H]thymidine-labeled virus, monolayers of RK cells were incubated for 24 or 48 h in EDS containing 5-fluorouracil (20 μg/ml) and thymidine (5 μg/ml) (a procedure that inhibits cellular DNA synthesis without affecting virus growth [8]). 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.

To label the virus with [^{35}S]cysteine, monolayers of RK cells were infected (multiplicity of infection, 5 PFU per cell) and incubated between 3 and 24 h postinfection in EDS without amino acids, except for arginine and [^{35}S]cysteine (20 μCi/ml).

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 SW27 rotor. 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 peak was diluted with TBSA and sedimented on a TBSA–30% sucrose cushion by centrifugation at 15,000 rpm for 1 h in a Beckman SW27 rotor.

Adsorption of labeled virus. Purified [^{3}H]thymidine-labeled virus in TBSA, prepared as described above, was added to cell monolayers which had been preincubated in TBSA for 15 min to minimize unspecific binding. The monolayers were incubated at 37°C for 1 h and washed extensively, the cells were scraped into TBS-SDS, and the amount of radioactivity that was associated with the cell monolayers was determined.

Immunoprecipitation. Immunoprecipitation was performed by the method of Kessler (9), as described previously (5). The characterization of the monoclonal antibodies against gI, gII, and gIII have been described (5). Mixtures of monoclonal antibodies against each protein were used. The monoclonal antibodies against gp50 were a gift of C. Marchioli, The Upjohn Co. The identities of the immunoprecipitated proteins were in all cases ascertained by analyses on polyacrylamide gels.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed as described previously (5).

Affinity chromatography of labeled virus. [^{3}H]thymidine-labeled virus (0.3 ml) was added to 1 ml of packed-volume heparin Sepharose (that had been prewashed with TBS), incubated at room temperature for 30 min with occasional shaking, and centrifuged at 5,000 × *g* for 2 min. The supernatant containing the unbound virus was collected. TBS-SDS (0.3 ml) was then added to the sample, which was heated at 60°C for 10 min and centrifuged, and the supernatant was collected. The percentage of the virus bound to the columns was calculated as [counts per minute bound/(counts per minute unbound + counts per minute bound)] × 100.

Elution of wild-type virus from heparin Sepharose CL6B by heparin. To determine the specificity of the adsorption of wild-type virus to heparin Sepharose CL6B, purified [^{3}H]thymidine-labeled wild-type PrV (in 0.3 ml of TBS; approximately 2 × 10^5 cpm) was added in duplicate to test tubes containing 1 ml of packed-volume heparin Sepharose CL6B beads. The samples were incubated at room temperature for 30 min with occasional shaking and centrifuged (5,000 rpm for 5 min), the supernatant was collected, and the amount of unbound ^{3}H-labeled virus was determined. The heparin Sepharose beads were then washed five times, either with 2 ml of TBS or 2 ml of TBS containing 100 μg of heparin per ml. The amount of radioactivity released by washing was determined. To ascertain the radioactivity that remained bound to the heparin Sepharose after washing, TBS-SDS (1 ml) was added to the samples, which were heated at 60°C for 15 min and centrifuged. The process was repeated three times to release most of the radioactivity that had remained adsorbed to the heparin Sepharose beads after washing. The amount of radioactivity eluted from the beads by TBS-SDS was determined.

Affinity chromatography of unlabeled virus. A suspension of virus was titrated and added to 1 ml of packed-volume heparin acrylic beads, incubated at room temperature for 30 min, and centrifuged. The supernatant containing the unbound virus was titrated.

Affinity chromatography of virus proteins. Purified [^{35}S]cysteine-labeled virus was incubated for 15 min at 37°C in TBS containing 0.2% Triton X-100 and centrifuged at 20,000 × *g* for 60 min. A portion (0.3 ml) of the supernatant containing the solubilized proteins was added to 1 ml of packed-volume heparin Sepharose beads that had been prewashed with either TBS or TBS–0.2% Triton X-100. The samples were incubated on ice for 30 min with occasional shaking and centrifuged at 5,000 × *g* for 2 min, and the supernatant was collected. The heparin Sepharose beads were washed twice with 2 ml of TBS–0.2% Triton X-100, and the number of counts released by washing as well as the proteins that were immunoprecipitated by various monoclonal antibodies were ascertained. TBS-SDS (0.3 ml) was added to the beads, the samples were heated at 60°C for 10 min and centrifuged, and the supernatant was collected.

RESULTS

Effect of heparin on adsorption of wild-type PrV and a gIII$^-$ mutant of PrV. Figure 1A shows the effects of different concentrations of heparin on plaque formation of wild-type PrV(Ka) and a gIII$^-$ mutant of that virus. (The isolation and characterization of this gIII$^-$ mutant has been previously described [23]). Plaque formation of wild-type PrV was sensitive to heparin; incubation of the virus with 1 μg of

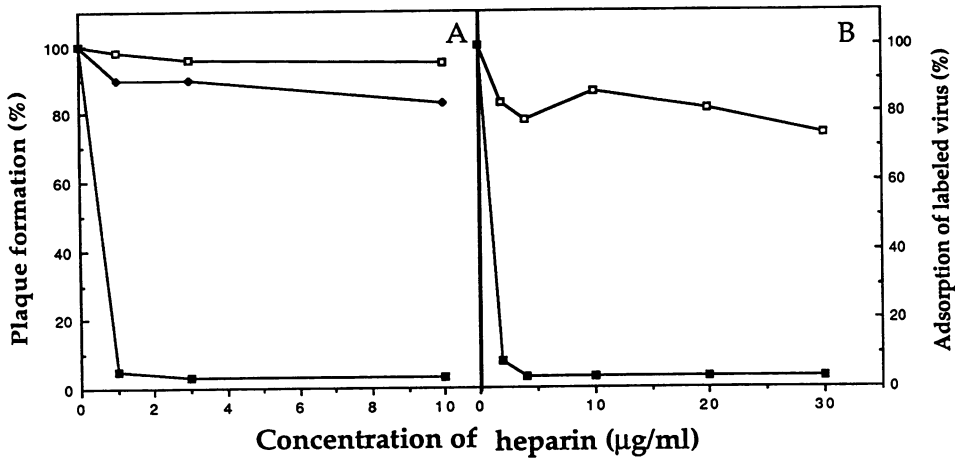

FIG. 1. Effect of heparin during virus adsorption on plaque formation and adsorption of radiolabeled virus. (A) Monolayers of MDBK cells were incubated with 1 ml of TBSA containing the indicated concentration of heparin for 10 min at 37°C. Approximately 200 PFU of either wild-type PrV (■) or a gIII⁻ mutant (□) was then added, and the monolayers were incubated at 37°C for 1 h. They were then washed extensively (to remove unadsorbed virus) and overlaid with agarose. One set of cultures (◆) was incubated with heparin for 1 h at 37°C, washed to remove the heparin, and incubated with approximately 200 PFU of wild-type virus in TBSA for 1 h. These monolayers were then washed again and overlaid with agarose. Plaques were counted 4 days later. (B) Monolayers of MDBK cells were incubated with 1 ml of TBSA containing the indicated concentrations of heparin for 10 min at 37°C. Approximately 10^5 cpm of purified [^{3}H]thymidine-radiolabeled wild-type PrV (■) or a gIII⁻ mutant (□) was then added. The cultures were incubated at 37°C for 1 h and washed extensively to remove unadsorbed virus. The cells were scraped into TBS-SDS, and the amount of radioactivity that had remained associated with the cells was determined. In this experiment, approximately 18% of the input wild-type virus and 1.1% of the gIII⁻ virus became associated with the cells after adsorption in the absence of heparin.

heparin per ml during the adsorption period reduced plaque formation by 95 to 99%. Treatment of the cells with heparin followed by its removal from the cultures prior to the addition of the virus did not significantly affect plaque formation. Thus, in order to be effective, heparin must be present in the cultures during the adsorption period. Furthermore, delaying the addition of heparin for 30 min after the addition of the virus reduced plaque formation by only approximately 25%, compared with a 95% inhibition if it was added at the time of infection (Fig. 2). These results indicate that heparin probably affects virus adsorption. Evidence that this is indeed the case was obtained from experiments in which the effect of heparin on the attachment of ^{3}H-labeled virus to the cells was ascertained (Fig. 1B); heparin effectively inhibited adsorption to the cells of radiolabeled wild-type virus.

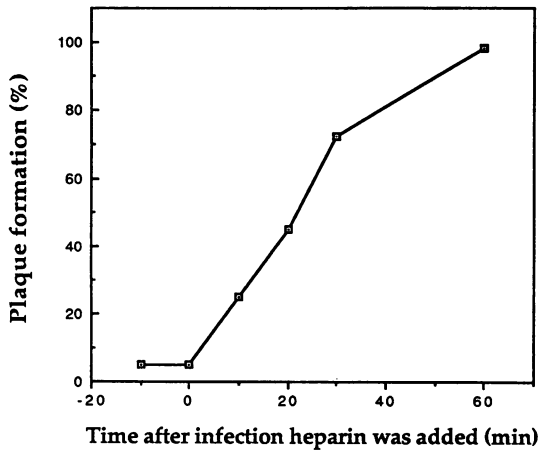

FIG. 2. Effect of addition of heparin at different times after virus inoculation of MDBK cultures. Heparin (50 μg/ml) was added to cultures of MDBK cells at various times after they had been inoculated with wild-type virus (approximately 200 PFU). At 1 h after virus inoculation, the cultures were washed extensively and overlaid with agarose. Plaques were counted 4 days later.

The effect of the incubation of a gIII⁻ mutant with heparin during the adsorption period was much less marked than it was on wild-type virus (Fig. 1A). Concentrations that reduced plaque formation of the wild type by more than 95% reduced plaque formation of the gIII⁻ virus by less than 5%. Furthermore, heparin was much less effective in inhibiting the adsorption of radiolabeled gIII⁻ mutants than of wild-type virus (Fig. 1B). (Although generally a low percentage only of ^{3}H-labeled gIII⁻ mutants remains associated with the cell monolayers, this virus most likely represents virus that has adsorbed to the cells because a consistent correlation exists between ^{3}H-labeled virus that remains cell associated under different conditions and the number of PFU formed [see below]). Two other independently isolated gIII⁻ mutants [a gIII⁻-gI⁻ mutant of PrV(Ka) and a gIII⁻ mutant (B10) of the Becker strain] were also much less sensitive to inhibition by heparin than was the wild-type virus (data not shown). We conclude that heparin interferes with the gIII-mediated mode of adsorption of wild-type virus but does not greatly affect the alternative mode of adsorption used by the gIII⁻ mutants.

Adsorption of wild-type PrV and a gIII⁻ mutant to heparin. Because heparin was effective in inhibiting the adsorption of wild-type PrV when it was present during the adsorption period but was less effective when the cells were treated with it prior to infection, it appeared likely that heparin may bind to the virus. Furthermore, since adsorption of gIII⁻ mutants was not greatly affected by heparin, these mutants should not bind heparin effectively.

The binding of ^{3}H-labeled wild-type virus and ^{3}H-labeled gIII⁻ mutants to heparin Sepharose beads was compared. Under similar conditions of adsorption, a much larger pro-

TABLE 1. Binding of wild-type PrV and a gIII$^-$ mutant of PrV to heparin[a]

Expt	% Unbound virus			
	[^{3}H]thymidine-labeled		Infectious	
	Wild type	gIII$^-$	Wild type	gIII$^-$
1	11	80	4	60
2	7	56	5	69
3	23	99		

[a] The experiment was performed as described in Materials and Methods. Either approximately 10^5 cpm of [^{3}H]thymidine-labeled virus or between 10^3 and 10^4 PFU of infectious virus was added to heparin Sepharose CL6B or to heparin acrylic beads. The amount of radioactivity or the number of PFU that remained unadsorbed was determined.

portion of ^{3}H-labeled wild-type virions than of gIII$^-$ virions adsorbed to heparin Sepharose beads (Table 1).

Similar results were also obtained when the binding of infectious wild-type virus and gIII$^-$ mutants to heparin was ascertained (Table 1). While only 5% of the wild-type input infectious virus was not retained by the heparin beads, more than 60% of the gIII$^-$ mutants was not retained (Table 1).

The binding of radiolabeled wild-type PrV to the heparin columns was specific; the virus could be eluted from the column by heparin. Almost all (95%) of the input virus (approximately 2×10^5 cpm) was initially retained by the column, and 67×10^3 cpm (32% of the input) was retained after extensive washing with PBS. However, when the washing was performed with PBS containing heparin (100 μg/ml), only 4×10^3 cpm (2% of input) was retained. Furthermore, other, unrelated Sepharose-bound substances (polyhistidine and polyproline) did not bind the virus (data not shown). These results indicate that wild-type PrV binds to heparin. Furthermore, because wild-type virus binds well to heparin but gIII$^-$ mutants bind much less effectively (Table 1), we conclude that gIII is necessary for the efficient binding of the virus to heparin.

Treatment of cells with heparinase affects adsorption of wild-type PrV but not of gIII$^-$ mutants. MDBK cells were treated for 1 h with different concentrations of heparinase. The cells were washed, and wild-type PrV or a gIII$^-$ mutant of PrV was assayed on these cells. Treatment of the cell monolayers with progressively higher concentrations of heparinase progressively decreased the titer of wild-type virus (Fig. 3A). On the other hand, the titer of the gIII$^-$ mutant did not decrease after treatment of the cells with heparinase but was consistently somewhat higher.

Similar results were also obtained when the adsorption of [^{3}H]thymidine-labeled virus to heparinase-treated and untreated MDBK cells was compared (Fig. 3B). Treatment of the cells with heparinase reduced the amount of radiolabeled wild-type virus but not of radiolabeled gIII$^-$ mutants that became associated with the cells. In fact, adsorption of the gIII$^-$ mutants was stimulated somewhat after pretreatment of the cells with heparinase (Fig. 3B), as was the infectious titer (Fig. 3A). The reason for this stimulation is unclear.

Comparison of the role of the heparinlike cellular moiety in the adsorption of PrV to different cell lines. The results described above show that in MDBK cells, a heparinlike molecule acts as the cellular attachment site for wild-type virus and that glycoprotein gIII is the viral component that plays a pivotal role in the ability of the virus to interact with this site. To ascertain whether this is also the case in another cell line, the experiments described above (Fig. 1 and 3) were repeated with RK cells.

The effects of different concentrations of heparin on the adsorption of wild-type virus or of gIII$^-$ mutants to RK and MDBK cells are summarized in Table 2. The results were qualitatively similar, although quantitative differences were observed. In both cell types, heparin had only a slight effect on the adsorption of gIII$^-$ virus but was quite effective in inhibiting the adsorption of wild-type virus. However, the inhibition by heparin of the adsorption of wild-type virus to MDBK cells was more marked (98%) than that to RK cells (90%).

The effect of pretreatment with heparinase on the subsequent abilities of RK and MDBK cells to adsorb wild-type PrV or a gIII$^-$ mutant of PrV was also qualitatively similar but, again, some quantitative differences between the two

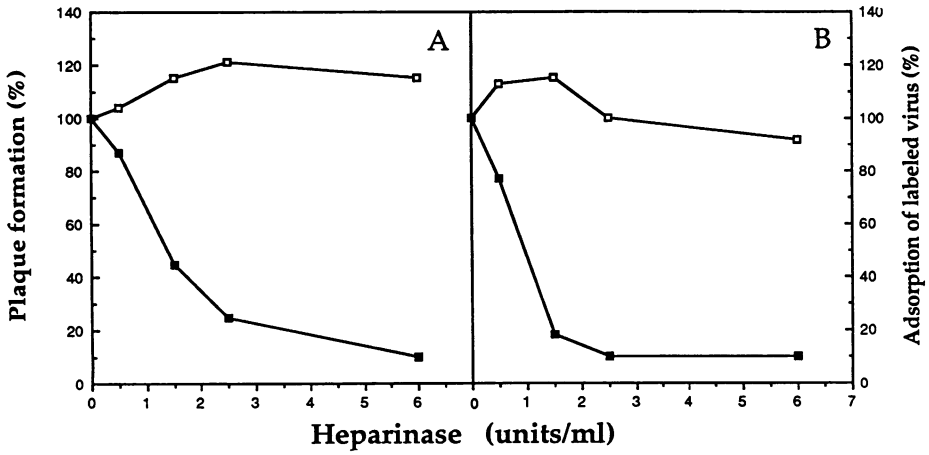

FIG. 3. Effect of treatment of MDBK cells with heparinase on the subsequent abilities of the cells to support plaque formation and adsorption of PrV. The cell monolayers were incubated with PBS containing 1% crystalline bovine albumin plus the indicated concentrations of heparinase for 1 h and then washed extensively. (A) Approximately 200 PFU of either wild-type virus (■) or a gIII$^-$ mutant (□) in 1 ml of TBSA was then added, and the cultures were further incubated for 1 h at 37°C, washed to remove unadsorbed virus, and overlaid with agarose. Plaques were counted 4 days later. (B) Approximately 10^5 cpm of [^{3}H]thymidine-labeled wild-type virus (■) or gIII$^-$ virus (□) in 1 ml of TBSA was then added; the cultures were further incubated for 1 h at 37°C and washed to remove unadsorbed virus, and the number of counts that remained associated with the cell monolayer was determined.

TABLE 2. Effect of different concentrations of heparin on adsorption of virus to RK and MDBK cells[a]

Concn of heparin (μg/ml)	Adsorption (cpm) of virus to:			
	RK cells		MDBK cells	
	Wild type	gIII⁻	Wild type	gIII⁻
0	9,670	1,760	30,380	760
1	6,040	1,210	1,575	690
3	2,469	1,290	525	670
10	900	1,610	465	530
30	620	1,680	510	690
100	860	1,280	520	680

[a] Cells were incubated in 1 ml of TBSA containing the indicated amount of heparin for 15 min. [^{3}H]thymidine-labeled virus (10^5 cpm in 50 μl) was then added, and the cultures were incubated for 1 h at 37°C. The cultures were then washed extensively to remove unadsorbed virus, and the amount of radioactivity that remained associated with the cell monolayers was ascertained, as described in Materials and Methods.

TABLE 3. Plaque assays of wild type PrV and gIII⁻ mutants on various cell types[a]

Expt and cell type	PFU/ml	
	Wild type	gIII⁻
1		
MDBK	5×10^8	4×10^6
CEF	4×10^7	2×10^7
RK	8×10^7	2×10^7
PK	1×10^8	3×10^7
2		
MDBK	9×10^8	8×10^6
CEF	3×10^8	4×10^7
RK	2×10^8	2×10^7

[a] Virus stocks of wild-type or a gIII⁻ mutant of PrV(Ka) were serially diluted (10-fold). A 1-ml portion of each dilution was inoculated in duplicate into confluent monolayers of the indicated cell types grown in 50-mm petri plates. After 1 h of adsorption, the monolayers were washed to remove unadsorbed virus and overlaid with agarose. Plaques were counted 3 to 4 days later.

cell types were observed (Fig. 4). Thus, the relative amounts of gIII⁻ mutants and wild-type virions that adsorbed to untreated cells as well as the relative decrease in the abilities of the cells to adsorb wild-type virus after treatment with heparinase differed in the two cell lines. In this experiment (Fig. 4), 60% more wild-type virus adsorbed to untreated MDBK cells than to untreated RK cells. In other experiments (Table 2), the difference was even greater. Conversely, almost twice as many gIII⁻ virions adsorbed to the untreated RK cells than to untreated MDBK cells (Fig. 4 and Table 2). Furthermore, after treatment with heparinase, the reduction in the amount of wild-type virus that adsorbed was much less marked in RK cells than in MDBK cells (Fig. 4A). However, in both cell types, treatment with heparinase inhibited adsorption of wild-type virus but not adsorption of the gIII⁻ mutants; on the contrary, adsorption of the gIII⁻ mutants was stimulated.

The difference in the relative amounts of wild-type and gIII⁻ virus that adsorbed to untreated RK and MDBK cells was confirmed by plaque assay of the viruses on these cell types. Table 3 shows the titers of wild-type virus and gIII⁻ mutant stocks assayed on four different cell types. As reported previously (23, 26), the titers of infectious virus of the gIII⁻ mutants are generally lower than are those of wild-type virus. However, the virus titers of both wild-type virus and the gIII⁻ mutants varied somewhat when assayed on the different cell lines. The differences in titer, though relatively small, were reproducible. Thus, we consistently obtained higher titers (between three and six times higher) of wild-type virus when it was assayed on MDBK cells than when it was assayed on RK, PK, or CEF cells. On the other hand, the titer of the gIII⁻ mutants was consistently higher when it was assayed on RK, PK, or CEF cells than when it was assayed on MDBK cells. Thus, the titers of the wild-type and gIII⁻ mutant stocks differed by a factor of approximately 100 when assayed on MDBK cells but only by a factor of less than 10 when assayed on RK, CEF, or PK cells. The relative differences in the titers of the virus obtained in MDBK and RK cells mimicked the different amounts of radiolabeled virus that adsorbed to these cells

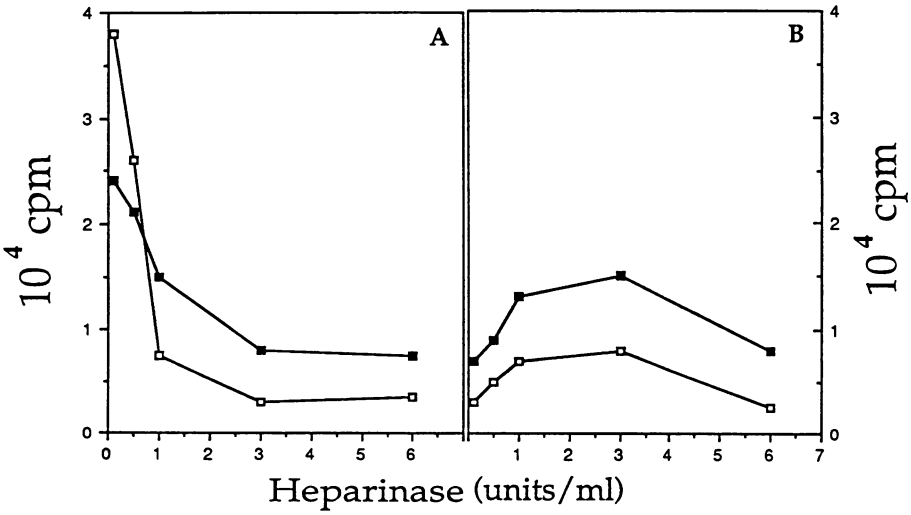

FIG. 4. Comparison of effects of treatment of RK (■) and MDBK (□) cells with heparinase on their subsequent abilities to adsorb wild-type (A) and gIII⁻ (B) PrV. The experiment was performed as described in the legend to Fig. 3 (panel B).

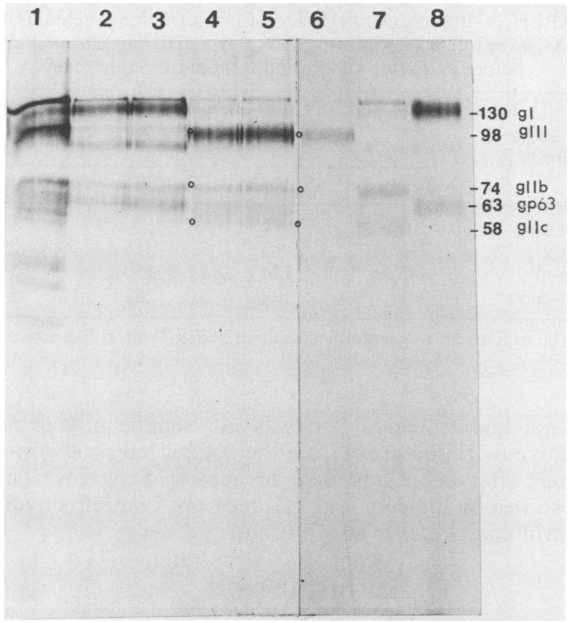

FIG. 5. Binding of ^{35}S-labeled PrV proteins to heparin Sepharose beads. The procedures used to prepare the Triton X-100 extracts of ^{35}S-labeled virus and to adsorb the proteins to heparin Sepharose beads are described in Materials and Methods. Lane 1, Triton X-100 extract of purified [^{35}S]cysteine-labeled wild-type virus; lane 2, unbound proteins obtained after incubation of the virus Triton X-100 extracts with heparin Sepharose beads which had been prewashed with TBS; lane 3, unbound proteins obtained after incubation of Triton X-100 extracts with heparin Sepharose beads prewashed with TBS–Triton X-100; lane 4, proteins released by treatment with TBS-SDS from heparin Sepharose beads prewashed with TBS; lane 5, proteins released by treatment with TBS-SDS from heparin Sepharose beads prewashed with TBS–Triton X-100; lane 6, viral proteins immunoprecipitated with anti-gIII serum (size marker for gIII); lane 7, viral proteins immunoprecipitated by antibodies against gII (size marker for gII); lane 8, proteins immunoprecipitated with antibodies against gI (size marker for gI). (gp63 is complexed to gI and is coprecipitated with gI [29].) The positions of gIII and gIIb and gIIc are indicated (○).

(Fig. 4). Thus, it appears that wild-type virus adsorbs better to MDBK than to RK cells and that gIII$^-$ virus adsorbs better to RK than to MDBK cells. Similar results were obtained with two other independently isolated gIII$^-$ mutants. These findings support the notion that in MDBK cells, the gIII-mediated mode of adsorption (which occurs by the binding of the virus to a heparinlike cellular receptor) is more efficient than in RK cells. Conversely, the gIII-independent, cellular heparinlike, receptor-independent mode of adsorption used by the gIII$^-$ mutants occurs more readily in RK cells than in MDBK cells. Furthermore, we have observed that the degree of confluence of each cell type also affects the adsorption of wild-type and gIII$^-$ virus somewhat differentially (data not shown). Thus, the adsorption of PrV is more or less dependent on the interaction between viral gIII and the cellular heparinlike receptor in different host cell types and is also somewhat affected by the physiological states of the cells.

Glycoprotein gIII adsorbs to heparin-Sepharose beads. The results described above indicate that wild-type PrV adsorbs to the cells by binding to a heparinlike substance and that gIII is necessary for this binding. To ascertain whether gIII is indeed the (or one of the) viral component(s) responsible for the binding of the virus to heparin, solubilized viral proteins were allowed to bind to heparin Sepharose beads and the identities of the proteins that had bound were determined. The results (Fig. 5) show that a protein with a molecular weight of approximately 98,000 (the approximate molecular weight of gIII) was depleted from the solubilized viral protein mixture by incubation with heparin beads (Fig. 5, compare lane 1 with lanes 2 and 3). This glycoprotein could be recovered from the heparin columns by treatment with SDS (lanes 4 and 5) or by elution with heparin (data not shown). Other protein bands were also present in the material that was eluted from the heparin Sepharose beads with SDS. Two of these bands were of interest because they had approximate molecular weights of 74,000 and 58,000, which are the molecular weights of two components of the gII complex. (The third component of gII [125,000] comigrates with gI; a band of approximately 125,000 to 130,000 is also present.)

TABLE 4. Immunoprecipitation of [^{35}S]cysteine-labeled PrV Triton X-100 extract before and after adsorption to heparin Sepharose[a]

Monoclonal antibody	Amt (cpm × 10^2)[b] of protein immunoprecipitated		% Depleted[c]
	Total viral	Unbound	
Anti-gIII	527	42	92
Anti-gII	215	78	64
Anti-gI	308	291	6
Anti-g50	405	420	0

[a] Triton X-100 extracts of ^{35}S-labeled wild-type viral protein were adsorbed to heparin Sepharose beads, and the proteins that did not bind were collected. An approximate equal number of counts of each (10^6) was immunoprecipitated as described in Materials and Methods.

[b] Background, obtained with preimmune serum (approximately 20 × 10^2 cpm), was subtracted.

[c] The amount of radioactivity precipitated by each of the monoclonal antibodies from the sample that had not been adsorbed to heparin was considered 100%, and the percentage of each protein that had been depleted from the unbound protein preparation was calculated.

To test whether the proteins that had bound to heparin were indeed glycoproteins gIII and gII, the relative amounts of gI, gII, gIII, and gp50 that could be immunoprecipitated by the appropriate antibodies from the original solubilized protein mixture (i.e., before it had been applied to the heparin Sepharose) and from the protein mixture that contained the unbound proteins (i.e., that remained after binding to heparin) were compared (Table 4).

After adsorption to heparin of the wild-type virus solubilized protein mixture, the amount of proteins that could be precipitated by monoclonal antibodies against glycoprotein gIII as well as gII (but not gI or gp50) was reduced considerably. The amount of proteins immunoprecipitated by monoclonal antibodies against gIII relative to those against gI or gp50 from the sample containing the proteins that had not bound to heparin was decreased by 92%, compared with that present in the original protein mixture. The amount of glycoprotein gII that remained in the unbound sample was also reduced, but only by 64%. Figures 6A and B show the polyacrylamide gel electrophoresis profiles of the immunoprecipitated proteins obtained from another similar experiment. A comparison of the results shown in Fig. 6A and B reveals that both gIII and gII were depleted to a much greater extent than were gI and gp50 from the protein mixture obtained from wild-type virus after it had been adsorbed to heparin. (In this gel, some contaminating

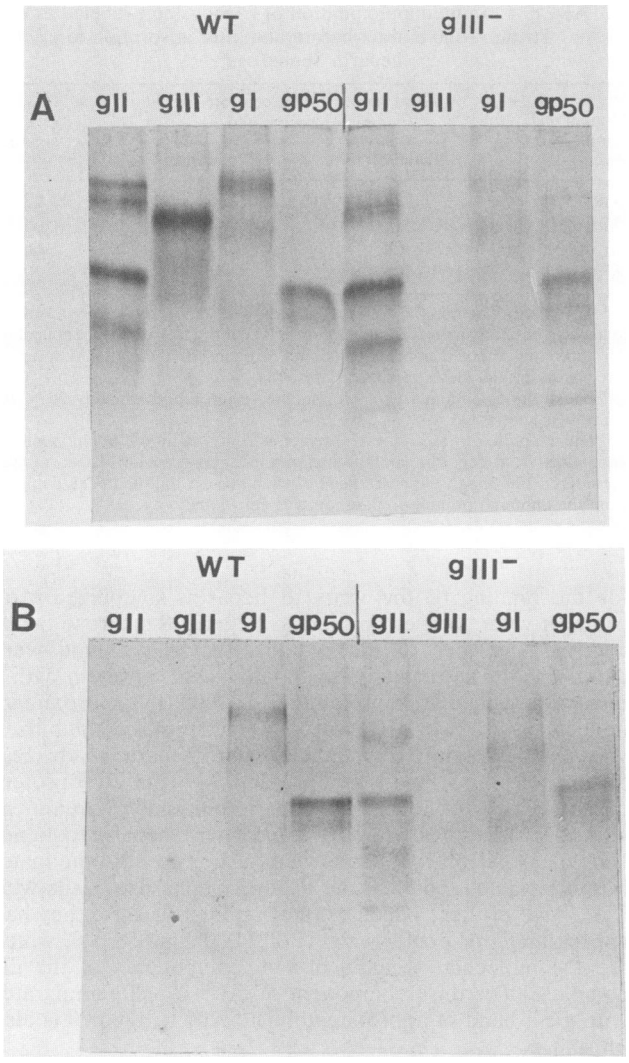

FIG. 6. PAGE of proteins immunoprecipitated from Triton X-100 extracts of ^{35}S-labeled virus proteins before and after they have been adsorbed to heparin Sepharose. The experiment was performed essentially as described in Table 4, footnote *a*. (A) Immunoprecipitates of proteins before adsorption to heparin Sepharose (approximately 8×10^5 cpm was immunoprecipitated with each monoclonal antibody). (B) Immunoprecipitates of proteins after adsorption to heparin Sepharose (approximately 3×10^5 cpm was immunoprecipitated with each antibody). An equal portion of the immuoprecipitated proteins was electrophoresed on acrylamide gels. The glycoproteins against which the monoclonal antibodies were directed are indicated at the top of each lane. Both gels were exposed for 7 days.

bands—possibly precursor or breakdown products—are observed in some of the other lanes. The presence of these contaminants does not, however, invalidate our conclusions, i.e., that gIII and gII were adsorbed to the heparin to a much greater extent than were gI and gp50.)

Since both glycoproteins gIII and gII bind to heparin, it was of interest to ascertain whether gIII and gII each binds autonomously to heparin or whether they bind as a complex. To this end, experiments similar to those illustrated for the wild-type virus were also performed with a gIII$^-$ mutant. The results (Table 5; Fig. 6A and B) showed that gII present in the solubilized proteins obtained from the gIII$^-$ mutants

TABLE 5. Immunoprecipitation of [^{35}S]cysteine-labeled Triton X-100 extracts of wild-type PrV and a gIII$^-$ mutant of PrV before and after adsorption to heparin Sepharose[a]

Monoclonal antibody	Amt (cpm × 10^2) of protein immunoprecipitated			
	Wild type		gIII$^-$	
	Total	Unbound	Total	Unbound
Anti-gIII	308	16		
Anti-gII	215	63	316	290
Anti-gI	143	114	150	150
Anti-gp50	201	194	251	240

[a] The experiment was performed as described in Table 4, footnote *a*.

did not bind effectively; it was not significantly depleted (relative to gI and gp50) from the solubilized protein preparations after being adsorbed to heparin Sepharose beads. These results indicate that gII does not bind effectively to heparin unless gIII is also present.

DISCUSSION

It has been known for some time that heparin inhibits the growth of herpesviruses but that it does not similarly inhibit the growth of several other viruses (19, 24, 25). Recently, WuDunn and Spear (27) showed that the initial interaction of HSV with its host cells is by binding to heparan sulfate. Our results show that the binding of PrV to its host cells is also in large part the result of the interaction of the virus with a cell surface component that includes a heparinlike moiety and that, furthermore, viral glycoprotein gIII is essential for this interaction.

We have shown previously that the adsorption of gIII$^-$ mutants of PrV is slower and less efficient than is that of wild-type virus (23). In the absence of gIII, the virus adsorbs to the cells by a process that differs from that of wild-type virus (29) and that does not appear to be mediated by specific viral proteins (it is insensitive to polyvalent sera against PrV). We show here that while the presence of heparin or treatment of the cells with heparinase markedly inhibits wild-type PrV adsorption, it does not similarly affect adsorption of gIII$^-$ mutants. Thus, while the initial interaction of most of the wild-type virus with the cell is by binding to a heparinlike cellular component, gIII$^-$ mutants adsorb to the cell (though with a relatively low efficiency) in a manner that bypasses this process.

While the majority of wild-type virions adsorb to the cells by the mode of adsorption that involves the interaction of viral gIII with the cellular heparinlike moiety, a small proportion of wild-type PrV can adsorb to the cells by an alternative mode of adsorption which is probably similar to that used by gIII$^-$ mutants. This can be deduced from the fact that heparin does not completely abolish the infectivity of wild-type virus and that some wild-type virus can initiate infection even after the cultures have been treated with high concentrations of heparinase.

The interaction between viral gIII and the cellular heparinlike moiety is clearly the main mode of wild-type PrV adsorption. However, the relative importance of this mode of adsorption differs in different cell types. Thus, PrV is more apt to adsorb to RK cells than to MDBK cells by a gIII-independent, heparin-independent pathway. This can be concluded from several observations. (i) Heparin is more effective in inhibiting the adsorption of wild-type virus to MDBK cells than to RK cells. (ii) Adsorption of wild-type

virus is reduced more after heparinase treatment of MDBK cells than after heparinase treatment of RK cells. On the other hand, the alternative gIII-dependent mode of adsorption occurs more efficiently on RK (as well as PK and CEF) than on MDBK cells.

Of the cell types that we have tested, the alternative gIII-independent mode of adsorption (resulting in a higher plaquing efficiency of gIII$^-$ mutants) occurred more readily in the two primary cell types (RK and CEF cells) and in one of the two established cell lines (PK) than in the other established cell line (MDBK). Thus, the difference in the adsorption patterns that were observed is not between primary and established cell lines. The basis for the difference in the adsorption patterns of the virus to different cells is not clear. It is interesting that we have observed some differences in the ability of a given cell line to adsorb PrV by the gIII-independent mode of adsorption and support plaque formation that depend on the physiological state (time after seeding) of the cells (unpublished observations).

Although gIII$^-$ mutants do not adsorb in cell culture as well as the wild-type virus does, they are as virulent for chicks and mice as is the wild-type virus (15; unpublished results). We have previously suggested that this indicates that the virus spreads mainly cell to cell in these systems (15). However, since different cells appear to differ in their abilities to adsorb the virus by a pathway that is independent of the interaction between viral gIII and the cellular heparin-like substance, it is possible that the virulence of the gIII$^-$ mutants may also be related, at least in part, to the ability of target cells in vivo to adsorb the virus efficiently by the gIII-independent mode of adsorption.

Glycoprotein gIII binds well to heparin Sepharose beads; other proteins, including glycoprotein gII, also bind but do so less effectively. While the binding of gIII to heparin is probably related to the pivotal role gIII plays in virus adsorption (i.e., in the binding of the virus to the heparinlike cellular receptor), the biological significance of the binding to heparin of glycoprotein gII remains to be ascertained. The fact that gII binds efficiently to heparin only in conjunction with gIII (and possibly vice versa) indicates that these two glycoproteins may form a complex. However, whether some gII is indeed complexed to gIII in the intact viral membrane and whether it plays a role in adsorption are uncertain.

Heparin is a polyanionic-sulfated polysaccharide (that is usually bound to proteins) that readily adsorbs cationic substances such as polyarginine. An arginine-rich region is present near the amino terminus of gIII (22). It remains to be ascertained whether this domain of glycoprotein gIII is responsible for the interaction of the protein with heparin.

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