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Porcine Circovirus 2 Uses Heparan Sulfate and Chondroitin Sulfate B Glycosaminoglycans as Receptors for Its Attachment to Host Cells

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Monocyte/macrophage lineage cells are target cells in vivo for porcine circovirus 2 (PCV2) replication. The porcine monocytic cell line 3D4/31 supports PCV2 replication in vitro, and attachment and internalization kinetics of PCV2 have been established in these cells. However, PCV2 receptors remain unknown. Glycosaminoglycans (GAG) are used by several viruses as receptors. The present study examined the role of GAG in attachment and infection of PCV2. Heparin, heparan sulfate (HS), chondroitin sulfate B (CS-B), but not CS-A, and keratan sulfate reduced PCV2 infection when these GAG were incubated with PCV2 prior to and during inoculation of 3D4/31 cells. Enzymatic removal of HS and CS-B prior to PCV2 inoculation of 3D4/31 cells significantly reduced PCV2 infection. Similarly, when PCV2 virus-like particles (VLP) were allowed to bind onto 3D4/31 cells in the presence of heparin and CS-B, attachment was strongly reduced. Titration of field isolates and low- and high-passage laboratory strains of PCV2 in the presence of heparin significantly reduced PCV2 titers, showing that the capacity of PCV2 to bind GAG was not acquired during in vitro cultivation but is an intrinsic feature of wild-type virus. When Chinese hamster ovary (CHO) cells were inoculated with PCV2, relative percentages of PCV2-infected cells were 27% ± 8% for HS-deficient and 12% ± 10% for GAG-deficient cells compared to wild-type cells (100%). Furthermore, it was shown using heparin-Sepharose chromatography that both PCV2 and PCV2 VLP directly interacted with heparin. Together, these results show that HS and CS-B are attachment receptors for PCV2.

Postweaning multisystemic wasting syndrome (PMWS) affects 5- to 14-week-old piglets and is associated with porcine circovirus type 2 (PCV2) infection (2, 7, 26, 29). PCV2 belongs to the genus *Circovirus* of the *Circoviridae* family along with psittacine beak and feather disease virus, columbid circovirus, goose circovirus, canary circovirus, and duck circovirus (3, 19, 38, 48, 60). PCV2 is a small nonenveloped single-stranded circular DNA virus with a 1.76-kb ambisense genome (59). The genome contains two major open reading frames (ORFs). ORF1 encodes the replicase protein (Rep and Rep′) involved in rolling circle PCV2 DNA replication, and ORF2 encodes the capsid protein which is the major immunogenic protein of PCV2 (11, 33, 46).

Virus attachment to host cell surface receptor(s) is the first step in establishing an infection, and in many cases, this step dictates the cell and tissue tropism and pathogenesis. Up till now, no receptor has been identified for PCV2. Cells of the monocyte/macrophage lineage have consistently been shown to be targets for porcine circovirus replication in vivo and appear to be important in the pathogenesis of PMWS (1, 52). Besides, a variety of cell types including hepatocytes, enterocytes, renal and alveolar epithelial cells, vascular endothelial cells, pancreatic acinar and ductular cells, lymphocytes, smooth muscle cells, and fibroblasts have also been shown to contain PCV2 antigens and/or nucleic acid (12). The porcine monocytic cell line 3D4/31 supports PCV2 replication in vitro, and attachment and internalization kinetics of PCV2 have been established in these cells (44). PCV2 has been shown to bind to all 3D4/31 cells and to internalize via clathrin-mediated endocytosis in a very low proportion of cells (44).

In the present study, the possible role of glycosaminoglycans (GAG) as PCV2 receptors was examined. Several hypotheses warranted the examination of the possible role of GAG in PCV2 attachment. First, many viruses have the ability to utilize surface GAG as cellular receptors for attachment, which enables them to take their first step toward establishing infection (9, 35, 50, 62). Second, the broad range of cells that support PCV2 infection and the fact that PCV2 genome and/or antigens have been detected in a wide variety of cells (12) indicates that PCV2 utilizes a broadly expressed receptor. Third, the XBBXBX heparan sulfate (HS) binding motif (where B stands for a basic amino acid and X stands for a neutral/hydrophobic amino acid), described by Cardin and Weintraub (10), is present on the PCV2 capsid protein as ^{98}IRKVKV^{103}. This sequence is conserved among PCV2 isolates.

Proteoglycans are expressed in all adherent cells and consist of a core protein and one or more covalently attached GAG chains (49). GAG include HS, chondroitin sulfate (CS), and keratan sulfate (KS). Each GAG differs in its disaccharide repeating units, which are the building blocks for the polysaccharides. The repeated disaccharide units are made up of an acidic sugar (uronic acid, either L-iduronic or D-glucuronic) alternating with an amino sugar (hexosamine, either D-glucosamine or D-galactosamine) (22, 27). Growing GAG chains are variably modified by epimerization and sulfation, and this can have a profound effect on binding of ligands. Most cell surface proteoglycans contain HS (e.g., the glypicans), but many are hybrid structures containing both HS and CS (e.g., the syndecans and beta-glycan) (50). A few cell surface proteoglycans

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contain exclusively CS (e.g., thrombomodulin, CD44, and NG2) (50).

Different experimental criteria have been used to establish a role for GAG in attachment and infection of host cells, including direct binding measurements, competition studies with defined soluble GAG, enzymatic removal of GAG from target cells, and altered adherence to animal cell mutants with defective GAG biosynthesis. In this study, these assays were performed to establish the role of GAG in PCV2 attachment.

MATERIALS AND METHODS

Virus, cells, and PCV2 virus-like particles. PCV2 strain Stoon-1010 isolated from a PMWS-affected piglet in Canada (15) was used in this study. A virus stock propagated in PK-15 cells with a titer of $10^{5.6}$ 50% tissue culture infective doses/ml was used at multiplicity of infection of 0.3 in (i) experiments to investigate the effect of soluble GAG and enzymatic removal of cell surface GAG on PCV2 infection of 3D4/31 cells and (ii) inoculation of Chinese hamster ovary (CHO) cells. Low- and high-passage PCV2 strains Stoon-1010 (15), VC2002 isolated from a PMWS-affected piglet in Belgium (42), and 1121 isolated from an aborted fetus in Canada (40) were used. Five other Belgian PCV2 isolates (VA88, VA94, VA302, VC4, and VC7371) obtained from lymphoid tissue homogenates of PMWS pigs were used without passaging to investigate the effect of in vitro cultivation of PCV2 on GAG affinity.

The continuous porcine monocytic cell line 3D4/31, developed from porcine alveolar mϕ (63), was grown in a 1:1 mixture of RPMI 1640 (GIBCO-BRL) and minimal essential medium containing Earle's salts (GIBCO-BRL) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 0.3 mg/ml glutamine (BDH Chemicals Ltd., Poole, England), 1% nonessential amino acids (100×; GIBCO-BRL), and antibiotics (monocyte/macrophage medium). Cells were seeded at 2×10^5 cells/ml of monocyte/macrophage medium in 96-well cell culture plates (Nunc, Roskilde, Denmark). Cells were incubated in a humidified incubator at 37°C in the presence of 5% CO_2. For experiments involving the use of PCV2 virus-like particles (VLP), cells were seeded at 1.5×10^5 cells/ml of monocyte/macrophage medium in eight-well Lab-Tek cover glass chamber slides (Nalge Nunc International, Naperville, IL). In both cases, cells were cultivated for 24 h before experimentation.

CHO cells were grown in Ham's F-12K medium (GIBCO-BRL) with 5% FBS, 1 mM sodium pyruvate, 0.3 mg/ml glutamine, and antibiotics (CHO medium). Cells were seeded at 2×10^5 cells/ml in 96-well cell culture plates for 24 h before they were inoculated with PCV2. In this study, three types of CHO cells were used. Wild-type CHO (CHO-K1) cells, which produce about 70% HS and 30% CS (34), were used. The mutant CHO *pgs*A-745 cells are deficient in the enzyme UDP-D-xylose:serine-1,3-D-xylosyltransferase, the first sugar transfer reaction in GAG formation, and thus, completely lack GAG (17). The mutant CHO *pgs*D-677 cells are deficient in *N*-acetylglucosaminyltransferase and glucuronosyltransferase activities required for HS polymerization and, thus, completely lack HS and produce three- to fourfold-higher levels of CS (34).

Porcine kidney epithelial (PK-15) cells were seeded in 96-well cell culture plates at 1.5×10^5 cells/ml in minimal essential medium with Earle's salts (GIBCO-BRL) supplemented with 5% FBS, 0.3 mg/ml glutamine, and antibiotics. Cells were maintained at 37°C in the presence of 5% CO_2 for 24 h to obtain 50% confluence.

For PCV2 attachment and internalization studies, PCV2 VLP were used as previously described (44). PCV2 VLP were purified in a cesium chloride gradient from *Spodoptera frugiperda* 9 (Sf9) insect cells infected with recombinant baculoviruses harboring PCV2 ORF2, the gene encoding the PCV2 capsid. PCV2 VLP used in this study have been characterized using PCV2-specific monoclonal antibodies and porcine polyclonal antibodies from PCV2-infected pigs and by fluorescence and electron microscopy (44).

Evaluation of the role of GAG in PCV2 infection of 3D4/31 cells. To investigate whether GAG are involved as receptors in PCV2 infection, defined soluble GAG were employed as soluble receptor analogues in competition experiments. Porcine intestinal mucosa heparin, bovine kidney HS, bovine trachea chondroitin sulfate A (CS-A), and porcine intestinal mucosa CS-B (dermatan sulfate) were obtained from Sigma. Bovine cornea KS was obtained from Seikagaku Corp. (Tokyo, Japan). GAG were dissolved in 1:1 RPMI 1640-Dulbecco's modified Eagle medium (DMEM) mixture. For competitive inhibition, PCV2 was incubated with either one GAG or a combination of two GAG at 37°C for 90 min before the mixture was added to 3D4/31 cells for 60 min at 37°C. The GAG-virus mixture was then washed off, and cells were further incubated with monocyte/macrophage medium until fixation after the first replication cycle of PCV2 at 36 h postinoculation (hpi) (41). Control cells were incubated with GAG for 90 min at 37°C followed by extensive washing. Control cells were then inoculated with PCV2 for 60 min at 37°C, the inoculum was washed off, and cells were incubated further in monocyte/macrophage medium until they were fixed at 36 hpi. PCV2-infected cells were stained as previously described (30). Briefly, cells were fixed and incubated with porcine polyclonal anti-PCV2 antibodies followed by horseradish peroxidase-conjugated polyclonal rabbit anti-swine immunoglobulins (DakoCytomation, Glostrup, Denmark), each for 1 h at 37°C. Substrate was added to stain-infected cells which were counted by examination with an Olympus light microscope (Olympus Optical Co., Hamburg, Germany).

Enzymatic removal of GAG from the cell surface of 3D4/31 cells. Heparinase I from *Flavobacterium heparinum* (heparinase, EC 4.2.2.7), chondroitinase AC from *Flavobacterium heparinum* (EC 4.2.2.5), and chondroitinase ABC from *Proteus vulgaris* (EC 4.2.2.4) were from Sigma. Heparinase I cleaves glycosidic linkages in HS and liberates it from the cell surface (37, 56). Chondroitinase AC cleaves glycosidic linkages in CS-A and CS-C, while chondroitinase ABC specifically cleaves glycosidic linkages in CS-A, CS-B, and CS-C (64). A single enzyme or a combination of two enzymes were added to 3D4/31 cells for 60 min at 37°C and 5% CO_2 in RPMI 1640-DMEM mixture. Cells were then washed and inoculated with PCV2 for 60 min at 37°C and 5% CO_2. Afterwards, the viral inoculum was washed off and cells were overlaid with monocyte/macrophage medium. Cells were further incubated at 37°C and 5% CO_2 until fixation at 36 hpi. Under the present assay conditions, enzymatic treatment did not cause cell death or detachment of cells from the culture plates. PCV2-infected cells were stained as described above.

PCV2 inoculation of CHO cells. CHO-K1 cells, HS-deficient CHO *pgs*D-667 cells, and GAG-deficient CHO *pgs*A-745 cells were inoculated with PCV2 at 37°C for 60 min. Afterwards, the viral inoculum was washed off and cells were overlaid with CHO medium. Cells were further incubated at 37°C in a humidified 5% CO_2 incubator until fixation at 36 hpi. PCV2-infected cells were stained as described above.

Binding of PCV2 and PCV2 VLP onto heparin-Sepharose. Purified PCV2 or PCV2 VLP were applied to a heparin-Sepharose column (Amersham Biosciences) at a flow rate of 1 ml per minute. The column was washed with phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} to remove unbound material. Bound proteins or virus particles were then eluted with 2,500 μg/ml heparin containing increasing concentrations of NaCl. High concentrations of salt disrupt the electrostatic interactions between heparin and heparin-binding proteins, and this technique can be used to determine the relative strength of binding. Original fractions and fractions of PCV2 and PCV2 VLP collected from the flowthrough, washing, and elution steps were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting using PCV2 capsid-specific monoclonal antibody F217 (39). The amount of infectious PCV2 virus in the different fractions was determined by virus titration as previously described (41).

Analysis of PCV2 VLP attachment to 3D4/31 cells. Analysis of PCV2 VLP binding to 3D4/31 cells using fluorescence confocal microscopy has been used before (44). To investigate the effect of GAG on binding of PCV2 VLP to 3D4/31 cells, PCV2 VLP were incubated with 2,500 μg/ml of heparin, CS-A, or CS-B at 37°C for 90 min. Control PCV2 VLP were incubated with RPMI 1640-DMEM mixture. The GAG/PCV2 VLP mixture or control VLP were then chilled on ice and added to 3D4/31 at 4°C for 60 min. Afterwards, cells were washed with RPMI 1640-DMEM mixture. Cells were then fixed with 3% paraformaldehyde in PBS with Ca^{2+} and Mg^{2+} for 20 min at room temperature. Cells were washed, and PCV2 VLP were stained using biotinylated anti-PCV2 swine antibodies followed by streptavidin-conjugated fluorescein isothiocyanate, each for 1 h. Images of bound PCV2 VLP on 3D4/31 cell were acquired by fluorescence confocal microscopy with a Leica TCS SP2 laser-scanning spectral confocal system. The total fluorescence area of bound PCV2 VLP was estimated using the image analysis software SigmaScan Pro 5.0. This software was used to calculate the total fluorescence of cell-bound PCV2 VLP for 20 cells under each experimental condition as previously described (44).

Statistical analysis. Results were tested for significance using the Mann-Whitney U test. Results that had P values of <0.05 were considered significant. Statistical analyses were performed using SPSS, version 12.0, software (SPSS Inc., Chicago, Illinois).

RESULTS

Heparin, heparan sulfate, and chondroitin sulfate B reduce PCV2 infection of 3D4/31 cells. To investigate the role of GAG as receptors in PCV2 infection of 3D4/31 cells, competition

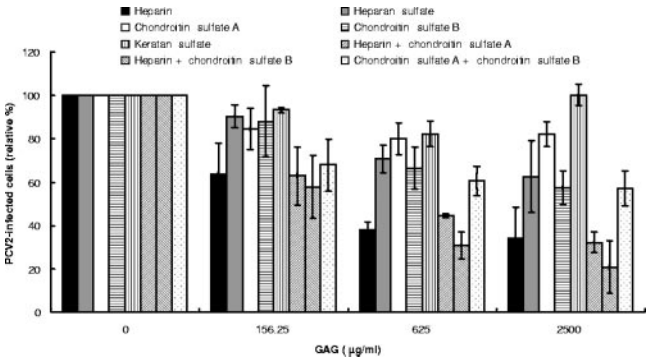

FIG. 1. Effect of GAG on virus infection. Equal amounts of PCV2 were mixed with various GAG or GAG combinations at indicated concentrations for 90 min at 37°C. The mixture was then added to 3D4/31 cells in serum-free conditions, and cells were incubated for 60 min at 37°C in a 5% CO_2 atmosphere. Cells were then washed and overlaid with monocyte/macrophage medium. After 36 h, cells were fixed and stained for PCV2 antigens. Points represent the relative percentages of PCV2 antigen-positive cells from three experiments ± standard deviations.

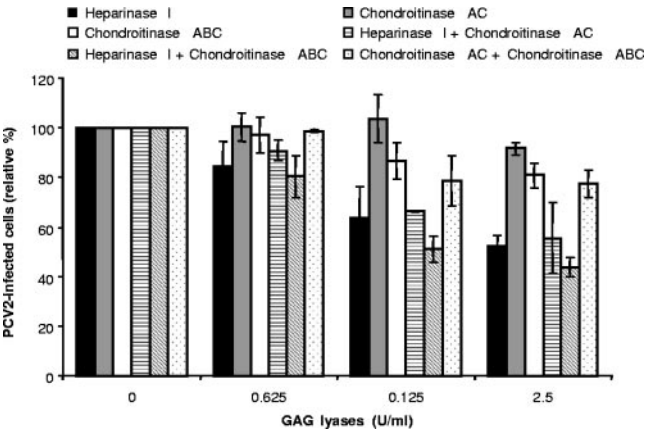

FIG. 2. Effect of enzymatic removal of GAG from the cell surface. 3D4 cells were digested with one or a combination of two GAG lyases for 60 min at 37°C. The enzymes were washed off, and cells were inoculated with PCV2 for 60 min at 37°C in a 5% CO_2 atmosphere. Cells were then washed and overlaid with monocyte/macrophage medium. After 36 h, cells were fixed and stained for PCV2 antigens. Points represent the relative percentages of PCV2 antigen positive cells from three experiments ± standard deviations.

studies with defined GAG were performed. PCV2 was mixed with GAG at different concentrations and inoculated into 3D4/31 cells. The number of infected cells was determined 36 h after inoculation. The effect of different GAG on the number of PCV2-infected cells is shown in Fig. 1. Heparin had the strongest inhibitory effect at the highest concentration tested, reducing PCV2 infection by 66% ± 14%, relative to mock treatment. The highest concentrations of HS and CS-B tested also reduced infection by 37% ± 17% and 43% ± 8%, respectively. In contrast, CS-A and KS had no significant effect on PCV2 infection (18% ± 6% and 0% ± 5% reduction of infection, respectively) at their highest concentrations used in this study. The effect of different GAG combinations was also investigated. At the highest concentration of GAG combinations, the relative percentages of PCV2-infected cells were 32% ± 5% for the heparin–CS-A mixture, 21% ± 12% for the heparin–CS-B mixture, and 57% ± 8% for the CS-A–CS-B mixture compared to mock treatment. The heparin–CS-B combination showed additive effect on reducing PCV2 infection, but infection was not completely blocked (Fig. 1).

Enzymatic removal of heparin sulfate and chondroitin sulfate B from the cell surface reduces PCV2 infection of 3D4/31 cells. To investigate the role of cell surface GAG on PCV2 infection of 3D4/31 cells, enzymes were used to cleave GAG and liberate them from the cell surface. The relative percentage of PCV2-infected cells following enzymatic removal of (i) HS from cells with heparinase I was 52% ± 5%, (ii) CS-A, CS-B, and CS-C with chondroitinase ABC was 81% ± 5%, and (iii) CS-A and CS-C with chondroitinase AC was 92% ± 3% (Fig. 2). The removal of HS and CS-B caused a significant reduction of PCV2 infection, while removal of CS-A and CS-C did not. A combination of enzymes was also used as shown in Fig. 2, and the effect of combined HS and CS-B removal was additive. However, using the combined treatments, infection could not be blocked completely.

PCV2 infects parental GAG-expressing CHO-K1 cells more efficiently than mutant CHO cells lacking GAG. To further verify the role of HS and other GAG in infection of PCV2, wild-type CHO-K1 cells expressing HS and CS and two mutant cell lines, *pgs*D-677 cells (deficient in HS but not CS) and *pgs*A-745 cells (deficient in both HS and CS) (17, 34), were inoculated with PCV2. CHO-K1 cells were shown to support PCV2 infection, since the capsid protein was localized in the nucleus. For the capsid protein to be localized in the nucleus, it needs to be expressed and transported to the nucleus guided by its nuclear localization signal (36, 53). The relative percentages of PCV2 infection of the *pgs*D-677 and *pgs*A-745 cells compared to CHO-K1 are shown in Fig. 3. CHO *pgs*A-745 cells showed 12% PCV2 infection compared to 100% in CHO-K1

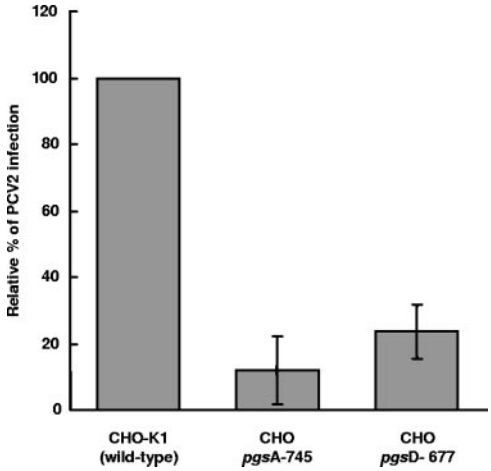

FIG. 3. Infection of PCV2 in CHO cell lines. Semiconfluent monolayers of wild-type CHO-K1 cells and of two CHO mutants, *pgs*D-667 (lacking HS but not CS) and *pgs*A-745 (lacking both HS and CS) were inoculated with PCV2 at 37°C for 1 h. Thereafter, the viral inoculum was washed off and cells were further incubated with CHO medium for 36 h. Cells were then fixed and stained, and PCV2-antigen positive cells were counted as described in Materials and Methods. Data represent means ± standard deviations of results from three experiments.

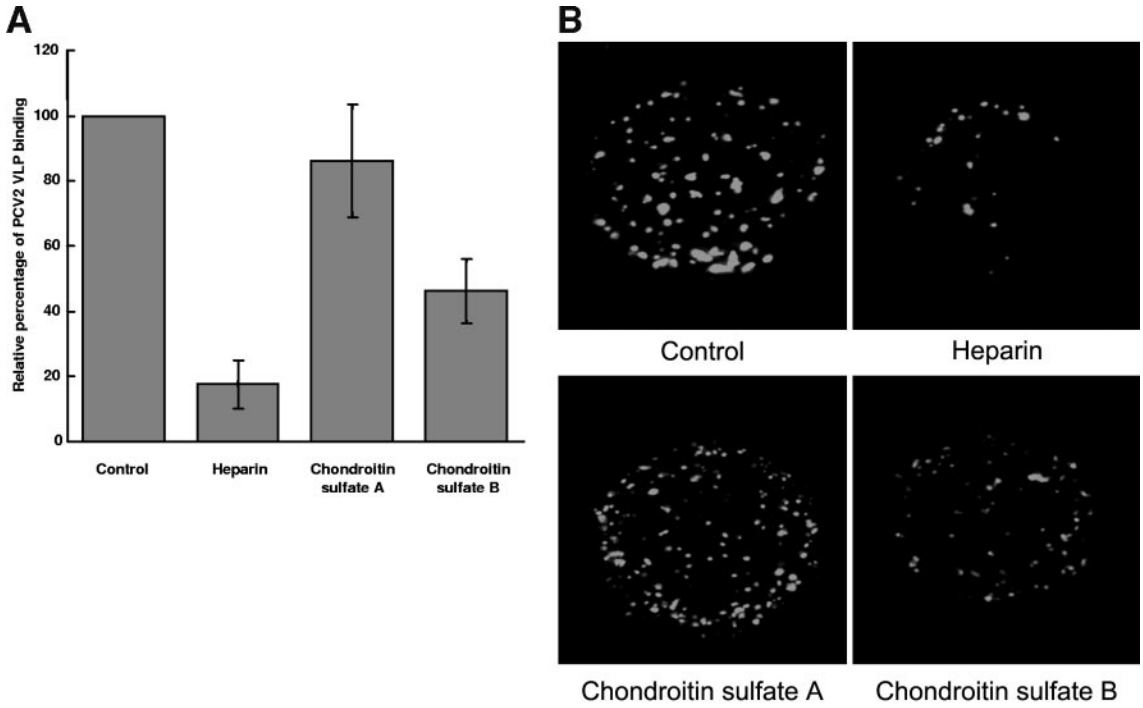

FIG. 4. PCV2 VLP were mixed with 2,500 μg/ml heparin, chondroitin sulfate A, or chondroitin sulfate B (dermatan sulfate) before they were allowed to bind to 3D4/31 cells for 60 min at 4°C. Cells were stained for PCV2 VLP. The amount of bound PCV2 VLP per cell was calculated from confocal images using SigmaScan Pro 5.0 and are represented on the left. The images shown on the right are representative confocal images of bound PCV2 VLP after incubating PCV2 VLP with either RPMI 1640 (control), heparin, CS-A, or CS-B (dermatan sulfate). Each panel represents an overlay of a series of confocal images taken from the apex to the base of a single cell.

cells, indicating that both HS and CS are PCV2 receptors. CHO *pgs*D-677 cells showed 24% PCV2 infection compared to 12% CHO *pgs*A-745 cells, indicating that HS was the PCV2 receptor compared to CS.

Effect of GAG on PCV2 VLP binding to 3D4/31 cells. To investigate whether heparin and CS-B reduce PCV2 infection of 3D4/31 cells by reducing virus attachment, the effect of GAG on PCV2 attachment to 3D4/31 cells was analyzed. PCV2 VLP were used in binding studies instead of PCV2 virions because of the difficulty in obtaining sufficient preparative amounts of the latter, due to low PCV2 titers. Binding assays of PCV2 VLP on 3D4/31 cells were performed as previously described (44) in the presence or absence of GAG. PCV2 VLP were mixed with either heparin, CS-B, or CS-A. Control PCV2 VLP attachment to cells was performed in the absence of GAG. Images of PCV2 VLP immunolabeled with fluorescein isothiocyanate were acquired using fluorescence confocal microscopy. Representative confocal images are shown in Fig. 4, right panel. Heparin strongly reduced PCV2 VLP binding to 3D4/31 cells (Fig. 4). A significant reduction of PCV2 VLP binding to 3D4/31 cells was also observed with CS-B. In contrast, CS-A did not cause any significant reduction of PCV2 VLP binding.

Titration of laboratory and field PCV2 isolates in PK-15 cells in the presence of heparin reduces PCV2 titer. For a number of viruses, including alphaviruses (5, 28), pestiviruses (20), picornaviruses (16, 51), and retroviruses (45, 47), it has been demonstrated that in vitro cultivation results in the selection of mutants that bind HS with high affinity. Sequence analysis of in vivo- and in vitro-grown viruses showed that cultivation rapidly induced amino acid substitutions that increase the net positive charge of their envelope proteins, which results in enhanced ability to bind HS or other GAG (31). Therefore, field PCV2 isolates isolated directly from PMWS-positive piglets without in vitro cultivation and low and high passages of laboratory PCV2 strains were titrated in the presence or absence of heparin. This allowed us to investigate the effect of in vitro cultivation and PCV2 passage on the interaction between PCV2 and heparin. Titration of field isolates and low and high passages of laboratory PCV2 strains in the presence of heparin significantly reduced PCV2 titers, as shown in Fig. 5. This indicated the absence of PCV2 cell culture adaptation toward a high-affinity binding to heparin.

Binding of PCV2 and PCV2 VLP to immobilized heparin. To examine whether PCV2 and PCV2 VLP binds directly to GAGs, PCV2 virions and PCV2 VLP were applied to a heparin-Sepharose column. After binding, the column was washed with PBS, and elution of bound material was done with 2,500 μg/ml heparin in PBS in the presence of increasing NaCl concentrations (0, 0.5, 1, and 2.5 mM). The fractions of both virus and VLP obtained were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting for the presence of PCV2 capsid protein. The fractions of PCV2 virus obtained were also analyzed by titration for the presence of infectious virus. Western blotting of original fractions and collected fractions of PCV2 and PCV2 VLP are shown in Fig. 6, top. PCV2 and PCV2 VLP could be specifically eluted by heparin, indicating that their interaction with

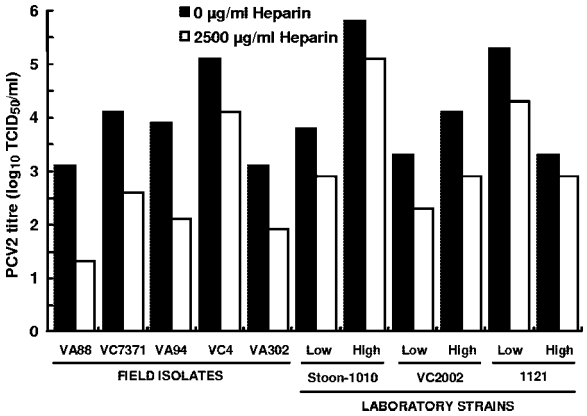

FIG. 5. PCV2 titers of laboratory and field PCV2 isolates titrated in PK-15 cells. Titration of low and high passages of laboratory PCV2 strains Stoon-1010, VC2002, and 1121 and field PCV2 strains obtained from lymphoid tissue suspension of PMWS-positive piglets (VA88, VC7371, VA94, VC4, and VA302) in the presence (□) or absence (■) of heparin.

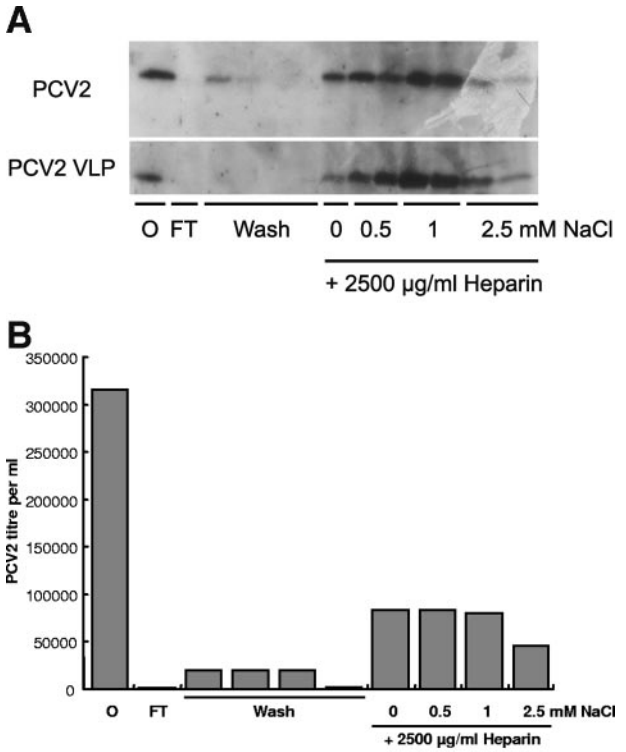

FIG. 6. Binding of PCV2 or PCV2 VLP on heparin-Sepharose. Purified PCV2 or PCV2 VLP were allowed to bind to a heparin-Sepharose column at 37°C for 60 min. Western blotting of both PCV2 virus and PCV2 VLP (top) and virus titration of PCV2 virus (bottom) of the original fraction (O), flowthrough fraction (FT), washing fractions (wash), and eluted fractions with 2,500 μg/ml heparin with increasing (0.5, 1, and 2.5 mM) NaCl concentrations were performed.

heparin in the column was specific. Only low levels of bound PCV2 or PCV2 VLP could be eluted at low NaCl concentrations, and complete elution required higher salt concentrations, indicating that the interaction between PCV2 and heparin is strong. Titration of the obtained PCV2 virus fractions confirmed that infectious virus was bound to and eluted from the heparin-Sepharose column (Fig. 6, bottom). Together, these data confirm that PCV2 virions and PCV2 VLP directly bind to heparin and are consistent with a direct interaction of PCV2 with HS present on the surface of cells.

DISCUSSION

Animal cell membranes are abundantly decorated with proteoglycans, which are made of a protein core with one or more covalently attached GAG chains that bind several different protein ligands (27, 50). These GAG have been shown to be exploited by several viruses for their attachment process to cell surfaces (35, 50). GAG have also been shown to mediate entry of viruses such as herpes simplex type 1 (54) and human T-cell leukemia virus type 1 (23). In the present study, using different approaches reviewed by Rostand and Esko (50), it was demonstrated that PCV2 uses HS and CS-B GAG for attachment to porcine monocytic 3D4/31 and porcine kidney epithelial PK-15 cells. Competition experiments were performed by allowing PCV2 to bind to soluble GAG before and during inoculation of 3D4/31 and PK-15 cells. PCV2 infection was significantly reduced in a dose-dependent manner by incubating PCV2 with heparin, HS, and CS-B but not CS-A and KS. The interaction between PCV2 and heparin, HS, or CS-B was shown to be specific, since two other GAG, CS-A and KS, had no significant effect on PCV2 infection. This means not only that the effect of heparin, HS, and/or CS-B on PCV2 infection was not merely a matter of attraction between negatively charged sulfate groups of GAG and positively charged basic amino acids on the PCV2 capsid but also that the structure of the GAG backbone is important for this interaction. In another approach, GAG lyases were used to enzymatically remove GAG from 3D4/31 cells before they were inoculated with PCV2. The removal of cell surface HS and CS-B but not of CS-A and CS-C significantly reduced PCV2 infection. Together, these experiments showed that PCV2 binds to cell surface HS and CS-B and that this binding is important for efficient infection of target cells.

Lower PCV2 infection inhibitions obtained with enzymatic removal of HS and CS-B compared to competition experiments with soluble HS and CS-B may be due in part to the lower efficiency of this method. Moreover, complete removal of all heparin moieties from the cell surface requires a combination of enzymes such as heparinase I and other enzymes such as heparitinase (25). Competitive inhibition with soluble GAG has also been shown to be more efficient than enzymatic GAG removal for other viruses using GAG as receptors such as porcine reproductive and respiratory syndrome virus (13), human parainfluenza virus type 3 (8), human respiratory syncytial virus (18), pseudorabies virus (43), bovine herpesvirus 4 (61), and human cytomegalovirus (25). The inability of soluble heparin and CS-B, or their combination, to completely prevent the virus infection could be due to the binding of PCV2 to other host cell surface molecules in the absence of HS and CS-B. Indeed, for most viruses using GAG as receptors, infection was never completely blocked using soluble GAG, and other membrane proteins were shown to be involved in virus entry.

To establish whether the inhibition of PCV2 infection by competition with soluble GAG and/or enzymatic GAG removal was a result of reduced PCV2 attachment to the cell surface receptors, PCV2 VLP were mixed with GAG before they were allowed to bind to 3D4/31 cells. Significant reduction in bound PCV2 VLP was observed with heparin, HS, and CS-B. Heparin is a more sulfated homologue of HS and is frequently used as a convenient analog of HS for experimental purposes (49). It can be concluded that PCV2 attaches to cell surface HS and CS-B, and not heparin, because heparin is expressed only in granules of connective tissue type mast cells, while HS is expressed in virtually all cell types (49). Reduction of PCV2 attachment with HS and CS-B was higher than reductions in infection, indicating that reduced infection is probably the result of reduced attachment. Previous studies have used CHO cell mutants with defective GAG biosynthesis to establish the role of GAG in virus binding. In this study, mutant CHO-derived cells lacking either HS or all GAG showed significantly lower levels of PCV2 infection compared to wild-type CHO cells, indicating that GAG were PCV2 receptors. By comparing relative PCV2 infection of mutant CHO cells, it can be concluded that HS is a major PCV2 receptor compared to CS-B. This hypothesis is also supported by the results obtained with competitive inhibition of PCV2 infection with soluble GAG and enzymatic removal of GAG from the cell surface. The fact that PCV2 could still infect mutant CHO cells that do not express GAG indicates that, apart from HS and CS-B, other yet unidentified cellular surface molecules participate in virus binding and subsequent virus entry. In this light, it is interesting that PCV2 has recently been shown to enter cells via clathrin-mediated endocytosis (44). Some membrane proteoglycans have been described to undergo endosomal cycling and, therefore, are potential internalizing receptors (6, 21). It will be of interest to investigate whether proteoglycans are involved in the internalization of PCV2 or if they enhance the efficiency of PCV2 internalization via another protein receptor.

To investigate specificity and strength of PCV2-heparin interaction, PCV2 virions and PCV2 VLP were applied to a heparin-Sepharose column. In these experiments, PCV2 and PCV2 VLP bound to a heparin-Sepharose column, and bound PCV2 or PCV2 VLP could be specifically eluted with free heparin alone or heparin containing increasing NaCl concentrations. PCV2 VLP showed a higher binding affinity than PCV2 virions. The ionic strength required for elution of PCV2 virions and PCV2 VLP from heparin-Sepharose was comparable to that seen for herpes simplex virus type 1 (58) and human papillomavirus type 11 (24).

PCV2 infection was reduced in competition experiments with heparin, HS, and CS-B, indicating that the virus utilizes HS and CS-B receptors for attachment. Several viruses, such as Sindbis virus, classical swine fever, foot-and-mouth disease virus, Japanese encephalitis virus, and West Nile virus have been shown to acquire the capacity to attach to GAG after in vitro cell cultivation (20, 28, 32, 47). In our experiments, infection of different field isolates of PCV2 obtained from lymphoid tissues and of low- and high-passage laboratory strains of PCV2 was competitively inhibited by heparin. These results indicate that the binding of PCV2 to GAG arises from neither cell culture passage nor adaptation of the virus.

The XBBXBX and XBBBXXBX sequences (where B is a basic amino acid and X is a hydrophobic residue) are putative heparin-binding motifs identified through molecular modeling of known heparin-binding proteins (10). For several viruses, such as human papillomavirus type 11 (24) and adenovirus (65), similar motifs have also been identified and proven to be essential in the interaction with HS. Analysis of PCV2 revealed a putative heparin-binding motif, ^{98}IRKVKV^{103}, in PCV2 capsid protein. This motif is highly conserved among different PCV2 isolates. The role of the putative heparin-binding motif in PCV2 capsid protein will be investigated in the future.

In conclusion, our results show that HS and CS-B are involved in PCV2 attachment and that PCV2 interaction with cell surface proteoglycans is probably the first ligand-receptor interaction leading to binding with other host cell receptors essential for the subsequent viral entry process. The hypothesis that PCV2 would require another cell entry receptor is supported by the fact that CHO cells completely lacking GAG could still be infected by PCV2. This view is similar to what has been described for several other viruses, such as herpes simplex virus (6, 55), porcine arterivirus (13, 14), adenovirus 2 (4) and adeno-associated virus 2 (57), where proteoglycans are used as coreceptors that mediate attachment and concentrate pathogens on the cell surface, thereby facilitating the interaction with other receptors that mediate internalization and/or fusion.

Knowledge on the interaction between PCV2 and target cells will allow us to define the binding region on the capsid protein which may allow the development of peptide vaccines and provide pharmaceutical strategies for prevention and treatment of PCV2 infection.

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