Interactions of bovine viral diarrhoea virus glycoprotein Erns with cell surface glycosaminoglycans

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Recombinant Erns glycoprotein of bovine viral diarrhoea virus (BVDV) has been tagged with a marker epitope or linked to an immunoglobulin Fc tail and expressed in insect and mammalian cell lines. The product was shown to be functional, both having ribonuclease activity and binding to a variety of cells that were permissive and non-permissive for replication of BVDV. Addition of soluble Erns to the medium blocked replication of BVDV in permissive cells. Binding of epitope-tagged Erns to permissive calf testes (CTe) cells was abolished and virus infection was reduced when cells were treated with heparinases I or III. Erns failed to bind to mutant Chinese hamster ovary (CHO) cells that lacked glycosaminoglycans (pgsA-745 cells) or heparan sulphate (pgsD-677 cells) but bound to normal CHO cells. Erns also bound to heparin immobilized on agarose and could be eluted by heparin and by a high concentration of salt. Flow cytometric analysis of Erns binding to CTe cell cultures showed that glycosaminoglycans such as heparin, fucoidan and dermatan sulphate all inhibit binding but dextran sulphate, keratan sulphate, chondroitin sulphate and mannan fail to inhibit binding. The low molecular mass polysulphonated inhibitor suramin also inhibited binding to CTe cells but poly-L-lysine did not. Furthermore, suramin, the suramin analogue CPD14, fucoidan and pentosan polysulphate inhibited the infectivity of virus. It is proposed that binding of Erns to cells is through an interaction with glycosaminoglycans and that BVDV may bind to cells initially through this interaction.

Introduction

Bovine viral diarrhoea virus (BVDV), classical swine fever virus (CSFV) and border disease virus (BDV) are members of the genus Pestivirus in the family Flaviviridae and are associated with a variety of disorders of cattle, pig, sheep, deer and giraffe (Thiel et al., 1991; Dekker et al., 1995; van Rijn et al., 1997; Becher et al., 1997; Wengler et al., 1995). The family Flaviviridae also includes viruses of major human importance including yellow fever virus, dengue virus and hepatitis C virus. Strains of BVDV can be classified into two genotypes, type 1 (BVDV-1) and type 2 (BVDV-2) (Pellerin et al., 1994; Ridpath et al., 1994). Viruses of either genotype may exist as one of two biotypes, non-cytopathogenic (NCP) or cytopathogenic (CP), as defined by the absence or presence of cytopathic effects in cell culture in which cells infected with CP virus die through apoptosis (Lee & Gillespie, 1957; Gillespie et al., 1960; Ridpath et al., 1994; Zhang et al., 1996). Co-infection with a CP virus and a related NCP virus can cause mucosal disease of cattle (Brownlie, 1991).

The pestivirus genome comprises positive-strand RNA that is translated to form a single virus polyprotein which is processed by a combination of cellular and viral proteases to produce the mature virus proteins (Collett et al., 1988a). The structural proteins comprise the nucleocapsid protein C and three envelope glycoproteins, Erns, E1 and E2 (Donis & Dubovi, 1987a, b; Collett et al., 1988b; Thiel et al., 1991). Studies on the interactions between the three glycoproteins have been carried out for CSFV. Initially, Erns and E1 are synthesized as a heterodimer (Erns/E1 precursor), but at later stages of polypeptide processing, Erns forms a disulphide-bonded homodimer (Rümenapf et al., 1993; König et al., 1995), whereas E2 forms a disulphide-linked homodimer and also a heterodimer with E1 (Weiland et al., 1990; Rümenapf et al., 1991). Erns and E2 are located at the surface of infected cells (Weiland et al., 1999), induce virus-neutralizing antibodies (Donis et al., 1988; Weiland et al., 1990, 1992) and both have

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been shown to elicit protective immunity (Hulst et al., 1993; König et al., 1995; Rümenapf et al., 1993; van Zijl et al., 1991).

The virus functions of glycoprotein E\textsuperscript{NS}, which is secreted into the extracellular environment (Rümenapf et al., 1993), are not yet fully understood and the mechanism by which this protein is bound to the virion surface remains to be elucidated (Weiland et al., 1992). E\textsuperscript{NS} has been identified as a ribonuclease and this enzymatic activity is inhibited by virus-neutralizing antibodies but not by reduction and deglycosylation (Windisch et al., 1996; Hulst et al., 1994; Schneider et al., 1993). CSFV E\textsuperscript{NS} is able to induce apoptosis in lymphocytes of several species (Bruscheck et al., 1997).

The initial events in the cycle of BVDV infection are not known, and therefore it is important to identify the initial virus-host interactions necessary for BVDV infection, not only for the general understanding of pestivirus infection but also for the effective control of this virus. Previous studies showed that monoclonal antibodies directed against two bovine cell surface proteins (60 and 90 kDa) were able to inhibit virus infection (Schelp et al., 1993) and, in addition, Xue & Minocha (1993) identified a 50 kDa cell surface protein as a putative E2-specific receptor. Recently, E\textsuperscript{NS} and E2 were found to inhibit the infection of cultured pig kidney cells by CSFV in two distinct ways (Hulst & Moormann, 1997) and E\textsuperscript{NS} also showed binding to cells that were non-permissive for virus replication (Hulst & Moormann, 1997). Here, we provide both biochemical and genetic evidence that cell surface glycosaminoglycans (GAGs) can serve as receptors for the BVDV E\textsuperscript{NS} glycoprotein.

**Methods**

**Cells and viruses.** Chinese hamster ovary cells (CHO-K1) and CHO-K1 mutant cells (GAG-deficient, pgSA-745; and heparan sulphate-deficient, pgSD-677) were obtained from the ATCC and grown in Ham's F-12 medium supplemented with 10% BVDV-free foetal calf serum (FCS). Insect (Schneider S2; Drosophila) cells were obtained from Invitrogen and grown in DES expression medium (Invitrogen) containing 10% FCS at room temperature. Calf testes cells (CTe) were maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% FCS, 5% lactalbumin hydrolysate and 0.1% penicillin G and streptomycin. Porcine (PK15) cells were grown in EMEM supplemented with 10% FCS, 1% non-essential amino acids (NEAA) and 1% tryptose phosphate broth. Bovine (MDBK), canine (MDCK) and human (Hep2) cells were grown in EMEM containing 10% FCS and 1% NEAA. Monkey (Cos-7), human (HEla) and murine (3T6) cells were maintained in Dulbecco's modified minimum essential medium supplemented with 10% FCS. All of these cells were free of BVDV contamination and maintained at the Institute for Animal Health, Compton, UK. Working stocks (passage no. 12) of CP and NCP BVDV strain Pe515 (Pocock et al., 1987) were prepared using CTe cells.

**Plasmid construction.** PCR was used to amplify a DNA fragment encoding E\textsuperscript{NS} of BVDV (NCP) strain Pe515 corresponding to amino acids 268–494 of the BVDV reference strain NADL polyprotein in a 12-cycle reaction using Pfu DNA polymerase (Stratagene). The primers used were: 5' ATTCAAGTTCAAGATCTGAAAAATAAAC 3', which corresponded to nucleotides 1176–1207 of the NADL genome sequence modified to include a BglII recognition site on the 5' end of the E\textsuperscript{NS} sequence; and 5' CAATAAGGGCTTAGCCCTATGTCCTCAAA 3', which corresponded to nucleotides 1862–1891 of the NADL genome sequence in an antisense orientation modified by the inclusion of XbaI recognition site. For the Drosophila expression system, the Xbal- and BglII-digested PCR product was cloned into pMT/Bilp/V5-His (Invitrogen) cut with Xbal and BglII. The shuttle vector (pMT/ Bilp/E\textsuperscript{NS}/V5-His; abbreviated to pE\textsuperscript{NS}V5) and control vector (pMT/Bilp/GFP/V5-His; abbreviated to pGFP/V5) were individually co-transfected with the hygromycin-responsive selection vector pCoHyGRO (Invitrogen) at a ratio of 29:1 into insect (S2) cells. Transformation was performed using a calcium phosphate transfection kit (Invitrogen) according to the supplier’s instructions.

The sense primer used for the mammalian expression system was 5' ATTCAGTTCAAGATCTGAAAAATAAAC 3', which corresponded to nucleotides 1178–1207 of the NADL genome incorporating a HindIII recognition site at the 5' end of the E\textsuperscript{NS} sequence. The reverse primer, with an Xbal site at the 3' end of the E\textsuperscript{NS} sequence, was 5' CAATAAGGGCTTAGCCCTATGTCCTCAAA 3', which corresponded to nucleotides 1862–1891 of the NADL genome sequence. The PCR product was digested with HindIII and Xbal and cloned into the Signal/plg/Fc plasmid (R&D Systems). The recombinant plasmid (Signal/plg/E\textsuperscript{NS}/Fc) and control plasmid (Signal/plg/CD44/Fc) were used for transient transfection of COS-7 cells using FuGENE6 transfection reagent (Roche Biochemicals) according to the supplier’s instructions.

**Expression and purification.** Drosophila S2 cells harbouring the pE\textsuperscript{NS}V5 were grown in DES expression medium without FCS in 175 cm² flasks and expression of V5-tagged E\textsuperscript{NS} (E\textsuperscript{NS}V5) was induced by the addition of copper sulphate (500 µM final concentration) when the cell density reached 4 × 10⁴–6 × 10⁵ cells/ml. After 2–3 days post-induction, the cells were harvested and the concentration of E\textsuperscript{NS}V5 was determined by ELISA with reference to known concentrations of purified protein. The culture supernatant was concentrated 10-fold by ultrafiltration and loaded onto a 2 ml metal affinity column (TALAN; Clontech) equilibrated and washed with buffer A (50 mM Tris–HCl, pH 8.0). A second wash was performed with buffer A containing 20 mM imidazole and the histidine-tagged protein was then eluted with buffer A containing 100 mM imidazole. Fractions containing E\textsuperscript{NS}V5 were pooled and dialysed against buffer A.

For the purification of E\textsuperscript{NS} linked to an immunoglobulin Fc (E\textsuperscript{NS}/Fc) expressed in mammalian cells, the COS-7 cell culture supernatant was adjusted to (pH 8.0) by adding 1/10 volume of 1 M Tris–HCl (pH 8.0) and applied to a 1 ml protein A-agarose (Sigma) column. The column was equilibrated and first washed with 100 mM Tris–HCl (pH 8.0) and then 10 mM Tris–HCl (pH 8.0). The E\textsuperscript{NS}Fc was eluted with 100 mM glycine, pH 3.0, and the fractions were immediately neutralized by adding 1/10 volume of 1 M Tris–HCl, pH 8.0.

**Immunoblot analysis.** Cell culture supernatants containing recombinant E\textsuperscript{NS} were subjected to SDS–PAGE in the presence or absence of 2-mercaptoethanol and electrophoretically transferred to a PVDF membrane. The membrane was blocked with 5% skimmed milk in PBS and incubated with an anti-V5-HRP antibody [specific for the V5 epitope (Invitrogen)] and detected by ECL (Amersham).

**Plaque reduction assay**

(i) Effect of E\textsuperscript{NS}. Confluent monolayers of CTe cells grown in 6-well tissue culture plates were washed twice with maintenance medium (EMEM) without FCS and pre-incubated for 1 h at 37 °C with 500 µl EMEM containing concentrations of E\textsuperscript{NS}V5 (0–50 µg/ml) or with
500 µl EMEM with a control protein (V5 epitope tagged GFP, GFP/V5His) at the same concentration. Following the preincubation period, 50 p.f.u. CP virus stock previously diluted in 500 µl EMEM was added to the cells, mixed and then incubated for 1 h. After 1 h, the virus inoculum was removed and the cells were rinsed once with EMEM and overlaid with maintenance medium containing 1% agarose. The cells were then incubated at 37 °C for 3 days, after which time they were stained with toluidine blue (0-1% toluidine blue, 4% formaldehyde in PBS) for 1 h at room temperature.

(ii) Effect of GAGs and GAG analogues. Confluent monolayers of CTe cells grown in 6-well tissue culture plates were washed twice with EMEM without FCS and pre-incubated for 1 h at 37 °C with 500 µl EMEM containing the individual GAG or GAG analogue. These were: 0-20 mg/ml heparin, chondroitin sulphate, dermatan sulphate, dextran sulphate and a suramin analogue CPD1 (Braddock et al., 1994); 0-10 mg/ml suramin and the suramin analogue CPD14 (Braddock et al., 1994); or 0-5 mg/ml fucoidan and pentosan polysulphate. Following the preincubation period, 50 p.f.u. CP virus stock previously diluted in 500 µl EMEM was added to the cells, mixed and then incubated for 1 h. After 1 h, the virus/GAG mixture was removed and the cells were rinsed once with EMEM and overlaid with maintenance medium containing 1% agarose. The cells were then incubated at 37 °C for 3 days and the plaques were detected as above. The concentration of GAGs or GAG analogues required to reduce virus binding by 50% (IC50) was calculated using the method of moving averages (Thompson, 1947).

■ Detection of RNase activity. RNase activity was determined using equal amounts of recombinant purified EFn/V5 and control protein GFP/V5His (expressed and purified in the same way as EFn/V5). The protein was incubated with 0-5 µg 16-235 RNA (Roche Biochemicals) for 1 h at 37 °C in 50 mM Tris–HCl (pH 8.0), 10 mM MgCl2 and 100 mM NaCl containing 4 units/ml RNase inhibitor (from human placenta, RNasin; Promega). Following electrophoresis on a 1% agarose gel, the samples were stained with ethidium bromide.

■ Binding of EFn to immobilized heparin. Culture supernatant containing EFn/V5 was concentrated 10-fold by ultrafiltration through a 10000 MW cut-off stirred filtration unit (Amicon) and 2 ml (4 µg/ml) was loaded onto a 1 ml heparin–agarose column (Sigma) previously equilibrated with PBS. The column was washed with PBS and eluted with 1000 µl (600 mg/ml) of heparin in PBS. In a second experiment, the heparin–agarose column was washed and equilibrated with 50 mM Tris–HCl (pH 8.0) and the EFn/V5 was eluted with a step-gradient of increasing concentration of NaCl (0–2 M) in equilibration buffer. Fractions were analysed by ELISA by coating a 96-well plate with dilutions of each chromatography fraction overnight at 4 °C. Following binding, the wells were blocked with 5% skimmed milk and incubated with peroxidase-conjugated anti-V5 antibody. Bound antibody was detected with tetramethyl benzidine (TMB) substrate and the absorbance was measured at 630 nm.

■ Detection of EFn binding by immunofluorescence. Cells were grown to approximately 60–80% confluency on 12-mm-round glass coverslips in 24-well tissue culture dishes. Coverslips were rinsed with PBS and then incubated at room temperature for 1 h with 1 µg/ml purified EFn/V5. After washing with PBS, the cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, then rinsed several times with PBS, and blocked with PBS containing 5% normal goat serum and 0.1% sodium azide (GS wash buffer). Cells were incubated with 10 µg/ml anti-V5 monoclonal antibody (Invitrogen) in blocking buffer for 1 h at room temperature in a humidified chamber. After rinsing several times with PBS, cells were treated with FITC-conjugated goat anti-mouse IgG (Sigma) diluted 1:60 in GS wash buffer. Coverslips were rinsed and then mounted in mounting buffer (80% glycerol, 2.5% DABCO (1,4-diazabicyclo[2.2.2]octane) in PBS) and were viewed with a Leica TCS confocal laser microscope.

■ Heparinase treatment of cell monolayers. To determine EFn binding, monolayers of CTe cells were washed with PBS and then incubated with heparinase I and/or III (Sigma) at a concentration of 10 Sigma units/ml (600 Sigma units equivalent to 1 IU) in PBS for 1 h at 37 °C. The cells were then incubated with EFn/V5, fixed and processed as described above.

To determine the effect of virus infection on the heparinase-treated cells, the cells were washed with PBS and incubated with heparinase I (Sigma) at a concentration of up to 600 Sigma units/ml in PBS containing 0.1% BSA and 0.1% glucose (digestion buffer) for 1 h at 37 °C. Following the preincubation period, heparinase I and mock-treated cells were incubated with 50 p.f.u. CP virus diluted in digestion buffer and further incubated for 20 min at room temperature. The virus inoculum was removed and the cells were washed twice with EMEM and overlaid with maintenance medium containing 1% agarose. Monolayers were stained as described above. The results were compared by Student’s t-test and expressed as the mean ± standard error of mean.

■ Flow cytometric analysis. CTe cells were grown overnight in 75 cm2 flasks, washed with EMEM and removed from the flask using a rubber policeman. The cells were resuspended in maintenance medium containing 1 µg/ml EFn/V5 and 0.5 mg/ml of the following GAGs: heparin from bovine lung; fucoidan from Fucus vesiculosus; dermatan sulphate from bovine mucosa; chondroitin sulphate A from bovine trachea; keratan sulphate from bovine cornea; mannan from Sacccharomyces cerevisiae; suramin (Sigma); poly-L-lysine (Sigma); and dextran sulphate (Sigma), and incubated at room temperature for 1 h. The cells were washed three times with GS wash buffer. Bound EFn/V5 was detected by incubation with anti-V5 antibody (10 µg/ml in GS wash buffer) for 30 min at 4 °C. After washing as above, the cells were incubated for 30 min with FITC-conjugated goat anti-mouse IgG antibody (1:200 dilution in GS wash buffer). Cells were then washed twice with GS wash buffer and resuspended in PBS containing 1% BSA and 0.1% sodium azide. Fluorescence intensity of single cells was measured using a FACScan flow cytometer (Becton Dickinson).

Results

Production and characterization of recombinant EFn

A cDNA fragment encoding the 227 amino acid polypeptide of EFn was obtained by PCR amplification from a cloned plasmid containing a cDNA from NCP BVDV strain PeS15. The PCR product was inserted into plasmids from Drosophila (pMT/Bip/V5-His) and mammalian (Signal/plg plus) expression systems. The resulting plasmids were pMT/Bip/EN/v5-V5-His (which produced EFn/V5) and Signal/plg/EN/Fc (which produced EFn/Fc), respectively. For expression in mammalian cells, transient transfection of COS-7 cells was performed; whereas insect (S2) cells were transfected and selected for stable expression of recombinant proteins. The overall yields of EFn in the mammalian and insect expression systems were approximately 100 and 400 µg/l, respectively. Purified recombinant EFn produced in insect (S2) and COS-7 cells showed similar cell surface binding and RNase activities as described below. In this report, we detail the results obtained using the insect (S2) cell line-derived recombinant EFn.
Fig. 1. Characterization of recombinant Erns produced in insect (S2) cells. (a) Western blot analysis of ErnsV5 (lanes 1 and 3) and control GFP/V5His (lanes 2 and 4) fusion proteins expressed in Drosophila (S2) cells and separated by 10% SDS–PAGE under non-reducing (lanes 1 and 2) and reducing (lanes 3 and 4) conditions, and probed with HRP-conjugated anti-V5 antibody in conjunction with a chemiluminescent substrate. (b) Detection of Erns ribonuclease activity. 16–23S rRNA (mock, lane 1) treated with ErnsV5 (lane 2) and with control protein GFP/V5His (lane 3). The samples were incubated for 1 h at 37 °C in 50 mM Tris–HCl, pH 8–0, 10 mM MgCl₂, 100 mM NaCl containing 4 units/µl RNase inhibitor and analysed on 1% agarose gel and stained with ethidium bromide.

Western blot analysis of the culture medium using anti-V5 epitope monoclonal antibodies revealed monomeric and dimeric forms of ErnsV5 under non-reducing conditions with apparent molecular masses of approximately 44 and 84 kDa, respectively. After reduction with 2-mercaptoethanol, the corresponding monomers show two bands of approximately 42–46 kDa (Fig. 1a).

Ribonuclease activity of insect cell-derived ErnsV5, purified on a metal chelating resin, was detected using a 16–23S rRNA as a substrate in the presence of RNasin. In contrast, no ribonuclease activity was observed with a control protein, GFP/V5His, produced in S2 cells and purified using the same protocol (Fig. 1b).

Recombinant ErnsV5 was further examined by testing its ability to inhibit plaque formation. Cells were incubated with ErnsV5 and control protein GFP/V5His and infected with CP BVDV virus. As shown in Fig. 2, the addition of ErnsV5 during the period of virus attachment reduced plaque formation in a concentration-dependent manner. The effect was specific for ErnsV5 as the control protein GFP/V5His has no effect on plaque number over the same concentration range. These results confirm that recombinant BVDV Erns similarly shares the characteristics of Erns of CSFV.

Fig. 2. Inhibition of infection of BVDV by Erns. CTe cells were pre-incubated for 1 h with 500 µl medium containing (a) ErnsV5 (●) and (b) control protein GFP/V5His (■) at the indicated concentrations. Subsequently, 500 µl medium containing CP virus was added and the cells were further incubated for 1 h, washed and overlaid with medium containing 1% agarose. After incubation for 3 days, the cells were fixed, and stained with toluidine blue.

Fluorescence intensity

Fig. 3. Flow cytometric analysis of interactions between cell surface GAGs and Erns. CTe cells were incubated with (a) ErnsV5 (filled-in histograms) or control protein GFP/V5His (open histograms) and examined by flow cytometry. (b–j) CTe cells were treated with 1 µg/ml ErnsV5 alone (filled-in histograms) or 1 µg/ml Erns V5 and 500 µg/ml GAGs or analogues (open histograms): (b) heparin, (c) suramin, (d) fucoidan, (e) dermatan sulphate, (f) chondroitin sulphate, (g) keratan sulphate, (h) dextran sulphate, (i) poly-L-lysine, (j) mannan. The cells were then washed and probed with anti-V5 antibody and FITC-conjugated secondary antibody and examined by flow cytometry.
BVDV glycoprotein binding to cell surface GAGs

Erns binding to the cell surface

Binding of BVDV ErnsV5 to the surface of a wide variety of cells, both permissive [CTe (exemplified in Fig. 3), MDBK and PK15, data not shown] and non-permissive [CHO-K1 (exemplified in Fig. 4), HeLa, MDCK, COS-7, 3T6 and Hep2, data not shown] for BVDV replication was examined. Detection of Erns on the cell surface was done with a monoclonal antibody to the V5 epitope and the results showed that ErnsV5 was able to bind to each cell type examined. A control protein with the same epitope tag, GFP-V5, did not bind any of the cells.

We hypothesized that Erns may bind to cell surface proteoglycans. To test this hypothesis, we analysed the ability of soluble GAGs to inhibit the binding of ErnsV5 to the cell surface. ErnsV5 (1 µg/ml) and GAGs (at a concentration of 500 µg/ml) were mixed and incubated with cultured cells for 1 h at room temperature. Following incubation, the cells were rinsed and assayed for ErnsV5 binding by flow cytometry (Fig. 3a) and by immunofluorescence microscopy (data not shown). Co-incubation of heparin, suramin, fucoidan and dermatan sulphate with ErnsV5 resulted in a marked inhibition of ErnsV5 binding (Fig. 3b, c, d, e) whereas chondroitin sulphate, keratan sulphate, dextran sulphate, poly-l-lysine and mannan had no significant effect on ErnsV5 binding (Fig. 3f, g, h, i, j).

Treatment of cells with heparinases

To investigate whether Erns binding to the cell surface was mediated directly through GAGs, CTe cells were treated with specific GAG lyases, followed by assessment of ErnsV5 binding. Heparinase I, which degrades heparin and highly sulphated domains in heparan sulphate and heparinase III (specific for heparan sulphate) were added to cells at a concentration of 10 units/ml in medium at 37 °C for 1 h and the cells were then fixed with paraformaldehyde. ErnsV5 binding to cells was clearly reduced following pre-treatment of the cells with either heparinase I or heparinase III (Fig. 4).

Binding to GAG-deficient mutant cell lines

To characterize further the nature of cell-surface GAGs as Erns receptors, the binding of ErnsV5 to wild-type CHO cells and mutant CHO cell lines that had defects in the production of specific GAGs was examined. Although binding of ErnsV5 to the wild-type CHO cells was easily detectable, no binding of ErnsV5 was observed on mutant cells pgsA-745 or pgsD-677 (Fig. 5). The reduced binding of ErnsV5 to the heparan sulphate/GAG-deficient mutant cells pgsA-745 and pgsD-677, indicated that the presence of heparan sulphate proteoglycan is a principal requirement for Erns attachment to the cell surface. Furthermore, no binding to pgsD-677 (which is
heparan sulphate/GAG-deficient but produces three times more chondroitin sulphate; Esko et al., 1985) demonstrated that E\textsuperscript{V5} exhibited at least some specificity for heparan sulphate or heparin.

These results indicated that the heparin or heparan sulphate and not the chondroitin sulphate moieties of cell surface proteoglycans serve as receptors for the BVDV E\textsuperscript{V5} glycoprotein. In addition, the reduced binding of E\textsuperscript{V5} to pgsD-677 cells and the inability of chondroitin sulphate and dextran sulphate to inhibit binding suggest that the degree of sulphation may be an important factor influencing binding of E\textsuperscript{V5} to proteoglycans.

### Inhibition of virus infection by GAGs, GAG analogues and heparinase treatment

To correlate the results obtained with recombinant E\textsuperscript{V5}, virus infection experiments were performed to determine whether soluble GAGs or GAG analogues could inhibit infection of CTe cells by BVDV. Suramin and the suramin analogues CPD1 and CPD14, fucoidan, pentosan polysulphate, heparin, chondroitin sulphate, dermatan sulphate and dextran sulphate were incubated with permissive cells both prior to, and during, virus binding. The effect of these compounds on virus infection was determined by reduction in the plaque titre. Results of these studies show that addition of suramin, the suramin analogue CPD14, fucoidan and pentosan polysulphate can reduce plaque number in a concentration-dependent manner (Fig. 6); the concentrations required to reduce virus infection by 50% were 2.87, 5, 0.15 and 0.62 mg/ml, respectively. In contrast, heparin, chondroitin sulphate, dermatan sulphate, dextran sulphate and the suramin analogue CPD1 did not have any significant effect on virus infectivity.

In addition, the effect of heparinase treatment of permissive cells on virus infectivity was examined. Treatment of cells with heparinase prior to infection reduced significantly (P < 0.01) the virus titre by approximately 50% compared to mock-treated controls (treated, 10\textsuperscript{-0.05} plaques per well; untreated, 19.62 ± 0.62 plaques per well).

### Binding of E\textsuperscript{V5} to immobilized heparin

To examine whether E\textsuperscript{V5} binds directly to GAGs, recombinant E\textsuperscript{V5} was applied to immobilized heparin–agarose. The majority of E\textsuperscript{V5} bound to the column and was eluted with 1000 units/ml (6.5 mg/ml) soluble heparin and by 0.5 M and 1 M NaCl (Fig. 7). These data confirmed that E\textsuperscript{V5} can bind to charged GAGs in vitro and is consistent with a direct interaction of E\textsuperscript{V5} with sulphated GAGs present on the surface of cells.
**Discussion**

To study the interaction of BVDV glycoprotein E\textsuperscript{rns} with the cell surface, we have cloned recombinant E\textsuperscript{rns} into mammalian and *Drosophila* expression vectors. As expected, the recombinant E\textsuperscript{rns} displayed very similar biological activities as those reported for E\textsuperscript{rns} from CSFV (Windisch et al., 1996; Schneider et al., 1993; Hulst et al., 1994; Hulst & Moormann, 1997). The glycoprotein was secreted into the culture medium as a monomer and as a disulphide-linked homodimer, it contained ribonuclease activity, it inhibited BVDV infection of cultured cells and bound to cells both permissive and non-permissive for BVDV replication.

We demonstrated that BVDV E\textsuperscript{rns} binding to cells was inhibited by soluble GAGs and we hypothesize that E\textsuperscript{rns} binds directly to GAGs on the cell surface since enzymatic treatment of cells with heparinase abolishes E\textsuperscript{rns} binding to cells. Moreover, recombinant E\textsuperscript{rns} can bind to heparin immobilized on agarose and could be eluted with heparin and relatively high salt concentration. A variety of GAGs and polyanions were tested for their ability to inhibit E\textsuperscript{rns} binding to cells and the results indicated that soluble GAGs (heparin, fucoidan and dermatan sulphate) inhibited binding whereas chondroitin sulphate, keratan sulphate and mannan failed to inhibit binding. From these experiments, we conclude that E\textsuperscript{rns} binding showed some specificity to GAGs. This conclusion is supported by the experiments that examined E\textsuperscript{rns} binding to mutant CHO cells in which GAG biosynthesis was defective. E\textsuperscript{rns} failed to bind to cells with greatly reduced levels of GAGs (pgsA-745), but also failed to bind to cells that were unable to make heparan sulphate (pgsD-677) but presented chondroitin sulphate on the cell surface (Esco et al., 1985; Lidholt et al., 1992). Overall, our data indicated that E\textsuperscript{rns} binding requires heparan sulphate and not chondroitin sulphate moieties, although we have not yet identified the specific sugar sequence required for E\textsuperscript{rns} binding. Heparan sulphate GAGs consist of repeating disaccharide units composed of alternating glucosamine monosaccharides. The specificity exhibited by E\textsuperscript{rns} for heparan sulphate moieties demonstrates that E\textsuperscript{rns} may interact with a glucosamine–hexuronic acid backbone. Further, since excess soluble dermatan sulphate could inhibit E\textsuperscript{rns} binding, E\textsuperscript{rns} may also interact with a heparan sulphate backbone that contains iduronic acid.

E\textsuperscript{rns} is secreted from infected cells but it is also a structural component of the virus particle (Rümenapf et al., 1993; Weiland et al., 1992). We have shown that the polysulphonated compounds suramin and fucoidan blocked both E\textsuperscript{rns} binding to cells and also inhibited virus replication in tissue culture. These observations suggest that E\textsuperscript{rns} binding to the cell surface plays a role in virus replication. However, we failed to observe inhibition of virus replication with heparan sulphate and heparinase treatment of CTe cells did not completely block virus infection. In the case of dengue virus, hepatitis C virus and adeno-associated virus type 2, highly sulphated forms of GAGs are required in order to inhibit infectivity (Chen et al., 1997; Garson et al., 1999; Summerford & Samulski, 1998). It is possible that the failure of heparin sulphate to block infection of CTe cells by BVDV may be similar determined by the degree of sulphation of heparan sulphate. The preparation of heparan sulphate used in our studies was clearly sufficient to inhibit monomeric or dimeric binding of E\textsuperscript{rns} but possibly not when E\textsuperscript{rns} was associated with the virus particle.

Heparinase treatment of CTe cells reduced infection by approximately 50%. This level of inhibition is somewhat lower than that observed with some other viruses that bind to heparan sulphate moieties. For example, although infectivity of cells treated with heparinase was reduced only by 50% for Sindbis virus (Mastromarino et al., 1991; Byrnes & Griffin, 1998), for other viruses the reduction of infectivity following heparinase treatment of cells was higher: reductions of infectivity of 80–90% for herpes simplex viruses (HSV) (WuDunn & Spear, 1989), of 66% for adeno-associated virus (Summerford & Samulski, 1998) and of 70–80% for foot-and-mouth disease virus (Jackson et al., 1996) have been reported. Nevertheless, reduction of BVDV infectivity following heparinase treatment of CTe cells was statistically significant. The reasons for the variation in the effect of heparinase treatment have not been systematically explored. The variable response may be caused by the differences in cell type and the degree to which virus may have adapted in culture to more efficient use of heparan sulphate (Sa-Carvalho et al., 1997). Indeed, HSV that fails to bind heparan sulphate through either gC or gB retains its ability to infect cells (Laquerre et al., 1998). The binding to heparan sulphate may be bypassed for BVDV infection of cultured CTe cells.

Our results show that cell lines from cattle, pig, human, mouse, monkey and dog interact with BVDV E\textsuperscript{rns}; similar results were shown by Hulst & Moormann (1997) for CSFV. However, BVDV infection of mammalian cells shows species specificity. This suggests that infection of cells by BVDV may be dependent on a multistep process in which virus attachment to cells and internalization are two distinct steps requiring different cell surface receptors. This conclusion is supported by the literature. Hulst & Moormann (1997) reported that CSFV glycoproteins E\textsuperscript{rns} and E2 interact with distinct cell surface receptors, whereas Schelp et al. (1995) identified two bovine-specific cell surface proteins of 60 and 90 kDa with monoclonal antibodies which efficiently inhibited the infection of bovine cells with BVDV, and Xue & Minocha (1993) identified a 50 kDa cell surface protein as a potential E2-specific receptor.

There is a growing list of viruses that enter cells by binding to two or more receptors. Herpesviruses, including HSV (WuDunn & Spear, 1989), human cytomegalovirus (Compton et al., 1993), bovine herpesvirus types 1 and 4 (Okazaki et al., 1991; Vanderplasschen et al., 1993), pseudorabies virus (Mettenleiter et al., 1990) and varicella-zoster virus (Zhu et al., 1995), have recently been reported to use GAGs as the initial site of binding to cells. Foot-and-mouth disease virus (Jackson et al., 1999) membrane proteins, and certain heparin-like molecules likely serve as cell attachment receptors for BVDV.
et al., 1996; Sa-Carvalho et al., 1997), dengue virus (Chen et al., 1997), respiratory syncytial virus (Krusat & Steckert, 1997), Sindbis virus (Klimstra et al., 1998), porcine reproductive and respiratory syndrome virus (Jusa et al., 1997), equine arteritis virus (Asagoe et al., 1997) and vaccinia virus (Chung et al., 1998) have also been shown to bind to cells through GAGs. Three-stage binding of virus to cells occurs in human immunodeficiency virus (HIV) infection, in which HIV glycoproteins bind to heparan sulphate proteoglycans and CD4 (Roderiguez et al., 1995; Sattentau & Weiss, 1991) followed by binding to chemokine receptors (Doms & Peiper, 1997). The demonstration here that a pestivirus E\textsuperscript{TM}s binds to cell surface GAGs leads us to postulate that pestivirus attachment and entry into cells is likely to be another example of a complex virus entry mechanism.

Recent work on E\textsuperscript{TM}s from CSF shows that it, like BVDV E\textsuperscript{TM}s, binds to GAGs, and virus replication in vitro is also inhibited by polysulphonated compounds and GAGs (M. M. Hulst & R. J. M. Moormann, personal communication).

Note added in proof. It has recently been shown by Agnello et al. (Proceedings of the National Academy of Sciences, USA 96, 12766–12771, 1999) that the low density lipoprotein receptor can mediate entry of several members of the Flaviviridae, including BVDV, and that BVDV is restricted at a stage beyond virus entry.

We thank Mrs Michelle Hill for providing the initial E\textsuperscript{TM} cDNA clone, Dr Terry Jackson for discussion and for providing GAG-deficient mutant cells, Dr R. Bicknell (John Radcliffe Hospital, Oxford, UK) for kindly providing suramin analogues, Drs Martin Fray and Sue Baigent for critical review of the manuscript and discussion, and Drs Gang Zhang, Ivan Morrison, Bryan Charleston, Trevor Collen and Mr Michael Clarke for comments and discussion.

References


Received 17 May 1999; Accepted 5 November 1999