Characterization of pseudorabies virus glycoprotein C attachment to heparan sulfate proteoglycans

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Pseudorabies virus first attaches to cells through an interaction between the envelope glycoprotein C (gC) and the cell surface heparan sulfate (HS) that is linked to proteoglycans (HSPGs). The HS-binding domain of gC is composed of three discrete heparin-binding domains (HBDs), designated HBD1, -2 and -3 for their proximity to the amino terminus of gC. Each HBD can independently mediate virus attachment to HS, yet each also exhibits a distinct binding preference for differentially sulfated derivatives of heparin. To demonstrate this, affinity columns composed of wild-type gC or mutant gC retaining a single HBD to capture several HSPGs from cultured pig and bovine kidney cells were used. The wild-type gC column bound all of the HSPGs well and, overall, bound more than 90% of the total sample applied to the column. Columns composed of either HBD2 or -3 bound intermediate amounts (40%) of the total sample applied, while the HBD1 column bound low amounts of HSPGs. HBD2 and -3 columns did not uniformly bind all of the HSPGs from bovine kidney cells, but the same HSPGs were bound with equal efficiency on each column. Thus, despite their different preferences for sulfation patterns on HS side-chains, HBD2 and -3 appear to bind the same proteoglycan cores. These results established a hierarchy of HBD2 > HBD3 > HBD1 in importance for HSPG binding. These in vitro-binding results correlated with the attachment phenotype of virus strains expressing gC with a single HBD in their envelopes.

Introduction

Pseudorabies virus (PRV) is a swine herpesvirus belonging to the sub-family Alphaherpesvirinae, along with herpes simplex virus types 1 (HSV-1) and 2, varicella-zoster virus and bovine herpesvirus type 1 (Roizman, 1990). PRV encodes about a dozen glycoproteins and, similar to HSV-1, requires four of them for cell entry, which is achieved through pH-independent fusion of the virus envelope with the plasma membrane (Klupp et al., 2000; Turner et al., 1998). In recent years, several cellular receptors have been identified for one of the required envelope components, glycoprotein D (gD) (Spear et al., 2000), CD155, the poliovirus receptor, and two related proteins, nectin-1α and nectin-2α, have been named as receptors for PRV gD and it appears that at least one of these proteins must be present for the virus to normally infect mucosal epithelial cells or neurons, two major targets of infection (Spear et al., 2000).

However, the various gD receptors are viewed as co-receptors, because initial PRV attachment is mediated by the interaction of glycoprotein C (gC) with cell surface heparan sulfate (HS) chains that are linked to proteoglycan cores (HSPGs) (Flynn et al., 1993; Flynn & Ryan, 1996; Mettenleiter et al., 1990). Surprisingly, this step is nonessential for infection, since gC-null strains are viable and PRV can infect some cells that are unable to express HS (Karger et al., 1995; Robbins et al., 1986). Infection is very inefficient under these circumstances, however, and a major role of PRV gC is to localize the virus to the cell surface and thus promote gD binding to a non-HSPG coreceptor (Mettenleiter, 1989; Spear et al., 2000). Additionally, gC plays a role in PRV entry, because null mutants are also delayed in virus penetration (Mettenleiter, 1989).

Our laboratory has shown previously that the HS-binding domain of PRV gC is composed of three discrete clusters of mostly basic amino acids that closely match proposed motifs of heparin-binding domains (HBDs) (Cardin & Weintraub, 1989; Flynn & Weintraub, 1989; Flynn & Ryan, 1996). Working with mutant viruses that retained only a single gC HBD, it was demonstrated that each HBD can independently mediate virus attachment that is resistant to PBS washes of the inoculated monolayers. A mutant lacking all three HBDs was removed from monolayers.
under the same conditions (Flynn & Ryan, 1996). Thus, the HBDs appear to function redundantly, but Trybala et al. (1998) showed that each differs in their ability to bind differentially sulfated derivatives of HS. Therefore, the different HBDs may bind to different HS ligands to mediate initial attachment of the various mutants. It has not been determined if different mutants bind to different proteoglycan cores.

There are two major families of membrane-bound HSPGs, the syndecans and the glypicans (reviewed by Bernfield et al., 1999). These HSPGs serve as coreceptors for a number of biologically important ligands and infectious agents (Bernfield et al., 1999; Rostand & Esko, 1997). Specificity of binding to HSPGs resides mostly in the sulfation patterns of the attached HS chains. The chains are generally 50–150 disaccharide units in length and can be sulfated at several positions on the disaccharide units (Bernfield et al., 1999; Lindahl et al., 1998). All HS chains from a given cell type are believed to be similarly modified, regardless of the proteoglycan core to which they are attached; the modifications result in a somewhat periodic clustering of lowly sulfated regions interspersed among highly sulfated disaccharides (Lindahl et al., 1998). It is a lack of distinguishing HS chains for the different HSPGs of a cell that may prevent any one from serving as a specific receptor and, hence, relegate it to a coreceptor status. As coreceptors, HSPGs are oligomerized through ligand binding and can subsequently concentrate or immobilize ligands on the cell surface or assist in signal transduction (Bernfield et al., 1999).

Karger & Mettenleiter (1996) have shown previously that PRV can bind both integral and peripheral HSPGs, but did not identify any specific species due to the high molecular mass of intact HSPGs. Here, we have resolved some of the specific HSPGs that are bound by wild-type and mutant forms of gC. We have uncovered preferences of individual HBDs for specific HSPGs, some of which are probably members of the syndecan family, and have demonstrated the importance of a diverse repertoire of HBDs for the overall ability of gC to bind HSPGs. In addition, we have been able to correlate the HSPG-binding capacities of columns composed of a single mutant gC with the binding kinetics of the corresponding PRV mutant strains.

Methods

- **Cells, virus and strain construction.** PK15 (porcine kidney), MDBK (bovine kidney) and mouse L cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% foetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin. Our wild-type strain, PRV-Becker, and mutant strains PRV580, -581, -582 and -583 have been described previously (Flynn & Ryan, 1996) and were propagated on PK15 cells.

- **Virus strains.** Virus strains expressing gC fused to the cellulose-binding domain (CBD) of exo-1,4,β-glucanase from *Cellulomonas fimi* were constructed as follows. To begin, the CBD-coding sequence in PET-38b( +) (Novagen) was modified by site-specific mutagenesis in two ways. First, five consensus sites for N-linked glycosylation addition were removed by replacing either threonine or serine in the sequence Asn-X-Seq/Thr with alanine. Second, a BamHI site was introduced at the 3′ end of the CBD-coding sequence along with an amber stop codon. All alterations were confirmed by DNA sequencing. A 377 bp XhoI–BamHI fragment encoding the modified CBD was then inserted into a plasmid containing the 3′ portion of gC and downstream sequences, replacing a XhoI–BamHI fragment corresponding to gC codons 421–460; this plasmid was named p3′gC–CBD. To generate full-length gC–CBD fusions encoding wild-type or mutant HS-binding domains, the upstream coding sequence and 5′ portion of gC alleles from PRV-Becker, PRV580, -581, -582 or -583 were inserted in-frame into p3′gC–CBD. Each plasmid was then cotransfected along with genomic DNA from PRV509 into PK15 cells by the calcium–phosphate method. As codons 2–458 of PRV509 gC had been deleted, PRV509 does not, therefore, express gC (Flynn et al., 1993). Furthermore, in screening for recombinants that restored gC expression, the extent of the gC deletion in PRV509 ensured that the full-length of each gC–CBD fusion was crossed into the virus genome. Recombinant viruses were identified using goat anti-gC serum 282 (Ryan et al., 1987) and horseradish peroxidase (HRP)-conjugated antibodies in the black plaque assay (Holland et al., 1983). Black plaques were purified for each construct and one was chosen for further use. The resulting strains and their encoded fusion proteins were: PRV612, gCWT–CBD; PRV613, gC<sub>HH</sub>D<sub>B</sub>–CBD; PRV614, gC<sub>HH</sub>D<sub>B</sub>–CBD; PRV615, gC<sub>HH</sub>D<sub>B</sub>D<sub>B</sub>–CBD; and PRV616, gC<sub>HH</sub>D<sub>B</sub>D<sub>B</sub>–CBD.

- **Production of gC–CBD affinity columns.** For each gC–CBD fusion strain, ten 100 mm dishes of confluent PK15 cell monolayers were inoculated with 20 p.f.u. per cell in DMEM/2% at 37 °C for 1 h. The inocula were then removed and monolayers were overlaid with 4 ml DMEM/2% and incubated at 37 °C. At 16 h post-infection, virus-free culture medium was prepared as described previously (Ryan et al., 1987) and the gC–CBD proteins were immunoprecipitated with monoclonal antibodies M1, M7 or M16 (Hampl et al., 1984). The immunoprecipitates were resolved by SDS–PAGE and subjected to fluorography and autoradiography (Ryan et al., 1987).

- **Extraction of HSPGs.** After 3 days of growth, confluent monolayers of PK15, MDBK or mouse L cells on 100 mm dishes were rinsed with cold PBS and extracted at 4 °C in 1 ml 10 mM Tris–HCl, pH 7.4, 150 mM NaCl and 1% Triton X-100 supplemented with 1 mM PMSF. After rocking the dishes constantly for 1 h, the extraction buffer was collected and centrifuged at 10000 g for 1 h at 4 °C. The resulting supernatant was diluted with 3 ml DMEM/2% if it was to be applied to gC–CBD columns. However, if it was to be digested directly with
glycosaminoglycans and then used in SDS–PAGE, it was left undiluted.

- **Application of cell extracts to gC–CBD columns.** A 600 µl aliquot of the diluted cell extracts was applied to each gC–CBD column and the flow-through fraction was collected and passed through each column twice more before being saved. Each column was rinsed with 5 vols of cold 20 mM Tris–HCl (pH 7.5) and the first 500 µl was collected and added to the flow-through fraction because this portion of the rinse contained a significant amount of HSPGs (data not shown). Five vols of 20 mM Tris–HCl, pH 7.5, 400 mM NaCl were then applied to the columns, but only the first 1 ml from each was collected as the eluate because nearly all of the HSPGs that eluted from the column were in this fraction (data not shown). In some experiments, elution was achieved by sequentially applying 5 vols of 20 mM Tris–HCl (pH 7.5) containing 200, 250, 300, 350 or 400 mM NaCl. After each use, the columns were washed with 5 vols of cold 20 mM Tris–HCl (pH 7.5) and 800 mM NaCl to prepare them for future experiments.

- **Heparitinase and chondroitinase digestion.** Samples were digested in 50 mM HEPES (pH 7.0), 1 mM CaCl₂, 0.1% Triton X-100 and 1 mM PMSF. Heparitinase (0.5 µlU, Sigma) and chondroitinase ABC (2.5 µlU, Sigma) were added and the samples were incubated at 37 °C for at least 2 h. After digestion, samples were precipitated with the addition of 40 µg tRNA (carrier) and 3 vols of ethanol. Precipitates were resuspended in SDS–PAGE sample buffer.

- **SDS–PAGE and Western blotting conditions.** Samples were resolved by 10% SDS–PAGE and then transferred electrophoretically to nitrocellulose membranes in 25 mM Tris, 192 mM glycine and 20% methanol. The buffer for all membrane washes and incubations was 20 mM Tris, 150 mM NaCl and 0.05% Tween-20 (TBS-T). Membranes were blocked in TBS-T containing 5% nonfat milk for 30 min at room temperature before incubating with primary antibodies in TBS-T containing 0.25% gelatin. The following antibodies were used: mouse monoclonal antibody 3G10 (obtained from G. David, Leuven University, Leuven, Belgium, and from Seikagaku), 281-2 rat anti-syndecan-1, MSE-2 rabbit anti-syndecan-2 and MSE-3 rabbit anti-syndecan-3 (all provided by M. Bernfield, Harvard Medical School, Boston, MA, USA). After 1 h at room temperature or overnight at 4 °C, the membranes were washed with TBS-T and then incubated with HRP-conjugated secondary antibodies. Afterwards, membranes were washed and chemiluminescence (SuperSignal West Pico, Pierce) was used to detect the protein bands on X-ray film.

- **Attachment assay.** For attachment assays, duplicate PK15 cell monolayers for each time-point were infected with approximately 100 p.f.u. of virus in 1 ml DMEM/2%. After incubation at 37 °C for 15, 30, 45 or 60 min, one monolayer of each pair was washed three times with prewarmed (37 °C) DMEM/2% containing 5 µg/ml heparin, while the other monolayer was washed three times with prewarmed (37 °C) DMEM/2% alone. All pairs were overlaid with DMEM/2% containing 1% methylcellulose to promote plaque formation. About 36 h later, plaques were counted and the number of plaques on the heparin-washed monolayers was expressed as a percentage of the number of plaques on the monolayers washed with medium alone.

**Results**

**PK15 and MDBK cells express multiple HSPGs**

We first determined the HSPG profiles of PK15 and MDBK cells, two cell lines used routinely in our laboratory. Detergent extracts of each cell line were digested with heparitinase and chondroitinase ABC to remove glycosaminoglycan chains and therefore allow the proteoglycans to migrate in SDS–polyacrylamide gels according to the molecular mass of their protein cores. HSPG cores were identified by Western blotting using a monoclonal antibody, 3G10, which specifically recognizes the HS ‘stub’ left on proteoglycans after heparitinase digestion (David et al., 1992). As expected (Kim et al., 1994), multiple protein bands were observed from both PK15 and MDBK cell extracts and, overall, the HSPG core profiles from the two cell lines were similar but distinct (Fig. 1). Both cell lines had prominent HSPG cores that migrated near 35, 45 and 70 kDa, but the smallest core migrated somewhat faster (at approximately 30 kDa) from PK15 cells compared to MDBK cells. In addition, the 70 kDa HSPG core appeared to be less abundant in PK15 cells compared to MDBK cells and a 60 kDa HSPG core found in MDBK cells was apparently absent in PK15 cell extracts. Both extracts contained multiple proteins of apparent molecular mass greater than 100 kDa. Most identified HSPGs have protein cores that are less than 100 kDa (Lories et al., 1989), thus the species migrating more slowly may represent oligomeric forms of smaller HSPGs or incompletely digested HSPGs.

**PK15 and MDBK cells encode syndecan family members**

The approximately 30 or 35, 45, 60 and 70 kDa HSPG cores (hereafter referred to as 30, 35, 45, 60 and 70 K, respectively) detected in PK15 or MDBK cells have apparent molecular masses similar to those reported for syndecans-4, -2, -3 and -1, respectively (Bernfield et al., 1992; Kim et al., 1994). Accordingly, antibodies recognizing murine syndecans were used in Western blot analyses of extracts from PK15 and...
MDBG cells to determine if any of the HSPG cores identified with antibody 3G10 were in fact syndecans. It was not known whether these antibodies would recognize porcine or bovine syndecans, so mouse L cell extracts were also analysed. Even though antibody 3G10 detected all four putative syndecans in extracts of MDBG cells (Fig. 1), only the 70 kDa syndecan-1 was confirmed using the various anti-murine syndecan antibodies (Fig. 2). In contrast, the antibody directed against murine syndecan-1 (Saunders et al., 1989) did not recognize 70 kDa from PK15 cells that was observed using antibody 3G10 (Fig. 2A). Anti-syndecan-2 and -3 antibodies (Kim et al., 1994) identified 45 kDa and 60 kDa species in PK15 and L cell extracts, respectively (Fig. 2, B and C). Interestingly, the 60 kDa protein was not detected in PK15 cell extracts when probed with antibody 3G10 (Fig. 1). Thus, we confirmed the expression of syndecan-2 and -3 in PK15 cells and the presence of syndecan-1 in MDBG cells. Despite generating the most intense signal with antibody 3G10, the putative syndecan-4 from PK15 and MDBG cells (migrating at 30–35 kDa in Fig. 1) did not react with the anti-murine syndecan-4 antibody. However, this antibody did not identify syndecan-4 from the control mouse L cells either (data not shown).

gC–CBD fusion proteins are secreted and retain wild-type, conformation-dependent epitopes

Previously, the three amino-terminal HBDs of wild-type gC were systematically rendered defective by replacing two basic amino acids with neutral residues in each HBD (Flynn & Ryan, 1996); the substitutions were predicted not to change the local secondary structure. Consequently, mutants PRV580, -581 and -582 encoded gC species that retained only HBD3, -2 or -1, respectively (Fig. 3). Nonetheless, each mutant gC could mediate virus attachment to HS. A fourth mutant, PRV583, produced a gC species in which all three HBDs bore substitutions and failed to bind HS.

We prepared affinity columns composed of wild-type or each of the mutant forms of gC, reasoning that columns containing mutant gC with a single HBD would allow us to determine which HSPGs were bound by that particular HBD. Our strategy was to fuse the extracellular domain of gC to a CBD and then bind the fusion protein to a cellulose column. Importantly, the fusion joint was located carboxy-terminal to the final cysteine residue in the gC ectodomain to facilitate proper folding of the fusion protein, but prior to the gC transmembrane domain to allow secretion (Fig. 3B). The hybrid proteins resulting from these manipulations of gC from wild-type, PRV580, -581, -582 and -583 were designated gCWT–CBD, gCBDD–CBD, gCBDD–CBD, gCBDD–CBD and gCBBBD–CBD, respectively.

To express the hybrid proteins for binding to cellulose columns, each gC–CBD fusion was recombined into the virus genome at the gC locus. As a result, the hybrid proteins received appropriate N- and O-linked glycosylation and were harvested from the culture medium of infected cells. To verify the authenticity of the gC–CBD hybrids, PK15 cells were infected with each hybrid strain and incubated with medium containing [35S]methionine/cysteine. The culture medium was then harvested, cleared of cells and virus and used in immunoprecipitations with a panel of monoclonal antibodies that recognize conformation-dependent epitopes of gC (Hampl et al., 1984). The samples were resolved by SDS–PAGE and the resulting autoradiogram is shown in Fig. 3(C). The hybrid proteins were secreted in similar amounts and appeared to be properly modified as they migrated at about 97 kDa, slightly larger than the wild-type species (Ryan et al., 1987). In all cases, the hybrid proteins were immunoprecipitated by each of the three monoclonal antibodies, indicating that the replacement of the gC transmembrane domain with the CBD had not drastically altered their conformations.

Individual HBDs of gC bind similar HSPGs, but to different extents

Equivalent amounts of the secreted hybrid proteins were bound to cellulose columns to create gC-affinity columns from wild-type and each of the mutant strains. To ascertain the binding capacities of the columns, equal amounts of PK15 or MDBG cell extracts were added to each column. The columns were washed with binding buffer and then eluted with 400 mM NaCl. Eluate and flow-through fractions were digested with heparitinase and chondroitinase ABC, resolved by SDS–PAGE and analysed by Western blot analysis using antibody 3G10. The results are shown in Fig. 4; both blots were scanned and relative amounts in each lane were determined using Scion Image software (Scion). The gCWT–CBD column bound greater than 90% of added HSPGs from either extract (Fig. 4A). The gCBDD–CBD and gCBDD–CBD columns bound about 40% of added HSPGs and the gCBDD–CBD column bound substantially less, about 10%. The gCBDD–CBD column, with no functional HBDs, bound less than 5% of the HSPGs added and these could be eluted at a low salt concentration of 50 mM NaCl (data not shown). This was
consistent with the HSPGs binding to the columns through their HS side-chains, since the gC_{HBD0}–CBD column exhibited little, if any, affinity. This was confirmed by first treating the extracts with heparitinase prior to their addition to a gC_{WT}–CBD column: all of the HSPGs shifted toward the flow-through fraction. However, if the extracts were first digested with chondroitinase ABC instead of heparitinase, the HSPGs retained their ability to bind to the column and appeared in the eluate (data not shown).

All of the columns, except the gC_{HBD0}–CBD column, bound 45 K and 30 or 35 K from both cells. The 60 kDa HSPG core and 70 K were detected inconsistently in PK15 cell extracts and were not observed in the blot shown in Fig. 4(A). MDBK cell extracts, however, consistently provided more intense signals in 3G10 Western blots and all of the HSPG cores were detected. As a result, the MDBK cell profiles revealed some noticeable differences in specificity among the columns. For example, in Fig. 4(B), 70 K was present mostly in the flow-through fractions of the gC_{HBD3}–CBD and gC_{HBD2}–CBD columns, while 60 K was mostly in the eluate fractions. The 45 kDa HSPG core and 35 K were partitioned equally between the eluate and flow-through fractions of the two columns. This indicated that different HSPGs from the same (MDBK) cells were bound differentially by specific HBDs, even though the HS chains of all of the HSPGs were, presumably, alike. Significantly, all of these HSPGs were bound efficiently to the gC_{WT}–CBD column.

A mixture of three mutant gCs binds HSPGs better than any single mutant alone

The efficient binding of HSPGs by the gC_{WT}–CBD column may have been due to the presence of all three different HBDs. Accordingly, we constructed a new column, gC_{MIX}–CBD, in which equal amounts of gC_{HBD3}–CBD, gC_{HBD2}–CBD and gC_{HBD1}–CBD were used. Importantly, the column was composed of the same total amount of fusion protein as the other columns.

The same amount of PK15 cell extract that was used in the experiment depicted in Fig. 4(A) was applied to the gC_{MIX}–CBD column. Eluate and flow-through fractions were
collected, digested, separated by SDS–PAGE and blotted with 3G10 antibody as before (Fig. 5). The gC_{MIX–CBD} column bound 67% of the total sample added, more than any of the mutant gC–CBD columns. Therefore, an expanded HBD repertoire appeared to increase the efficiency of HSPG binding. Still, the efficiency of the gC_{MIX–CBD} column fell short of the gC_{WT–CBD} column efficiency. This may have been due to the presence, at least potentially, of three functional HBDs in every wild-type species compared to only one in each of the mutants.

**HSPGs bind different gC fusion proteins with different affinities**

Comparing the amounts of HSPGs in eluate and flow-through fractions measures the capacity of gC columns to bind syndecans, but it does not determine how tightly they are bound to the gC columns. To compare the affinities of the columns, bound HSPGs were eluted with NaCl in 50 mM increments. Eluted samples were digested, separated by SDS–PAGE and detected by Western blot analysis using 3G10 antibody. The resulting chemiluminescence images were scanned and protein band intensities were quantified. The amount of HSPGs that eluted at a particular salt concentration is represented graphically in Fig. 6 as a percentage of total HSPGs eluted. At NaCl concentrations ≥ 300 mM, 65% of the HSPGs eluted from the gC_{WT–CBD} column. The gC_{HBD$-$CBD} column bound HSPGs with equal, if not higher, affinity. HSPGs eluted at lower salt concentrations from the gC_{HBD$^+$CBD} column, with a majority eluting at ≤ 250 mM NaCl and all eluting by 300 mM. HSPGs bound to the gC_{HBD$^-$CBD} column eluted at much lower salt concentrations, with more than 90% eluting at ≤ 200 mM.

The affinity of the gC_{MIX–CBD} column for HSPGs was representative of the affinities of each of the gC–CBD mutants of which it was composed. A majority of the HSPGs eluted from the gC_{MIX–CBD} column between 200 and 300 mM NaCl, as was the case with the gC_{HBD$^-$CBD} and gC_{HBD$^+$CBD} columns. A minor fraction of the HSPGs eluted at ≤ 200 mM, which may be attributed to gC_{HBD$^+$CBD} binding. There was also a minor fraction of HSPGs that bound with higher affinity and eluted at salt concentrations between 300 and 400 mM. Because the highest affinities were not observed with any of the mutant columns alone, this may have been the result of individual HSPGs binding simultaneously to different hybrid proteins.

**Reduced binding ability for HSPGs can impair mutant virus entry of cells**

PRV gC-null mutants are defective not only for initial virus attachment, but for virus penetration as well (Flynn et al.,...
Our goal was to resolve specific HSPGs bound by gC and characterize any binding preferences that a particular gC HBD might have for HSPGs expressed in PK15 or MDBK cells. We were able to limit our survey to gC–HSPG interactions because gC is the only heparin-binding protein in the PRV envelope (Flynn & Ryan, 1996; Karger et al., 1995). We demonstrated that several of the bound HSPGs were probably syndecan family members. Therefore, our results potentially define the pertinent syndecan species in initial PRV attachment to cells and may represent the first specific identification of primary receptors for any alphaherpesvirus.

Wild-type gC proficiently bound all of the HSPGs that we detected by Western blot analyses with antibody 3G10. However, HBD2 and -3 preferentially bound 60 K, but bound 45 K poorly and, to an even worse extent, 70 K from MDBK cells. These were somewhat surprising results as it is generally assumed that all HS chains of a given cell type are modified by the same cellular machinery to the same extent (Carey, 1997). Our results suggest that either this is not the case in MDBK cells or that gC binds some HSPGs through additional, core-specific features, which has been suggested for other HSPG–ligand interactions (Rapraeger & Ott, 1998). Alternatively, topological determinants may influence the accessibility of the HS chains. Various heparin-binding proteins have been shown to bind specific HS sequences (Bernfield et al., 1999); our findings support the idea that topological differences might also preferentially direct the ligand to a particular proteoglycan core (Carey, 1997).

It is not uncommon for heparin-binding proteins to contain multiple HBDs, although it appears in some cases that one of the HBDs is responsible for the majority of binding activity (Barkalow & Schwarzbauer, 1991). This may explain the presence of three functional HBDs in the amino terminus of PRV gC. Even though our laboratory has shown previously that any one of the three HBDs can lead to virus attachment that is resistant to PBS washes of the inoculated monolayers (Flynn & Ryan, 1996), results here suggest that HBD2 and -3 are the dominant domains, leaving HBD1 to serve an ancillary role in virus binding. The overall binding capacities of columns composed of gC<sub>HBD2</sub>–CBD or gC<sub>HBD3</sub>–CBD fusion proteins were equivalent and significantly higher than the binding capacity of a gC<sub>HBD1</sub>–CBD column. However, the gC<sub>HBD2</sub>–CBD and gC<sub>HBD3</sub>–CBD columns were distinguished from one another by their NaCl elution profiles. While we described the elution of HSPGs at higher salt concentrations as indicative of higher binding affinity for the gC<sub>HBD3</sub>–CBD column, this characterization may be somewhat misleading. As noted by Trybala et al. (2000), protein–HS bonds are more stable in high salt buffers as the relative contribution of nonionic interactions increases. Protein–HS bonds that are dependent mostly on ionic interactions are broken more readily at lower salt concentrations. Thus, the NaCl elution profiles of the gC<sub>HBD2</sub>–CBD and gC<sub>HBD3</sub>–CBD columns may indicate that HBD2 bound HS predominantly through ionic interactions, while HBD3 relied more on nonionic interactions. This is consistent with past analyses showing that PRV581 virions, which contain only HBD2 of gC, preferentially bound highly sulfated derivatives of heparin, while PRV580 virions used HBD3 only to bind undersulfated heparin derivatives with greater affinity (Trybala et al., 1998). Because HS chains exhibit clusters of lowly sulfated stretches intermingled with highly sulfated saccharides (Lindahl et al., 1998), the dual nature of the
sulfation preferences of HBD2 and -3 may ensure that a suitable ligand is always nearby for gC-mediated attachment. Results obtained with the gC-MIX–CBD column emphasized further the importance of a diverse repertoire of HBDs in mediating binding to HS. Overall, the salt elution profile of the mixed column was representative of the individual profiles and was shifted to elution at lower salt concentrations compared to the wild-type column. Still, some HSPGs eluted at the highest salt concentrations and we would suggest that these represented individual HSPGs bound simultaneously to multiple fusion proteins bearing different HBDs. If this is true, such cross-linking was apparently stronger than any cross-linking that occurred on a column composed uniformly of a single HBD. How intermolecular cross-linking would compare to any that occurred on a column composed uniformly of a single HBD cannot be determined clearly by our assays.

The major function of gC is to mediate the initial attachment of PRV to cells and our in vitro-binding assays correlated with the attachment phenotype of each gC mutant. If gC–HSPG interactions only facilitated initial virus attachment, then the identification of specific HSPGs bound by gC might be inconsequential. However, gC-null mutants are also defective in virus entry and we have been able recently to separate the role of gC in virus penetration from its role in attachment (unpublished data). Thus, the identity of bound HSPGs is potentially important because different properties, such as cytoskeletal association and the mediation of signal transduction, have been attributed to the various syndecans (Bernfield et al., 1999; Rapraeger & Ott, 1998). While we cannot point to a particular HSPG yet, it is possible that a property associated with a specific HSPG facilitates PRV penetration, if not attachment, through gC.

We thank T. Hatch (UT) for the gift of mouse L cells and are grateful to G. David (Leuven University, Belgium) for antibody 3G10 and to M. Bernfield (Harvard Medical School, Boston, USA) for anti-syndecan antibodies. We would also like to thank A. Rapraeger (University of Wisconsin, Madison, USA), H. Jarrett (UT) and the members of the Cox and Whitt laboratories (UT) for very helpful discussions during the course of this work. Special thanks to T. Higgins (UT) for preparing the Figures.

References


PRV gC–HSPG interactions


Received 8 August 2001; Accepted 16 October 2001