Passage of Classical Swine Fever Virus in Cultured Swine Kidney Cells Selects Virus Variants That Bind to Heparan Sulfate due to a Single Amino Acid Change in Envelope Protein E\textsuperscript{rms}

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Infection of cells with Classical swine fever virus (CSFV) is mediated by the interaction of envelope glycoprotein E\textsuperscript{rms} and E2 with the cell surface. In this report we studied the role of the cell surface glycoaminoglycans (GAGs), chondroitin sulfates A, B, and C (CS-A, -B, and -C), and heparan sulfate (HS) in the initial binding of CSFV strain Brescia to cells. Removal of HS from the surface of swine kidney cells (SK6) by heparinase I treatment almost completely abolished infection of these cells with virus that was extensively passaged in swine kidney cells before it was cloned (clone C1.1.1). Infection with C1.1.1 was inhibited completely by heparin (a GAG chemically related to HS but sulfated to a higher extent) and by dextran sulfate (an artificial highly sulfated polysaccharide), whereas HS and CS-A, -B, and -C were unable to inhibit infection. Bound C1.1.1 virus particles were released from the cell surface by treatment with heparin. Furthermore, C1.1.1 virus particles and CSFV E\textsuperscript{rms} purified from insect cells bound to immobilized heparin, whereas purified CSFV E2 did not. These results indicate that initial binding of this virus clone is accomplished by the interaction of E\textsuperscript{rms} with cell surface HS. In contrast, infection of SK6 cells with virus clones isolated from the blood of an infected pig and minimally passaged in SK6 cells was not affected by heparinase I treatment of cells and the addition of heparin to the medium. However, after one additional round of amplification in SK6 cells, infection with these virus clones was affected by heparinase I treatment and heparin. Sequence analysis of the E\textsuperscript{rms} genes of these virus clones before and after amplification in SK6 cells showed that passage in SK6 cells resulted in a change of an Ser residue to an Arg residue in the C terminus of E\textsuperscript{rms} (amino acid 476 in the polyprotein of CSFV). Replacement of the E\textsuperscript{rms} gene of an infectious DNA copy of C1.1.1 with the E\textsuperscript{rms} genes of these virus variants proved that acquisition of this Arg was sufficient to alter an HS-independent virus to a virus that uses HS as an E\textsuperscript{rms} receptor.

Classical swine fever virus (CSFV), Bovine viral diarrhea virus (BVDV), and Border disease virus (BDV) are members of the Pestivirus genus within the family of Flaviviridae (10). The viruses are structurally, antigenically, and genetically closely related. BVDV and BDV can infect ruminants and pigs. CSFV infections are restricted to pigs (5). Pestiviruses are small, enveloped, positive-stranded RNA viruses (28). The RNA genome is approximately 12.5 kb in length (2, 7, 26, 29) and contains a single large open reading frame (ORF) (2, 8, 26, 29). This ORF is translated into a polyprotein which is further processed into mature proteins by viral and host cell proteases (33). The envelope of the pestivirus virion contains three glycoproteins: E\textsuperscript{rms}, E1, and E2 (40). In infected animals antibodies are raised against E\textsuperscript{rms} and E2 (25, 45). Until now, no antibodies have been detected against E1 in infected animals.

Glycoaminoglycans (GAGs) are unbranched polysaccharide chains composed of repeated disaccharide sequences that carry sulfate groups in various positions (23). These sulfate groups give the GAG chains a net negative charge. Multiple chains are covalently linked to a protein core forming complex structures (proteoglycans) which are present on the surface of virtually all types of cells and in the extracellular matrix (20, 23). The classification of GAGs is mainly based on the composition of their disaccharide repeats. Common GAGs are chondroitin sulfates (CSs) A, B (dermatan sulfate), and C; keratan sulfate; and heparan sulfate (HS). The sulfate groups in CSs are O linked. The sulfates groups in HS and heparin, a GAG chemically related to HS, are O and N linked. The main difference between heparin and HS is that heparin contains more N- and O-linked sulfate groups (9, 14). In contrast to HS, heparin is not present on the cell surface (23). Besides the interaction of positively charged arginine and lysine-rich amino acid regions with negatively charged sulfate groups of the GAG chains, more specific interactions of amino acids with GAG chains may also be important for binding of proteins to proteoglycans (6, 23, 38).

A wide variety of pathogens, including many viruses, bind to GAGs (32). Examples of viruses that bind to HS are herpes simplex virus (HSV) (47), human immunodeficiency virus type 1 (31), Sindbis virus (SV) (4), and foot-mouth disease virus (FMDV) (21). In most cases, however, binding of these viruses to HS is not sufficient to enter the host cell, and additional, more-specific cell surface receptors are needed to mediate entry (references 4 and references therein). Moreover, for several of these viruses it was demonstrated that passage in cell culture selects virus variants that use HS as receptor to attach to the surface of cells (24, 34).

Entry of pestiviruses into cells is mediated by the interaction of envelope proteins E\textsuperscript{rms} and E2 with the cell surface. Inhibition studies with E\textsuperscript{rms} and E2 produced in insect cells showed that E\textsuperscript{rms} and E2 interact with different cell surface components and that E\textsuperscript{rms} mediates initial binding of pestiviruses to cells.
A 50-kDa, uncharacterized surface protein has been identified as a putative E2 receptor (48). Recently, Iqbal et al. (19) showed that a recombinant E2 protein of BVDV interacts with membrane-associated HS. In the virion, E2 is present as a homodimer with a molecular mass of about 100 kDa (40). About 50% of the mass of E2 is made up of N-linked glycosyl groups (33, 46). E2 lacks a transmembrane-spanning domain, and association with the envelope is accomplished by an as-yet-unknown mechanism. Considerable amounts of E2 are secreted into the extracellular environment (33). In vitro studies showed that recombinant E2 induces apoptosis in infected cells, indicating that it contributes to the immunosuppressive action of pestiviruses (3). The fact that a structural protein of an RNA virus possesses RNase activity makes E2 a unique viral protein (16, 36). Recently, the function of this RNase activity in the replication of pestiviruses was studied using reverse genetics. Inactivation of the RNase activity of E2 in the noncytopathogenic CSFV strain C (a vaccine strain) led to the production of viable cytopathogenic virus which induced apoptosis in infected cells (18). This observation suggests that the RNase activity of E2 is involved in regulation of RNA synthesis in infected cells. Furthermore, inactivation of this RNase activity in a virulent background led to attenuation of CSFV (27).

The fact that E2 of a CSFV vaccine strain also binds to the surface of cells originating from various species and unsusceptible to pestivirus infection (17) suggests that E2 interacts with a widely expressed surface molecule. We studied the role of cell surface GAGs in initial binding of CSFV to cells. We show here that interaction of CSFV E2 with membrane-associated HS facilitates the binding of virus to the cell surface. In addition, we demonstrate that in vitro cultivation of native CSFV in swine kidney cells selects these HS-binding virus variants.

MATERIALS AND METHODS

Cells and viruses. Swine kidney cells SK6 (22) and bovine kidney cells MDBK (ATCC, CCL22) were maintained as described previously (17). Fetal bovine serum (FBS) and cells were free of BDV; the FBS was free of anti-BDV antibodies.

CSFV strain Brescia was isolated from a pig infected with a virulent field isolate (43). To isolate clone C1.1.1, blood of this pig was used to infect PK15 cells (ATCC, CCL33). Virus was grown in PK15 cells for 24 passages before it was cloned by repeating endpoint dilution (three times) on PK15 cells. The cloned virus (C1.1.1) was amplified by three additional passages in PK15 cells and adapted to growth in SK6 cells by two passages. Animal experiments showed that clone C1.1.1 is avirulent (43). A virus stock of CSFV strain C (a vaccine strain) was used to infect SK6 cells and “C” (44). Plaques of BVDV strain B2 were detected using a MAb directed against NS2-3 (17). TGEV plaques were detected using an MAb directed against the spike protein (17). Positive plaques in a well were counted with a microscope. When more than 250 plaques per well were present, a minimum of 100 plaques in a fixed area (at a magnification of 40 times) was counted to calculate the total number of plaques in these wells. The percentage of infection of M24 cells was calculated using the formula: 100 × (1 – [e]), where e is the number of plaques in a well to which no inhibitor was added (control well) and r is the number of plaques in wells to which inhibitor was added.

Treatment of cells with enzymes. Confluent monolayers of SK6, grown in 2-cm² tissue culture wells (M24 plates; Costar), were washed twice with binding buffer (phosphate-buffered saline [PBS] containing 0.2% bovine serum albumin, 0.5 mM CaCl₂, and 0.5 mM MgCl₂) and incubated with 200 μl of binding buffer containing different concentrations of heparinase I or chondroitinase ABC. After incubation for 2 h at room temperature with gentle shaking, the enzyme solutions were removed, and the cells were washed twice with 0.5 ml of binding buffer. The cells were infected with 200 μl of an appropriate virus dilution in binding buffer. After 30 min of infection at 37°C, the virus was removed, and the cells were washed twice with binding buffer, supplied with overlay medium, and further treated as described for a plaque assay. The percentage of infection of infected cells was calculated with the same formula as described above.

Preparation of cell-free virus. Confluent monolayers of SK6 or MDBK cells grown in 175-cm² tissue culture flasks were infected for 90 min with C1.1.1 (SK6) or strain Karevaa (MDBK) virus stocks (see above) at a multiplicity of infection of 1. The virus was removed, and the cells were washed once with complete medium. Fresh medium was added, and the cells were grown for 2 days at 37°C. The culture fluid was collected and clarified by centrifugation for 15 min at 3000 × g. Tired on a 1.10-ml cushion of 2.1 M sucrose in 10 mM Tris-Cl (pH 7.2)–150 mM NaCl and centrifuged at 4°C for 24 h in a Beckmann SW 28 rotor at 85,000 × g. The virus pellet was suspended gently in 0.6 ml of ice-cold 10 mM Tris-Cl (pH 7.2)–150 mM NaCl and used directly for heparin-chromatography or stored in aliquots at −80°C.

Binding to immobilized heparin. Prepacked heparin columns (1 ml, Hitrap-Sepharose; Pharmacia) were pre-eluted with 5 ml of 10 mM phosphate buffer (pH 7.0). A total of 200 μg of purified E2 or E3 was diluted to 1 ml with 10 mM phosphate buffer (pH 7.0). Cell-free virus preparations (300 μl) were diluted with 1.2 ml of 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl. Purified E2, E3, or virus preparations were loaded on Hitrap columns at a flow rate of 1 ml/min using a peristaltic pump. Bound material was eluted at a flow rate of 1 ml/min and concentrated by centrifugation at 1 ml (1.5 ml) and assayed for E2, E3, or virus. A portion (100 μl) of the fractions collected from the chromatography of virus was diluted directly in EMEM supplemented with antibiotics and 10% FBS and titrated in a plaque assay. The osmolarity of 10 mM phosphate (pH 7.0) solutions was measured with a model 3D3 Osmometer (Advanced Instruments, Inc.). The concentration NaCl in fraction 1 was determined by measuring the osmolarity with a model 3D3 Osmometer (Advanced Instruments, Inc.). The concentration NaCl was calculated from a standard curve prepared by measuring the osmolarity of 10 mM phosphate (pH 7.0) solutions with known NaCl concentrations.

Isolation, passage, and analyses of virus clones of CSFV strain Brescia. EDTA-blood of a pig infected with CSFV strain Brescia (see above) was used to infect a 2-cm² tissue culture well with SK6 cells. The well was infected with a dilution of blood in EMEM that corresponded to 5–50 PFU. After 90 min of infection at 37°C, cells were washed twice, supplied with fresh medium, and grown for 3 h at 37°C. Cells were treated with trypsin, suspended in medium, and divided among 480 M96 wells. For 4 days of growth the medium was harvested and infected cells were detected using immunostaining. The medium of seven positive wells (clones) was used to infect SK6 cells grown in 2-cm² tissue culture wells. For 4 days of growth, cells and medium were freeze-thawed twice and clarified to prepare a virus stock (passage number 2, clones Ap2 to Gp2). Clone Ap2 yielded a very low virus titer (<100 PFU/ml). Therefore, clone A was passaged in SK6 cells (passage number 3). Clone Ap2 was passaged for four additional rounds in SK6 cells. For every round of amplification, 100 μl of this virus stock was used to infect SK6 cells grown in 25-cm² tissue culture flask. Cells were grown for 3 days before freezing. The percent inhibition of virus replication (50% reduction of infected cells) was used as the endpoint for these virus clones (at different passage numbers) by 200 μg of heparin per ml or per treatment of cells with 12.5 μl of heparinase I per ml as determined was as...
described above. To determine the sequence of the E^NS genes, RNA was isolated from SK6 cells infected with virus clones. SK6 cells, grown in 2-cm² wells, were infected with 100 μl of virus stocks Ap3, Ap4, Bp2, Bp3, Ep2, and Ep3 diluted in 300 μl of EMEM at 30 min at 37°C. The virus was removed, and the cells were washed twice and supplied with overlay medium. After 2 days of growth, cytoplasmic RNA was extracted and used to determine the sequence of the complete E^NS genes (**18**).

**Construction, generation, and characterization of recombinant viruses.** A full-length DNA copy of clone C1.1.1 of CSFV Brescia strain was constructed by joining cDNA fragments, isolated from pUC19 subclones (**29**), in the low-copy number plasmid pOK12. Construction was performed in the same manner as that described for the full-length DNA of CSFV strain C (**30**). Digestion of this full-length cDNA in pOK12 (named pflc1.1.1) with SfI generated the exact 3' terminus of the RNA genome of C1.1.1. To construct the recombinant viruses flc1.1.1 E^NS (S-ST) and flc1.1.1 E^NS (S-RT) in a standard reverse transcription-PCR reaction, cDNA fragments were generated using RNA isolated from virus clones Bp2 and Bp3 as the template (see above). An 18-mer, 5'-GGGGAGGAGGAGCAGATACAA-3' (nucleotides 527 to 544 in the sequence of CSFV strain Brescia C1.1.1 [**29**]), was used as the forward primer and a 21-mer, 5'--CTTCTCAGGGTGGTAGTGACAC-3' (complementary to nucleotides 2514 to 2534 of C1.1.1), was used as the reverse primer. The amplified DNA fragments, covering the C-terminal part of Npro, the capsid protein (C), E^NS, and E1, were sequenced. After digestion with ClaI and NgoMIV fragments were isolated from agarose gel and used to replace the ClaI-NgoMIV fragment of pflc1.1.1 to give full-length plasmids pflc1.1.1 E^NS (S-ST) and pflc1.1.1 E^NS (S-RT). Sequence analysis showed that the sequence of the ClaI-NgoMIV regions of these plasmids were identical to that of pCR fragments.

SfI-linearized DNA (250 ng) of full-length plasmids pflc1.1.1, pflc1.1.1 E^NS (S-ST), and pflc1.1.1 E^NS (S-RT) was transfected to SK6.T7a5 cells as described recently (**41**). Two days after transfection the medium was harvested and stored at −70°C. 663-hp/tB cells were isolated from agarose gel and used to replace the ClaI-NgoMIV fragment of pflc1.1.1 to give full-length plasmids pflc1.1.1 E^NS (S-ST) and pflc1.1.1 E^NS (S-RT). Sequence analysis showed that the sequence of the ClaI-NgoMIV regions of these plasmids were identical to that of pCR fragments.

RESULTS

**Inhibition of CSFV infection by GAGs.** Binding of E^NS to the cell surface is not limited to porcine and bovine cells susceptible to pestivirus infection (**17**). E^NS of CSFV strain C produced in insect cells binds also tightly, in large amounts, to baby hamster kidney cells, monkey kidney cells, insect cells, and lymphocytes, indicating that C strain E^NS interacts with a widely expressed surface molecule. Therefore, we tested whether the most common GAGs found on the cell surface, CS-A, CS-B, CS-C, and HS, were able to inhibit infection of SK6 cells with CSFV strain Brescia clone C1.1.1. This virus clone was extensively passaged in swine kidney cells before it was cloned. In addition, heparin and DS, a highly sulfated artificial polysaccharide), were tested. In a plaque assay, up to 200 μg of HS and CS-A, -B, and -C per ml did not inhibit the infection of SK6 cells with CSFV strain Brescia clone C1.1.1. This virus clone was extensively passaged in swine kidney cells before it was cloned. In addition, heparin and DS (a highly sulfated artificial polysaccharide) were tested. In a plaque assay, up to 200 μg of HS and CS-A, -B, and -C per ml did not inhibit the infection of SK6 cells with CSFV strain Brescia clone C1.1.1. (Fig. 1; results for CS-A, -B, and -C not shown). In contrast, heparin and DS inhibited C1.1.1 infection in a dose-dependent manner. Nearly 100% inhibition of infection was achieved at 50 μg of heparin and 12.5 μg of DS per ml. Similar concentrations of heparin and DS also inhibited infection of SK6 cells with CSFV strain C, a vaccine strain, almost completely. High concentrations of heparin (200 μg/ml) and DS (100 μg/ml) did not affect the infection of SK6 cells with TGEV, a coronavirus (results not shown). Infection of cultured cells with other viruses that initially bind to cell surface HS, like SV (4) and HSV type 1 (47), was inhibited efficiently by heparin and DS, whereas HS was unable to reduce infection significantly. These studies clearly showed that the degree of sulfation of the heparin-HS-type polysaccharide chain is critical for inhibition of these viruses in cell culture. The fact that completely N-desulfated heparin was unable to inhibit C1.1.1 infection efficiently confirmed that this is also true for CSFV (**Fig. 1**). Thus, initial binding of CSFV strain Brescia C1.1.1 is likely accomplished by interaction with membrane-associated HS.

**Removal of GAGs from the cell surface.** To prove that C1.1.1 initially binds to HS before entering the cell, GAGs were removed from the cell surface of SK6 cells. Cells were treated with heparinase I and chondroitin ABC. Heparinase I hydrolyses the (1→4)glycosidic linkages between glucosamine and iduronic acid, a specific disaccharide repeat of heparin and HS. Chondroitin ABC degrades CS-A, -B, and -C but not heparin or HS. SK6 cells were treated with up to 100 μIU of enzyme per ml for 2 h at 20°C. Chondroitin ABC treatment had no significant effect on the infection of SK6 cells with C1.1.1 (Fig. 2). In contrast, treatment with 6 μIU of heparinase I per ml reduced the infection of SK6 cells with C1.1.1 to 10%. Treatment of SK6 cells with up to 100 μIU of chondroitin ABC or heparinase I per ml did not affect TGEV infection (results not shown).

**Heparin inhibits infection of C1.1.1 particles bound to the cell surface.** CSFV E2 of strain Brescia (clone C1.1.1) and E^NS of CSFV strain C, both produced in insect cells, were shown to inhibit pestivirus infection in cell culture (**17**). In that study, complete inhibition of infection of SK6 cells with C1.1.1 was achieved when E^NS was only present during virus adsorption. In contrast, inhibition of infection by E2 appeared to be re-
versible. To achieve 100% inhibition of infection with C1.1.1, E2 was also needed in the overlay medium after the virus was removed from the cells. When E2 was omitted, about 50% inhibition of infection was achieved (17). Those results showed that, after removal of the virus from the cells, virus particles, which were already attached to the cell surface but were prevented from entering the cell due to competition with E2, were again able to infect cells in the absence of E2. Treatment with E\textsuperscript{rms} released these already-bound virus particles from the cell surface, indicating that E\textsuperscript{rms} and not E2 is responsible for the initial binding of C1.1.1 particles to the cell surface of SK6 cells (17). To determine whether heparin could interfere with the infection of virus particles, which were already bound to the surface of SK6 cells, we performed a similar experiment (Fig. 3). Six 2-cm\textsuperscript{2} tissue culture wells with SK6 cells were infected with C1.1.1 at 4°C. At 4°C virus particles bind to the cell surface but do not penetrate the cell. Subsequently, unbound particles were removed, and three wells were treated (chased) with medium containing 100 μg of heparin per ml and three wells with medium without heparin (Fig. 3, H and V respectively) at 4°C. These chase media were removed from the cells, diluted 10 times, and assayed for virus in a plaque assay. To allow penetration of bound virus particles, cells were supplied with fresh medium and incubated for 60 min at 37°C. After this period, cells were washed and supplied with overlay medium. The average number of plaques in these wells after 24 h of growth (open bars) and the average number of plaques recovered from the chase media (shaded bars) are presented. Two-thirds of the virus particles bound to the cell surface at 4°C were no longer able to infect cells after treatment with heparin. Moreover, most of these virus particles were recovered from the heparin chase medium, indicating that they were released from the cell surface by treatment with heparin. This clearly demonstrated that heparin, like E\textsuperscript{rms} (17), directly interfered with the binding of C1.1.1 particles to the cell surface.

**Binding of recombinant proteins and C1.1.1 virus to immobilized heparin.** The observation that heparin, like E\textsuperscript{rms}, was able to strip bound virus particles from the cell surface, strongly suggested that E\textsuperscript{rms} is responsible for the interaction with cell surface HS rather than E2. To further prove this, in a separate experiment E\textsuperscript{rms} and E2, purified from insect cells, were applied to heparin-Sepharose columns and eluted with increased concentrations of NaCl (Fig. 4A). The NaCl concentration at which proteins elute from the column gives an indication of the strength of the electrostatic interaction. Due to the heterogeneous nature of heparin, affinities of ligands for heparin are in reality average values (9, 11). Therefore, heparin columns from the same batch number were used for all experiments performed in this study. E2 applied to the column at a concentration of 0 mM NaCl eluted also at this NaCl concentration, indicating that E2 did not bind to heparin. E\textsuperscript{rms} eluted as a broad peak at an NaCl concentration of about 750 mM. No residual E\textsuperscript{rms} was recovered when the column was eluted with a higher concentration NaCl (see fraction 16) or with 1 M NaCl containing 4 mg of heparin per ml. This relatively high concentration of NaCl needed to elute E\textsuperscript{rms} demonstrated that positively charged amino acid domains of E\textsuperscript{rms} bind with high affinity to the negatively charged heparin.

To demonstrate that a heparin-HS-type polysaccharide chain alone (without additional cell surface molecules) is sufficient to bind virus particles, C1.1.1 was tested for binding to heparin-Sepharose (Fig. 4B). For this experiment virus was partially purified from the culture fluid and applied to the column at a concentration of 100 mM NaCl. Hundred percent of the virus present in the preparation C1.1.1 bound to heparin and eluted as a single peak at 260 mM NaCl. A partially purified preparation of BVDV strain Korevaar (isolated from cattle, not cloned, and minimally passaged in cell culture) eluted at 100 mM NaCl, indicating that this virus preparation did not bind to heparin (results not shown). These results indicate that binding of C1.1.1 under these circumstances is not an artifact and that a heparin-HS-type polysaccharide chain is able to immobilize CSFV virus particles.
Characterization of virus variants. To determine whether passage in SK6 cells selects for CSFV variants that have a high affinity for HS, viruses were biologically cloned from the blood of a pig infected with CSFV strain Brescia. After cloning and one or two additional passages in SK6 cells, seven virus clones (clone A, passage number 3 [p3], and clones B to G, passage number 2 [p2]) were tested for inhibition by heparin. Two-hundred micrograms of heparin per milliliter did not inhibit infection of SK6 cells with all these virus clones seriously (shown for clones Ap3, Bp2, and Ep2; Fig. 5). Three clones, A, B, and E, were further passaged in SK6 cells and tested for heparin inhibition after each round of amplification. Surprisingly, after one additional round of passage in SK6 cells, infection with all three clones was inhibited almost completely by 200 μg of heparin per ml (Ap4, Bp3, and Ep3; Fig. 5). In addition, heparinase I treatment reduced infection of SK6 cells with clones Ap4, Bp3, and Ep3 efficiently, whereas infection with viruses of one passage less was not affected. This indicated that passage in SK6 cells changed these clones to viruses that infected cells by an HS-dependent mechanism. Furthermore, after 2 days of growth in medium with 1% methylcellulose, the diameters of Ap4, Bp3, and Ep3 plaques were about three times smaller than those of Ap3, Bp2, and Ep2 plaques (results not shown). When grown under agar, HS-dependent SV (4) and FMDV (34) also produce smaller plaques compared to their HS-independent phenotypes. Binding of HS-dependent virus to sulfated polysaccharides present in methylcellulose appears to reduce the spread of virus in this environment. To locate genetic differences between virus clones Ap3, Bp2, and Ep2 and their once-extra-passaged counterparts, the Env genes of these viruses were sequenced. The nucleotide sequences of the Env genes of Ap3, Bp2, and Ep2 were identical to each other. Compared to this consensus sequence, all three HS-dependent counterparts (Ap4, Bp3, and Ep3) shared an identical nucleotide mutation in the C-terminal part of the Env gene. Due to this mutation a Ser residue (AGC) at position 476...
in the ORF changes to an Arg residue (AGA). In Fig. 6, the amino acid sequence of Erns of these clones is compared to the published Erns sequence of C1.1.1 (29). These results indicated that passage in SK6 cells selected virus variants, which acquired a high affinity for HS due to the replacement of a neutral Ser residue by a positively charged Arg residue in the C terminus of Erns.

Construction and characterization of HS-dependent and HS-independent recombinant viruses. To prove that the Ser-to-Arg change in the C-terminal part of Erns solitarily is responsible for the change to an HS-dependent phenotype the Erns genes of clone Bp2 (S-ST) and Bp3 (S-RT) were inserted in a full-length DNA copy of Brescia clone 1.1.1. This full-length cDNA, pf1c.1.1.1, was constructed in a similar fashion as the full-length clone of CSFV strain C (30). Virus derived from pf1c.1.1.1 grows as fast and to the same titer as native C1.1.1 (30). Virus derived from the full-length clone of Brescia clone 1.1.1. This full-length cDNA, pf1c.1.1.1, was constructed in a similar fashion as the full-length clone of CSFV strain C (30). Virus derived from pf1c.1.1.1 grows as fast and to the same titer as native C1.1.1 (30). Reverse transcription-PCR fragments, covering the complete C, Erns. and E1 genes (see Materials and Methods) were generated using RNA isolated from virus clone Bp2 and Bp3 as the template. Sequence analysis showed that the point mutation that resulted in the Ser-to-Arg change in the C-terminal part of the Erns gene is the only difference between the Bp2 and Bp3 fragments. Replacement of the corresponding cDNA fragment in pf1c.1.1.1 with those of Bp2 and Bp3 resulted in full-length cDNA vectors pf1c.1.1.1.Erns (S-ST) and pf1c.1.1.1.Erns (S-RT), respectively. Transfection of SrfI-linearized vector DNAs into SK6.T7a5 cells (41) yielded the infectious recombinant viruses pf1c.1.1.1.Erns (S-ST) and pf1c.1.1.1.Erns (S-RT). The transfection medium (passage number 1) was used to infect SK6 cells in order to prepare a virus stock with passage number 2. Virus flc.1.1.1.Erns (S-ST) was passaged for three additional rounds in SK6 and PK15 cells (p5). Virus stocks were titrated by endpoint dilution and in a plaque assay and were tested for reduction of virus infection after heparinase I treatment of cells and for inhibition by heparin (Table 1). All tests were performed with SK6 cells. Like the control recombinant virus C1.1.1.1 (R-RI), the S-RT virus reacted as an HS-dependent phenotype. Infection with this virus was almost completely abolished by heparin and heparinase I treatment. As observed for virus clone Bp2, infection with the S-ST recombinant virus was not inhibited by heparin and was not affected by heparinase I treatment. Also, compared to the other recombinant viruses, this virus produced relatively large plaques. When p2 virus stocks were prepared, immunostaining with E2-specific MAbs showed 100% infected cells with a similar intense staining for all three viruses. However, the virus titer of the S-St p2 stock was significantly lower than the titers of S-RT p2 and C1.1.1.1. p2. Within two additional rounds of amplification in SK6 or in PK15 cells the S-ST p2 virus changed from an HS-independent to an HS-dependent phenotype (results not shown for PK15 cells). Passage number 5 virus stock, derived from the S-ST recombinant virus by passage in SK6 cells, was further characterized. This virus stock achieved a virus titer that was equivalent to that of S-RT p2 and C1.1.1.1. p2. Within two additional rounds of amplification in SK6 or in PK15 cells the S-ST p2 virus changed from an HS-independent to an HS-dependent phenotype (results not shown for PK15 cells). Passage number 5 virus stock, derived from the S-ST recombinant virus by passage in SK6 cells, was further characterized. This virus stock achieved a virus titer that was equivalent to that of S-RT p2 and C1.1.1.1. p2. Sequence analysis of the Erns gene of this p5 virus showed that Ser 476 was changed to an Arg. Compared to its parent virus (S-ST p2), no additional nucleotide mutations were present in the Erns gene of the p5 virus. Surprisingly, Erns of strain C produced in insect cells (with an Arg at position 476), which binds tightly to the cell surface (17) and to immobilized heparin, was not able to inhibit infection of SK6 cells with S-ST recombinant virus.

**TABLE 1. Characterization of recombinant virus**

<table>
<thead>
<tr>
<th>Virus</th>
<th>% Inhibition or reduction of virus infectiona</th>
<th>Relative plaque sizeb</th>
<th>Titer (log10/ml)c</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Heparin Heparinase I Erns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>flc.1.1.1.Erns (R-RI) p2</td>
<td>95 90</td>
<td>Small 6.8</td>
<td>6.4</td>
</tr>
<tr>
<td>flc.1.1.1.Erns (S-RT) p2</td>
<td>95 85</td>
<td>Small 6.6</td>
<td>5.7</td>
</tr>
<tr>
<td>flc.1.1.1.Erns (S-ST) p2</td>
<td>0 5</td>
<td>Large 4.7</td>
<td>4.1</td>
</tr>
<tr>
<td>flc.1.1.1.Erns (S-ST) p5</td>
<td>95 90</td>
<td>Small 7.0</td>
<td>6.3</td>
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<th>Virus</th>
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<tr>
<td></td>
<td>Heparin Heparinase I Erns</td>
<td></td>
<td></td>
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<tr>
<td>flc.1.1.1.Erns (R-RI) p2</td>
<td>95 90</td>
<td>Small 6.8</td>
<td>6.4</td>
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<td>95 85</td>
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<tr>
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<td>4.1</td>
</tr>
<tr>
<td>flc.1.1.1.Erns (S-ST) p5</td>
<td>95 90</td>
<td>Small 7.0</td>
<td>6.3</td>
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</table>

a Inhibition (heparin) and plaque reduction (heparinase I) experiments were performed as described in the legend to Fig. 5.
b Relative plaque size observed after 2 days of growth (see legend to Fig. 5).
c Virus titer was determined by endpoint dilution (50% tissue culture infective dose [TCID50]) or determined in a plaque assay (PFU).

FIG. 6. Erns amino acid sequence of Brescia virus clones with different passage numbers. Differences compared to the published sequence of Erns of Brescia clone C1.1.1 (29) are listed. Identical amino acid sequences were obtained for clones Ap3, Bp2, and Ep2 and for clones Ap4, Bp3, and Ep3. RNase domains are underlined.
virus p2, even when E'NS was included in the overlay medium. In contrast, all other recombinant viruses that have an Arg at position 476, including the p5 virus derived from virus S-ST p2, were efficiently inhibited by C-strain E'NS (see Discussion).

DISCUSSION

In this report we demonstrated that initial binding of CSFV to cells can be accomplished by the interaction of envelope protein E'NS with HS. Removal of HS from the cell surface and addition of heparin to the culture medium led to the abolishment of infection of swine kidney cells with CSFV strains Brescia (C1.1.1) and “C.” The effective inhibition by DS and the lack of inhibition by de-N-sulfated heparin indicated that electrostatic interactions between positively charged amino acid domains on the surface of virions and negatively charged sulfate groups of HS play a major role in the binding of these viruses to the cell surface. Genetic analysis of virus variants, combined with construction of recombinant viruses, clearly showed that envelope protein E'NS and not E2 is responsible for interaction with HS. Moreover, purified E'NS of strain C binds with high affinity to immobilized heparin, whereas purified E2 of strain Brescia (C1.1.1) did not. These results and the fact that CSFV particles bind to heparin indicated that binding of E'NS to HS alone is sufficient to sequester virus particles to the cell surface. Recently, Iqbal et al. (19) showed that a recombinant Erns protein, generated from a cloned BVDV, also interacts with HS. However, binding of this recombinant E'NS to the cell surface was inhibited by heparin but not by the highly negatively charged DS. Their results suggest that the interaction of this BVDV E'NS with HS is less dependent on electrostatic forces and perhaps more specific than we observed for CSFV E'NS.

For several viruses it was demonstrated that in vitro cultivation selects virus variants which use HS as a receptor (24,34). Here, we showed that passage in SK6 cells selects an HS-binding CSFV variant. As observed for FMDV (34) and SV (24), adaptation of CSFV is also accompanied by the replacement of an uncharged residue by a highly positively charged residue in one of the surface proteins. For CSFV, the substitution of an Ser for an Arg residue in the C-terminal part of envelope protein E'NS increases the net positive charge of this region. This increase probably results in the tight binding of virions to the negatively charged HS chains. This also suggests that Arg 476 is exposed on the surface of virions and is involved in direct binding to HS. However, without this extra Arg, the C terminus of E'NS (Arg 459 to Lys 487) is already the most positively charged region of the protein (see Fig. 6). The increase in the net positive charge due to one additional Arg in this region is probably not dramatic. For FMDV type O, acquisition of an Arg in the antigenic site of the capsid made direct binding of several adjacent residues to HS possible (13). Therefore, acquisition of Arg in this region may alter the conformation of E'NS and/or distribution of positive charges on the surface of E'NS. Such changes could facilitate the interaction of amino acid residues located in other parts of the protein with HS. Besides basic amino acids in the vicinity of Arg 476, residues in a more N-terminally located positive domain (Arg 396 to Lys 409) are good candidates. This region, conserved for pestiviruses (28,26,29), contains the sequence KKG, which is similar to the Cardin and Weintraub (6) heparin-binding motif XBBXBXB (B, basic; X, any amino acid).

As observed for SV (24), only a few passages in cultured SK6 cells were needed to select HS-dependent CSFV variants. The rapid change of the S-ST recombinant virus to an S-RT HS-dependent virus was accompanied by a 100-fold increase in virus titer on SK6 and PK15 cells. Obviously, binding to HS is advantageous for infection of SK6 cells. Virions are immobi-

lized at the cell surface, and diffusion is reduced to a relatively small two-dimensional space. (35). The probability for virions to encounter a nonabundant and more specific surface receptor, such as the E2 receptor, is increased, resulting in a higher infection efficiency. Such a mechanism for infection is consistent with the results presented in Fig. 3 and published recently (17). C1.1.1 virions bound to the cell surface and prevented from entering the cell by incubation at 4°C or by blocking of the E2 receptor with exogenous E2 (17) could be released from the cell surface by disconnecting the virus-HS binding with heparin or with exogenous E'NS (17). This clearly indicates that C1.1.1 virions bind to HS before they interact with the E2 receptor and successfully penetrate the cell. If no other surface molecules sequester HS-independent virus to the surface of swine kidney cells (see also below), diffusion in the much-larger three-dimensional space of cells and medium reduces the probability to encounter an E2 receptor.

For most viruses for which binding to HS has been reported, interaction with additional, more-specific cell surface receptors are needed to mediate entry (reference 4 and references there-in). Moreover, for most these viruses, natural isolates infect cultured cells by an HS-independent mechanism. As mentioned above and as indicated in several studies, interaction of E2 with a probably more specific receptor is essential for pestivirus infection (12,17,48). Furthermore, we showed here that CSFV is able to infect cells by an HS-independent mechanism. Thus, for pestiviruses the question arises as to whether an HS-independent interaction of E'NS with a specific cell surface receptor is essential for infection of cells with both HS-inde-

pendent and HS-dependent virus variants. Remarkably, insect cell-derived E'NS of strain C, which showed a high affinity for HS-heparin, failed to inhibit infection with the HS-inde-

pendent S-ST virus. Several explanations for this failure are plaus-

ible. First, SK6 cells may not express a more specific surface receptor for E'NS and the S-ST virus may not utilize E'NS to mediate infection of these cells. Second, by sequestering a large amount (17) of insect cell-derived E'NS in the network of HS chains on the cell surface movement to and/or saturation of a specific receptor may become impossible. Third, due to differences in protein processing between insect cells and mam-

malian cells, insect cell-derived E'NS may have a lower affinity for such a receptor than does virus-bound E'NS. Finally, as mentioned above, the conformation of E'NS with an Arg at position 476 could be different from E'NS with a Ser at this position. This could also result in no affinity or a low affinity for a specific receptor. Irrespective of this failure, there are also several data suggesting that an E'NS-specific receptor exists. However, none of these data provide solid evidence. For example, MAbs directed against E'NS are able to neutralize CSFV infection, including infection with an HS-independent genotype (42). Furthermore, the cytotoxic action of unbound E'NS specifically directed toward lymphocytes suggests that a cell-specific receptor present on particular subsets of lymphocytes exists (3,27). Further studies regarding interaction of HS-inde-

pendent viruses with native pig cells, including inhibition ex-

periments with recombinant E'NS derived from HS-inde-

pendent viruses, may provide insight about the specific functions and the existence of a possible E'NS receptor on the surface of various cell types.

For FMDV (34) and SV (24) reverse genetics proved that the affinity of virus binding to HS-heparin is inversely corre-

lated with virulence in vivo. These studies suggested that se-

questering of HS-binding viruses to sites that are not favorable for replication slow down the spread of virus in the animal.
This could also be the case for CSFV. Brescia C1.1.1 binds with high affinity to HS-heparin and is avirulent in pigs (43). However, to correlate HS binding and virulence, the recombinant viruses derived from C1.1.1 and characterized here are not ideal tools. Although the amino acid sequence of S-RT E\textsuperscript{rms} is identical to the consensus sequence of virulent Brescia isolated from the blood of a pig, compared to this consensus sequence C1.1.1 contains more different amino acids than the three (R-R) located in E\textsuperscript{ms} (H. G. P. van Gennip and R. J. M. Moormann, personal communication). One is located in E2 and several others are located in the nonstructural proteins. All could have an impact on replication in vivo. Our results show that differences at positions 276 (Ser-Arg) and 477 (Thr-Ile) in E\textsuperscript{rms} did not lead to a measurable difference (with the tests performed here) in the strength of the virus-HS interaction. However, this does not rule out the possibility that changes at these positions could also affect replication in vivo. Construction and in vivo testing of a fully virulent virus derived from an infectious full-length DNA copy, combined with testing of an S-RT E\textsuperscript{ms} variant derived from this DNA copy, is needed to properly correlate HS-binding with the virulence of CSFV. Irrespective of the outcome of these experiments, the information generated here is valuable for in vivo studies regarding biological properties of E\textsuperscript{ms} and other viral proteins using reverse genetics. Limited passage in swine kidney cells or culture of additional clones isolated from organ suspensions of a pig in vivo, replication in a more controlled environment, such as in cultured SK6 cells, a more homogeneous virus population may be produced. Such a population may no longer be able to infect SK6 cells by an HS-dependent mechanism due to their genetic background. Binding of CSFV to HS may be more complex than acquisition of a positively charged residue in E\textsuperscript{ms}. More specific interactions with characteristic HS structures have been reported for several proteins (38), including for the HSV glycoprotein gD (37). Therefore, more studies with native virus isolates and different cell types are needed to evaluate the role of HS binding for pestiviruses in vivo.

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