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A Heterologous Heparin-Binding Domain Can Promote Functional Attachment of a Pseudorabies Virus gC Mutant to Cell Surfaces

SHANNON J. FLYNN AND PATRICK RYAN*

Department of Microbiology and Immunology, University of Tennessee at Memphis, Memphis, Tennessee 38163

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The efficient attachment of pseudorabies virus to cultured cells is dependent on an electrostatic interaction between negatively charged cell surface heparan sulfate and the viral envelope glycoprotein gC. Deletion of the first one-third of gC severely impairs virus attachment, but the mutant virions are still capable of entering cells and establishing an infection via a gC-independent pathway. This region of gC contains three clusters of positively charged amino acids that exactly or nearly conform to proposed consensus motifs for heparin-binding domains (HBDs), and the loss of one or more of these potential HBDs may be responsible for the observed attachment defect. To more directly show the involvement of HBDs in pseudorabies virus attachment to cells, we replaced the first one-third of gC with a single, biochemically defined HBD from apolipoprotein B-100. On the basis of the results of attachment, penetration, and heparin competition assays, the heterologous HBD mediated heparan sulfate-dependent virus attachment, but not to fully wild-type levels. Although the intermediate phenotype is not understood, the apolipoprotein B-100 HBD may represent the smallest defined amino acid sequence that promotes functional herpesvirus attachment to cultured cells.

Herpesviruses gain entry into cells through a cascade of events that involve at least five different viral envelope glycoproteins (7). The initial step for many herpesviruses has been shown to include an electrostatic binding of virus particles to negatively charged cell surface heparan sulfate (17, 18, 20, 32). This attachment is mediated principally by glycoprotein gC but has been found to be unnecessary, at least in cell culture, in that strains lacking their gC homolog are still infectious (10, 19, 23). Additionally, biochemical studies on several herpesviruses have demonstrated that gC and a second envelope glycoprotein, gB, are capable of binding to heparin, a species closely related to heparan sulfate (10, 17, 27). Despite the similarities among the various herpesviruses, though, differences have emerged. For example, virions of pseudorabies virus (PRV) lacking or containing mutant forms of gC (formerly gIII) are readily removed from cell surfaces by washing infected monolayers with phosphate-buffered saline (PBS) (6, 29). The virions that do manage to infect cells do so independently of heparan sulfate (6, 17). In contrast, virions of herpes simplex virus type 1 gC mutants are resistant to washes with PBS, although many fewer mutant particles than wild-type particles bind to cells (10). In addition, herpes simplex virus type 1 gC mutant particles still attach to cells via heparan sulfate but do so through gB; this may explain their resistance to PBS washes (9).

Numerous heparin-binding proteins have been characterized in other systems, and two consensus motifs for heparin-binding domains (HBDs) have emerged from these studies (1). It has been suggested that basic amino acids arranged either -X-B-B-X-B-X- or -X-B-B-B-X-X-B-X- (where B is a basic amino acid and X is a hydropathic residue) mediate specific protein-heparin interactions. These motifs are often found to occur more than once in known heparin-binding proteins.

PRV gC is a 479-amino-acid polypeptide in which the first 22 residues constitute a signal peptide that is removed during translocation across the endoplasmic reticulum to yield a mature species that has also acquired N-linked glycosylation (4, 22). Ultimately, gC is found as a 92-kDa species in the virus envelope and plasma membrane of an infected cell with the bulk of the amino-terminal portion exposed to the outside (25). Inspection of the predicted amino acid sequence of gC reveals seven exact or near matches to the proposed HBD consensus motifs (6, 22). We have previously shown that a deletion of the first one-third, but not deletions of the back two-thirds, of mature gC prevents the mutant PRV particles from functionally attaching to PK15 or Vero cells (6). This led us to suggest that at least a principal component of the PRV attachment domain resides in the first one-third of gC and may involve some or all of three potential HBDs in this region. Work by others implicated the middle one-third of PRV gC as necessary for virus binding to two other cell lines; they did not assay mutations in the first one-third (34). In addition, the middle one-third may be involved in the transition of bound particles from a heparin-sensitive to a heparin-resistant state (35). These studies indicate that different portions of PRV gC may mediate virus binding to different cell types, perhaps through redundancy that is conferred by multiple HBDs.

To more directly demonstrate the role of HBDs in mediating PRV attachment to cells, we have effectively replaced the first one-third of mature gC with a single, biochemically defined HBD from apolipoprotein B-100 (apoB-100) (11). This HBD was introduced as a part of just 24 amino acids, yet it functionally replaced the first one-third of wild-type gC. The apoB-100 HBD promoted heparan sulfate-dependent attachment of PRV virions and may represent the smallest sequence of amino acids demonstrated to mediate herpesvirus attachment.

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* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Tennessee at Memphis, 858 Madison Ave., Rm. 201, Memphis, TN 38163. Phone: (901) 448-8764. Fax: (901) 448-8462.

MATERIALS AND METHODS

Cells, virus, and DNA. PK15 (porcine kidney) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. PRV-Becker (PRV-Be), our wild-type strain, and PRV523 have been described previously (6); they were propagated on PK15 cells. Plasmids were constructed by standard recombinant DNA techniques (15) and were maintained in *Escherichia coli* KK2186 (6).

Construction of pIG529 and pIG537. Plasmid pIG523 contains a 3.3-kbp fragment of PRV DNA that encodes a mutant *gC* allele (6). In pIG523, *gC* codons 25 to 157 have been replaced with a *Sph*I restriction site that is unique to the plasmid. To construct pIG529, the following oligonucleotides were treated with kinase and annealed to each other: 5′-TATAAGCTGCAGGGTACTAC CCGTCTCACTCGTAAGCGAGGTCTTAAGCTTGCTACGGCCATG-3′ and 5′-GCCGTAGCAAGCTTAAGACCTCGCTTACGAGTGAGACGGGT AGTACCCTGCAGCTTATACATG-3′. The double-stranded linker encoded an HBD from apoB-100 and provided *Sph*I-compatible overhangs at each end. To introduce the HBD coding sequence in-frame into *gC*, pIG523 was digested with *Sph*I, treated with shrimp alkaline phosphatase, and then ligated to the kinase-treated linker. To ensure that only one linker was inserted, the ligation products were digested with *Afl*II (a unique site in the linker and underlined above), religated, and transformed into competent KK2186 cells, with selection for ampicillin-resistant colonies.

Plasmid pIG537 was constructed in an identical manner with the following two oligonucleotides: 5′-TATAAGCTGCAGGGTACTACCCGTCTCACTGCTAG CAGTTATCTTAAGCTTGCTACGGCCATG-3′ and 5′-GCCGTAGCAAGC TTAAGATAACTGCTAGCAGTGAGACGGGTAGTACCCTGCAGCTTA TACATG-3′. The linker produced by these oligonucleotides encoded a mutant form of the HBD, which is described in Results.

Plasmid DNA was prepared from several isolates of each transformation and screened by restriction enzyme and dideoxy-DNA sequence analyses (26, 33) for having a single linker inserted in the correct orientation. One such construct was chosen for each linker, and the plasmids were designated pIG529 and pIG537.

Construction and identification of PRV mutants. Plasmid pIG529 or pIG537 was cotransfected with PRV56 genomic DNA by calcium phosphate precipitation (8) to produce mutant virus strains. Recombinant viruses were identified with gC-specific antiserum 282 in an immunohistochemical plaque assay, the black-plaque assay (12, 25). In this assay, PRV56 plaques did not stain (remained "white") because the gC encoded by PRV56 lacked a signal sequence and was not exported to infected cell surfaces (4). Recombinant virus plaques stained black in the assay, the result of restoring a wild-type signal sequence coding region to *gC* while concomitantly introducing the linker sequences contained in pIG529 or pIG537. Several black plaques were picked from each cotransfection lysate and purified. Lysates of each isolate were then produced by standard methods.

Southern blot analysis. To identify correct recombinant viruses, a Southern blot analysis was performed on viral DNAs as previously described (23). Viral DNAs were digested with *Pst*I and probed with [^{32}P]pGBe4.3. Plasmid pGBe4.3 is a pGEM-3Zf(+) derivative containing a 4.3-kbp *Pst*I fragment that encodes wild-type *gC* and possesses about 1 kbp of PRV DNA on either side of *gC* (6).

Radiolabeling of viral glycoproteins. Viral glycoproteins were radiolabeled with [^{3}H]glucosamine and immunoprecipitated as previously described (25). Immune precipitates were then resolved on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and visualized by fluorography and autoradiography (25).

Virus assays. Attachment, penetration, and soluble heparin competition assays, as well as the measurement of attachment on heparinase-treated cells, were all performed exactly as described by Flynn et al. (6).

Chondroitin sulfate A and C and dermatan sulfate (chondroitin sulfate B) were obtained from Sigma Chemical Co. Competition assays with these compounds were performed in triplicate as follows. PK15 cell monolayers on 60-mm dishes were inoculated at 4°C with approximately 150 PFU of wild-type or mutant strains in the presence of 0 or 20 μg of either compound per ml. After a 2-h incubation at 4°C, the infected monolayers were washed vigorously with ice-cold PBS and overlaid with Dulbecco's modified Eagle's medium containing 1% methylcellulose. The infected monolayers were then incubated for 36 h at 37°C to allow plaque formation. The average number of plaques per plate was determined from treated infections, divided by the average number of plaques per plate from untreated infections of the same strain, and multiplied by 100 to represent relative resistance as a percentage of the control value.

All assays were performed multiple times for each strain, and standard deviations were measured and are reported. To determine if observed differences between strains were significantly different, Student's *t* tests were performed (24).

Protein sequence alignment. The HBD sequences of various gC homologs were compared by the PileUp program of the University of Wisconsin Genetics Computer Group (3).

RESULTS

Construction and characterization of PRV strains that have replaced the first one-third of mature gC with just 24 amino acids. We have previously described a plasmid, pIG523, that contained a cloned copy of *gC* in which codons 25 to 157 were replaced with an *Sph*I restriction site that maintained the proper reading frame. When this *gC* allele was recombined into an otherwise wild-type PRV genome, the resulting strain, PRV523, was found to be attachment defective, probably owing to the loss of one or more of three potential HBDs that reside in the first one-third of wild-type gC (6). To determine if a single, heterologous HBD could functionally replace the missing gC sequences, an oligonucleotide linker containing 24 codons that encoded a biochemically defined HBD from apoB-100 (11) was inserted in-frame into the unique *Sph*I site of pIG523 (see Materials and Methods). The resulting plasmid was named pIG529. A second oligonucleotide linker was independently introduced into the *Sph*I site of pIG523 as a control; this plasmid was termed pIG537. The linker in pIG537 was identical to the first except that it no longer coded for a consensus HBD; codons for three basic amino acids and one neutral residue were replaced by codons for neutral amino acids only. The resulting peptide sequence was predicted to maintain the secondary structure of the apoB-100 HBD (2) and was chosen to test whether the substitution of such a structure for the first one-third of mature gC could by itself restore attachment function, perhaps by leading to the activation of a cryptic HBD harbored in PRV523 gC. Plasmid DNA from pIG529 or pIG537 was cotransfected with PRV genomic DNA to recombine each *gC* allele into the virus genome. As described and referenced in Materials and Methods, recombinant viruses were identified and plaque purified, resulting in strains PRV529 and PRV537 (corresponding to the progenitor plasmid). The predicted *gC* products of PRV529 and PRV537 are depicted in Fig. 1.

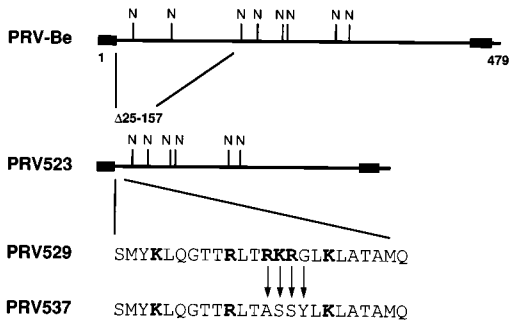

FIG. 1. Depiction of the gC protein from wild-type (PRV-Be) and mutant PRV strains. At the top, the 479-amino-acid wild-type *gC* product is illustrated, with black boxes representing the signal sequence at the amino terminus and the transmembrane anchor near the carboxy terminus. The N's designate the relative positions of N-linked glycosylation sites. PRV523-encoded gC is drawn to indicate the deletion of amino acids 25 through 157. The *gC* products of PRV529 and PRV537 have 24 foreign amino acids in place of the residues deleted in PRV523 gC. The amino acid sequences of both substitutions are indicated by the single-letter code. The boldface letters denote positively charged amino acids, and the arrows highlight the alterations between the two inserted sequences.

The identity of each isolate was verified by a Southern blot analysis (Fig. 2). As previously shown, wild-type *gC* of PRV-Be was contained on a single 4.3-kbp *Pst*I fragment whereas PRV523 produced a *gC*-bearing *Pst*I fragment of 3.9 kbp as a result of the deletion of codons 25 to 157 (6). Correct isolates of PRV529 and PRV537 harbored their *gC* alleles on two *Pst*I fragments of 2.1 and 1.8 kbp. This was because the oligonucleotide linkers used to construct the mutant plasmids possessed an internal *Pst*I site. This resulted in an asymmetric cleavage of the 3.9-kbp *Pst*I fragment and facilitated the correct identification of each mutant virus strain.

It was also important to demonstrate that each mutant gC

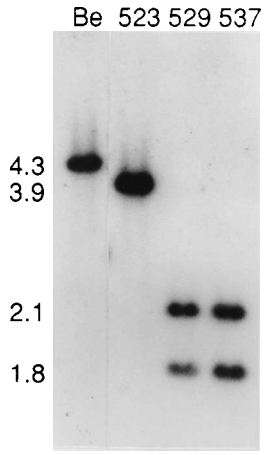

FIG. 2. Southern blot of genomic DNA from wild-type and mutant strains. Viral DNAs were digested with *Pst*I and hybridized to a *gC*-specific probe as described and referenced in Materials and Methods. Migration of the molecular mass markers (in kilobase pairs) is indicated on the left, and the strain designations are shown at the top. All lanes in this figure are from the same autoradiogram; a lane was removed for presentation purposes.

was correctly localized to the virus envelope. This was achieved by immunoprecipitating [^{3}H]glucosamine-labeled gC from virions of wild-type and mutant strains as previously described (25). Immunoprecipitations of glycoprotein gB (formerly gII) were also included in the experiment. This glycoprotein was wild type for all of the strains and was used as a relative measure of the radiolabeling efficiency. As shown in Fig. 3, wild-type gC from PRV-Be appeared as an abundant, heterogeneous 92-kDa species. Similarly, each of the mutants produced a heterogeneous form of gC that migrated with an apparent molecular mass of 65 kDa as a result of deletion of the first one-third of the glycoprotein. However, all three mutants appeared to localize slightly smaller amounts of gC to the virus envelope than did the wild type. We have previously described this reduction for PRV523 but would also note that

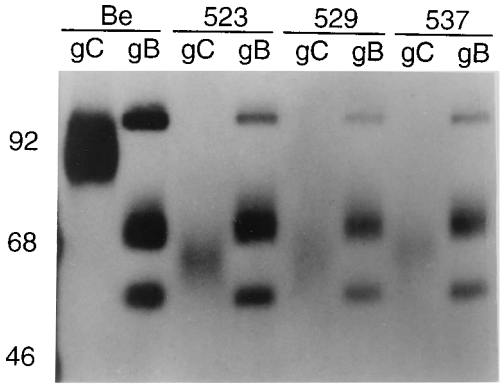

FIG. 3. Wild-type and mutant forms of gC that are localized to virus envelopes. PK15 cells were infected at a multiplicity of 10 with wild-type or mutant virus and incubated for 16 h at 37°C in Dulbecco's modified Eagle's medium containing 50 μg of [^{3}H]glucosamine per ml. Virus particles were then purified from the culture medium as previously described (25). Antiserum 282 (25) was used to immunoprecipitate gC, while gB was immunoprecipitated with antiserum 284 (31). The immunoprecipitates were resolved on an SDS–10% polyacrylamide gel followed by autoradiography. Strain and glycoprotein designations are shown above the lanes, and the migration of molecular mass markers (in kilodaltons) is indicated to the left. Only the relevant portion of the autoradiogram is shown.

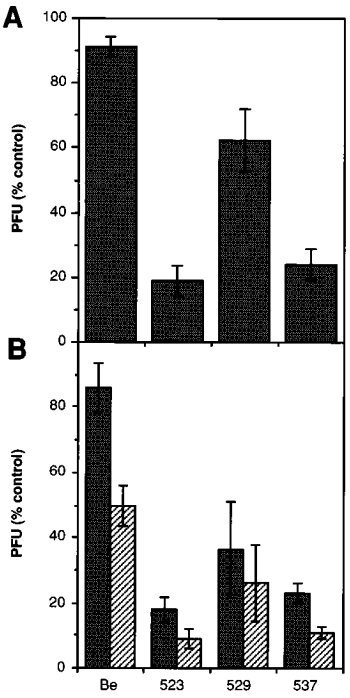

FIG. 4. Attachment and penetration profiles of wild-type and mutant strains. (A) Relative attachment efficiency of each strain after 2 h at 4°C. The assay was performed as described in the text and by Flynn et al. (6). (B) Relative absorption and penetration efficiencies of each strain. For each strain, the relative absorption (i.e., attachment and penetration) efficiency is indicated by a shaded bar and the relative penetration efficiency is indicated by a hatched bar. Monolayers were incubated with about 150 PFU of virus for 1 h at 37°C and then, after removal of each inoculum, left untreated (control), washed twice with PBS (absorption), or treated with low-pH citrate buffer for 2 min at room temperature and washed twice with PBS (penetration). All monolayers were then overlaid with methylcellulose medium to allow plaque formation. For details, see Flynn et al. (6). For panels A and B, the *y* axis is the average number of plaques per plate expressed as a percentage of untreated control values. Strain designations are shown along the *x* axis. Each bar represents the mean of at least three independent experiments, and the error bars show the standard deviation.

earlier work has shown that gC-mediated attachment is independent of the levels of gC in the virus envelope (6).

PRV529, but not PRV537, is attachment proficient on PK15 cells. The ability of each virus to attach to PK15 cells was determined as previously described (6). Briefly, about 150 PFU of each virus was incubated at 4°C for 2 h on PK15 cells prior to the removal of each inoculum. Monolayers were then either overlaid with methylcellulose-containing medium or washed twice vigorously with ice-cold PBS before the addition of methylcellulose medium. Attachment proficiency was expressed as the percentage of virus that was resistant to the wash treatment; the results are shown in Fig. 4A. Consistent with previous findings (6), more than 90% of the input wild-type virus was wash resistant whereas less than 20% of PRV523 virions remained attached to cells after the wash treatment. The replacement of the first one-third of gC with the apoB-100 HBD promoted a substantial but intermediate attachment proficiency for PRV529 of more than 60%. This was contrasted by the failure to restore attachment to strain PRV537 through the introduction of 24 similar amino acids that did not encode a consensus HBD. The 24% attachment proficiency of PRV537 was not significantly different from that of PRV523.

Strains lacking gC are generally defective for penetration as well as attachment (6, 16). Therefore, the penetration efficiency of each virus was measured after a 1-h incubation period

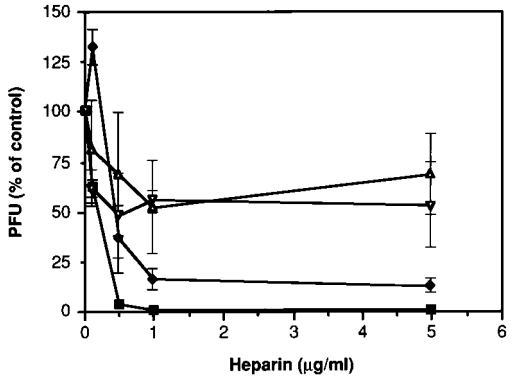

FIG. 5. Soluble heparin competition with wild-type and mutant strains. Monolayers were infected with about 150 PFU of virus in Dulbecco's modified Eagle's medium containing 0, 0.01, 0.1, 0.5, 1.0, or 5.0 μg of heparin per ml. After 2 h at 4°C, inocula were removed, and then monolayers were washed twice with PBS and overlaid with methylcellulose medium to allow plaque formation. For details, see Flynn et al. (6). The *y* axis is the average number of plaques per plate expressed as a percentage of the 0-μg/ml heparin result obtained for each mutant. The *x* axis is the concentration of heparin in micrograms per milliliter. Symbols: ■, PRV-Be; △, PRV523; ◆, PRV529; ▽, PRV537. Each point on the graph represents the mean of values generated from at least two independent experiments, and the error bars are the standard deviations. Where no error bar is evident, the standard deviation was less than the extent of the symbol.

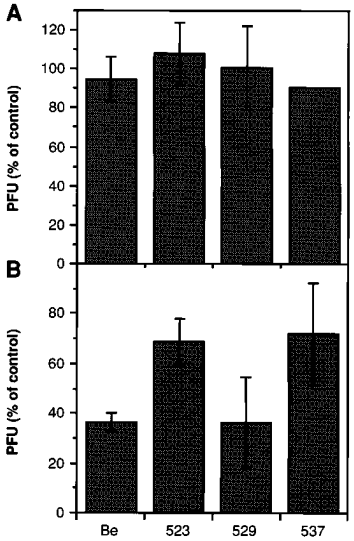

FIG. 6. Competition of wild-type or mutant strains with chondroitin sulfate (A) or dermatan sulfate (B). Viruses were inhibited with 20 μg of either GAG per ml at 4°C as described in Materials and Methods. The *y* axis is the average number of plaques per plate expressed as a percentage of a control value obtained in the absence of competitor. Strain designations are shown along the *x* axis. Each bar represents the mean of two independent experiments, and the error bars show the standard deviations. Two independent chondroitin competition studies with PRV537 gave identical results; therefore, no standard deviation exists for this data set.

on PK15 cells at 37°C by inactivating any surface-exposed virions with a citrate buffer (pH 3) (6). Penetration proficiency was expressed as the percentage of virus that was resistant to citrate treatment when compared with untreated infections. As shown in Fig. 4B (hatched bars), PRV-Be was about fivefold more efficient in penetration than was either attachment-defective strain (PRV523 or PRV537), with half of the input wild-type virions having penetrated PK15 cells after 1 h. Thus, as described for PRV523 (6), PRV537 proved to be both attachment and penetration defective. However, Fig. 4B indicates that the percentage of virions that penetrated was proportional to the percentage that attached for all of the strains assayed. Therefore, as noted by others working with PRV (16), the reduced rate of penetration was probably a consequence of, not independent of, the attachment defect. PRV529 exhibited an intermediate penetration efficiency that was significantly different from that of either PRV523 or PRV537 and which was consistent with its attachment phenotype. The profile obtained for PRV529 was unusual, though, in that the citrate-resistant virions that had penetrated did not increase the apparent overall resistance to PBS wash treatment (Fig. 4B, shaded bars). Because wash resistance at 37°C is a measure of both virus attachment and penetration (i.e., absorption), this value is generally at least as great as that obtained at 4°C (e.g., compare the profiles obtained for PRV-Be, PRV523, and PRV537 in Fig. 4A and B). In fact, the 36% absorption efficiency obtained for PRV529 at 37°C was significantly lower than the 62% observed at 4°C (Fig. 4A). In other experiments not shown, we have assayed the attachment proficiency of PRV529 after a 1-h incubation at 4 or 37°C and found no difference in the PBS wash resistance of this virus after 1 h at each of these temperatures (5). Therefore, we cannot attribute the results in Fig. 4B to a temperature-dependent attachment phenotype for PRV529.

Soluble heparin and heparinlike molecules affect the attachment of PRV-Be and PRV529 similarly. To demonstrate that the restored attachment phenotype of PRV529 was due to an interaction with heparinlike molecules on cell surfaces, wild-type and mutant strains were subjected to attachment assays in the presence of increasing amounts of exogenous heparin. As shown in Fig. 5, soluble heparin acted as an effective competitor for binding by PRV-Be and PRV529. Productive attachment of PRV-Be to PK15 cells was reduced more than 95% in the presence of 0.5 μg of heparin per ml, while PRV529 attachment was inhibited more than 60% by the same level of heparin. Moreover, PRV529 attachment could be reduced by 88% in the presence of 5 μg of heparin per ml, the highest concentration used. The residual binding activity of PRV523 and PRV537 was relatively unaffected by heparin competition, with these strains exhibiting better than 50% of their maximal attachment proficiency even at the highest concentration of heparin. The efficiencies observed for PRV523 and PRV537 compared favorably with those previously described for a PRV *gC* null mutant, and they probably reflect some level of nonspecific inhibition by soluble heparin on the gC-independent attachment process (6).

If the apoB-100 HBD in PRV529 gC was functionally mimicking the first one-third of wild-type gC, PRV529 should behave similarly to PRV-Be when challenged with glycosaminoglycans (GAGs) other than heparin. Accordingly, wild-type or mutant viruses were challenged with either chondroitin sulfate or dermatan sulfate, two GAGs closely related to heparan sulfate (13). In the assay (see Materials and Methods), a single concentration (20 μg/ml) of soluble GAG was chosen that represented 20-fold the amount of soluble heparin necessary for virtually complete inhibition of wild-type binding. None of the strains was inhibited for functional attachment to PK15 cells by chondroitin sulfate (Fig. 6A). Dermatan sulfate, which is more highly sulfated than chondroitin sulfate (13), did compete somewhat with the two attachment proficient strains: the number of PRV-Be and PRV529 plaques was reduced by 64% (Fig. 6B). As with all other GAGs tested, dermatan sulfate was relatively ineffective in inhibiting the residual attachment of PRV523 or PRV537.

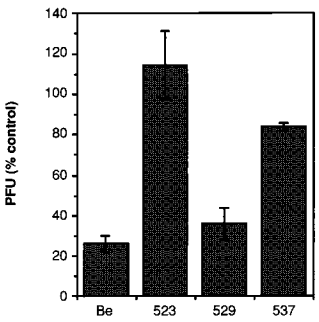

FIG. 7. Attachment of wild-type and mutant viruses to heparinase-treated cells. PK15 cells were incubated with heparinase for 1 h at 37°C and then washed three times with PBS. Approximately 100 PFU of virus was then added to each monolayer at 4°C. After 2 h, monolayers were washed twice with PBS and overlaid with methylcellulose medium to allow plaque formation. See Flynn et al. (6) for details. The *y* axis is the average number of plaques per plate expressed as a percentage of control values obtained on untreated monolayers. Strain designations are given along the *x* axis. Each bar represents the mean of at least two independent experiments, and the error bars show the standard deviations.

TABLE 1. Alignment of gC HBDs implicated in attachment

Location[a]	Sequence[b]	Reference(s)[c]	
		Mapping studies	Peptide binding/ competition
PRV 75–82	------SRRKPPRN---	6	
PRV 95–101	----AHGRKRI------	6	
PRV 132–142	VRFYRRGRFRS------	6	28
PRV 263–271	--PRRSVRLRW------	14, 34	14
HSV-1 142–152	----IRCRFRNSTRM--	30	30
HSV-1 246–254	-----WGRMDSPHE---	30	30
BHV-1 130–139	SKAPPKERKW-------	14	14
BHV-1 176–186	---QRVGRFRSTRG---	14	14
BHV-1 256–262	----TKSQRKV------	14, 19	14
BHV-1 298–308	---PRSTRLHWFRN---	14, 19	14
apoB-100 HBD III	------TRLTRKRGLKL		

[a] Numbers are amino acid positions of the sequence range that is presented.
[b] Boldface letters indicate basic amino acids.
[c] Citations are listed for each category.

PRV529 specifically attaches via heparan sulfate to PK15 cells. The enzymatic removal of heparan sulfate moieties from cell surfaces has demonstrated the dependence of gC-mediated attachment on these molecules (6, 17). As a final phenotypic assay of PRV529, wild-type or mutant strains were used to infect PK15 cells that had been pretreated with heparinase as previously described (6). The resulting infectivity was compared with that obtained with untreated cells to measure attachment proficiency; the results are depicted in Fig. 7.

Both PRV-Be and PRV529 were greatly diminished in their attachment ability on heparinase-treated cells, with wild-type virus appearing more sensitive than the mutant strain to the loss of cell surface heparan sulfate. This was consistent with our findings for the relative sensitivity of these two strains to competition by exogenous heparin. As expected, the residual binding activity of PRV523 and PRV537 was largely unaffected by the pretreatment of cells with heparinase.

DISCUSSION

Mapping studies have identified the first two-thirds of PRV gC, the first half of herpes simplex virus type 1 gC, and the middle two quarters of bovine herpesvirus 1 (BHV-1) gC as containing viral attachment domains, but the precise sequences that function as a receptor domain in these homologs have not been identified (6, 14, 21, 34). Clusters of positively charged amino acids, some conforming to HBD consensus motifs (1), are present in these regions of the various gC homologs, and it has recently been shown that the deletion or alteration of some of these clusters leads to an attachment defect or the loss of heparin-binding activity (5, 19, 30). In addition, peptides derived from positively charged regions of at least three gC homologs can bind to heparin and in some cases can competitively inhibit virus binding (14, 28, 30).

To directly implicate clustered basic amino acids in PRV attachment, we effectively replaced the first one-third of gC carboxyl to the signal peptide with 24 amino acids containing HBD III of apoB-100 (11). This sequence promoted virus binding to the surface of PK15 cells in a heparan sulfate-dependent manner. However, the attachment phenotype of PRV529, the strain bearing the heterologous HBD, was intermediate. The intermediate sensitivity of PRV529 virions for attachment to heparinase-treated cells, in particular, suggests that some fraction of the virus population continued to rely on a gC-independent mode of entry.

Significantly, we found that the replacement of the first one-third of mature gC with 24 similar amino acids that did not encode a consensus HBD failed to promote virus attachment. The attachment-defective phenotype of this strain, PRV537, was important because this virus and its parent, PRV523, both retain four potential HBDs in the final two-thirds of gC (6, 22). Thus, PRV537 demonstrated that simply inserting a peptide with the predicted secondary structure of the apoB-100 HBD does not activate any existing HBDs that might be lying dormant in the context of deletion of the first one-third of gC. The potential for more than one functional HBD in wild-type PRV gC has hampered the identification of discrete amino acid sequences involved in PRV gC-mediated attachment (6, 14, 34). The same concern of multiple, perhaps redundant, HBDs cannot be overlooked in this study, either. We cannot unequivocally state that the attachment of PRV529 virions is mediated exclusively by the apoB-100 HBD. Instead, the heterologous HBD may be contributing a sufficient number of positively charged amino acids to lead to functional virus binding to cells when the amino acids are present in combination with HBDs native to the carboxy two-thirds of gC. In fact, we have found that PRV529 is not attachment proficient on Madin-Darby bovine kidney cells (5), suggesting that the apoB-100 HBD cannot promote functional binding to the heparan sulfate of these cells.

An alignment of sequences implicated in gC-mediated attachment is presented in Table 1; we have also included the apoB-100 HBD III sequence to better relate it to the wild-type situation. We have maximized the alignment of the central core of basic amino acids in each potential HBD and, as a result, can generate the following consensus sequence: X-b-b-X-B-b-B-X-X-b-X, where B is almost always a basic amino acid, b is often a basic amino acid, and X is usually an uncharged, often hydrophobic residue. This sequence is quite large compared with those previously proposed as HBDs, and it may represent two divergent consensus sequences with a common core element. Whether orientation plays a role in HBD function has not been established. We offer this consensus only as a working model and even so with extreme caution for the following reasons: (i) certain of these sequences have been more strongly implicated than others, and thus some of the sequences may not belong in this grouping; (ii) we do not yet understand the context in which the sequences func-

tion—in fact, evidence suggests that the two sequences derived from HSV-1 gC may work in concert and, at least, probably lie close to each other in the folded protein (30); and (iii) as noted previously, different portions of the gC homologs may mediate virus attachment to different cells and their sulfation patterns on heparan (34).

Why did the apoB-100 HBD only partially restore attachment on PK15 cells? The intermediate phenotype of PRV529 may have been due to one or more of the following: (i) the apoB-100 HBD could exhibit a generally lower affinity for heparan sulfate than the wild-type attachment domain; (ii) functional PRV attachment may require that gC bind a specific heparan sulfate moiety on PK15 cells that is not efficiently bound by the apoB-100 HBD; (iii) the replacement of multiple HBDs with a single HBD of equal affinity could lessen overall binding; or (iv) the presentation of the HBD by such a grossly altered gC (deleted for the first one-third) might not provide the proper context for full binding activity. Nonetheless, while acknowledging the intermediate phenotype of PRV529 and the possible contribution of other HBDs residing in gC, this work represents the first direct demonstration that an HBD can promote functional herpesvirus attachment to cells. Moreover, we would suggest that the 24 amino acids derived from apoB-100 represent the smallest, albeit artificial, attachment domain reported for a herpesvirus to date.

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