Genogroup II Noroviruses Efficiently Bind to Heparan Sulfate Proteoglycan Associated with the Cellular Membrane

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Norovirus (NV), a member of the family *Caliciviridae*, is one of the important causative agents of acute gastroenteritis. In the present study, we found that virus-like particles (VLPs) derived from genogroup II (GII) NV were bound to cell surface heparan sulfate proteoglycan. Interestingly, the VLPs derived from GII were more than ten times likelier to bind to cells than were those derived from genogroup I (GI). Heparin, a sulfated glycosaminoglycan, and suramin, a highly sulfated derivative of urea, efficiently blocked VLP binding to mammalian cell surfaces. The reagents known to bind to cell surface heparan sulfate, as well as the enzymes that specifically digest heparan sulfate, markedly reduced VLP binding to the cells. Treatment of the cells with chlorate revealed that sulfation of heparan sulfate plays an important role in the NV-heparan sulfate interaction. The binding efficiency of NV to undifferentiated Caco-2 (U-Caco-2) cells differed largely between GI NV and GII NV, whereas the efficiency of binding to differentiated Caco-2 (D-Caco-2) cells did not differ significantly between the two genogroups, although slight differences between strains were observed. Digestion with heparinase I resulted in a reduction of up to 90% in U-Caco-2 cells and a reduction of up to only 50% in D-Caco-2 cells, indicating that heparan sulfate is the major binding molecule for U-Caco-2 cells, while it contributed to only half of the binding in the case of D-Caco-2 cells. The other half of those VLPs was likely to be associated with H-type blood antigen, suggesting that GII NV has two separate binding sites. The present study is the first to address the possible role of cell surface glycosaminoglycans in the binding of recombinant VLPs of NV.

Norovirus (NV), a member of the family *Caliciviridae*, is a causative agent of acute gastroenteritis, and NV is known to have been responsible for both sporadic cases and epidemic outbreaks of the disease in both developing and developed countries (23, 55). As many as 95% of nonbacterial gastroenteritis outbreaks in the world are reported to be caused by this agent (20). Most of the outbreaks are associated with the ingestion of contaminated food, in particular raw shellfish, or contaminated drinking water (24, 39, 55). The predominant clinical manifestations are nausea, vomiting, and diarrhea (38). In 1972, 27-nm-diameter viral particles of the prototype NV strain (Hu/NV/GI/Norwalk/1968/US, NV/68) were identified by immune electron microscopy in fecal specimens collected from a patient during a gastroenteritis outbreak at an elementary school in Norwalk, Ohio, in the year 1968 (1, 37).

NV is a nonenveloped virus containing an approximately 7.5-kb, positive-sense, single-stranded RNA genome with a polyadenylated tail at the 3' end (33). The genome contains three open reading frames (ORFs), with ORF1 encoding non-structural proteins and ORF2 and ORF3 encoding structural proteins (18). Currently, NV is classified into two genogroups,

genogroup I (GI) and GII, according to the nucleotide and amino acid sequences (4, 21, 40).

In spite of extensive efforts, biochemical studies of NV have been hampered by the lack of a cell culture system in which the virus will grow, although one animal model has recently been reported (67). Only chimpanzees developed serologic responses when inoculated with NV/68, but they usually underwent an asymptomatic infection (82). Most of our understanding of NV infection has been obtained from epidemiological and volunteer studies (25, 52, 57, 64).

Recombinant baculoviruses harboring the gene encoding the NV capsid protein have been constructed, and the proteins were expressed in insect cells (34). An approximately 58-kDa capsid protein appeared to be self-assembled into virus-like particles (VLPs). The fine structure of the recombinant VLPs of NV/68 (rNV/68) was elucidated by electron cryomicroscopy and X-ray crystallography, and it was found that rNV/68 is composed of 180 capsid proteins that form an icosahedron that is 38 nm in diameter (59, 60). Green and coworkers determined that rNV/68 is morphologically and antigenically similar to the native virions (22). Hyperimmune sera against rNV/68 were subsequently prepared, and enzyme-linked immunosorbent assays were established for the detection of NV/68 in stool specimens (19). It was also found that rNV/68 was immunogenic, and rNV/68 has been used for oral immunization to evaluate its ability to stimulate mucosal immunity (6, 26, 50). VLPs have also been useful for studying virus-cell interactions in vitro (7, 58, 80).

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It remains unclear where NV infects the host and subsequently multiplies, although the jejunum from volunteers with NV infection exhibited histopathological lesions (63, 64). Knowledge of the molecular basis for the interaction between NV and target cells may facilitate the development of vaccines and provide pharmaceutical strategies for the prevention and treatment of NV infection. In a previous study, we prepared VLPs from Ueno virus (UEV), a GII NV, and found a 105-kDa protein as a candidate receptor molecule (71). However, attempts to extract and purify the 105-kDa protein have remained unsuccessful. Recently, the association between NV infection and histo-blood group antigens present on host secretor intestinal cells has been reported (28, 31, 46, 49), and hemagglutination by rNV/68 was observed (32).

In the present study, we compared the binding of VLPs from both GI NV and GII NV to intestinal cells and found that the binding of GII NV VLPs to the cells occurred more efficiently than those of GI NV. We used ileum Intestine 407 cells, from human small intestine, to investigate the cell surface binding molecules. Assays using various inhibitors and enzymatic modifications of the cell surface revealed that NV binding was mediated by heparan sulfate proteoglycan, and this interaction was ubiquitously observed in various cell lines.

MATERIALS AND METHODS

Cells. Intestine 407 (human ileum) cells were grown at 37°C with Dulbecco's modified Eagle's medium (D-MEM) (Sigma Chemical Co., St. Louis, Mo.) containing recombinant human insulin (10 µg/ml; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, Kans.). CHO (Chinese hamster ovary) cells, HeLa (human cervix) cells, Vero (African green monkey kidney) cells, and A549 (human alveolar type II-derived carcinoma) cells were grown at 37°C with D-MEM containing 10% FBS. Tn5 cells, derived from the insect *Trichoplusia ni* (30), were grown at 27°C with Ex-CELL 400 (JRH Biosciences). Caco-2 (human colon) cells were grown at 37°C with GIT medium (Wako) containing 3% FBS. Caco-2 cells are known to spontaneously differentiate into enterocyte-like cells more than 6 days post-confluency. The cells showed biochemical and morphological features of differentiation, such as sucrose activity and the presence of domes (2, 80). The cells were incubated for at least 10 days postconfluency and were used as differentiated Caco-2 (D-Caco-2) cells, which represented typical domes.

Recombinant VLPs. Two strains from GI NV (Seto 124 virus [SEV] [43] and Funabashi virus [FUV] [AB078335] [unpublished data]) and three strains from GII NV (Ueno 7k virus [UEV] [AB078337] [71], Chitta 76 virus [CHV] [42], and Kashiwa 47 virus [KAV] [AB078334] [unpublished data]) were used. ³⁵S-labeled or nonlabeled VLPs were prepared by utilizing baculovirus expression systems with Tn5 insect cells, as described previously (71). Briefly, Tn5 cells in a 250-ml flask were infected with a recombinant baculovirus harboring NV ORF2 at a multiplicity of infection of 5 to 10, and the cells were incubated for 24 h at 27°C. The NV capsid proteins were metabolically radiolabeled with L-[³⁵S]methionine (Tran³⁵S-Label metabolic labeling reagent [30 µCi/ml]; ICN Biomedicals, Inc.) for 12 h at 27°C. Seven days after infection, the cells were harvested and the ³⁵S-labeled rNV were purified by CsCl equilibrium density gradient centrifugation followed by 5-to-30% sucrose gradient centrifugation. The specific activities of the purified ³⁵S-labeled-rSEV, -rFUV -rUEV, -rCHV, and -rKAV were 2.2×10^4 , 1.2×10^4 , 9.6×10^3 , 5.9×10^3 , and 2.7×10^3 cpm/µg, respectively.

Inhibitory agents and enzyme digestion. Heparin sodium salt, suramin sodium salt, protamine sulfate grade X (from salmon), poly-L-lysine hydrobromide (molecular weight, 30,000 to 70,000), magainin I, and cecropin A were obtained from Sigma Chemical Corporation. Glycosaminoglycans, heparan sulfate sodium salt (from bovine kidney), chondroitin sulfate C sodium salt (from shark cartilage), and hyaluronic acid (from bovine vitreous humor) were obtained from Sigma Chemical Co. Dermatan sulfate I (from porcine intestinal mucosa) was obtained from ICN Pharmaceuticals, Inc. (Costa Mesa, Calif.). Blood group H disaccharide, blood group A trisaccharide, and blood group B trisaccharide were obtained from Calbiochem-Novabiochem Co. (La Jolla, Calif.). Those chemicals were dissolved in either distilled water or phosphate-buffered saline (pH 7.5; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan).

Heparinase I, heparinase III (heparitinase), and chondroitinase ABC (from *Proteus vulgaris*) were obtained from Sigma Chemical Co. α1,2-Fucosidase (from *Xanthomonas* sp.) was obtained from Calbiochem-Novabiochem Co. They were dissolved in serum-free D-MEM. Enzyme digestion was carried out in serum-free D-MEM at 37°C for 60 min. Under the present assay conditions, none of these enzymatic treatments caused any detachment of cells from the monolayers.

Binding assay. Confluent monolayers of various mammalian cells in a 24-well collagen-coated plate (Asahi Techno Glass Co. Ltd., Tokyo, Japan) (10^5 to 10^6 cells/well) were incubated with 15 µg of ³⁵S-labeled VLPs in either the presence or absence of inhibitors for 1 h at 4°C. All reactions were performed in a final volume of 200 µl of serum-free D-MEM. Free ³⁵S-labeled-VLPs were eliminated by washing the cells three times with serum-free D-MEM, and the cells were solubilized with radioimmunoprecipitation assay (RIPA) buffer (0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.01% aprotinin, 10 mM Tris-HCl [pH 7.2]). The radioactivity was then measured with a liquid scintillation counter. Assays were performed in triplicate.

Inhibition of glycosaminoglycan sulfation. Low-sulfate medium D-MEM/F-12 was obtained from GIBCO BRL (Gaithersburg, Md.) and supplemented with 10% FBS. Vero cells were cultured in D-MEM or low-sulfate D-MEM/F-12 for 48 h in 24-well plates in the presence of the sulfation inhibitor sodium chlorate (5). Replicate cells were supplemented with sodium sulfate in order to assess the sulfation-specificity of inhibition.

RESULTS

The binding of VLPs of GI NV and GII NV to cells. NV is classified into two genogroups, GI and GII, according to the nucleotide and amino acid sequences (45, 78). Each genogroup contains many genetic clusters forming the various genotypes. We used a virus-binding assay to compare the NV binding efficiency between the two genogroups, as described previously (71). ³⁵S-labeled VLPs were prepared from five NV strains, and we compared the binding efficiency to Intestine 407 cells, a cell line derived from human intestine, and CHO cells, a cell line derived from Chinese hamster ovary. The binding of three recombinant VLPs of GII NV (rUEV, rKAV, and rCHV) to Intestine 407 cells appeared to be more efficient than those of either of the two VLPs of GI NV (rSEV and rFUV) (Fig. 1A). Similar results were obtained when CHO cells were used. The binding of rUEV to CHO cells was also more efficient than that of rSEV and rFUV, indicating that VLPs of GII NV were able to bind more efficiently to these cells than did the VLPs of GI NV (Fig. 1B).

The binding of rUEV to Intestine 407 cells via negatively charged cell surface molecule(s). To account for the discrepancies between the binding efficiencies observed between GI NV and GII NV, we investigated cell surface molecule(s) involved in the binding process. We used six potential binding inhibitors to study the binding of rUEV to Intestine 407 cells (Fig. 2). Heparin and suramin were known to bind to polycations on the surfaces of various virus particles and cell surface molecules (11, 53, 75). Poly-L-lysine and protamine sulfate, heparin antagonists, are known to bind to polyanions. Magainin I, a positively charged peptide (85), and cecropin A (66) are antimicrobial and antiviral peptides.

As depicted in Fig. 2A, when the assay was performed in the presence of heparin, the binding of rUEV to Intestine 407 cells was decreased in a dose-dependent manner. However, this inhibition of binding was not observed when the cells were pretreated with heparin (2.5 µg/ml), indicating that heparin blocks the binding of rUEV to cells by associating with rUEV, and not with the cells. Similar results were observed when suramin was used (Fig. 2B). Poly-L-lysine and protamine sulfate similarly inhibited the binding of rUEV in a dose-depend-

FIG. 1. Comparison of binding efficiency. Fifteen micrograms of ^{35}S -labeled VLPs from GI (rSEV and rFUV) and GII (rUEV, rKAV, and rCHV) was incubated for 1 h at 4°C with the cells placed in 24-well plates. The cells were washed, and the radioactivity was measured. The results are expressed as the percentage of ^{35}S -labeled rUEV binding. The mean values were plotted (error bars, standard deviation ranges). The experiments were performed in triplicate and were reproduced at least twice. (A) Intestine 407 cells were incubated with ^{35}S -labeled rUEV, rKAV, rCHV, rSEV, and rFUV. (B) CHO cells were incubated with ^{35}S -labeled rUEV, rSEV, and rFUV.

dent manner (Fig. 2C and D). However, strong inhibition was also observed when the cells were treated before being exposed to rUEV, demonstrating that these two reagents caused an inhibitory effect by directly binding to the cells. This observation was in contrast to that of heparin and suramin. Cecropin A, magainin I, and bovine serum albumin (BSA) had no effect on the binding of rUEV (Fig. 2E and F).

Although heparin is not a constituent of cell membranes, this molecule is a structural homologue of heparan sulfate, a negatively charged glycosaminoglycan (56). Heparan sulfate is widely expressed on the cell surface and in extracellular matrices (29, 36). Poly-L-lysine and protamine sulfate are known to bind to cell surface polyanions. Therefore, negatively charged Intestine 407 cell surface molecules, such as sulfated glycosaminoglycans, are likely to be involved in the binding of rUEV.

rUEV interacts with negatively charged glycosaminoglycans.

To determine whether a negatively charged cell surface glycosaminoglycan is associated with binding to the cell surface, we

selected four major soluble glycosaminoglycans (i.e., heparan sulfate, chondroitin sulfate C, dermatan sulfate I [chondroitin sulfate B], and hyaluronic acid) as competitive antagonists. As depicted in Fig. 3, heparan sulfate, chondroitin sulfate C, and dermatan sulfate I showed dose-dependent inhibition, which was distinguished at doses of $50\text{ }\mu\text{g/ml}$ and higher (Fig. 3A to C). These three glycosaminoglycans have a negatively charged sulfate group (27). No inhibition was observed when the cells were pretreated with any one of these compounds at a concentration of $50\text{ }\mu\text{g/ml}$, indicating that they all successfully block binding by associating with rUEV, and not with Intestine 407 cells. In contrast, hyaluronic acid, which has no charged group in the molecule, had no effect, even when concentrations up to $200\text{ }\mu\text{g/ml}$ were tested (Fig. 3D). These results indicated that negatively charged glycosaminoglycans are capable of easily binding to rUEV and that they inhibit the binding of rUEV to Intestine 407 cells. The findings therefore suggest that glycosaminoglycans on the cell surface play a role in the binding of rUEV to the cell surface.

The cell surface molecule involved in binding is heparan sulfate glycosaminoglycan. To determine whether or not cell surface glycosaminoglycan is actually used in the binding process, we pretreated Intestine 407 cells with glycosaminoglycan-specific lyases and measured the extent of ^{35}S -labeled rUEV binding. The cells were exposed to three different enzymes capable of digesting cell surface glycosaminoglycan moieties, namely, heparinase I, heparinase III, and chondroitinase ABC. Heparinase I cleaves glycosidic linkages in both heparin and heparan sulfates, while heparinase III selectively cleaves the linkages in heparan sulfate (47, 56). Chondroitinase ABC specifically cleaves glycosidic linkages in chondroitin sulfates A, B, and C (83), which are also major glycosaminoglycans on cell surfaces.

Digestion with either heparinase I or heparinase III resulted in a marked reduction of the binding of ^{35}S -labeled rUEV in a dose-dependent manner (Fig. 4A and B), while digestion with chondroitinase ABC had no influence on the binding (Fig. 4C). We also confirmed that digestion with heparinase I (10 U/ml) resulted in a reduction of more than 90% of the ^{35}S -labeled rCHV binding to Intestine 407 cells (data not shown). These results indicated that cell surface heparan sulfate glycosaminoglycan is the major molecule involved in the binding, at least as regards these two strains of GII NV.

Sulfation of glycosaminoglycans is required for binding. Hyaluronic acid was not associated with an inhibitory effect on cell surface binding (Fig. 3D), and this molecule is the only nonsulfated glycosaminoglycan among the potent inhibitors (27). This result suggested that the sulfation of glycosaminoglycan is important for rUEV binding to cells. To further clarify this point, we cultivated Vero cells in low-sulfate medium D-MEM/F-12 with a specific sulfation inhibitor, sodium chlorate (5), and measured the binding of ^{35}S -labeled rUEV to the cells. The addition of sodium chlorate led to a marked dose-dependent loss of binding (Fig. 5). The lack of rUEV binding due to desulfation by sodium chlorate was recovered dose dependently by supplementing the cells with sodium sulfate (Fig. 5). These results thus demonstrated that sulfation of glycosaminoglycan plays an important role in the binding of rUEV.

FIG. 2. Inhibition of binding with various inhibitory molecules. Intestine 407 cell monolayers were incubated with ^{35}S -labeled rUEV in the presence of increasing concentrations of potential inhibitors: heparin (A), suramin (B), poly-L-lysine (C), protamine sulfate (D), cecropin A (100 $\mu\text{g/ml}$) and magainin I (100 $\mu\text{g/ml}$) (E), and BSA (F). The cells were also preincubated with each compound at concentrations of 2.5 $\mu\text{g/ml}$ (heparin and suramin) and 50 $\mu\text{g/ml}$ (poly-L-lysine and protamine sulfate), washed three times, incubated with 15 μg of ^{35}S -labeled rUEV, and washed three times, and the radioactivity remaining in the cells was measured (column Pre at the far right in panels A to D). Control values in the absence of inhibitor ranged from 430 to 2,800 cpm. Experiments were performed in triplicate and were reproduced at least twice. The plotted data represent the means (error bars, standard deviation ranges).

The binding profile differs between undifferentiated and differentiated Caco-2 cells. White et al. reported that rNV/68 was able to bind more significantly to D-Caco-2 cells than to any other mammalian cell lines tested, although the cell surface binding factor was unknown (80). To assess the contribution of heparan sulfate glycosaminoglycan in the process of rUEV binding to Caco-2 cells, we investigated the binding of GI NV and GII NV to Caco-2 cells using a virus-binding assay. We prepared ^{35}S -labeled VLPs from four NV strains and compared the efficiencies of each strain in terms of ability to bind to undifferentiated Caco-2 (U-Caco-2) cells and to D-Caco-2 cells.

As depicted in Fig. 6A, the GI NV and GII NV strains differed greatly in terms of their ability to bind to U-Caco-2 cells. In contrast, the level of binding to D-Caco-2 cells did not differ significantly between the two genogroups, although slight differences between strains were observed (Fig. 6B). To determine whether or not cell surface heparan sulfate is necessary for this type of binding, we treated U-Caco-2 and D-Caco-2 cells with heparinase and measured the level of binding of ^{35}S -labeled rUEV to these cells. Digestion with heparinase I

resulted in a dose-dependent reduction of up to 90% in the case of binding to U-Caco-2 cells (Fig. 6C). However, the corresponding reduction with heparinase I amounted to only 50% in the case of the D-Caco-2 cells (Fig. 6D), indicating that although heparan sulfate is the major binding molecule for U-Caco-2 cells, it contributed to only half of the amount of binding in the case of the D-Caco-2 cells.

Contribution of H-type blood antigen to rUEV binding to D-Caco-2 cells. A recent study has shown that D-Caco-2 cells have a high level of expression of H-type 1 blood group antigen on the cell surface (2). Other related studies have suggested that rNV/68 is associated with H-type histo-blood antigen (32, 49). Therefore, we investigated the inhibitory activity of the blood group H disaccharide, blood group A trisaccharide, and blood group B trisaccharide. As depicted in Fig. 7A, only incubation of ^{35}S -labeled rUEV with blood group H disaccharide reduced the binding to D-Caco-2 cells by more than 40%. Next, we used $\alpha 1,2$ -fucosidase to determine whether the 1,2-fucosylated structure was important for NV binding (49). Pre-treatment of D-Caco-2 cells with $\alpha 1,2$ -fucosidase also dose dependently reduced the amount of binding by approximately

FIG. 3. Inhibition of binding of rUEV by glycosaminoglycans. Intestine 407 cell monolayers were incubated with ^{35}S -labeled rUEV in the presence of increasing concentrations of each glycosaminoglycan: heparan sulfate (A), chondroitin sulfate C (B), dermatan sulfate I (C), and hyaluronic acid (D). The cells were also preincubated with each glycosaminoglycan at a concentration of 50 $\mu\text{g/ml}$, washed three times, incubated with 15 μg of ^{35}S -labeled rUEV, and washed three times, and the radioactivity of the cells was measured (column Pre at the far right in panels A to C). Control values in the absence of the glycosaminoglycan ranged from 570 to 1,270 cpm. The experiments were performed in triplicate and were reproduced at least twice. The plotted data represent the means (error bars, standard deviation ranges).

40% (Fig. 7B). These experiments indicated that approximately half of the bound rUEV was associated with heparan sulfate and the other half was associated with H-type blood group antigen on the D-Caco-2 cells (Fig. 6D and Fig. 7). Although it remains unclear whether rUEV could bind to blood group A antigen or blood group B antigen based on this assay, our data suggested that rUEV particles probably possess separate binding sites for at least heparan sulfate and H-type blood antigen.

Cell surface heparan sulfate for rUEV binding to various cells. To determine whether the interaction between rUEV and heparan sulfate is limited to Intestine 407 and Caco-2 cells (Fig. 4 and 6), we treated five cell lines derived from different species with heparinase I and measured the extent of rUEV binding (Fig. 8). Digestion with heparinase I (20 U/ml) resulted in a marked reduction in binding to all five cell lines, although the degree of binding to Tn5 insect cells was relatively high (Fig. 8). These results suggested that the interaction between rUEV and heparan sulfate is ubiquitously shared by cells from various species, although the structure of heparan sulfate differs from tissue to tissue and species to species (14, 72).

Cell surface heparan sulfate for GI NV binding. To determine whether the weak binding of GI VLPs to cells is associated with cell surface heparan sulfate (Fig. 1), we performed binding assays in the presence of heparin and also after treat-

ment with heparinase I (Fig. 9). The inhibitory effects of heparin differed in a comparison of the GI and the GII NVs (Fig. 2A), although the binding of rSEV and rFUV to Intestine 407 cells decreased in both cases in a dose-dependent manner, respectively (Fig. 9). No inhibition was observed when the cells were pretreated with heparin at 2.5 $\mu\text{g/ml}$ (Fig. 9). Digestion with heparinase I (20 U/ml) resulted in an incomplete reduction of the binding of ^{35}S -labeled rSEV and rFUV (~60 and ~40%, respectively) (Fig. 9). These results suggested that the cell surface heparan sulfate glycosaminoglycan might not be the major molecule involved in the binding process of at least two strains of GI NV.

DISCUSSION

Heparan sulfate, a long polyanionic carbohydrate chain consisting of repeating disaccharides, is found in a wide variety of tissues in many animal species (14, 73). This molecule has a core protein that is rooted in the lipid bilayer of the plasma membrane of most types of vertebrate and invertebrate cells (41, 51). A number of studies have indicated the potential roles of heparan sulfate proteoglycans in the regulation of cell growth and transformation (15, 61, 77, 84), differentiation processes (10), cell adhesion (12, 44), and neuromuscular junction formation (3). In addition, heparan sulfate acts as a receptor,

FIG. 4. Effect of treatment with lyase on binding. Intestine 407 cell monolayers were pretreated with increasing concentrations of each enzyme for 1 h, and the binding of ³⁵S-labeled rUEV was measured. Control values in the absence of the enzyme ranged from 1,050 to 1,520 cpm. The experiments were performed in triplicate and were reproduced at least twice. The plotted data represent the means (error bars, standard deviation ranges). (A) Heparinase I (0 to 20 U/ml); (B) heparinase III (0 to 20 U/ml); (C) chondroitinase ABC (0 to 10 U/ml).

not only for viruses, but also for bacteria and parasites (11, 62, 65, 69).

Our series of experiments demonstrated that rUEV and rCHV, recombinant VLPs from GII NV, predominantly bound to heparan sulfate on most mammalian cell surfaces tested, except for on D-Caco-2 cells. The first indication leading to our present conclusions was obtained from experiments using the binding inhibitors heparin and suramin. These molecules had been shown to block the binding of rUEV to Intestine 407 cells by associating with the VLPs and not with the cells. The binding to rUEV presumably occurred via the sulfated portion of these inhibitors, which occupied the sites on the VLPs that were otherwise necessary for binding to cell surface heparan sulfate (Fig. 2A and B). Heparin, a functional analogue of heparan sulfate, is a polymer containing repeated disaccharides and suramin, a complex derivative of urea. Other than their respective sulfate groups, there was no other common feature shared by these two molecules. Unexpectedly, chondroitin sulfate C and dermatan sulfate I also exerted inhibitory effects on binding at higher doses (Fig. 3B), which is a finding that differs from that in studies of dengue virus; the discrepancy is due to the fact that these two compounds had no inhibitory effect on dengue virus. The binding of dengue virus was exclusively blocked by highly sulfated heparan sulfate (11). Poly-L-lysine and protamine sulfate directly inhibited the binding of rUEV to Intestine 407 cells by associating with the cell

surface (Fig. 2C and D). The binding of heparan sulfate, chondroitin sulfate C, and dermatan sulfate I to rUEV is thought to be due to their glycosaminoglycan-specific structures, such as sulfated sugars, rather than being the result of a simple non-specific effect, since hyaluronic acid did not show any inhibitory effect, even at doses up to 200 µg/ml. In contrast with all other glycosaminoglycans, hyaluronic acid contains no sulfated sugars (27). Under the present assay conditions, Intestine 407 cell surface heparan sulfate was shown to be associated with more than 90% of the binding that took place, as indicated by treatment with heparinase I or III at 20 U/ml (Fig. 4A and B). NV presumably interacts with other cell surface glycosaminoglycans in vivo; for instance, chondroitin sulfate exists abundantly in the human intestinal tract (73).

Removal of heparan sulfate by heparinase III, a heparan sulfate-specific enzyme, clearly indicated that this molecule is specifically associated with the binding between Intestine 407 cells and rUEV (Fig. 4B). Although rUEV selectively binds to heparan sulfate on Intestine 407 cells, we cannot rule out the possibility that rUEV interacted with other cell surface polyanions. In a previous study, we suggested a 105-kDa protein as a candidate receptor molecule in various mammalian cells (71), but this 105-kDa molecule did not correspond to cell surface heparan sulfate (data not shown).

The sulfation of heparan sulfate is important for the binding of rUEV to Intestine 407 cells, as shown in Fig. 5. These results

FIG. 5. Effect of sulfation of glycosaminoglycan in the binding of rUEV. Vero cells were incubated in the presence of increasing concentrations (0, 1, 5, and 25 mM) of sodium chlorate. After the cells were washed, the binding of ^{35}S -labeled rUEV was examined. Cells incubated in the presence of 25 mM sodium chlorate were supplemented with either 1 or 5 mM sodium sulfate for 48 h. After the cells were washed, the binding of ^{35}S -labeled rUEV was measured. The experiments were performed in triplicate and were reproduced at least twice. The plotted data represent the means (error bars, standard deviation ranges).

may explain the inhibition by suramin, a compound not related structurally to glycosaminoglycans but one that contains six sulfate groups (Fig. 2B). Recent studies have shown that the structure of heparan sulfate is highly heterogeneous in terms of both the pattern and level of sulfation, as well as in terms of the primary sequence of disaccharides, depending on the tissues and species from which this molecule is extracted (48, 72). Such differences may explain the tropism of pathogenic microorganisms that exploit heparan sulfate structures as a target for binding (11). In our previous study, we did not observe an association of rUEV with glycoproteins, since the binding of rUEV was not influenced by treatment with sodium periodate (71). We are of the opinion that oxidation by sodium periodate is unlikely to affect the sulfate group of heparan sulfate (16).

Although heparan sulfate appeared to play a role in the binding of rUEV to Intestine 407 cells, the structure of heparan sulfate is known to be heterogeneous, depending on the cell type (51, 73). Therefore, U-Caco-2, CHO, HeLa, A549, and Vero cells were digested with heparinase I, and the subsequent extent of rUEV binding was examined. As indicated in Fig. 8, treatment with enzymes drastically reduced the extent of binding, indicating that heparan sulfate was also used for the binding process in these other types of cell. However, the binding of rUEV to D-Caco-2 (Fig. 6D) and Tn5 (Fig. 8) cells reflected some degree of resistance to heparinase I digestion. These results therefore suggest that a cell surface molecule(s) other than heparan sulfate participated in the binding process in these cases.

A difference in the binding efficiency was detected between GI and GII VLPs, whereby GI VLPs were found to inefficiently bind to the cells, when they were compared to GII VLPs (Fig. 1). A low level of binding of rSEV and rFUV was observed, compared with that of GII VLPs (Fig. 1A), and the inhibition by both heparin and heparinase I was only half

FIG. 6. Comparison of binding of VLPs to U-Caco-2 and D-Caco-2 cells. Fifteen micrograms of ^{35}S -labeled VLPs was incubated with either U-Caco-2 or D-Caco-2 cells for 1 h at 4°C . After the cells were washed, the binding of VLPs to the cells was measured. (A) U-Caco-2 cells were incubated with ^{35}S -labeled rUEV, rSEV, and rFUV. (B) D-Caco-2 cells were incubated with ^{35}S -labeled rUEV, rCHV, rSEV, and rFUV. The effect of heparinase I treatment on the binding of rUEV to U-Caco-2 and D-Caco-2 cells was also measured. Monolayers of U-Caco-2 (C) and D-Caco-2 (D) were pretreated with increasing concentrations of heparinase I for 1 h before ^{35}S -labeled rUEV was added. After washing, the radioactivity of the cells was measured. The experiments were performed in triplicate and were reproduced at least twice. The plotted data represent the means (error bars, standard deviation ranges).

complete (Fig. 9). A similar incomplete inhibitory effect was observed in the case of treatment with suramin (data not shown). Therefore, we concluded that both rSEV and rFUV also bind to cell surface heparan sulfate very weakly or non-specifically. This finding may account for previous data by White et al. that NV/68 did not bind to most cell lines, with the exception of Caco-2 cells (80). Most human cell lines used in their assay would have expressed heparan sulfate proteoglycan on the cell surface. However, GI NV is likely to bind to heparan sulfate nonspecifically, which would result in a low level of binding to those cell lines. As regards the binding to insect Sf9 cells, NV/68 possibly bound to molecules other than heparan sulfate, as was observed in our experiments with another insect cell, Tn5 (Fig. 8). It is of note in this context that the hemagglutination profiles of GI and GII were also different (32).

Generally, binding assays are typically carried out at 4°C in order to avoid changes in physiological conditions. However, most viruses infect human hosts at body temperature, 37°C , in

FIG. 7. Blood antigens on binding of rUEV to D-Caco-2 cells. (A) D-Caco-2 cell monolayers were incubated with ^{35}S -labeled rUEV in the presence of increasing concentrations (1 to 100 $\mu\text{g/ml}$) of blood group H disaccharide, blood group A trisaccharide, or blood group B trisaccharide. The cells were washed, and the radioactivity of the cells was measured. (B) D-Caco-2 cell monolayers were pretreated with increasing concentrations (0 to 10 mU/ml) of $\alpha 1,2$ -fucosidase for 1 h at 37°C before 15 μg of ^{35}S -labeled rUEV was added. The radioactivity of the cells was then measured. Control values in the absence of the enzyme ranged from 190 to 710 cpm. The experiments were performed in triplicate and were reproduced at least twice. The plotted data represent the means (error bars, standard deviation ranges).

vivo. Interestingly, ^{35}S -labeled rUEV associated equally to Intestine 407 and CHO cells at both 4 and 37°C (data not shown). In contrast, ^{35}S -labeled rUEV bound neither to U-Caco-2 nor D-Caco-2 cells at 37°C (data not shown). In early studies using coxsackievirus B3 and poliovirus, more than 50% of the cell-

FIG. 8. Heparinase I treatment of several cell lines and the binding of rUEV. CHO, HeLa, A549, Vero, and Tn5 cell monolayers were pretreated with heparinase I (20 U/ml) for 1 h, and 15 μg of ^{35}S -labeled rUEV was added. After the cells were washed, the radioactivity of the cells was measured. The experiments were performed in triplicate and were reproduced at least twice. The plotted data represent the means (error bars, standard deviation ranges).

FIG. 9. Effects of heparin and heparinase I on GI VLP adsorption to Intestine 407 cells. Intestine 407 cell monolayers were incubated with ^{35}S -labeled rSEV (A) or ^{35}S -labeled rFUV (B) in the presence of increasing concentrations (0, 0.05, 0.25, 0.5, and 2.5 $\mu\text{g/ml}$) of heparin. Cells were also preincubated with heparin at a concentration of 2.5 $\mu\text{g/ml}$ before being incubated with 15 μg of ^{35}S -labeled rSEV (A) or ^{35}S -labeled rFUV (B), and the radioactivity of the cells was measured (columns labeled Pre). In another experiment, the cells were treated with heparinase I (20 U/ml) for 1 h before 15 μg of ^{35}S -labeled rSEV (A) or ^{35}S -labeled rFUV (B) was added (Hep I). Control values in the absence of heparin or the enzyme ranged from 80 to 95 cpm. The experiments were performed in triplicate and were reproduced at least twice. The plotted data represent the means (error bars, standard deviation ranges).

associated viruses were shown to be eluted from the cells at 37°C (13, 35), and a recent kinetic analysis suggested that the poliovirus receptor functions like an enzyme (74). These observations raised two different possibilities regarding the interaction between heparan sulfate and VLPs: (i) heparan sulfate is only activated in the company of a Caco-2 cell-specific component, or (ii) the temperature sensitivity of heparan sulfate glycosaminoglycan of Caco-2 cells differs from that of other cell lines. Some ligand-heparan sulfate interactions require a particular structure of heparan sulfate (11). Further investigation will be required to resolve this issue.

In this study, we demonstrated that cell surface heparan sulfate could efficiently associate with GII NV. Intestinal glycosaminoglycans are likely to be associated with NV infection,

since abundant glycosaminoglycans are observed in the human intestine (73). It is also likely that the binding of NV to glycosaminoglycans serves merely to concentrate the virus on the cell surface, such that binding to another receptor is facilitated (81). Alternatively, the binding of a virion component with glycosaminoglycans is necessary for a virion-cell interaction, e.g., the interactions observed in neural cells (12, 84). It is unclear whether heparan sulfate functions as the NV receptor, because the expression of heparan sulfate does not always support viral infection (54, 68). Further investigation will be needed to identify the NV receptor.

In a recent study, we found that histone H1 inhibits the binding of NV VLPs to the mammalian cell surface by associating with both VLPs and the cell surface (70). Various interactions between histones and the glycosaminoglycans in the nuclei and those on the cell surface have been described (8, 9, 17, 76, 79). Such findings, in combination with our data, suggest that histone H1 may directly bind cell surface heparan sulfate and consequently block the binding of NV to the cell surface.

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REFERENCES

- Adler, J. L., and R. Zickl. 1969. Winter vomiting disease. *J. Infect. Dis.* **119**:668–673.
- Amano, J., and M. Oshima. 1999. Expression of the H type 1 blood group antigen during enterocytic differentiation of Caco-2 cells. *J. Biol. Chem.* **274**:21209–21216.
- Anderson, M. J., F. G. Klier, and K. E. Tanguay. 1984. Acetylcholine receptor aggregation parallels the deposition of a basal lamina proteoglycan during development of the neuromuscular junction. *J. Cell Biol.* **99**:1769–1784.
- Ando, T., J. S. Noel, and R. L. Fankhauser. 2000. Genetic classification of “Norwalk-like viruses.” *J. Infect. Dis.* **181**:S336–S348.
- Baeuerle, P. A., and W. B. Huttner. 1986. Chlorate—a potent inhibitor of protein sulfation in intact cells. *Biochem. Biophys. Res. Commun.* **141**:870–877.
- Ball, J. M., M. E. Hardy, R. L. Atmar, M. E. Conner, and M. K. Estes. 1998. Oral immunization with recombinant Norwalk virus-like particles induces a systemic and mucosal immune response in mice. *J. Virol.* **72**:1345–1353.
- Basavappa, R., A. Gomez-Yafal, and J. M. Hogle. 1998. The poliovirus empty capsid specifically recognizes the poliovirus receptor and undergoes some, but not all, of the transitions associated with cell entry. *J. Virol.* **72**:7551–7556.
- Bilozur, M. E., and C. Biswas. 1990. Identification and characterization of heparan sulfate-binding proteins from human lung carcinoma cells. *J. Biol. Chem.* **265**:19697–19703.
- Bornens, M. 1973. Action of heparin on nuclei: solubilization of chromatin enabling the isolation of nuclear membranes. *Nature* **244**:28–30.
- Cassaro, C. M. F., and C. P. Dietrich. 1977. Distribution of sulfated mucopolysaccharides in invertebrates. *J. Biol. Chem.* **252**:2254–2261.
- Chen, Y., T. Maguire, R. E. Hileman, J. R. Fromm, J. D. Esko, R. J. Linhardt, and R. M. Marks. 1997. Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat. Med.* **3**:866–871.
- Cole, G. J., A. Loewy, and L. Glaser. 1986. Neuronal cell-cell adhesion depends on interactions of N-CAM with heparin-like molecules. *Nature* **320**:445–447.
- Crowell, R. L., and L. Philipson. 1971. Specific alterations of coxsackievirus B3 eluted from HeLa cells. *J. Virol.* **8**:509–515.
- Dietrich, C. P., H. B. Nader, and A. H. Straus. 1983. Structural differences of heparan sulfates according to the tissue and species of origin. *Biochem. Biophys. Res. Commun.* **111**:865–871.
- Esko, J. D., K. S. Rostand, and J. L. Weinke. 1988. Tumor formation dependent on proteoglycan biosynthesis. *Science* **241**:1092–1096.
- Fransson, L. A., I. Sjöberg, and B. Havsmark. 1980. Structural studies on heparan sulphates. Characterization of oligosaccharides; obtained by periodate oxidation and alkaline elimination. *Eur. J. Biochem.* **106**:59–69.
- Frenster, J. H. 1965. Nuclear polyanions as de-repressors of synthesis of ribonucleic acid. *Nature* **206**:680–683.
- Glass, P. J., L. J. White, J. M. Ball, I. Leparc-Goffart, M. E. Hardy, and M. K. Estes. 2000. Norwalk virus open reading frame 3 encodes a minor structural protein. *J. Virol.* **74**:6581–6591.
- Gray, J. J., X. Jiang, P. Morgan-Capner, U. Desselberger, and M. K. Estes. 1993. Prevalence of antibodies to Norwalk virus in England: detection by enzyme-linked immunosorbent assay using baculovirus-expressed Norwalk virus capsid antigen. *J. Clin. Microbiol.* **31**:1022–1025.
- Green, K. Y. 2000. Summary of the first international workshop on human caliciviruses. *J. Infect. Dis.* **181**:S252–S253.
- Green, K. Y., R. M. Chanock, and A. Z. Kapikian. 2001. Human caliciviruses, p. 841–874. *In* D. M. Knipe and P. M. Howley (ed.), *Fields virology*, vol. 1. Lippincott Williams & Wilkins, Philadelphia, Pa.
- Green, K. Y., J. F. Lew, X. Jiang, A. Z. Kapikian, and M. K. Estes. 1993. Comparison of the reactivities of baculovirus-expressed recombinant Norwalk virus capsid antigen with those of the native Norwalk virus antigen in serologic assays and some epidemiologic observations. *J. Clin. Microbiol.* **31**:2185–2191.
- Greenberg, H. B., J. Valdesuso, R. H. Yolken, E. Gangarosa, W. Gary, R. G. Wyatt, T. Konno, H. Suzuki, R. M. Chanock, and A. Z. Kapikian. 1979. Role of Norwalk virus in outbreaks of nonbacterial gastroenteritis. *J. Infect. Dis.* **139**:564–568.
- Griffin, M. R., J. J. Surowiec, D. I. McCloskey, B. Capuano, B. Pierzynski, M. Quinn, R. Wojnarski, W. E. Parkin, H. Greenberg, and G. W. Gary. 1982. Foodborne Norwalk virus. *Am. J. Epidemiol.* **115**:178–184.
- Grohmann, G. S., A. M. Murphy, P. J. Christopher, E. Auty, and H. B. Greenberg. 1981. Norwalk virus gastroenteritis in volunteers consuming depurated oysters. *Aust. J. Exp. Biol. Med. Sci.* **59**:219–228.
- Guerrero, R. A., J. M. Ball, S. S. Krater, S. E. Pacheco, J. D. Clements, and M. K. Estes. 2001. Recombinant Norwalk virus-like particles administered intranasally to mice induce systemic and mucosal (fecal and vaginal) immune responses. *J. Virol.* **75**:9713–9722.
- Hardingham, T. E., and A. J. Fosang. 1992. Proteoglycans: many forms and many functions. *FASEB J.* **6**:861–870.
- Harrington, P. R., L. Lindesmith, B. Yount, C. L. Moe, and R. S. Baric. 2002. Binding of Norwalk virus-like particles to ABH histo-blood group antigens is blocked by antisera from infected human volunteers or experimentally vaccinated mice. *J. Virol.* **76**:12335–12343.
- Hedman, K., M. Kurkinen, K. Alitalo, A. Vaheri, S. Johansson, and M. Hook. 1979. Isolation of the pericellular matrix of human fibroblast cultures. *J. Cell Biol.* **81**:83–91.
- Hink, W. F. 1970. Establish insect cell line from the cabbage looper, *Trichoplusia ni*. *Nature* **226**:466–467.
- Hutson, A. M., R. L. Atmar, D. Y. Graham, and M. K. Estes. 2002. Norwalk virus infection and disease is associated with ABO histo-blood group type. *J. Infect. Dis.* **185**:1335–1337.
- Hutson, A. M., R. L. Atmar, D. M. Marcus, and M. K. Estes. 2003. Norwalk virus-like particle hemagglutination by binding to H histo-blood group antigens. *J. Virol.* **77**:405–415.
- Jiang, X., D. Y. Graham, K. Wang, and M. K. Estes. 1990. Norwalk virus genome cloning and characterization. *Science* **250**:1580–1583.
- Jiang, X., M. Wang, D. Y. Graham, and M. K. Estes. 1992. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. *J. Virol.* **66**:6527–6532.
- Joklik, W. K., and J. E. Darnell, Jr. 1961. The adsorption and early fate of purified poliovirus in HeLa cells. *Virology* **13**:439–447.
- Kanwar, Y. S., and M. G. Farquhar. 1979. Presence of heparan sulfate in the glomerular basement membrane. *Proc. Natl. Acad. Sci. USA* **76**:1303–1307.
- Kapikian, A. Z., R. G. Wyatt, R. Dolin, T. S. Thornhill, A. R. Kalica, and R. M. Chanock. 1972. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. *J. Virol.* **10**:1075–1081.
- Kaplan, J. E., G. W. Gary, R. C. Baron, N. Singh, L. B. Schonberger, R. Feldman, and H. B. Greenberg. 1982. Epidemiology of Norwalk gastroenteritis and the role of Norwalk virus in outbreaks of acute nonbacterial gastroenteritis. *Ann. Intern. Med.* **96**:756–761.
- Kaplan, J. E., R. A. Goodman, L. B. Schonberger, E. C. Lippy, and G. W. Gary. 1982. Gastroenteritis due to Norwalk virus: an outbreak associated with a municipal water system. *J. Infect. Dis.* **146**:190–197.
- Katayama, K., H. Shirato-Horikoshi, S. Kojima, T. Kageyama, T. Oka, F. Hoshino, S. Fukushi, M. Shinohara, K. Uchida, Y. Suzuki, T. Gojibori, and N. Takeda. 2002. Phylogenetic analysis of the complete genome of 18 Norwalk-like viruses. *Virology* **299**:225–239.
- Kjellen, L., I. Pettersson, and M. Hook. 1981. Cell-surface heparan sulfate:

- an intercalated membrane proteoglycan. *Proc. Natl. Acad. Sci. USA* **78**: 5371–5375.
42. Kobayashi, S., K. Sakae, Y. Suzuki, H. Ishiko, K. Kamata, K. Suzuki, K. Natori, T. Miyamura, and N. Takeda. 2000. Expression of recombinant capsid proteins of chitta virus, a genogroup II Norwalk virus, and development of an ELISA to detect the viral antigen. *Microbiol. Immunol.* **44**:687–693.
 43. Kobayashi, S., K. Sakae, Y. Suzuki, K. Shinozaki, M. Okada, H. Ishiko, K. Kamata, K. Suzuki, K. Natori, T. Miyamura, and N. Takeda. 2000. Molecular cloning, expression, and antigenicity of Seto virus belonging to genogroup I Norwalk-like viruses. *J. Clin. Microbiol.* **38**:3492–3494.
 44. Laterra, J., J. E. Silbert, and L. A. Culp. 1983. Cell surface heparan sulfate mediates some adhesive responses to glycosaminoglycan-binding matrices, including fibronectin. *J. Cell Biol.* **96**:112–123.
 45. Lew, J. F., A. Z. Kapikian, J. Valdesuso, and K. Y. Green. 1994. Molecular characterization of Hawaii virus and other Norwalk-like viruses: evidence for genetic polymorphism among human caliciviruses. *J. Infect. Dis.* **170**:535–542.
 46. Lindesmith, L., C. Moe, S. Marionneau, N. Ruvoen, X. Jiang, L. Lindblad, P. Stewart, J. Le Pendu, and R. Baric. 2003. Human susceptibility and resistance to Norwalk virus infection. *Nat. Med.* **9**:548–553.
 47. Lohse, D. L., and R. J. Linhardt. 1992. Purification and characterization of heparin lyases from *Flavobacterium heparinum*. *J. Biol. Chem.* **267**:24347–24355.
 48. Maccarana, M., Y. Sakura, A. Tawada, K. Yoshida, and U. Lindahl. 1996. Domain structure of heparan sulfates from bovine organs. *J. Biol. Chem.* **271**:17804–17810.
 49. Marionneau, S., N. Ruvoen, B. Le Moullac-Vaidye, M. Clement, A. Cailleau-Thomas, G. Ruiz-Palacios, P. Huang, X. Jiang, and J. Le Pendu. 2002. Norwalk virus binds to histo-blood group antigens present on gastroduodenal epithelial cells of secretor individuals. *Gastroenterology* **122**:1967–1977.
 50. Mason, H. S., J. M. Ball, J. J. Shi, X. Jiang, M. K. Estes, and C. J. Arntzen. 1996. Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. *Proc. Natl. Acad. Sci. USA* **93**:5335–5340.
 51. Medeiros, G. F., A. Mendes, R. A. B. Castro, E. C. Bau, H. B. Nader, and C. P. Dietrich. 2000. Distribution of sulfated glycosaminoglycans in the animal kingdom: widespread occurrence of heparin-like compounds in invertebrates. *Biochim. Biophys. Acta* **1475**:287–294.
 52. Meeroff, J. C., D. S. Schreiber, J. S. Trier, and N. R. Blacklow. 1980. Abnormal gastric motor function in viral gastroenteritis. *Ann. Intern. Med.* **92**:370–373.
 53. Mitsuya, H., D. J. Looney, S. Kuno, R. Ueno, F. Wong-Staal, and S. Broder. 1988. Dextran sulfate suppression of viruses in the HIV family: inhibition of virion binding to CD4+ cells. *Science* **240**:646–649.
 54. Montgomery, R. I., M. S. Warner, B. J. Lum, and P. G. Spear. 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* **87**:427–436.
 55. Morse, D. L., J. J. Guzewish, J. P. Hanrahan, R. Stricof, M. Shayegani, R. Deibel, J. C. Grabau, N. A. Nowak, J. E. Herrmann, G. Cukor, and N. R. Blacklow. 1986. Widespread outbreaks of clam- and oyster-associated gastroenteritis. Role of Norwalk virus. *N. Engl. J. Med.* **314**:678–681.
 56. Nader, H. B., C. P. Dietrich, V. Buonassisi, and P. Colburn. 1987. Heparin sequences in the heparan sulfate chains of an endothelial cell proteoglycan. *Proc. Natl. Acad. Sci. USA* **84**:3565–3569.
 57. Parrino, T. A., D. S. Schreiber, J. S. Trier, A. Z. Kapikian, and N. R. Blacklow. 1977. Clinical immunity in acute gastroenteritis caused by Norwalk agent. *N. Engl. J. Med.* **297**:86–89.
 58. Payne, E., M. R. Bowles, A. Don, J. F. Hancock, and N. A. J. McMillan. 2001. Human papillomavirus type 6b virus-like particles are able to activate the Ras-MAP kinase pathway and induce cell proliferation. *J. Virol.* **75**:4150–4157.
 59. Prasad, B. V. V., M. E. Hardy, T. Dokland, J. Bella, M. G. Rossmann, and M. K. Estes. 1999. X-ray crystallographic structure of the Norwalk virus capsid. *Science* **286**:287–290.
 60. Prasad, B. V. V., R. Rothnagel, X. Jiang, and M. K. Estes. 1994. Three-dimensional structure of baculovirus-expressed Norwalk virus capsids. *J. Virol.* **68**:5117–5125.
 61. Roberts, R., J. Gallagher, E. Spooner, T. D. Allen, F. Bloomfield, and T. M. Dexter. 1988. Heparan sulphate bound growth factors: a mechanism for stromal cell mediated haemopoiesis. *Nature* **332**:376–378.
 62. Rostand, K. S., and J. D. Esko. 1997. Microbial adherence to and invasion through proteoglycans. *Infect. Immun.* **65**:1–8.
 63. Schreiber, D. S., N. R. Blacklow, and J. S. Trier. 1973. The mucosal lesion of the proximal small intestine in acute infectious nonbacterial gastroenteritis. *N. Engl. J. Med.* **288**:1318–1323.
 64. Schreiber, D. S., N. R. Blacklow, and J. S. Trier. 1974. The small intestinal lesion induced by Hawaii agent acute infectious nonbacterial gastroenteritis. *J. Infect. Dis.* **129**:705–708.
 65. Shukla, D., J. Liu, P. Blaiklock, N. W. Shworak, X. Bai, J. D. Esko, G. H. Cohen, R. J. Eisenberg, R. D. Rosenberg, and P. G. Spear. 1999. A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. *Cell* **99**:13–22.
 66. Steiner, H., D. Hultmark, A. Engstrom, H. Bennich, and H. G. Boman. 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* **292**:246–248.
 67. Subekti, D. S., P. Tjaniadi, M. Lesmana, J. McArdle, D. Iskandriati, I. N. Budiarsa, P. Walujo, I. H. Suparto, I. Winoto, J. R. Campbell, K. R. Porter, D. Sajuthi, A. A. Ansari, and B. A. Oyofo. 2002. Experimental infection of Macaca nemestrina with a Toronto Norwalk-like virus of epidemic viral gastroenteritis. *J. Med. Virol.* **66**:400–406.
 68. Subramanian, G., D. S. McClain, A. Perez, and A. O. Fuller. 1994. Swine testis cells contain functional heparan sulfate but are defective in entry of herpes simplex virus. *J. Virol.* **68**:5667–5676.
 69. Summerford, C., and R. J. Samulski. 1998. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J. Virol.* **72**:1438–1445.
 70. Tamura, M., K. Natori, M. Kobayashi, T. Miyamura, and N. Takeda. 2003. Inhibition of attachment of virions of Norwalk virus to mammalian cells by soluble histone molecules. *Arch. Virol.* **148**:1659–1670.
 71. Tamura, M., K. Natori, M. Kobayashi, T. Miyamura, and N. Takeda. 2000. Interaction of recombinant Norwalk virus particles with the 105-kilodalton cellular binding protein, a candidate receptor molecule for virus attachment. *J. Virol.* **74**:11589–11597.
 72. Toida, T., H. Yoshida, H. Toyoda, I. Koshiishi, T. Imanari, R. E. Hileman, J. R. Fromm, and R. J. Linhardt. 1997. Structural differences and the presence of unsubstituted amino groups in heparan sulphates from different tissues and species. *Biochem. J.* **322**:499–506.
 73. Toledo, O. M. S., and C. P. Dietrich. 1977. Tissue specific distribution of sulfated mucopolysaccharides in mammals. *Biochim. Biophys. Acta* **498**:114–122.
 74. Tsang, S. K., B. M. McDermott, V. R. Racaniello, and J. M. Hogle. 2001. Kinetic analysis of the effect of poliovirus receptor on viral uncoating: the receptor as a catalyst. *J. Virol.* **75**:4984–4989.
 75. Vaheri, A. 1964. Heparin and related polyionic substances as virus inhibitors. *Acta Pathol. Microbiol. Scand.* **171**(Suppl.):S1–S98.
 76. Villeponteau, B. 1992. Heparin increases chromatin accessibility by binding the trypsin-sensitive basic residues in histones. *Biochem. J.* **288**:953–958.
 77. Vlodavsky, I., J. Folkman, R. Sullivan, R. Fridman, R. Ishai-Michaeli, J. Sasse, and M. Klagsbrun. 1987. Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. *Proc. Natl. Acad. Sci. USA* **84**:2292–2296.
 78. Wang, J., X. Jiang, H. P. Madore, J. Gray, U. Desselberger, T. Ando, Y. Seto, I. Oishi, J. F. Lew, K. Y. Green, and M. K. Estes. 1994. Sequence diversity of small, round-structured viruses in the Norwalk virus group. *J. Virol.* **68**:5982–5990.
 79. Watson, K., N. J. Gooderham, D. S. Davies, and R. J. Edwards. 1999. Nucleosomes bind to cell surface proteoglycans. *J. Biol. Chem.* **274**:21707–21713.
 80. White, L. J., J. M. Ball, M. E. Hardy, T. N. Tanaka, N. Kitamoto, and M. K. Estes. 1996. Attachment and entry of recombinant Norwalk virus capsids to cultured human and animal cell lines. *J. Virol.* **70**:6589–6597.
 81. WuDunn, D., and P. G. Spear. 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J. Virol.* **63**:52–58.
 82. Wyatt, R. G., H. B. Greenberg, D. W. Dalgard, W. P. Allen, D. L. Sly, T. S. Thornhill, R. M. Chanock, and A. Z. Kapikian. 1978. Experimental infection of chimpanzees with the Norwalk agent of epidemic viral gastroenteritis. *J. Med. Virol.* **2**:89–96.
 83. Yamagata, T., H. Saito, O. Habuchi, and S. Suzuki. 1968. Purification and properties of bacterial chondroitinases and chondrosulfatases. *J. Biol. Chem.* **243**:1523–1535.
 84. Yayon, A., M. Klagsbrun, J. D. Esko, P. Leder, and D. M. Ornitz. 1991. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* **64**:841–848.
 85. Zasloff, M. 1987. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc. Natl. Acad. Sci. USA* **84**:5449–5453.