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The Receptor-Binding Domain of Pseudorabies Virus Glycoprotein gC Is Composed of Multiple Discrete Units That Are Functionally Redundant

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Many herpesviruses attach to cells in a two-step process, using the glycoprotein gC family of homologs to bind the primary receptor, heparan sulfate (HS) proteoglycan, and glycoprotein gD homologs to bind an unknown secondary receptor. We have previously shown by deletion analysis that the amino-terminal one-third of gC from pseudorabies virus (PRV), a swine herpesvirus, includes at least the principal HS receptor-binding domain. This portion of PRV gC contains three discrete clusters of basic residues that exactly or nearly match proposed consensus sequences for heparin-binding domains (HBDs); four additional potential HBDs lie in the distal two-thirds of the glycoprotein. We now specifically implicate each of the three amino-terminal HBDs in virus attachment. Mutational analysis demonstrated that any one of the three HBDs could mediate efficient virus infectivity; HS-dependent PRV attachment to cells was eliminated only after all three amino-terminal HBDs were altered. Furthermore, the binding dysfunction was due to a disruption of the specific HBDs and not to total charge loss. Thus, unlike previously described viral receptor-binding domains, the PRV gC receptor-binding domain is composed of multiple, discrete units that can function independently of one another. These units may function redundantly either to increase binding affinity or perhaps to effectively increase the virus's host range.

Pseudorabies virus (PRV) is a veterinary alphaherpesvirus that causes Aujeszky's disease in swine. Similar to the prototypical herpes simplex virus type 1 (HSV-1), PRV enters the cell through a multistep process that involves at least five different virus-encoded glycoproteins, all of which have homologous counterparts in most other alphaherpesviruses studied to date (40, 66). Initial virus binding to the cell is mediated by glycoprotein gC, which specifically interacts with heparan sulfate (HS) but not other glycosaminoglycans (43). Interestingly, this primary attachment event is not an absolute requirement for infection: *gC* null mutants are viable, although they produce lysates with lower titers (55, 73). In addition, PRV *gC* null mutants are easily removed from cell surfaces with phosphate-buffered saline (PBS) washes under conditions in which the wild-type virus is largely unaffected, and null mutants generally exhibit a significant delay in entering almost all cell types assayed (12, 39). This is consistent with the hypothesized role of the gC-HS interaction, which is effectively to reduce the three-dimensional search for a cellular receptor to two dimensions (20, 66). In the proposed two-step binding model, the gC-HS interaction facilitates the movement of the virus particle to a secondary receptor which is bound via glycoprotein gD, an event that is essential for virus infection (28, 33, 38, 50, 52). However, gD-mediated attachment is not well understood, and to date, only for HSV-1 has a potential secondary receptor bound by gD been tentatively identified: the mannose 6-phosphate receptor (3). Following the dual binding events, the virus envelope and plasma membrane eventually fuse in a pH-independent step that requires at least glycoproteins gB, gH, and gL (66).

So far, seven different herpesviruses have been shown to use HS as a primary receptor (8, 43, 46, 71, 76, 79). As a participant in the initial virus-cell interaction, HS represents a very heterogeneous substrate for binding. The heterogeneity is in part the result of incomplete sulfation of the polysaccharide backbone, which is composed of repeating hexuronic acid and D-glucosamine disaccharide units (34). Thus, although some limits on variation exist, HS offers the infecting virus a vast array of negative charge distribution for electrostatic binding. The specificity of binding appears to depend upon the arrangement of basic amino acids found in the cognate HS-binding protein. By analyzing proteins that bind heparin, a glycosaminoglycan closely related to HS, Cardin and Weintraub (4) have proposed two consensus motifs for heparin-binding domains (HBDs) of polypeptides. Each is composed of a core of basic amino acids flanked by an additional positively charged residue that is separated by hydrophobic amino acids: either XBBXBX or XBBBXXBX, where B is a basic amino acid and X is a hydrophobic residue. Heparin-binding proteins, which often contain more than one of these sequences, have been implicated in a number of processes, including target cell adherence by a number of pathogens, cell-to-cell adhesion, growth stimulation, and thrombosis (1, 4, 14, 19, 30, 35, 44, 49, 77, 78).

PRV gC (formerly gIII) is predicted to be a 479-amino-acid glycoprotein containing eight sites for N-linked glycosylation (54). Glycoprotein gC is found in the virus envelope and the infected-cell plasma membrane, where it is oriented with the amino terminus residing extracellularly and is anchored by a single membrane-spanning domain near its carboxy terminus (59, 65). There is no evidence that PRV gC forms a multimeric complex with itself or any other viral component. We previously mapped the HS receptor-binding domain to the amino-

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terminal one-third of gC, and we noted that three exact or near matches to consensus HBD sequences lie in this portion of the glycoprotein; four other potential HBDs exist in the distal two-thirds of gC (12). Others, using peptides derived from the wild-type sequence, have shown that at least two of the amino-terminal HBDs can indeed bind heparin (32, 62). Our goal has been to further resolve the HS receptor-binding domain harbored by PRV gC and, in particular, to address the roles of the three potential HBDs that reside in the amino-terminal one-third of the glycoprotein. Once we implicated the HBDs in virus attachment, we hoped to determine whether each HBD mediated binding through its specific arrangement of basic residues or simply by its total charge contribution. The results of our mutational analysis have revealed a distinctive feature of a virus-cell receptor interaction: PRV gC contains multiple HBDs that do not necessarily contribute to a single receptor-binding domain but which instead may function redundantly to mediate virus attachment to cell surface HS.

MATERIALS AND METHODS

Cells, virus, and DNA. PK15 (porcine kidney) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (P/S). Our wild-type strain is PRV-Becker (PRV-Be), and it and PRV2, PRV505, PRV509, and PRV523 have been previously described (12, 59). All strains were propagated on PK15 cells in DMEM supplemented with 2% fetal bovine serum and 1% penicillin-streptomycin (DMEM/2%). When plaque formation was required, the DMEM/2% also contained 1% methylcellulose. Restriction enzyme digestions and ligations of plasmid DNA were performed by standard recombinant DNA techniques (36). Plasmids were maintained in *Escherichia coli* KK2186 (12).

Construction and identification of PRV gC mutants. Site-specific mutagenesis was performed as previously described (69) by using a modification of the Altered Sites system of Promega Corp. The template for all of the reactions was plasmid pAXXHB, a derivative of pALTER-1 that possesses an approximately 900-bp *Xho*I-*Bse*AI restriction fragment insert. The *Xho*I-*Bse*AI fragment contains about 200 bp of PRV DNA lying upstream of *gC* and the contiguous 5′ portion of the *gC* coding sequence through codon 226; the *Xho*I site, which lay upstream of *gC*, was destroyed during the cloning procedure. The cloned *gC* sequence was obtained from the wild-type allele but contained two additional restriction sites, unique to pAXXHB, whose introduction altered codons but not the encoded amino acids. Specifically, a *Xho*I restriction site was introduced at *gC* codons 74 to 76, and a *Hin*dIII site was engineered at codons 116 to 118. These sites were flanked by naturally occurring unique sites recognized by restriction enzymes *Sal*I at codons 23 to 25 and *Sac*I at codons 157 to 158. Thus, it was possible to conveniently subclone a restriction fragment, e.g., a *Hin*dIII-*Sac*I fragment containing mutations in the coding sequence of HBD 3, into a nonmutagenized preparation of pAXXHB or into another HBD mutant derivative of pAXXHB, the latter to generate *gC* alleles with alterations in more than one HBD-coding sequence. Subcloning occurred only after each restriction fragment was completely sequenced to confirm that only the intended alteration was produced by the site-specific mutagenesis protocol. Once a desired *gC* allele was confirmed, the remainder of the wild-type *gC* gene was inserted distal to the *Bse*AI site to provide sufficient homology to introduce the mutant allele into the virus genome. Each resulting plasmid was cotransfected with PRV509 genomic DNA into PK15 cells to allow recombination (17). Recombinant viruses were identified by using gC-specific antiserum 282 in an immunohistochemical plaque assay, the black plaque assay (25, 59). In this assay, PRV509 plaques did not stain, but recombinant virus plaques stained black because they contained gC on their infected-cell surfaces. Several black plaques were picked from each cotransfection lysate and plaque purified. Lysates of each isolate were then produced by standard methods, and a Southern analysis was performed with viral DNA as previously described (12) to confirm that each strain resulted from a correct homologous recombination event.

The nomenclature for the alleles contained by the mutant viruses was based on the location and nature of the alteration. For example, a deletion of codons 75 to 82 was designated *gC Δ75-82*, and a replacement of an arginine with a glycine at position 77 was designated *gC R77G*.

Virus assays. Except noted otherwise, attachment assays, heparin competition assays, and attachment assays with heparinase-treated cells were all performed exactly as described by Flynn et al. (12). All assays were performed at least twice for each strain, and standard deviations were calculated. To determine if observed differences between strains were significantly different, analysis of variance and Student's *t* tests were performed (58).

Neutralization assays. Neutralization assays with antisera 282 (gC specific) and 284 (gB specific) were performed exactly as described by Powers and Ryan (51). Briefly, virus lysates were diluted in DMEM/2% to approximately 100 PFU per ml. Heat-inactivated 282 or heat-inactivated 284 antiserum was then added at 25 μl/ml, and the aliquots were incubated at 37°C for 1 h, after which cell monolayers were overlaid with the aliquots and incubated at 37°C. After 1 h, the overlays were removed and replaced with DMEM/2% containing 1% methylcellulose. After 2 days, plaques were counted, and the number of plaques arising from antiserum-treated aliquots was compared with the number derived from inocula that had not been preincubated with antisera. The experiment was independently performed three times; the results of a single, representative experiment are presented.

TABLE 1. PRV gC HBD mutants[a]

Strain	HBD 1	HBD 2	HBD 3
	75	102	133 143
PRV-Be	...**SRRKPPRN**NNRTRVHGDKAT**AHGRKRI**V...RF**YRRGRF**RSP...		
PRV565	Δ75-82		
PRV566		Δ95-102	
PRV567			Δ133-143
PRV568	Δ75-82	Δ95-102	
PRV569	Δ75-82		Δ133-143
PRV570		Δ95-102	Δ133-143
PRV571	Δ75-82	Δ95-102	Δ133-143
PRV580	R77G,K78T	R98A,K99N	
PRV581	R77G,K78T		R136A,R137G
PRV582		R98A,K99N	R136A,R137G
PRV583	R77G,K78T	R98A,K99N	R136A,R137G
PRV584	R76S,R77G,K78T,R81G		R136A,R137G
PRV585	R77G,K78T,R85A,R87N		R136A,R137G

[a] The HBDs of PRV-Be are in boldface. Deletions and substitutions in the mutant strains are indicated.

Radiolabeling and precipitation of viral glycoproteins with antisera and heparin agarose. Viral glycoproteins were radiolabeled with [^{3}H]glucosamine and immunoprecipitated from purified virus particles with goat antisera recognizing gC or gB as previously described (59). Precipitation of radiolabeled glycoproteins with heparin agarose was performed by first lysing purified virus particles in TNX (10 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100) and clarifying each sample by centrifugation at 14,000 × *g* for 10 min. Half of the resulting supernatant was incubated at 4°C with 20 μl of heparin agarose beads (binding capacity, 0.5 to 1.2 mg of heparin per ml of beads; Gibco-BRL) that had been previously washed twice with TE (10 mM Tris, 1 mM EDTA [pH 8.0]). After 1 h, the glycoprotein-heparin agarose complexes were washed seven times with a Tris-saline solution (150 mM NaCl, 50 mM Tris [pH 7.4]) (61) through a series of centrifugations and resuspensions of the beads. The final heparin agarose pellet was resuspended in standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled to release the precipitated glycoproteins. Heparin agarose precipitation of intact virus particles was performed identically except that the TNX lysis step was omitted. All precipitates were then resolved by SDS-PAGE and visualized by fluorography and autoradiography (59).

RESULTS

Multiple HBDs must be deleted to disrupt PRV's efficient attachment to cells and its sensitivity to a soluble heparin competitor. The three potential HBDs that reside in the amino-terminal one-third of PRV gC lie at amino acid positions 75 to 82 (HBD 1), 95 to 101 (HBD 2), and 135-140 (HBD 3); these are indicated in boldface in Table 1. HBD 3 is an exact match with one of the proposed consensus sequences for HBDs, while HBD 1 contains the proper basic amino acid arrangement but lacks significantly hydrophobic spacer residues. HBD 2 contains both basic and hydrophobic amino acids,

but it conforms to an HBD motif only if the amino-to-carboxy-terminal orientation is unimportant. To determine whether any or all of these sequences contributed to the receptor-binding domain previously mapped to this portion of gC (12), deletions, each encompassing a single HBD-coding sequence, were introduced individually and in all possible combinations into the PRV genome (see Materials and Methods). Once a plaque-purified lysate was obtained for each strain, virions were purified and the encoded gC species were immunoprecipitated and resolved by SDS-PAGE to confirm that the mutant glycoprotein was correctly localized to the virus envelope (data not shown).

The precise residues removed in each viral strain are indicated in Table 1. Functional attachment by these strains was determined by calculating the percentage of total input PFU that was not removed from monolayers by vigorous washing with PBS after an initial 2-h adsorption period at 4°C (12). As shown in Fig. 1A, the loss of any single HBD (strain PRV565, PRV566, or PRV567) did not decrease the attachment efficiency of the mutant strains to below wild-type (PRV-Be) levels. Essentially wild-type attachment proficiency was also observed for PRV568, a strain whose gC had both HBD 1 and HBD 2 deleted. In contrast, strains with HBD 3 and either HBD 1 or HBD 2 deleted (strains PRV569 and PRV570, respectively) exhibited a pronounced attachment defect comparable to that observed for PRV509, a *gC* null mutant strain with codons 2 to 458 deleted (12). Not surprisingly, then, strain PRV571, whose gC had all three HBDs deleted, was also found to be attachment defective, with greater than 80% of its input virions removed by the PBS wash. Importantly, no statistically significant differences in attachment deficiency could be discerned among strains PRV509, -569, -570, and -571.

The binding of wild-type PRV to cell surface HS can be inhibited by using soluble heparin as a competitor (12, 43, 81). However, the results depicted in Fig. 1A suggested that certain of the HBD deletion mutants had lost the ability to interact with heparinlike substrates. We reasoned, therefore, that the residual infectivity of these mutants should be relatively immune to heparin competition, a phenotype that has been previously described for other attachment-defective strains of PRV (12, 43). Accordingly, wild-type and mutant viruses were used in attachment assays in the presence of increasing concentrations of exogenous heparin; the results are shown in Fig. 1B. As expected, the wild type and the attachment-proficient HBD deletion mutants were fully inhibited by 1 μg of heparin per ml. By comparison, the residual infectivity of the *gC* null mutant PRV509 was incompletely diminished. In the presence of the highest concentration of heparin, 5 μg/ml, 70% of the wash-resistant PFU of PRV509 were removed, but this was still 30-fold-more-efficient infectivity than that observed for the wild type at the same concentration of heparin. Two of the attachment-defective HBD deletion mutants, PRV569 and PRV571, were just as, if not more, resistant to heparin competition as the *gC* null mutant, a result consistent with their inability to effectively interact with heparinlike substances. The remaining attachment-defective mutant, PRV570, revealed a somewhat curious heparin competition profile. This strain's residual infectivity appeared to be slightly stimulated by very low concentrations of exogenous heparin, but overall, PRV570 exhibited an intermediate sensitivity. This perhaps indicated that its encoded gC, which contained HBD 1 but not HBD 2 or HBD 3, retained some ability to interact with heparinlike moieties.

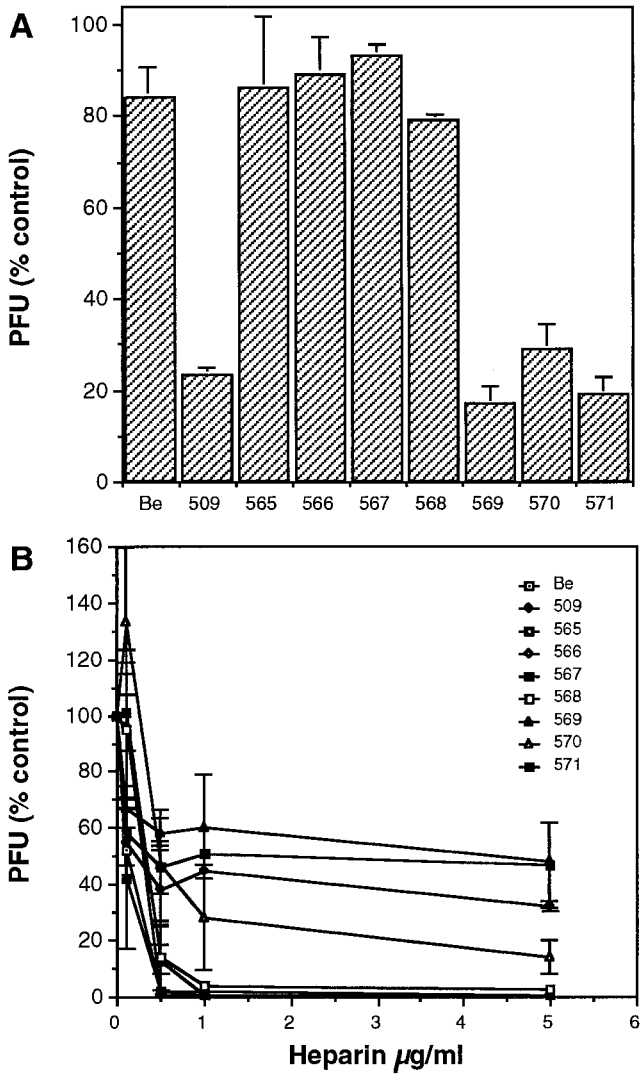

FIG. 1. Attachment and heparin competition profiles of HBD deletion strains. (A) Relative attachment efficiencies. For each strain, each of six monolayers was inoculated with approximately 150 PFU in DMEM/2% and incubated for 2 h at 4°C. After removal of the inocula, half of the monolayers were left untreated and the other half were washed twice with ice-cold PBS. They were then overlaid with methylcellulose-containing medium and incubated for 48 h at 37°C to allow plaque formation. The *y* axis shows the average number of wash-resistant PFU per plate, expressed as a percentage of values for untreated controls. 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 deviations. (B) Sensitivities to soluble-heparin competition. Triplicate monolayers were each inoculated with approximately 150 wash-resistant PFU of a particular strain and incubated for 2 h at 4°C in DMEM/2% containing 0, 0.01, 0.1, 0.5, 1.0, or 5.0 μg of heparin (Sigma Chemical Co.) per ml. After removal of the inocula, the monolayers were washed twice with PBS. They were then overlaid with methocellulose-containing medium and incubated for 48 h at 37°C to allow plaque formation. The *y* axis shows the average number of plaques per plate, expressed as a percentage of the average number of plaques per plate obtained for each strain in the absence of competitor. The *x* axis indicates the concentration of heparin. Each datum point represents the mean of at least two independent experiments, and the error bars show the standard deviation at each concentration. Where no error bar is evident, the standard deviation was less than the extent of the symbol.

Substitutions of basic residues with neutral amino acids eliminate receptor-binding activity only if they occur in all three HBDs of the amino-terminal third of gC. Our results obtained with HBD deletion mutants indicated that more than one HBD contributed to the receptor-binding domain of gC. Specifically, the region in or near HBD 3 appeared to act independently of, or redundantly to, the portion containing

TABLE 2. Reactivities of gC mutants to antibodies

Strain[a]	Reactivity with antibody:			
	282	M1	M7	M16
PRV-Be	+	+	+	+
PRV509	−	−	−	−
PRV523	+	−	−	−
PRV2	+	−	−	−
PRV505	+	+	−	+
PRV565	+	+	+	+
PRV566	+	−	+	+
PRV567	+	−	−	−
PRV568	+	−	+	+
PRV569	+	−	−	−
PRV570	+	−	−	−
PRV571	+	−	−	−
PRV580	+	+	+	+
PRV581	+	+	+	+
PRV582	+	+	+	+
PRV583	+	+	+	+
PRV584	+	+	+	+
PRV585	+	+	+	+

[a] Genotypes are as indicated in Table 1 except as follows: PRV509 (*gC Δ2-458*), PRV523 (*gC Δ25-157*), PRV2 (*gC Δ158-291*), and PRV505 (*gC Δ265-411*).

HBD 1 and HBD 2. Less clear was the relationship between HBD 1 and HBD 2. These two sequences are separated by only 12 amino acids, several of which are basic residues (Table 1). Our finding that the deletion of HBD 1 or HBD 2, in combination with the deletion of HBD 3, resulted in an attachment defect suggested that the first two HBDs were contributing to a single receptor-binding component that could be disrupted by the loss of either HBD. Thus, our designation of the highly basic region between positions 75 and 102 as two domains because of the identification of consensus sequence motifs may have been premature. Conversely, the rather sizable deletion that removed either of the first two HBDs may have adversely affected the conformation and function of the remaining member of the pair. Thus, it was still plausible that each of the amino-terminal three HBDs functioned independently.

Knowledge of the interrelationship of the three HBDs was further refined by introducing amino acid substitutions into each one (Table 1). In each case, two basic residues within the core element of the consensus sequence were replaced with two neutral amino acids whose introduction was not predicted to alter the wild-type secondary structure of the region (5). Such substitutions were likely to disrupt any heparin-binding ability of an HBD, as judged by our previous results obtained for other PRV *gC* mutants (13). Because our deletion analysis indicated that the loss of multiple HBDs was required in order to impair virus attachment, only strains harboring amino acid substitutions in more than one HBD were constructed (Table 1). Once again, the correct localization of the mutant gC species to the virus envelope was established by performing immunoprecipitations from purified virions (data not shown).

Concerns that our alterations were affecting the conformation of gC were addressed by assaying for the presence of epitopes that were recognized by a panel of monoclonal antibodies. Table 2 lists the reactivities, as determined by a histochemical plaque assay (25), of *gC* mutants to three monoclonal antibodies, M1, M7, and M16 (18), and to a polyclonal goat antiserum, 282 (12). PRV509 did not react with any of the antibodies, confirming the specificity of the assay for gC. The epitopes recognized by the three monoclonal antibodies are not well defined, but it has been demonstrated that generation of the M1 epitope is dependent in part upon gC's acquisition of complex-modified N-linked glycosylation or some other Golgi-derived addition (18, 59). Moreover, all three of the monoclonal antibodies fail to recognize denatured gC in immunoprecipitations, suggesting that none of the antibodies recognizes a linear epitope (11). Additional characterization of the epitopes is provided in Table 2, for which three strains, PRV523, -2, and -505 (12, 59), whose gC species contain deletions of approximately the amino-terminal third, middle third, and carboxy-terminal third of the ectodomain, respectively, have been included in the plaque assay. Deletion of the first or middle third of gC destroyed all three epitopes, while removal of the final third of gC resulted in the loss of the M7 epitope only. When the HBD deletion mutants were assayed, only the gC species with only HBD 1 deleted (encoded by PRV565) retained all three epitopes. This observation supported the possibility that unintended conformational changes in gC could have contributed to some of the attachment defects that were detected. However, the same result also distinguished HBD 1 from HBD 2, in that only deletion of the latter domain affected the integrity of the M1 epitope, suggesting that deletion of either HBD 1 or HBD 2 did not elicit strictly interchangeable consequences for gC. Moreover, there was no correlation between the loss of a specific epitope and the loss of attachment efficiency: gC encoded by PRV567 had lost all three epitopes, yet the strain remained fully attachment proficient. Nonetheless, it is important to note that none of the gC species harboring amino acid substitutions had lost any of the epitopes. While this did not rule out the existence of minor conformational changes, the retention of all three epitopes by these gC mutants reduced the likelihood of significant alterations in folding.

The attachment and heparin competition assays were repeated with the HBD amino acid substitution mutants. Strains with alterations in any two of the first three HBDs (PRV580, -581, or -582) attached with wild-type proficiency (Fig. 2A). Only PRV583, a strain with substitutions in all three HBDs, proved to be attachment defective. The same general trend held true in heparin competition studies (Fig. 2B), with all of the attachment-proficient mutants exhibiting at least a 98% reduction in plaque numbers in the presence of 5 μg of heparin per ml. However, it was found that any strain whose gC contained substitutions in HBD 3 was less sensitive to competition with 1 μg of heparin per ml than the wild type. As noted for two of the HBD deletion strains, the attachment-defective strain PRV583 appeared to be slightly more resistant to soluble heparin than the *gC* null strain. Collectively, the results indicated that each of the three amino-terminal HBDs could independently mediate wild-type levels of attachment and infectivity on cultured cells.

The amino-terminal HBDs appear to mediate receptor binding through their specific charge arrangements and not by total charge contribution. HBD 1 and HBD 2 seemed to be functionally resolved by our analysis of the HBD amino acid substitution mutants: substitutions in both, and not just in one or the other, were required in conjunction with a dysfunctional HBD 3 to impair virus attachment. However, this reasoning was valid only if HBD 1 and HBD 2 interacted with heparinlike moieties exclusively through their specific cationic charge arrangements that adhere to the consensus motif. In fact, our data were also consistent with a model in which the region between amino acids 75 and 102 could still have been considered a single component of the gC receptor-binding domain, lending activity through its total charge contribution. Consider, for example, that all of the alterations in this region which led to an attachment defect, in the presence of a faulty HBD 3,

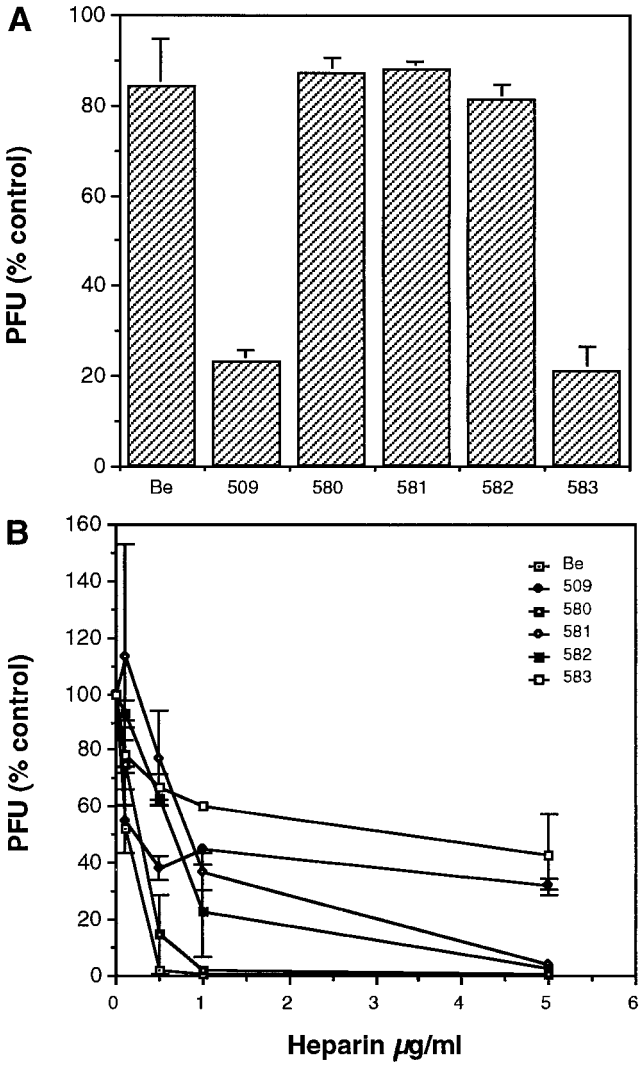

FIG. 2. Attachment and heparin competition profiles of HBD amino acid substitution strains. For details, see the legend to Fig. 1.

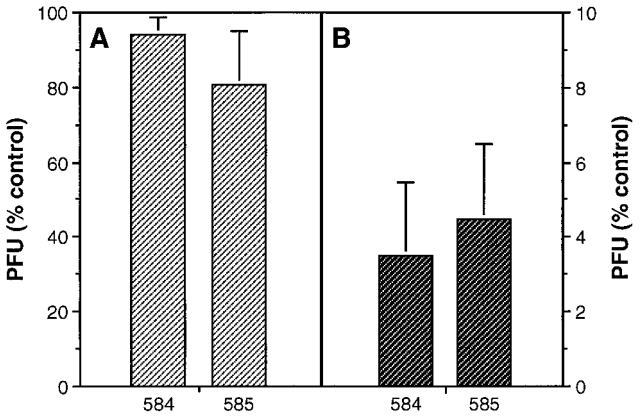

FIG. 3. Attachment and heparin competition profiles of PRV584 and PRV585. (A) Relative attachment efficiencies. For details, see the legend to Fig. 1A. (B) Sensitivities to 5 μg of a soluble heparin competitor per ml. The *y* axis shows the average number of plaques per plate obtained from challenged infections, expressed as a percentage of the average number of plaques per plate obtained from infections in the absence of a heparin competitor. Strain designations are shown along the *x* axis. Each bar represents the mean of at least two independent experiments, and the error bars show the standard deviations. Note the reduction in the scale of the *y* axis compared with that in panel A.

removed four basic residues through either deletion or substitution; those that failed to impair attachment removed only two positively charged amino acids. Thus, a threshold positive charge density, and not the specific arrangement of the basic residues in a consensus motif, might have been the operative feature of this region. To distinguish between these two possibilities, two final mutants were constructed such that their encoded gC species would lack four basic residues in the region between amino acids 75 and 102 but would retain one intact HBD motif (Table 1). Both mutants were derivatives of PRV581, whose gC already harbored double amino acid substitutions in HBD 1 and HBD 3 but still mediated proficient attachment. One derivative, PRV584, encoded a gC species that contained two additional amino acid substitutions in HBD 1 so that all four of the original basic residues had been replaced with neutral amino acids. The other, PRV585, coded for a mutant gC in which two arginines residing between HBD 1 and HBD 2 were both replaced with neutral amino acids. Note that in each mutant, HBD 2 remained intact. As before, the alterations were designed to maintain the predicted secondary structure of the region, and both mutants proved to be reactive to all three of the monoclonal antibodies used to monitor conformational changes in gC (Table 2).

When PRV584 and PRV585 were assayed for their attachment proficiencies, both strains mimicked the wild type, with greater than 80% of the input PFU remaining after the PBS wash (Fig. 3A). These strains were also subjected to heparin competition studies, but the format was altered to measure only their sensitivities to the highest concentration of heparin used in the previous experiments, i.e., 5 μg/ml. The infectivities of both strains were reduced by greater than 95% under these conditions (Fig. 3B), a result that again was consistent with an HS-dependent, attachment-proficient phenotype. Thus, a simple reduction in total charge density was not sufficient to eliminate the receptor-binding function within the region between amino acids 75 and 102 of gC. Instead, amino acid substitutions that specifically removed both of the HBD motifs in this region were required.

The residual binding activity of PRV583 is HS independent and does not involve gC. With just six amino acid substitutions, the gC encoded by PRV583 is the least altered form of this PRV glycoprotein reported to cause an attachment defect. Still, as much as 20% of the input PFU of PRV583 acquire PBS wash resistance after 2 h of incubation at 4°C (Fig. 2A). To determine if this residual binding was to HS, we first removed cell surface HS by enzymatic digestion with heparinase and then performed attachment assays with digested and undigested monolayers to compare the effects of the treatment on attachment proficiency. When this experiment has been performed in the past with the wild type and *gC* null mutants, wild-type attachment levels have been greatly diminished, while the residual, PBS wash-resistance binding of the null strain has been relatively unaffected (12, 43). Here, we included the wild type, PRV509, and, in addition to PRV583, mutants containing double amino acid substitutions in two of the three amino-terminal HBDs to see if the retention of different individual HBDs results in different sensitivities to heparinase treatment of cell monolayers. The results are shown in Fig. 4.

The wild type and PRV509 behaved as expected, revealing almost reciprocal responses to heparinase-treated monolayers.

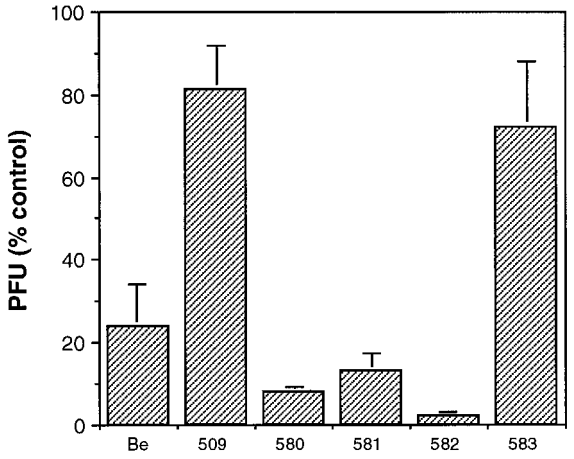

FIG. 4. Attachment profiles of HBD amino acid substitution mutants on heparinase-treated cells. Cells were incubated with 1 U of heparinase (ICN Pharmaceuticals Inc.) per ml for 1 h at 37°C and washed with PBS. Monolayers were chilled and inoculated with approximately 100 PFU of a particular strain. Attachment efficiencies after 2 h at 4°C were then determined in triplicate for each strain. The *y* axis shows the average number of plaques per plate obtained with heparinase-treated monolayers, expressed as a percentage of control values obtained with undigested cells. 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.

The *gC* mutant strains that retained a single intact HBD (PRV580, -581, and -582) had greatly reduced infectivity on heparinase-treated cells, more so, in fact, than the wild type. In addition, trends consistent with potential significant differences among these strains were observed, with PRV582 showing greater than a 95% reduction in plaque numbers on digested monolayers. In contrast, the profile for PRV583 closely resembled that for PRV509, indicating that little, if any, of the residual attachment by virions of this strain was to cell surface HS.

Further evidence that PRV583 gC did not mediate virus attachment to cell surface HS was gained through the use of neutralizing antibodies. Neutralizing antibodies have been used previously to order the steps of herpesvirus entry into target cells: specific antibodies can inhibit the event mediated by a particular glycoprotein (15, 16). We employed polyclonal antiserum 282, which has been shown to bind all of the HBD mutants listed in Table 2, to see whether the residual binding activity of PRV583 could be inhibited by neutralizing antibody; a failure to do so would be indicative of PRV583 gC's exemption from the entry process. The wild type and PRV509 were used as control strains along with PRV580, which was included to demonstrate that an attachment-proficient mutant containing just one intact amino-terminal HBD could still be efficiently neutralized. The results are given in Table 3. The wild type and PRV580 showed an 86% or greater reduction in plaque numbers when incubated with 282 antiserum compared with incubation in the absence of antibodies. Conversely, the residual infectivity of PRV509 was not at all neutralized by the 282 antiserum, confirming the specificity of the assay for gC. Similarly, PRV583 showed less than a 10% reduction in infectivity when incubated with antiserum 282, supporting the position that the encoded gC plays little, if any, role in this strain's entry into cells. A control antiserum, 284 (53), was also included in the study. This antiserum is directed against essential glycoprotein gB (53), which is present in its wild-type form in all of the strains tested. Virtually complete neutralization was observed for all of the strains, suggesting that the gC-independent pathway was not an aberrant one and still relied on the proposed fusogenic properties of gB (42) to enter cells.

TABLE 3. Neutralization of gC mutants by polyclonal antibodies

Strain	% Neutralization with antiserum[a]:	
	282	284
PRV-Be	91	99
PRV509	—[b]	100
PRV580	86	99
PRV583	9	92

[a] Neutralization was calculated by determining the percentage of PFU inhibited by the presence of the antibody.
[b] —, The titer was slightly higher in the presence of the antibody.

PRV583 gC does not bind heparin. The six amino acid substitutions contained by PRV583 gC eliminated its receptor-binding activity in functional assays that ultimately measured virus infectivity. Still, this mutant form of gC retained four potential HBDs in its carboxy-terminal two-thirds, and a peptide containing one of these potential HBDs has been shown to bind heparin (32). Therefore, it was of interest to determine whether PRV583 gC had lost all ability to physically bind to heparin, or specifically, the capability to mediate functional virus attachment. Previous studies have used immobilized heparin to demonstrate the affinity of herpesvirus glycoproteins for heparin (8, 23, 43, 46, 61). We chose to use heparin agarose beads to precipitate radiolabeled glycoproteins from detergent-lysed envelopes of purified PRV particles (see Materials and Methods), using the same strains employed in the neutralization studies. Proteins that bound to the beads were then resolved by SDS-PAGE and visualized by autoradiography. The results are shown in Fig. 5A.

In previous work on PRV, gB and gC were the predominant, perhaps only, glycoproteins that bound heparin (43, 61). Therefore, we have provided immunoprecipitated gC and gB from purified wild-type particles in the first two lanes of Fig. 5A to show the migrations of these glycoproteins. As expected, gC migrated as a diffuse 92-kDa band, while gB was represented by three species of 110, 68, and 56 kDa, with the two smaller forms constituting normal cleavage products of the largest polypeptide (18). For the heparin binding studies with each strain, half of the detergent-lysed virion preparation was used to immune precipitate gC with antiserum 282, and the other half was incubated with heparin agarose beads. A protein comigrating with gC could be readily detected in the heparin agarose precipitations that were obtained from wild-type and PRV580 virus particles. In contrast, no gC species were observed in the heparin agarose precipitations obtained from PRV509 or PRV583, even though a large amount of gC was easily immune precipitated from the lysed virus particles of PRV583. Thus, it would appear that PRV583 gC had lost all affinity for heparin despite retaining four potential HBDs. In all of the strains tested, the heparin agarose precipitated polypeptides that comigrated with gB species, even through Mettenleiter et al. (43) have reported that the heparin-binding ability of wild-type gB is selectively lost in a PRV strain that lacks gC. The heparin agarose profile of several strains also contained two additional bands: (i) a high-molecular-mass species that was probably a complex of gB molecules (61, 74) and (ii) a low-molecular-mass protein that was smaller than any reported PRV glycoprotein and which may have been a degradation product.

Our results from the heparin agarose experiment raised a significant question: if gB can bind to the beads, then why does

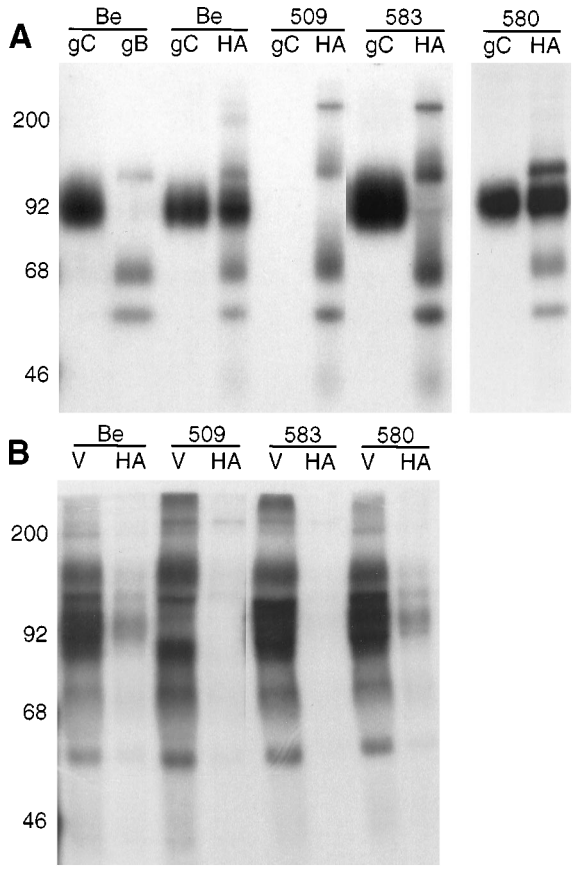

FIG. 5. Binding of PRV glycoproteins and intact virions to heparin agarose. (A) Heparin-bound glycoproteins from lysed viral envelopes. PK15 cells were inoculated at a multiplicity of infection of 10 with the strains indicated at the top. After 1 h at 37°C, the inocula were removed and replaced with DMEM/2% containing 50 μCi of [^{3}H]glucosamine per ml. After a 16-h incubation at 37°C, virus particles were purified from the culture medium, and the glycoproteins were precipitated from lysed envelopes with antisera or heparin agarose as described in Materials and Methods. The precipitates were then resolved by SDS-PAGE; the resulting autoradiograms are shown. The leftmost two lanes contain immunoprecipitations of gC (with antiserum 282) and gB (with antiserum 284) from wild-type virus particles. All other samples were halved and immunoprecipitated with antiserum 282 (lanes gC) or precipitated with heparin agarose (lanes HA). Extraneous lanes between the PRV509 and PRV583 profiles have been removed for presentation purposes. Precipitates obtained from PRV580 virus particles were resolved on a separate SDS-PAGE run. Migrations of molecular mass markers (in kilodaltons) are indicated on the left. (B) Heparin-bound intact virus particles. Virus particles containing radiolabeled glycoproteins were obtained as described for panel A. Half of the intact virus particles in each preparation were lysed in SDS-PAGE sample buffer, and the other half were precipitated with heparin agarose prior to lysis in sample buffer. All samples were then resolved by SDS-PAGE; the resulting autoradiogram shown is a composite of two different exposures of the same gel. Positions of molecular mass markers (in kilodaltons) are indicated on the left. V, control virus particles; HA, heparin agarose-precipitated virus particles.

it not mediate virus attachment to HS? We addressed this question by repeating the heparin agarose precipitation experiment but this time with intact rather than detergent-lysed virus particles. Half of each purified virus preparation was incubated with heparin agarose beads and the precipitate was prepared for SDS-PAGE along with the other half of each preparation, which provided a whole virus glycoprotein profile for each strain; the results are shown in Fig. 5B. Intact virus particles of the wild type and PRV580 were precipitated by the heparin agarose, although the precipitation appeared to be inefficient compared with the intensity of the corresponding whole virus profiles. Still, this result was consistent with the attachment-proficient phenotypes of these two strains. In contrast, little or no heparin agarose precipitation of PRV509 or PRV583 virus particles was apparent, even though sufficient amounts of radiolabeled particles were incubated with the beads. Therefore, gB obtained from detergent-lysed virus particles was bound by heparin, but the same glycoprotein, when still a component of intact PRV envelopes, did not appear to be available for binding. A similar conclusion was recently reached by others using a cell-binding assay (29).

DISCUSSION

A fairly large number of virus receptors have now been identified (for a review, see reference 20), and while the cognate attachment protein(s) has been determined for many viruses, the receptor-binding domain in these instances is most often poorly delineated. Still, through X-ray crystallographic, mutational, and antibody neutralization studies, a few receptor-binding domains have been described, including those for the nonenveloped human rhinoviruses, poliovirus, and foot-and-mouth disease virus and for the enveloped influenza virus (6, 7, 37, 48, 57, 72). In work perhaps more closely related to our own, Roderiquez et al. (56) have determined that the V3 region of the gp120-gp41 envelope glycoprotein complex of human immunodeficiency virus type 1 mediates binding to HS; specific amino acids responsible for attachment have not been described. This is the same region that contains the CD4 receptor-binding domain, for which several key amino acids have been identified by mutational analysis (47), and its has been suggested that the V3 region can simultaneously bind HS and CD4 (56). In these various examples, when multiple proteins or multiple regions of a single polypeptide have been implicated in receptor binding, they have proved to contribute to a single functional domain. More directly stated, appropriate substitutions at any one of the implicated residues have resulted in altered binding activity. When the structure of a binding protein is known, the pertinent residues tend to reside spatially near one another, regardless of their linear position in the amino acid sequence of the polypeptide.

The PRV gC receptor-binding domain exhibits several features that distinguish it from those previously described. The three-dimensional structure of PRV gC is not known, but our results clearly establish that the HS-binding domain consists of multiple discrete units that function redundantly and which can act independently of one another. Alterations in any one component do not eliminate functional virus attachment; instead, all three contributors must be impaired to render the virus defective for binding to cell surface HS. Our results obtained with mutants containing amino acid substitutions in or near HBD 1 and HBD 2 indicated that this region mediates virus attachment through specific arrangements of basic residues which conform to consensus HBD motifs and not simply by the total charge contribution. By extension, we believe this to be true also for HBD 3. We have not yet identified other important features of the regions that contain the HBDs, but our finding that deletion of either HBD 1 or HBD 2 is sufficient to impair both sites suggests that local secondary or tertiary structure is important. In addition, the fact that gC contains four potential, but apparently inactive, HBDs in its carboxy-terminal two-thirds suggests that the PRV gC HBDs do function in a context-dependent manner. However, the overall structure of gC does not appear to be critical for receptor-binding activity. In this study, we found no correlation between the preservation of conformation-dependent epitopes for monoclonal antibodies and the retention of receptor-binding

activity. Previously, we have shown that PRV strains lacking the middle or final one-third of the gC ectodomain remain attachment proficient for HS even with the gross conformational alterations that accompany the loss of 134 or 147 amino acids, respectively (12). In addition, a PRV mutant encoding a chimeric gC in which the first 133 residues behind the signal peptide have been replaced by just 24 amino acids containing a biochemically defined HBD from apolipoprotein B-100 (24) was found to attach to PK15 cells despite the absence of the principal wild-type attachment domain (13). It seems unlikely that any of these mutant species resemble the wild-type protein, yet each possesses some receptor-binding activity.

Thus, the PRV gC receptor-binding domain can be characterized by its need for an intact HBD and by a seeming lack of other significant constraints. This latitude in receptor-binding domain requirements is reflected among the various herpesvirus gC species, which are not highly homologous. For example, we can compare PRV gC with its homologs in HSV-1 and bovine herpesvirus type 1 (BHV-1), glycoproteins whose receptor-binding domains have also been somewhat defined (45, 70). On the basis of the deduced amino acid sequence of each glycoprotein (10, 54, 67), PRV gC is only 46% similar to HSV-1 gC and 54% similar to BHV-1 gC when compared by the Bestfit program of the University of Wisconsin Genetics Computer Group (9). In addition, an alignment of these homologs by their cysteine residues reveals that the HBD motifs harbored by each do not always coincide. Deletion analyses performed with transiently expressed BHV-1 gC indicated that a region between positions 172 and 337 of the 521-amino-acid glycoprotein is required for heparin-binding activity; a smaller deletion of residues 172 to 211 allows the mutant gC to retain two-thirds of the activity (45). By measuring the heparin-binding abilities of synthetic peptides corresponding to specific regions of BHV-1 gC, it has been further suggested that four discrete regions between positions 129 and 310 are involved in virus attachment to HS; one peptide, representing amino acids 297 to 310, actually competes with BHV-1 for attachment to cells (32). It has been concluded from these studies that BHV-1 gC, like PRV gC, contains multiple HBDs that contribute to the receptor-binding domain (32, 45).

This may not be true for HSV-1 gC, even though as for PRV gC, a fairly large portion of the amino terminus of the glycoprotein has been linked to receptor binding (68, 70). Trybala et al. (70) showed that monoclonal antibodies recognizing antigenic site II of HSV-1 gC disrupt gC-HS interactions in a hemagglutination assay; monoclonal antibodies recognizing other epitopes do not. Moreover, certain monoclonal antibody-resistant (*mar*) mutants mimic a *gC* null mutant in their attachment phenotypes. All of the mutants have alterations that are confined to two different regions contributing to antigenic site II. The first region begins at residue 143 and consists of the sequence RCRFR. Among the *mar* mutants, all three positions containing arginine are replaced by neutral amino acids; however, in each mutant only a single arginine is replaced. As with PRV gC, the specific charge arrangement appears to be important because a peptide containing the RCRFR sequence, but not a scrambled version of it, successfully competes with HSV-1 for attachment to cells. The second site, some 100 amino acids carboxy terminal to the first, contains fewer basic residues, and the single alteration that impairs gC-HS binding, a change of a glycine to an aspartate, does not disturb an obvious HBD motif. The disruption of gC-mediated attachment by single alterations at either site indicates that HSV-1 gC does not contain multiple, independent receptor-binding domains. However, the virus as a whole appears to maintain two mechanisms for binding HS, using gB to mediate HS-dependent attachment of *gC* null strains (22). This distinguishes HSV-1 and PRV, since PRV gB from solubilized virus envelopes binds heparin but intact PRV particles lacking functional gC fail to do so, indicating that PRV gB does not facilitate HS-dependent attachment (29, 43, 61; this study).

Why does PRV gC contain more than one HBD? Perhaps the multiple HBDs function to strengthen binding, as has been suggested for other HS-binding proteins (2, 64, 75). Consistent with this possibility, we have previously suggested that wild-type PRV can become "hyperbound" to HS on certain cell surfaces because penetration of Vero cells is considerably slower for the wild type than for *gC* mutant strains; pretreatment of these cells with heparinase allows the wild type to penetrate more rapidly (12). But this is an exceptional case: the wild type rarely becomes encumbered with HS and clearly penetrates almost all cultured cells tested more rapidly than do *gC* mutants (12, 28, 39). Moreover, this example also illustrates that achieving high-affinity binding to HS can be detrimental and perhaps should not be the "goal" of the redundant HBDs. If herpesviruses bind their two receptors sequentially, and not simultaneously, then virus particles must be able to dislodge themselves from the first receptor, HS, to bind the second receptor and eventually fuse with the plasma membrane. Since events subsequent to the initial binding still require envelope glycoproteins, the virus particle cannot leave the HS receptor by shedding its envelope. Thus, a relatively low initial binding avidity would seem appropriate, a need supported by the recent findings of Li et al. (31), who determined the binding kinetics to cell monolayers of purified BHV-1 gC. They found that gC's affinity for HS is far lower than that typically measured for other virus-receptor interactions, with a dissociation constant of only 1×10^{-7} to 5×10^{-7} M.

Therefore, perhaps the multiple HBDs are present not to increase binding affinity but to extend the range of potential HS substrates, with each HBD recognizing a different arrangement of negative charges on the polysaccharide backbone. Herold et al. (21) have recently demonstrated that HSV-1 gB and gC recognize different heparin structures, and there is suggestive evidence that this is the case for the individual HBDs of PRV gC. The three PRV gC HBDs involved in receptor binding differ from one another in the arrangement of their basic amino acids, differing either in the consensus motif to which they match or in the orientation of the motif. Less-conjectural evidence is provided by heparin-binding assays using peptides that contain HBD 1 or HBD 3, which have shown that HBD 1 binds heparin with at least 10-fold-greater affinity than HBD 3 (60). In some of our assays, we noted differences in infectivity among various mutants that suggested that the intact HBDs remaining in each mutant interacted differently with the heparin competitor or with the remnants of HS left after heparinase treatment. Admittedly, it seems unlikely that PRV could encounter a cell type that lacks a suitable HS substrate for any one of its functional HBDs, considering the extreme diversity of negative charge presentation offered by HS on any given cell (34). This view is supported experimentally by the fact that all of our mutants retaining a single intact HBD are attachment proficient not only on the PK15 cells used here but also on Vero, rabbit kidney (RK13), and Madin-Darby bovine kidney (MDBK) cells (11). However, our earlier replacement of the entire wild-type receptor-binding domain with a single HBD from apolipoprotein B-100 allowed attachment of the mutant virus to PK15 but not MDBK cells, suggesting that the lone HBD was unable to locate its cognate HS receptor on the latter cells (13). In addition, we have recently isolated spontaneous revertants of PRV583, our least altered attachment-defective strain, that restore HS-dependent virus

attachment to PK15 cells but in some cases not to Vero, RK13, or MDBK cells (11). Thus, it seems possible that a lack of multiple HBDs can limit the range of HS-dependent attachment. We cannot yet say whether this is due to a critical reduction in binding affinity or to an actual absence of an appropriate receptor for the lone HBD.

It should be noted that our results conflict somewhat with previous reports. Zsak et al. (80) reported that PRV2, the strain with the middle one-third of *gC* deleted, is attachment defective on primary rabbit kidney cells. The proposal that this region therefore contained a receptor-binding domain appeared to be substantiated when it was shown that a peptide containing a potential HBD found in the middle one-third (HBD 4 in our nomenclature) does indeed bind heparin and also competes with virus for attachment to MDBK cells (32). We have subsequently assayed PRV2 on RK13 cells and found the virus to be attachment proficient (11). Moreover, we have selectively deleted HBD 4 from a number of our amino-terminal HBD mutants and found no significant effect on their attachment phenotypes on PK15, RK13, or MDBK cells (11). We currently believe that HBD 4 plays a minor role at best in mediating PRV attachment to all of the cells we have tested, and we suggest that the peptide harboring HBD 4 competes with the first three gC HBDs, not its native site in the glycoprotein, for cell surface HS. In fact, our mutational analysis perhaps sends the cautionary message that mapping HS receptor-binding domains through competitive heparin-binding peptides can generate misleading results. Of course, this can also be said of mutational analyses: our HBD deletion mutants initially led us to believe that HBD 1 and HBD 2 functioned as a single unit.

Significantly, PRV583 is attachment defective on every cell line we have tested (PK15, Vero, MDBK, and RK13), making the six amino acid substitutions found in PRV583 gC the fewest alterations yet described that eliminate PRV attachment to HS. The apparently minor impact of these changes on the structure of the glycoprotein is of particular importance because gC is reportedly multifunctional (63). In addition to its role in receptor binding, gC is involved in virus release (along with glycoproteins gE and gI) and may help stabilize virus particles (63). PRV gC also binds the C3 component of complement, a property that it shares with its homolog in HSV (26, 27). Given the versatility of PRV gC, it is somewhat surprising that virions lacking gC are only modestly attenuated (41). However, mutants with *gC* and either *gE* or *gI* deleted are significantly attenuated, although the reason for this has not been established (41). The use of discretely altered *gC* mutants such as PRV583 should facilitate the resolution of gC's various properties into structural domains and assist in determining how important the attachment function alone is to the virulence of PRV and perhaps other herpesviruses.

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