

Identification of the glycosaminoglycan-binding site on the glycoprotein E^{rns} of bovine viral diarrhoea virus by site-directed mutagenesis

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Bovine viral diarrhoea virus (BVDV) envelope glycoprotein E^{rns} interacts with highly sulphated heparin-like glycosaminoglycans (GAGs) located on the cell surface as an early step in virus infection of cells. Site-directed mutagenesis of recombinant E^{rns} was undertaken and analysis of mutants by heparin-affinity chromatography and cell surface binding showed that a cluster of basic amino acids (⁴⁸⁰KKLENKSK⁴⁸⁷) near the C terminus of E^{rns} was essential for binding. Mutants with amino acid substitutions of lysine residues 481 and 485 in E^{rns} reduced the binding of E^{rns} to immobilized heparin and cellular GAGs but retained ribonuclease activity. In contrast to normal E^{rns}, E^{rns} that was unable to bind to cells also failed to inhibit BVDV infection of cells when the cells were pre-incubated with E^{rns}. It is proposed that the cluster of basic residues (⁴⁸⁰KKLENKSK⁴⁸⁷) localized at the C-terminal end of E^{rns} constitutes a GAG-binding site.

Introduction

Bovine viral diarrhoea virus (BVDV) is a major cattle pathogen with a worldwide distribution, which, together with classical swine fever virus (CSFV) of pigs and border disease virus (BDV) of sheep, belongs to the genus *Pestivirus* of the family *Flaviviridae* (Thiel *et al.*, 1996). Many members of this family can cause severe human disease, such as hepatitis C virus, dengue virus and yellow fever virus (Pringle, 1998). The pestivirus genome comprises positive-stranded RNA that is translated to form a single virus polypeptide, which, through cleavage by both host and virus proteases, gives rise to 11 or 12 mature viral proteins (NH₂-N^{pro}-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH) (Rice, 1996). E^{rns}, E1 and E2 are glycoproteins found on the surface of the virion. E^{rns} lacks a typical transmembrane domain and a proportion of this protein is secreted from infected cells into the extracellular environment (Rumenapf *et al.*, 1993) and also contains a ribonuclease activity (Hulst *et al.*, 1994; Schneider *et al.*, 1993; Windisch *et al.*, 1996). The role of ribonuclease activity in the virus life cycle is not clear. It has been reported that a genetically engineered strain of CSFV, which lacks ribonuclease activity, induced cell death in swine kidney cells but the parent

virus did not (Hulst *et al.*, 1998). However, another mutant CSFV lacking ribonuclease activity was not cytopathogenic (Meyers *et al.*, 1999). Recently we have assigned an additional function to E^{rns}: BVDV glycoprotein E^{rns} binds to glycosaminoglycans (GAGs) on the cell surface (Iqbal *et al.*, 2000); subsequently, this was also shown for E^{rns} of CSFV (Hulst *et al.*, 2000).

Heparin is present in the form of heparan sulphate proteoglycans on the cell surface and a large number of viruses utilize heparan sulphate to mediate attachment and infection of target cells. Examples can be found within many virus families: for example, members of the families *Flaviviridae* (Chen *et al.*, 1997; Hilgard & Stockert, 2000; Su *et al.*, 2001), *Herpesviridae* (Terry-Allison *et al.*, 2001) and *Picornaviridae* (Jackson *et al.*, 1996); human immunodeficiency viruses (Di Caro *et al.*, 1999), human respiratory syncytial virus (Martínez & Melero, 2000), adenovirus types 2 and 5 (Dehecchi *et al.*, 2001); and members of the genus *Alphavirus* (Byrnes & Griffin, 1998). The binding of proteins and glycoproteins to GAGs is often mediated through heparin-binding domains (HBDs), which are found in a wide range of proteins and are characterized by an overall positive charge. Common structural motifs have been proposed: the sequences BBXB, XBBXB and XBBBXXB (where B designates a basic amino acid and X designates any other residue) have been suggested to act as heparin-binding regions (Hileman *et al.*, 1998; Sobel *et al.*, 1992).

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The amino acid sequence of E^{rns} has two conserved sequences, ⁴⁰⁶KKGK⁴⁰⁹ and ⁴⁸⁰KKLENKSK⁴⁸⁷, which closely match consensus HBD sequences. Here we examine whether these putative heparin-binding consensus sequences present in E^{rns} are involved in E^{rns}–GAG binding; we describe their relative affinities for heparin and cell surface binding; and the ability of mutated E^{rns} to block BVDV infection.

Methods

■ **Cells and viruses.** Calf testes (CTe) cells were maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% BVDV-free foetal calf serum (FCS), 5% lactalbumin hydrolysate and 0.1% penicillin G and streptomycin (100 µg/ml). Insect cells (Schneider S2; *Drosophila*) were obtained from Invitrogen and grown at room temperature in DES expression medium (Invitrogen) containing 10% FCS. Working stock (passage no. 12) of BVDV cytopathogenic (CP) virus strain Pe515 (Pocock *et al.*, 1987) was prepared in CTe cells.

■ **Site-directed mutagenesis of heparin-binding sites.** The E^{rns} gene was cloned previously into a *Drosophila* expression vector to produce pMT/BiP/E^{rns}/V5-His, which represented the full-length E^{rns} cDNA of BVDV (non CP) strain Pe515 corresponding to amino acids 268–494 of the BVDV polyprotein (Iqbal *et al.*, 2000). Specific mutation of positively charged amino acids in putative heparin-binding motifs of E^{rns} was carried out to substitute lysine for uncharged amino acids (alanine, glycine or threonine). Mutagenesis was done by PCR using the Quick Change Site-Directed Mutagenesis kit (Stratagene) following the manufacturer's guidelines. The sequences of the mutagenic primers used for the constructs can be obtained from the authors upon request. Sequencing of mutagenized plasmids was carried out commercially (MWG-Biotech). A deletion mutant of E^{rns} (Δ480–494 E^{rns}), where the C-terminal 15 amino acids of mature E^{rns} were deleted, was constructed by PCR and cloned into the DES expression vector pMT/BiP/V5-His (Invitrogen). The primers used were: 5' ATCAAGTTACAAGATCTGAAAATATAACA 3' (sense orientation), which corresponds to nucleotides 1178–1207 of the BVDV genome sequence modified to include a *Bgl*II recognition site (bold letters) on the 5' end of the E^{rns} sequence; and 5' CTTGTTTTCCAATCTAGATCCTAGTATCCC 3' (anti-sense orientation), which corresponds to nucleotides 1820–1849 of the BVDV genome sequence modified by the inclusion of an *Xba*I recognition site (bold letters). Plasmids (pMT/BiP/E^{rns}/V5-His) containing the genes encoding wild-type, mutated or deleted E^{rns} were individually co-transfected with a hygromycin-resistant selection vector pCoHYGRO (Invitrogen) at a ratio of 19 (pMT/BiP/E^{rns}/V5-His) to 1 (pCoHYGRO) into insect (S2) cells using a Calcium Phosphate Transfection kit (Invitrogen), according to the supplier's instructions.

■ **Expression and purification of wild-type and mutant E^{rns}.** Wild-type and mutant E^{rns} glycoproteins were expressed and purified as described previously (Iqbal *et al.*, 2000). Briefly, *Drosophila* S2 cells harbouring the E^{rns} gene were grown in Ultimate Insect serum-free medium (Invitrogen) and expression of V5-tagged E^{rns} (E^{rns}V5) was induced by the addition of 5 µM copper sulphate. The culture supernatants collected 72 h post-induction were dialysed against equilibration buffer (20 mM Tris–HCl, pH 7.8, and 500 mM NaCl) and subjected to nickel affinity chromatography (Probond, Invitrogen). The column was washed with 20 mM Tris–HCl, pH 6.0, containing 1.0 M NaCl and 50 mM imidazole and E^{rns} was eluted with 20 mM Tris–HCl, pH 6.0, containing 300 mM imidazole. Fractions containing E^{rns}V5 were

pooled and the concentration of protein was determined using the Coomassie reagent (Pierce).

■ **Binding of E^{rns} and its mutants to heparin.** The relative affinity of wild-type and mutant E^{rns} to immobilized heparin was determined by loading the S2 cell culture supernatants containing wild-type or mutant proteins onto a 1.0 ml heparin–agarose column (Sigma) equilibrated previously with 20 mM Tris–HCl, pH 7.8. The column was washed with equilibration buffer and eluted in 1.0 ml fractions with a stepwise gradient of increasing concentrations of NaCl (0.1–1.0 M) in equilibration buffer. Dilutions of each fraction were coated onto a 96-well ELISA plate at 4 °C overnight and then blocked with 5% skimmed milk in PBS. E^{rns} was detected using an anti-V5 antibody (a generous gift from Professor R. Randall, University of St. Andrews, Scotland, UK).

■ **Cell surface binding assay.** Confluent cell monolayers of CTe cells in 96-well plates were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 45 min at room temperature and blocked overnight at 4 °C with blocking buffer (5% normal goat serum and 0.01% NaN₃ in PBS). The cells were then incubated with 0.3 µg wild-type or mutant E^{rns} in PBS in a total volume of 50 µl for 1 h at room temperature. Subsequently, the cells were washed three times with PBS. Bound wild-type and mutant E^{rns} were detected with an anti-E^{rns} monoclonal antibody (WB210, Veterinary Laboratories Agency) or an anti-V5 monoclonal antibody (ibid).

■ **Inhibition of BVDV infection of cells by E^{rns}.** Confluent monolayers of CTe cells growing in 6-well plates were rinsed twice with serum-free EMEM and pre-incubated with 500 µl serum-free EMEM containing recombinant wild-type E^{rns}, mutant E^{rns} or control protein at 37 °C. After 1 h, dilutions of 500 µl CP BVDV (75 p.f.u.) in serum-free EMEM were added to the wells. When the virus solution was added, the concentration of protein (wild-type E^{rns}, mutant E^{rns} or GFP/V5) was diluted to 20 µg/ml (the concentration at which inhibition was measured). The virus–protein mixture was further allowed to adsorb for 1 h at 37 °C, after which the cells were washed three times with EMEM and overlaid with maintenance medium containing 1% agarose. The cells were then incubated at 37 °C for 3 days. The cells were stained with toluidine blue (0.1% toluidine blue and 4% formaldehyde in PBS) and the number of CP virus plaques was counted.

■ **Ribonuclease activity of E^{rns}.** Ribonuclease activity of wild-type and mutant E^{rns} was carried out essentially as described by Hulst *et al.* (1994). Briefly, the assay mixture (25 µl in total) contained 0.2 µg of purified wild-type or mutant E^{rns} and 12.5 µg of yeast 16–23S RNA in 20 mM sodium acetate buffer, pH 4.5. The mixture was incubated at 37 °C for varying time-points (5–45 min) and the enzyme reaction was stopped by acid precipitation using 5 µl of 25% (v/v) HClO₄ containing 0.75% (w/v) uranyl acetate. After cooling on ice for 10 min, the reaction mixture was centrifuged for 5 min at 10 000 *g* and the absorbance at 260 nm of the supernatant was measured. The specific ribonuclease activity was expressed as A₂₆₀ units per min per mg of E^{rns}.

Results

Construction of E^{rns} mutants

The BVDV glycoprotein contains two clusters of basic amino acids: ⁴⁰⁶KKGK⁴⁰⁹ (HBD-1), which exactly matches various viral and mammalian heparin-binding site sequences (Feldman *et al.*, 1999; Hileman *et al.*, 1998; Proudfoot *et al.*, 2001), and ⁴⁸⁰KKLENKSK⁴⁸⁷ (HBD-2) in the polyprotein of

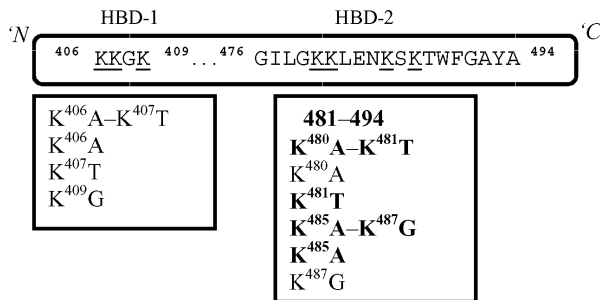


Fig. 1. Amino acid sequence of potential HBDs of BVDV E^{ns}. The lysine residues altered by site-directed mutagenesis are underlined and the resulting single and double mutants are shown in the boxes below. Mutant clones, which lost heparin-binding affinity, are indicated in bold.

BVDV strain Pe515, both may be potential heparin-binding sites. Both of these regions of basic amino acids are conserved among all the BVDV strains for which data have been deposited in sequence databases. To determine whether the basic amino acid residues in these potential heparin-binding motifs are indeed responsible for E^{ns}-GAG-binding activity, a series of single and double mutants, generated by site-directed mutagenesis by substituting lysine with neutral residues, and a single deletion mutant (a deletion of 15 amino acids, 480–494) were made. A total of 11 single, double and deletion mutants was constructed (Fig. 1).

Effect of E^{ns} mutation on heparin-binding affinity

The relative affinities of the recombinant proteins for heparin were assessed by chromatography on heparin-agarose columns. Wild-type and mutant E^{ns} proteins were expressed in *Drosophila* S2 cells and culture supernatants were applied to heparin-agarose columns and eluted with a stepwise gradient of an increasing concentration of NaCl (0.1–1.0 M). Wild-type and E^{ns} mutants, K⁴⁰⁶A–K⁴⁰⁷T, K⁴⁰⁶A, K⁴⁰⁷T and K⁴⁰⁹G, in which basic amino acids had been substituted in the first potential HBD, HBD-1, and two mutants, K⁴⁸⁰A and K⁴⁸⁷G, from the second potential HBD, HBD-2, eluted between 0.5 and 1.0 M NaCl. These results indicated that the heparin-binding affinity of these E^{ns} mutants was like wild-type E^{ns} (Fig. 2a). In contrast, the other mutants from HBD-2, K⁴⁸⁰A–K⁴⁸¹T, K⁴⁸⁵A–K⁴⁸⁷G, K⁴⁸¹T and K⁴⁸⁵A and the deletion mutant Δ 480–494 eluted from the matrix at 0.2–0.4 M NaCl (Fig. 2b) and showed reduced heparin binding. From these results we deduce that two basic residues of E^{ns}, Lys⁴⁸¹ and Lys⁴⁸⁵, are critical for binding to the heparin matrix.

E^{ns} mutants that lack heparin-binding ability retain their RNase activity

To ensure that the changes introduced into E^{ns} did not induce extensive conformational change to the molecule, the ribonuclease activity of E^{ns} was examined. The specific ribonuclease activities of the purified wild-type and E^{ns}

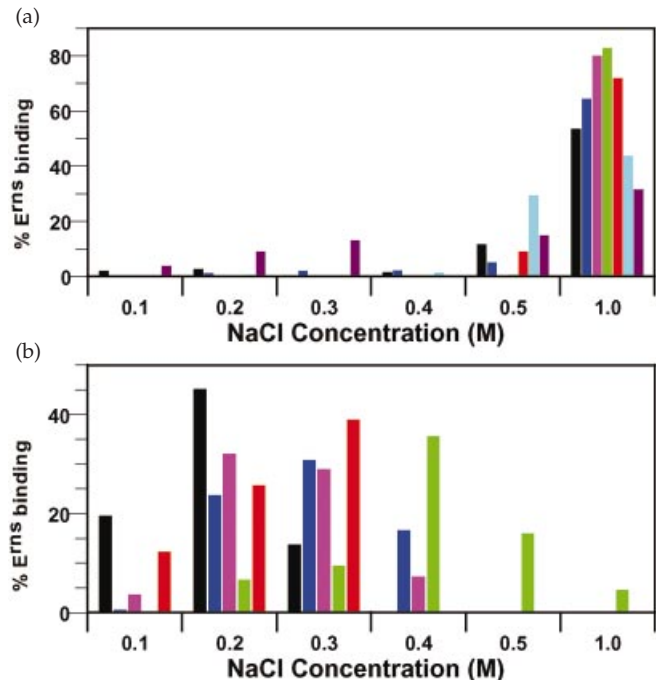


Fig. 2. Binding activities of E^{ns} mutants to heparin-agarose. The relative binding of E^{ns} mutants to heparin was estimated by the concentration of NaCl required for elution from a heparin-agarose column. Culture supernatants from *Drosophila* S2 cells expressing wild-type and mutant E^{ns} proteins were applied to a heparin-agarose column and the E^{ns} protein was eluted with a stepwise NaCl gradient (0.1–1.0 M). The fractions were assayed for E^{ns} by ELISA. Total eluted E^{ns} for each mutant from each NaCl concentration is shown. (a) E^{ns} mutants that had no effect on their binding to heparin-agarose: wild-type (black), K⁴⁰⁶A–K⁴⁰⁷T (blue), K⁴⁰⁶A (pink), K⁴⁰⁷T (green), K⁴⁰⁹G (red), K⁴⁸⁰A (cyan) and K⁴⁸⁷G (purple). (b) E^{ns} mutants that showed altered binding to heparin-agarose: Δ 480–494 (black), K⁴⁸⁰A–K⁴⁸¹T (blue), K⁴⁸⁵A–K⁴⁸⁷G (pink), K⁴⁸¹T (green) and K⁴⁸⁵A (red).

mutants K⁴⁸⁰A–K⁴⁸¹T, K⁴⁸⁵A–K⁴⁸⁷G, K⁴⁸¹T and K⁴⁸⁵A and the deletion mutant Δ 480–494 were: 756, 822, 634, 712, 568 and 613 A₂₆₀ units per min per mg, respectively. These results demonstrated that mutant E^{ns} showed no significant loss of ribonuclease activity.

Effect of E^{ns} mutations on binding to the cell surface

The E^{ns} mutants were examined for their differences in cell surface binding. Purified recombinant proteins were incubated with paraformaldehyde-fixed CTe cells and the relative binding of mutant proteins was analysed by ELISA. The data, shown in Fig. 3, demonstrated that wild-type and six mutants of glycoprotein E^{ns} (K⁴⁰⁶A–K⁴⁰⁷T, K⁴⁰⁶A, K⁴⁰⁷T, K⁴⁰⁹G, K⁴⁸⁰A and K⁴⁸⁷G) showed similar binding to CTe cells but mutants K⁴⁸⁰A–K⁴⁸¹T, K⁴⁸⁵A–K⁴⁸⁷G, K⁴⁸¹T and K⁴⁸⁵A and the deletion mutant Δ 480–494 had lost their ability to bind to the surface of cells. These results show that residues Lys⁴⁸¹ and Lys⁴⁸⁵ are important residues involved in binding to cell surface GAGs. Notably, the ability of E^{ns} to bind to cells correlates with their affinity for immobilized heparin.

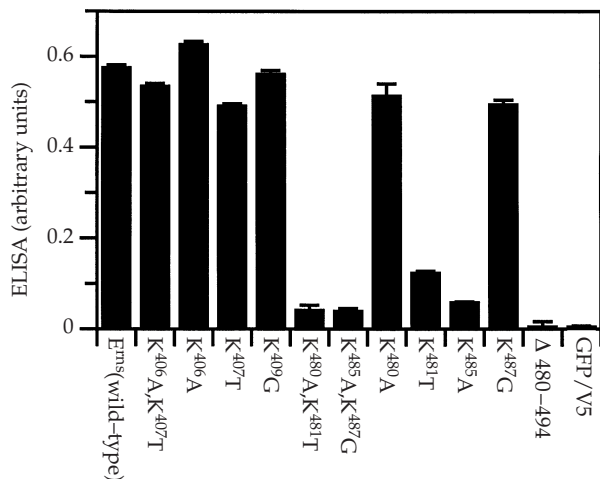


Fig. 3. Binding profiles of E^{rns} mutants to CTe cells. Cell surface binding of wild-type and mutant E^{rns} was measured by incubation of E^{rns} with paraformaldehyde-fixed CTe cells in 96-well plates for 1 h at room temperature. E^{rns} bound to cells were detected by ELISA using an anti-V5 monoclonal antibody. Each bar represents the mean of at least two independent experiments and error bars show the standard deviations from triplicate data points.

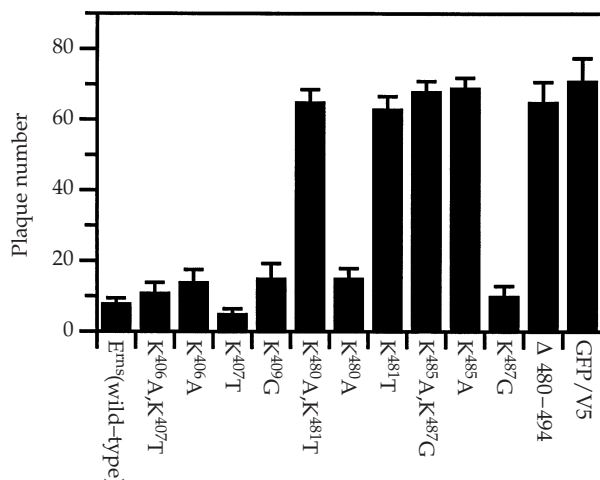


Fig. 4. Effect of amino acid substitutions in the E^{rns} protein on its ability to inhibit BVDV infection. E^{rns} mutants were tested for their ability to block virus infection by treating the CTe cells with wild-type E^{rns}, mutant E^{rns} or GFP/V5 (control) for 1 h at 37 °C. Cells were then inoculated with approximately 75 p.f.u. of CP BVDV. The final concentration of E^{rns}, mutant E^{rns} or GFP/V5 was diluted to 20 µg/ml and incubated for a further 1 h. The cells were then washed and overlaid with medium containing 1% agarose. After incubation for 3 days, the cells were fixed and stained and the plaques counted. Error bars show the standard deviations from triplicate data points.

Ability of mutant E^{rns} to block virus infectivity

To determine whether the observed impairment of E^{rns} mutants in cell surface binding and binding to immobilized heparin also correlated with inhibition of virus infection, CTe cells were incubated with wild-type and mutant proteins and subsequently infected with CP BVDV virus. As shown in Fig. 4, the addition of wild-type E^{rns} and mutant E^{rns}, K⁴⁰⁶A–K⁴⁰⁷T,

K⁴⁰⁶A, K⁴⁰⁷T, K⁴⁰⁹G, K⁴⁸⁰A and K⁴⁸⁷G during the period of virus attachment reduced plaque formation. In contrast, E^{rns} mutants K⁴⁸⁰A–K⁴⁸¹T, K⁴⁸⁵A–K⁴⁸⁷G, K⁴⁸¹T and K⁴⁸⁵A and the deletion mutant Δ480–494 showed no significant inhibitory effect on virus infection compared to the GFP/V5 negative control protein. Hence, we conclude that E^{rns} mutants with lysine residues at positions 481 and 485 changed to neutral amino acid residues were defective in heparin and cell surface binding and also lost their ability to block virus infection.

Discussion

Recently we showed that BVDV glycoprotein E^{rns} binds to immobilized heparin and interacts with cell surface heparan sulphate-like GAGs and proposed that BVDV interacts initially with the infected cell through this mechanism (Iqbal *et al.*, 2000). In this report, we localized a functional site on E^{rns} that is involved in binding to cell surface GAGs and we determined the relative importance of positively charged residues in two potential HBDs (⁴⁰⁶KKGK⁴⁰⁹ and ⁴⁸⁰KKLENKSK⁴⁸⁷) of E^{rns}. The results from site-directed mutagenesis of E^{rns} presented here indicated that binding of the E^{rns} to GAGs involves lysine residues at positions 481 and 485 of the virus polyprotein, the motif at residues 406–409 had no effect on GAG binding.

In CSFV, strain Brescia, Hulst *et al.* (2000) observed that the affinity of the virus for heparin increased after passage in tissue culture. These authors showed that residue 476 was involved in determining the affinity of the virus for heparin, since, on adaptation to tissue culture, this residue changed from Ser to Arg; this residue is adjacent to the heparin-binding motif that we have identified here for BVDV. At position 476, strains of CSFV vary with lysine, arginine or serine residues present but all BVDV and BDV sequences deposited in sequence databases are distinct and have glycine at that position. (In this study, we have not examined the effect of changing the conserved glycine at position 476 of BVDV E^{rns}.) Like residue 476, the region we have identified (⁴⁸⁰KKLENK⁴⁸⁶) as a GAG-binding site is totally conserved in all BVDV strains sequenced to date. As a comparison with the examples from sequence databases, we have examined the amino acid sequence of this region of BVDV E^{rns} directly from the serum of three persistently infected calves. RT-PCR and sequencing the cDNA products prepared from serum samples showed a conserved amino acid sequence between residues 470 and the C terminus of E^{rns} in the region of the GAG-binding motif we have identified (data not shown). We submit, therefore, that adaptation of BVDV to tissue culture does not select for mutations around the GAG-binding motif and changes in binding to GAGs; this contrasts with observations made on CSFV (Hulst *et al.*, 2000). This GAG-binding motif is also highly conserved in BDV isolates: the only variation has been observed is residue 485. In BDV, residue 485 may be asparagine (as observed in BVDV) or histidine, which makes the BDV consensus KKLE^N/_HK. This region is somewhat different in CSFV isolates. The sequence

reported most frequently in this region for CSFV isolates is KRLEGR but the consensus sequence can be described as $K/R^K/R^K/LE^G/s/R^K/R$. Notably, in all pestiviruses the LE motif is totally conserved. The significance of the change between CSFV and the BVDV/BDV group is unclear but it may correlate with the fine specificity of GAG binding and the important influence of residue 476 in CSFV.

The GAG-binding motif for all pestiviruses can be summarized as BBLEXBSB. This sequence does not exactly match any of the motifs for heparin binding adduced by Hileman *et al.* (1998). Similarly this region of E^{ns} does not match the other motifs: the XBBBXXB motif, typified by IL8, or the TXXBXXTBXXXBTBB motif, typified by fibroblast growth factors.

There is a wide range of diverse interactions between cell surface proteoglycans and proteins or glycoproteins. Normal physiological interactions range from coagulation cascades, growth factor signalling and cell adhesion to lipase binding (Davies *et al.*, 2001; Feldman *et al.*, 2000; Hirose *et al.*, 2001; Hussain *et al.*, 2000; Koyama *et al.*, 1991; Wuppermann *et al.*, 2001). Likewise, viruses interact with proteoglycans in a number of ways. Several viruses may use cell surface proteoglycans as a step in virus attachment to cells but other virus molecules are not involved in the binding of virus to cells. Two well-studied examples of virus particles binding to GAGs are worth considering.

The importance of binding to cell surface GAGs of members of the family *Herpesviridae* in virus entry has recently been reviewed (Rajcani & Vojvodova, 1998). Herpes simplex virus (HSV) contains at least two glycoproteins that bind to GAGs, gB and gC, and the residues involved in binding to GAGs have been identified. In the alphaherpesviruses pseudorabies virus and bovine herpesvirus-1 the gC glycoproteins contain multiple HBDs (Flynn & Ryan, 1996; Liang *et al.*, 1993), whereas the gC of HSV-1 appears to have a single GAG-binding region (Mårdberg *et al.*, 2001; Tal-Singer *et al.*, 1995; Trybala *et al.*, 1994). Clearly, at least some herpesviruses interact with GAGs through multiple proteins in multiple interactions.

On the other hand, the crystal structure of foot-and-mouth disease virus (FMDV) associated with GAGs has revealed that there is a single site of interaction between GAGs and the virus particle. The interaction is formed by three virus capsid proteins (Fry *et al.*, 1999) with special importance associated with arginine at position 56 of VP3. This residue in field strains is histidine but arginine is selected on passage in tissue culture, which parallels an increased affinity for heparin but attenuation for cattle (Sa-Carvalho *et al.*, 1997). The adaptation of FMDV to tissue culture may be analogous to the adaptation of CSFV to tissue culture. There is no structural information available for pestivirus glycoproteins and whether variation in the affinity of the E^{ns} glycoprotein of BVDV for GAGs affects virulence is not known. For BVDV and BDV, we do not know whether there is a selective pressure in culture to increase the

affinity of the virus for GAGs. However, the highly conserved nature of the GAG-binding region in both BVDV and BDV and the absence of the variant amino acids in that region observed in CSFV prompts us to suggest that adaptation of GAG binding in tissue culture is not a major issue. However, the mutation of a single region in BVDV E^{ns} and the alteration of the affinity for heparin in CSFV close to this site suggest that there is a single GAG-binding motif in E^{ns}.

The binding of cytokines and growth factors to GAGs is thought to be an important mechanism favouring a paracrine action rather than a systemic action. It is hypothesized that binding to sulphated polysaccharides may focus the action of soluble polypeptides locally. Examples include many interleukins (for example, see Mummery & Rider, 2000) and IFN- γ (Lortat-Jacob & Grimaud, 1991; Lortat-Jacob *et al.*, 1995). Interaction with proteoglycans local to the site of infection also may be an important way for viruses to influence their local environment. This strategy seems to have been utilized by some poxviruses. Many secreted poxvirus proteins bind to GAGs; examples include a complement control protein (Smith *et al.*, 2000) and the chemokine antagonists MC148 from *Molluscum contagiosum* (Damon *et al.*, 1998) and MT-1 from myxoma virus (Seet *et al.*, 2001). The GAG-binding activity of these virus proteins may well retain the activity of these polypeptides local to the site of virus infection acting in a paracrine fashion.

E^{ns} is a structural component of the virus particle but it is also secreted from infected cells. E^{ns} has the opportunity to act not only in virus entry but also as a paracrine mediator of an undefined activity. It is possible that an unknown function of E^{ns} as a secreted glycoprotein may require its GAG-binding activity. Whether such a function is required during the acute phase of infection or in establishing a persistent infection is open to investigation. Identification of the residues involved in the interaction between E^{ns} and glycosaminoglycan proteoglycans should promote the design of experiments to dissect the function of this glycoprotein in the natural history of pestiviruses.

Note added in proof. Langedijk (*Journal of Biological Chemistry* **277**, 5308–5314, 2002) has recently shown that peptides corresponding to the C-terminal domain of E^{ns} were able to enter the cell and localize to nucleoli. In his study, deletion of the region identified here as the GAG-binding motif of E^{ns} abrogated that activity.

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