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Heparan Sulfate Is a Selective Attachment Factor for the Avian Coronavirus Infectious Bronchitis Virus Beaudette

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SUMMARY. The avian coronavirus infectious bronchitis virus (IBV) strain Beaudette is an embryo-adapted virus that has extended species tropism in cell culture. In order to understand the acquired tropism of the Beaudette strain, we compared the S protein sequences of several IBV strains. The Beaudette strain was found to contain a putative heparan sulfate (HS)-binding site, indicating that the Beaudette virus may use HS as a selective receptor. To ascertain the requirements of cell-surface HS for Beaudette infectivity, we assayed for infectivity in the presence of soluble heparin as a competitor and determined infectivity in mutant cell lines with no HS or glycosaminoglycan expression. Our results indicate that HS plays a role as an attachment factor for IBV, working in concert with other factors like sialic acid to mediate virus binding to cells, and may explain in part the extended tropism of IBV Beaudette.

RESUMEN. El heparán sulfato es un factor de unión para la cepa Beaudette del virus de bronquitis infecciosa.

La cepa Beaudette del virus de bronquitis infecciosa aviar (coronavirus), es un virus adaptado a embrión que tiene tropismo por un amplio rango de especies en cultivo celular. Con la finalidad de comprender el tropismo adquirido por la cepa Beaudette, se compararon las secuencias de la proteína S de varias cepas de bronquitis infecciosa aviar. Se demostró que la cepa Beaudette contiene un sitio de unión putativo para el heparán sulfato, lo que indica que el virus Beaudette debe utilizar el heparán sulfato como un receptor selectivo. Para confirmar que el heparán sulfato en la superficie celular es requerido para la infectividad del virus Beaudette, se analizó la infectividad del virus en la presencia de heparina soluble como competidor y se determinó la infectividad en líneas celulares mutantes que no expresan heparán sulfato o glicosaminoglicanos. Los resultados indican que el heparán sulfato juega un papel como un factor de unión para el virus de bronquitis infecciosa, trabajando en conjunto con otros factores como el ácido siálico para mediar la unión del virus con la célula y puede explicar en parte el amplio tropismo de la cepa Beaudette del virus de bronquitis infecciosa.

Key words: infectious bronchitis virus, receptor, heparan sulfate, sialic acid

Abbreviations: ACE2 = angiotensin-converting enzyme 2; ATCC = American Type Culture Collection; BHK = baby hamster kidney; CEK = chick embryo kidney; CHO = Chinese hamster ovary; CK = chick kidney; CoV = coronavirus; CS = chondroitin sulfate; EDTA = ethylenediaminetetraacetic acid; fAPN = feline aminopeptidase N; FFU = fluorescence focus unit; GAG = glycosaminoglycan; HS = heparan sulfate; IBV = infectious bronchitis virus; M41 = Massachusetts 41; NA = neuraminidase; NCBI = National Center for Biotechnology Information; PBS = phosphate-buffered saline; RT-PCR = reverse transcription–polymerase chain reaction; SARS = severe acute respiratory syndrome

Infectious bronchitis virus (IBV) is a coronavirus infecting domestic fowl. It was originally isolated in the 1930s and continues to cause major problems in the poultry industry (11). Coronaviruses are enveloped positive-stranded RNA viruses that replicate in the cytoplasm (23). They have a distinctive set of club-shaped spikes on their envelope, which surrounds the viral core shell and nucleocapsid. The coronavirus (CoV) S protein comprises a large, heavily glycosylated ectodomain that can be cleaved during biosynthesis into two subdomains (S1 and S2) by a furin-like enzyme in the Golgi apparatus (13). Not all coronaviruses are cleaved, yet even without cleavage the basic subdomain structure of the S protein is conserved (25). S1 comprises the receptor-binding domain (24), and S2 comprises the fusion domain (5). The S protein of IBV is fully cleaved at the S1/S2 boundary (10), especially in chicken embryo systems.

Coronaviruses show strong species and tissue tropism, and a major factor in this specificity is the virus receptor on host cells (19). Receptor binding is mediated by the viral S protein, and this has been studied in some detail for many coronaviruses, a principal exception being group 3 coronaviruses such as IBV. Group 1 coronaviruses (e.g., HCoV 229E, transmissible gastroenteritis virus, and feline infectious peritonitis virus) are well established to use aminopeptidase N (CD13) as a primary receptor (14,22,36,42); indeed most, if not all, members of this group can apparently use the feline aminopeptidase N (fAPN) for productive entry (35). An exception to this is the recently identified human coronavirus NL63, which uses angiotensin-converting enzyme 2 (ACE2), the severe acute respiratory syndrome (SARS)-CoV receptor (18). In the case of group 2 coronaviruses, glycoproteins in the carcinoembryonic antigen-related glycoprotein family act as receptors (12,39) and SARS-CoV uses ACE2 likely in combination with CD209L (20,25). There is one published report that fAPN may act as a receptor for the IBV strain Arkansas 99 (30); however, it is unclear whether APN is a general receptor for IBV. Recently, sialic acid has been identified as a receptor determinant for the IBV strains Massachusetts 41 and Beaudette-US (40). The coronavirus receptor is generally considered to be the primary determinant of host range, and this has recently been shown in some detail for SARS-CoV. Viruses isolated during the 2002–2003 SARS outbreak, during the much less severe 2003–2004 outbreak, and from palm civets were analyzed. All three S proteins bound to and used palm civet ACE2 efficiently, but the latter two S proteins used human ACE2 markedly less efficiently, and the lower affinity S proteins could be complemented by incorporating specific mutations (26).

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Like most coronaviruses, clinical isolates of IBV show distinct tropism both *in vivo* and in cell culture. The prototype IBV strain (Massachusetts 41 [M41]) causes an acute, highly contagious respiratory disease in chickens. The virus can also replicate in the gastrointestinal tract, oviduct, and kidney, where it can be highly nephropathogenic with the potential to cause up to 44% mortality (8,11); in some cases infection of the proventriculus leads to 75%–100% mortality in young birds (43). IBV is distributed worldwide, and in the United States several serotypes (e.g., Arkansas and Delaware) are currently circulating in addition to the originally identified Massachusetts type. Most isolates of IBV replicate well in the developing chicken embryo following inoculation of the allantoic cavity, and high titers of virus can be isolated from the allantoic fluid. The IBV strain Beaudette was derived from the prototypic M41 strain following at least 150 passages in chick embryos (2,16,29). IBV Beaudette is no longer pathogenic for adult birds but rapidly kills embryos. In terms of host cells, IBV M41 is normally restricted to infection of primary chicken cells; however, the Beaudette strain of IBV is known to be able to infect a range of cells in culture, including Vero and baby hamster kidney (BHK) (1,17,31). IBV Beaudette therefore represents a significant virus that has extended its host range based on very limited changes in its S protein. IBV M41 and Beaudette are closely homologous (having 96% amino acid identity in their S proteins) and the replacement of the Beaudette S protein with that of M41 shows that the S protein (and by implication the receptor-binding properties of the virus) is the sole determinant of its extended species tropism (7).

Here we carried out a bioinformatic analysis of the S protein of IBV M41 and Beaudette and identified a unique heparin-binding consensus sequence on the Beaudette S protein. We show that IBV Beaudette infection is specifically inhibited by soluble heparin and is restricted for infection in heparan sulfate (HS)-deficient cells, indicating that the specific use of heparan sulfate as an attachment factor is involved in the extended species tropism of IBV Beaudette, most likely in conjunction with sialic acid moieties as well as an additional specific, but unidentified, receptor protein used by all strains of IBV.

MATERIALS AND METHODS

Virus growth and cell culture. IBV (strains Beaudette 42 and Massachusetts 41) were obtained from Dr. Benjamin Lucio-Martinez, Unit of Avian Health, Cornell University, and propagated in 11-day-old embryonated chicken eggs. Virus was harvested from the allantoic fluid after 24 hr of infection for Beaudette and 48 hr of infection for Massachusetts 41). BHK cells were obtained from the American Type Culture Collection (ATCC) and grown in Dulbecco minimal essential medium containing 10% fetal calf serum. Wild-type Chinese hamster ovary (CHO) cells, as well as the glycosaminoglycan (GAG)-deficient mutants CHO-677 and CHO-745, were obtained from ATCC and grown in αMEM containing 10% fetal calf serum. Cells were infected with 1–5 fluorescence focus units (FFU) per cell of IBV Beaudette or M41, with a fluorescent focus scored as single infected cell by immunofluorescence assay following infection of cells for 8 hr. Heparin, sodium salt, was obtained from Sigma. Neuramindase (from *Vibrio cholera*) was obtained from Roche Applied Science.

Preparation of primary chick kidney (CK) and chick embryo kidney (CEK) cells. For preparation of CK cells, specific-pathogen-free white leghorn chicks (11–14 days of age) were placed in a CO_2 chamber and kidneys removed from each side of the chick. Kidney tissue was placed in 25–50 ml sterile phosphate-buffered saline (PBS), and the container was shaken gently to remove clots and red blood cells. The supernatant containing the cells was removed, and cells were rinsed a second time with an equivalent volume of sterile PBS. Twenty-five milliliters of trypsin/ethylenediaminetetraacetic acid (EDTA) was added and allowed to rinse/digest for approximately 2 min with a stir bar on a stir plate (or by hand swirling). The trypsin/EDTA was decanted or aspirated and a further 10 ml trypsin/EDTA added. This was then allowed to digest for 2 min with a stir bar on stir plate (or by hand swirling). The supernatant was poured through sterile cheesecloth into a sterile beaker with 1 ml fetal calf serum on ice, and the trypsin/EDTA digest repeated 1–2 more times until all chunks of tissue were digested. The supernatant was placed into a 50-ml Falcon tube and centrifuged at 1000 rpm for 2 min. Cells were resuspended in 25 ml M20 media and counted on a hemocytometer. Cells were adjusted to a concentration of 1×10^6 to 1.5×10^6 per milliliter with M25 media containing 5% FBS.

Preparation of CEK cells was essentially as for CK cells, except that kidneys were removed from 19-day-old chick embryos.

Sequence alignment of the region, including the potential heparin-binding site of IBV Beaudette. The following IBV S protein sequences were obtained from the National Center for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/entrez/) and aligned using Clustal W (European Bioinformatics Institute, http://www.ebi.ac.uk/clustalW/): Bdtt_Cornell accession number DQ830981, IBV_Bdtt accession number DQ001342, Mass41_Cornell accession number DQ830980, IBV_Mass41 accession number AY851295, IBV_HK accession number AY761141, IBV_Holt accession number AF334785, IBV_Iowa accession number AF334684, IBV_Ark99 accession number AF094814, IBV_CU-72_Ark accession number U49858, IBV_Belgian B1648 accession number X87238, IBV_Florida accession number AF094819, IBV_Connecticut46 accession number AF094818, IBV_Cal99 accession number AY514485, IBV_Ark_DPI accession number AF335555, IBV_Netherlands accession number X15832, IBV_Partridge accession number AY646238.

Reverse transcription–polymerase chain reaction (RT-PCR) and DNA sequencing. Viral RNA (vRNA) was extracted from 0.2 mg of sucrose purified virus stock using an RNeasy Plus Mini kit (Qiagen, Valencia, CA). Ten nanograms of vRNA was subjected to RT-PCR reaction using the forward primer (5′-TGAAAACTGAACAAAA-GACAG-3′) and reverse primer (5′-CTTGTATTAGTTGTTG-GAGCG-3′) using a OneStep RT-PCR kit (Qiagen) according to manufacturer's instructions. The full-length cDNA product of the IBV spike glycoprotein gene was then purified using QIAquick Gel Extraction kit (Qiagen) and sequenced by the Biotechnology Resource Center (Cornell University). Individual sequences were further analyzed and aligned using the ClustalW online software from the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw).

Immunofluorescence microscopy. This was essentially performed as described previously (34), except with methanol fixation for IBV antibodies. IBV was identified using anti-S1 monoclonal antibody 15:88 (21). Secondary antibodies used were Alexa 488-labeled or Alexa 568-labeled goat anti-mouse or chicken IgG (Molecular Probes, Carlsbad, CA). Nuclei were counterstained with 7.5 μg/ml Hoechst 33258 (Molecular Probes), which was included as a marker of the nucleus of all cells, with the goal of clearly identifying the presence of any noninfected cells in the image(s). Cells were viewed on a Nikon Eclipse E600 fluorescence microscope and images captured with a Sensicam EM camera and IPLab software before transfer into Adobe Photoshop 7 and determination of infection frequency.

Flow cytometry. For flow cytometry preparation, cells were scraped gently from the dish, washed in PBS, fixed in 3% paraformaldehyde/PBS, permeabilized in 0.1% Triton X-100, and blocked in 10% goat serum/PBS. To detect virus infection, cells were incubated with the monoclonal antibody 15:88 for 30 min, followed by Alexa 488-labeled goat anti-mouse IgG for 30 min. Cells were analyzed on a FACSCalibur cytometer using CellQuest 3.1f software (Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ). Data analysis was performed with Flow Jo 4.6 software (Treestar Inc., Ashland, OR). At least 10^4 cells were analyzed for each sample.

RESULTS

Whereas most strains of IBV have restricted tropism and only efficiently infect embryonated eggs and primary chicken cells in

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                                                XBBXBX - heparin consensus
Bdtt_Cornell        FYSSTKPAGFNTPVLSNVSTGEFNISLLLTTPSSRRKRSLIEDLLFTSVESVGLPTNDAY 712
IBV_Bdtt            FYSSTKPAGFNTPVLSNVSTGEFNISLLLTTPSSRRRRSVIEDLLFTSVESVGLPTDDAY 712
Mass41_Cornell      FYSSTKPAGFNTPFLSNVSTGEFNISLLLTTPSSPRRRSFIEDLLFTSVESVGLPTDDAY 712
IBV_Mass41          FYSSTKPAGFNTPFLSNVSTGEFNISLLLTTPSSPRRRSFIEDLLFTSVESVGLPTDDAY 712
IBV_HK              FYSSTKPAGFNTPVLSNVSTGEFNISLFLTTPSSPRRRSFIEDLLFTSVESVGLPTDDAY 712
IBV_Holte           FYSSTKPAGFNTPVLSNVSTGEFNISLLLTPPSSPSGRSFIEDLLFTSVESVGLPTDDAY 180
IBV_Iowa            FYSFTKPAGFNTPVFNNISTGDFNISLLLTPPSTPSGRSFIEDVLFTSVESVGLPTDDAY 180
IBV_Ark99           FYSSTKPSGFNTPVFSNLSTGEFNISLLLTTPSSPRGRSFIEDLLFTSVESVGLPTDEAY 180
IBV_CU-72_Ark       FYSSTKPSGFNTPVFSNFSTGEFNISLFLTTPSSPRGRSFIEDLLFTSVESVGLPTDEAY 718
IBV_Belgian         FYSSTKPRDYNTPLFSNVSTGDFNISLLLTPPSSPNGRSFIEDILFTSVESVGLPTDAEY 716
IBV_Florida         FYSSTKPSGFNTPVFSNLSTGDFNISLLLTPPSSTTGRSFIEDLLFTSVESVGLPTDEAY 180
IBV_Connecticut46   FYSSTKPSGFNTPVFSNLSTGDFNISLLLTPPSSTTGRSFIEDLLFTSVESVGLPTDEAY 180
IBV_Cal99           FYSSTKPFGFNTPILSNLSTGDFNISLLLTPPSSTTGRSFIEDLLFTSVESVGLPTDDAY 719
IBV_Ark_DPI         FYSSTKPARFNTPVFSNLSTGEFNISLLLTPPSSPRRRSFIEDLLFTSVESVGLPTDDAY 180
IBV_Netherlands     FYSSTKPSGFNTPVLSNVSTGEFNISLLLTPPSSASGRSFIEDLLFTSVESVGLPTDDAY 713
IBV_Partridge       FYSSTKPAGYNAPVFSNISTGDFNISLLLTPPSSPSGRSFIEDLLFTSVETVGLPTDAEY 715
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Fig. 1. Sequence alignment of the region including the potential heparin-binding site of IBV Beaudette. The single letter amino acid code is used. Asterisk indicates that the residues are identical in all sequences in the alignment, colon indicates that conserved substitutions are present relative to Bdtt_Cornell, period indicates that semiconserved substitutions are present relative to Bdtt_Cornell. The heparin-binding consensus sequence, XBBXBX, is indicated with a gray box, where B = a basic residue and X = any residue. Amino acid residue numbers based on the GenBank submissions are indicated.

tissue culture, IBV Beaudette has extended species tropism in cell culture. As a way to examine possible differences in tropism, we carried out a bioinformatics analysis of the spike protein sequences for these viruses, with the goal of identifying possible novel receptor-binding sites on the Beaudette virus. We first performed DNA sequence analysis of the Beaudette and prototype Massachusetts (M41) viruses used in our laboratory. The S gene from each virus was reverse transcribed, PCR amplified from purified virions, and sequenced. These sequences were designated Bddt_Cornell and Mass41_Cornell and showed a high degree of similarity to published Beaudette and M41 sequences. Sequence information for these viruses is available at NCBI (accession numbers DQ830981 and DQ830980). The predicted amino acid sequences of Bddt_Cornell and Mass41_Cornell were then aligned with the S protein sequences from a number of other representative IBV strains (Fig. 1). We observed that IBV Beaudette encoded a novel sequence (SRRKRS or SRRRRS) between residues 686 and 691 that may comprise a heparin-binding site—having a consensus sequence of XBBXBX, where B is a basic residue and X is any amino acid (6). The precise sequence (SRRKRS or SRRRRS) varied between different Beaudette strains, but the very conservative K-R change (both arginine and lysine are basic residues) is unlikely to change either the chemistry of the individual residue or the overall function of the domain. The consensus was present in all Beaudette sequences available in the NCBI GenBank database, but not present for any other IBV strain analyzed. Because of the known propensity of HS to act as a viral coreceptor (28), we therefore investigated the possible role of the HS as a cofactor in the extended tropism of IBV Beaudette.

We first examined whether IBV Beaudette infection could be competed by soluble exogenous heparin. BHK cells were infected with approximately 1 FFU/cell IBV Beaudette, and infection was analyzed at 8 hr after infection by immunofluorescence microscopy using an anti-IBV S1 monoclonal antibody. In the absence of soluble heparin, approximately 90% of the cells were infected; however, in the presence of excess soluble heparin, infectivity was not detectable (Fig. 2A). To quantify the effect of soluble heparin, we carried out

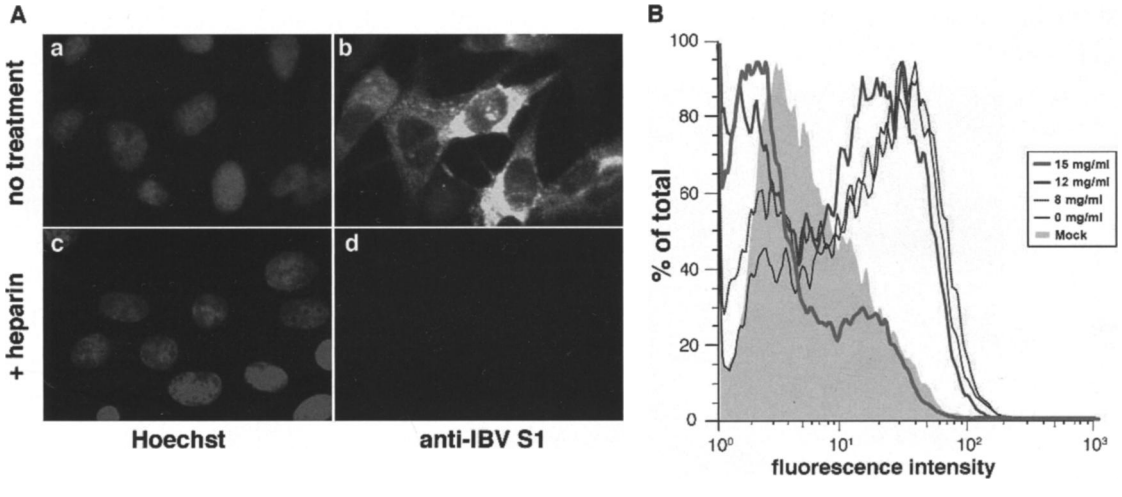

Fig. 2. Soluble heparin prevents infection by IBV Beaudette. (A) BHK cells were infected with IBV Beaudette that had been treated with 15 mg/ml heparin (c and d) or were untreated (a and b). Cells were analyzed at 8 hr after infection by immunofluorescence microscopy using an anti-S1 monoclonal antibody to detect IBV infection (b and d) and nuclei counterstained with Hoechst 33258. (B) BHK cells were infected with IBV Beaudette that had been treated with varying concentrations of heparin, or were untreated. Cells were also mock infected. Cells were analyzed at 8 hr after infection by flow cytometry using an anti-S1 monoclonal antibody.

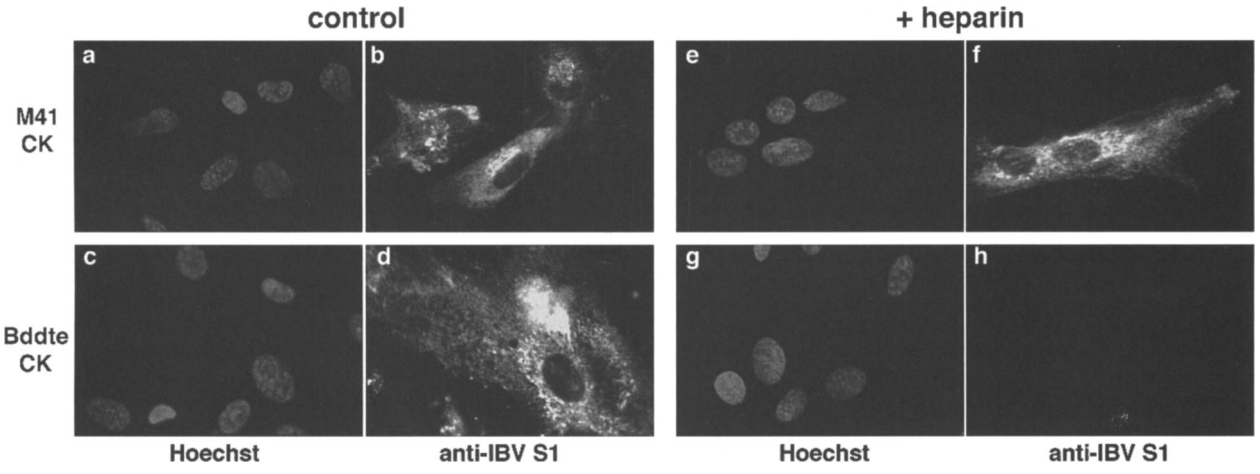

Fig. 3. Soluble heparin does not prevent infection by IBV M41. Primary chick kidney (CK) cells were infected with IBV M41 (a, b, e, and f) or Beaudette (Bddte) (c, d, g, and h) that had been treated with 15 mg/ml heparin (e–h) or were untreated (a–d). Cells were analyzed at 8 hr after infection by immunofluorescence microscopy using an anti-S1 monoclonal antibody to detect IBV infection (b, d, f, and h) and nuclei counterstained with Hoechst 33258 (a, c, e, and g).

a dose-response experiment of IBV Beaudette infection in BHK cells by flow cytometry (Fig. 2B). We found that whereas 15 mg/ml soluble heparin gave a complete inhibition of viral infectivity, 12 mg/ml gave an approximately 50% block, and 8 mg/ml or lower gave only a limited or undetectable inhibition of infection. To confirm that the effects of the higher levels of soluble heparin were specific for virus entry, we repeated the experiments adding the heparin after the first 60 min of virus infection. Under these conditions, we observed no significant inhibition of IBV infection (data not shown).

To determine whether the effects of soluble heparin were specific for IBV Beaudette, we repeated the experiments in primary CK cells and compared the infectivity of IBV Beaudette with the prototype Massachusetts 41 (M41) strain. In the absence of soluble heparin, addition of approximately 1 FFU/cell IBV M41 gave approximately 60%–70% infectivity of CK cells, a level of infection that was essentially unchanged during incubation with excess soluble heparin (Fig. 3). As with BHK cells, approximately 90% of the CK cells were infected by IBV Beaudette in the absence of excess soluble heparin, whereas infectivity of CK cells with IBV was not detectable after incubation with heparin (Fig. 3). These data show a specific requirement of HS for infection by IBV Beaudette.

HS is an abundant cell-surface GAG that is widely used as a viral attachment factor (28); however, other cell-surface GAGs are also expressed at high levels (notably chondroitin sulfate [CS]) and can be used as viral receptor moieties (32). To assess the specific contribution of cell-surface HS for infection by IBV Beaudette, we used two mutant CHO cells lines, CHO-745, which is deficient in overall GAG biosynthesis as a result of a mutation in the xylotransferase I gene, and CHO-677, which is specifically deficient in HS biosynthesis as a result of mutations in the N-acetylglucosaminyl transferase and glucuronosyl transferase genes (15,37). We first examined CHO-677 cells, which express no detectable cell-surface HS, but have a threefold higher expression of CS (15). Infection of wild-type CHO cells with approximately 1 FFU/cell IBV Beaudette resulted in approximately 70% of the cells being infected. However, infection of CHO-677 cells was significantly reduced or undetectable (<5% infection) (Fig. 4), confirming the important role for HS in infection by IBV Beaudette.

We next examined CHO-745 cells, which have no detectable cell-surface GAG (15). Unexpectedly, these cells showed no significant reduction in the level of IBV Beaudette infection; for both wild-type and CHO-745 cells we observed approximately 70%–75% infection

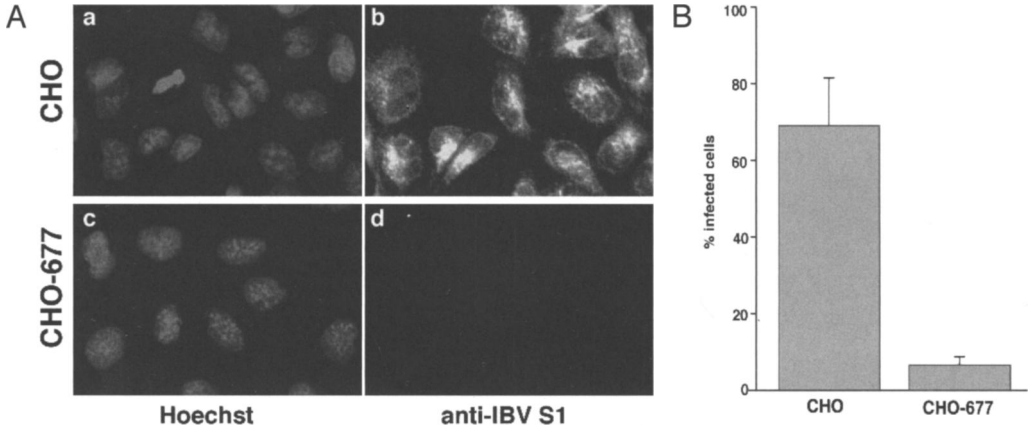

Fig. 4. IBV Beaudette does not infect heparan sulfate deficient cells. (A) CHO-677 cells (c and d) or wild-type CHO cells (a and b) were infected with IBV Beaudette cells. Cells were analyzed at 8 hr after infection by immunofluorescence microscopy using an anti-S1 monoclonal antibody to detect IBV infection (b and d) and nuclei counterstained with Hoechst 33258 (a and c). (B) Quantification of immunofluorescence data. More than 300 cells were scored for infection. Error bars represent the standard deviation of the mean.

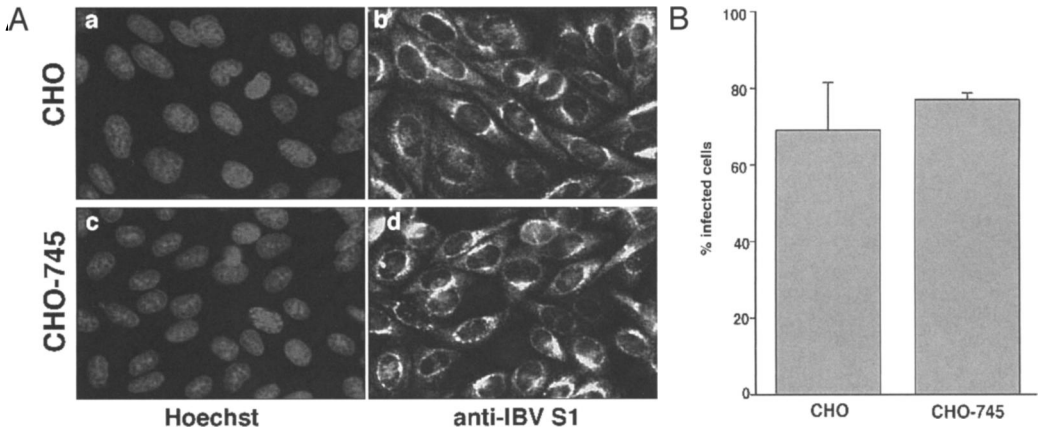

Fig. 5. IBV Beaudette infects glycosaminoglycan-deficient cells. (A) CHO-745 cells (c and d) or wild-type CHO cells (a and b) were infected with IBV Beaudette cells. cells (c and d) or wild-type CHO cells (a and b). Cells were analyzed at 8 hr after infection by immunofluorescence microscopy using an anti-S1 monoclonal antibody to detect IBV infection (b and d) and nuclei counterstained with Hoechst 33258 (a and c). (B) Quantification of immunofluorescence data. More than 300 cells were scored for infection. Error bars represent the standard deviation of the mean.

(Fig. 5). To account for the apparent discrepancy in the data from CHO-677 *vs.* CHO-745 cells, we considered the possibility that although elimination of specific HS-binding sites led to a reduction in IBV Beaudette infection by virtue of the fact that IBV Beaudette–HS interactions were prevented, elimination of all cell-surface GAGs in CHO-745 cells exposed an alternative virus binding site(s), which could act as a virus receptor. As sialic acid residues have recently been shown to be involved in binding and entry of IBV (40), we reasoned that CHO-745 cells may have increased sialic acid binding capacity, which overcomes the lack of HS. We therefore assessed the effect of neuraminidase (NA) treatment on infection of CHO-745 cells, along with the effects of NA on CHO-677, wild-type CHO, and BHK cells. Cells were infected with approximately 1 FFU/cell IBV Beaudette. In all cases treatment with NA reduced infection to a level $<5\%$ of that in

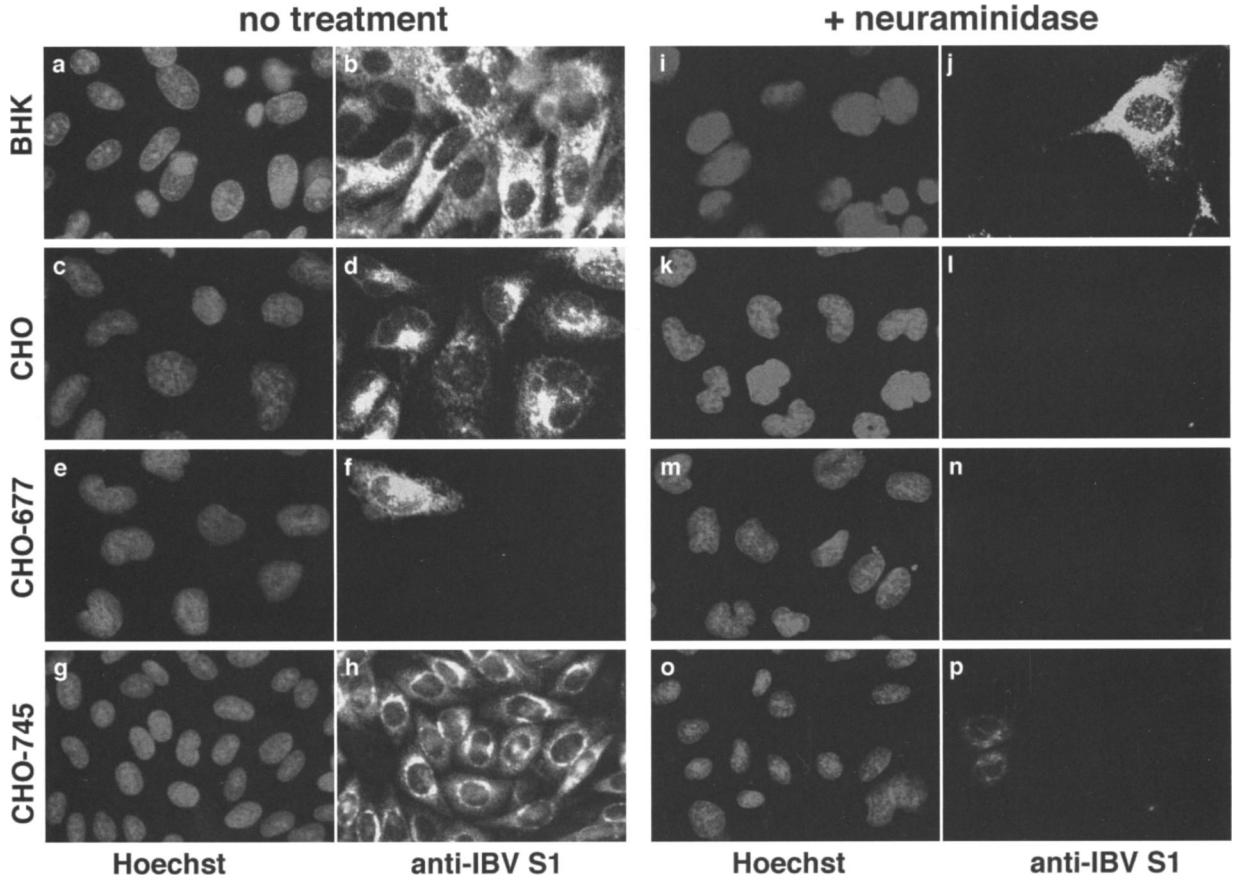

Fig. 6. Neuraminidase treatment prevents IBV Beaudette infection of glycosaminoglycan-deficient cells. BHK cells (a, b, i, and j), wild-type CHO (c, d, k, and l), CHO-677 (e, f, m, and n), or CHO-745 cells (g, h, o, and p) were infected with IBV Beaudette that had been treated with 200 mU/ml neuraminidase (i–p) or were untreated (a–h). Cells were analyzed at 8 hr after infection by immunofluorescence microscopy using an anti-S1 monoclonal antibody to detect IBV infection (b, d, f, h, j, l, n, and p) and nuclei counterstained with Hoechst 33258 (a, c, e, g, i, k, m, and o).

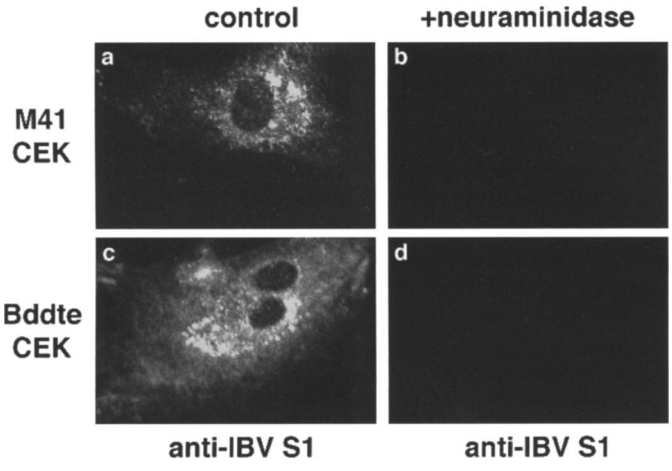

Fig. 7. Neuraminidase treatment prevents IBV M41 and Beaudette infection of primary chick cells. Primary chick embryo kidney (CEK) cells were infected with IBV Massachusetts 41 (M41) (a and b) or Beaudette (Bddte) (c and d) that had been treated with 200 mU/ml neuraminidase (b and d) or were untreated (a and b). Cells were analyzed at 8 hr after infection by immunofluorescence microscopy using an anti-S1 monoclonal antibody to detect IBV infection.

the absence of NA (Fig. 6). Therefore, we consider that HS acts in conjunction with sialic acid for infection of cells by IBV Beaudette.

To confirm a general role for sialic acid in IBV infection, we analyzed infection by IBV M41 and Beaudette of chicken embryo kidney (CEK) cells that had been treated with NA. Cells were infected with approximately 1 FFU/cell IBV Beaudette or IBV M41, and in both cases pretreatment of cells with NA reduced infection to undetectable levels compared to untreated cells (Fig. 7). Identical results were obtained with primary CK cells (data not shown). Overall, these data confirm a general role for sialic acid as an attachment factor for IBV, acting in concert with specific HS-mediated binding of IBV Beaudette.

DISCUSSION

IBV Beaudette is an embryo-adapted avian coronavirus with an extended host range in cell culture. We show here that this extended tropism may in part be due to the use of HS as a selective attachment factor; excess soluble heparin competed with IBV Beaudette infection. Excess soluble heparin competed with IBV M41 infection. HS is well established as a cofactor in entry of a wide variety of viruses (28,32). However the levels of heparin needed for inhibition of IBV Beaudette infection were relatively high (approximately 12 mg/ml), which is significantly higher than for other viruses where heparin typically gives inhibition of infectivity, for example with Dengue virus and herpes simplex virus (27,41). However, in other cases, virus binding is only inhibited by high levels of heparan sulfate, e.g., a human rhinovirus type 89 variant was inhibited at heparin concentrations of 2 mg/ml (38) and adeno-associated virus type 3H required pretreatment of cells with 50–100 mg/ml to prevent infections (4). Our data indicate that the affinity of IBV Beaudette–HS interactions is quite low. However, owing to the multivalent nature of virus–cell interactions, it is not possible to determine a value for this low-affinity interaction.

To further characterize the heparin binding of IBV Beaudette, we carried out experiments with CHO cells that are deficient in HS/GAG expression, as well as carrying out experiments on BHK cells where surface HS was enzymatically removed with heparinase treatment (data not shown). However, in contrast to the situation where the virus receptor–binding sites are blocked by excess soluble heparin, we believe that the absence of HS/GAG on the cells surface can expose additional binding sites for the virus (i.e., sialic acid), and so these treatments do not inhibit infection with IBV Beaudette. In other words, blockage of a HS-binding site on the virus is not equivalent to removal of HS from the cell surface.

One distinctive feature of IBV Beaudette that may account for the specific interaction with HS is the presence of a novel sequence (SRRKRS or SRRRRS) between residues 686 and 691, which fits the heparin-binding consensus sequence of XBBXBX, where B is a basic residue and X is any amino acid (6). This consensus was present in all Beaudette sequences available in the database but not present for any other IBV strain analyzed. It is presently unclear whether this sequence is responsible for HS binding of IBV Beaudette. The consensus sequence identified by Cardin and Weintraub is based on the assumption that all GAG-binding proteins interact with the same oligosaccharides within heparin, a strategy that has been suggested to be overly simplistic (37). In fact examination of the consensus binding sites for heparin in basic fibroblast growth factor turned out to be incorrect once the crystal structure was determined. In the case of IBV Beaudette, the SRRKRS or SRRRRS motif is actually present in the S2 domain of the spike protein and not the S1 domain typically associated with receptor interactions. However, in the absence of detailed structural information, it cannot be ruled out that this region of S2 (between 686 and 691) is not exposed for use in viral attachment; the use of this as a receptor-binding site awaits experimental analysis.

As shown recently by Winter and colleagues (40), we confirm that sialic acid is an attachment factor for both IBV Beaudette and M41 and likely acts in a similar manner for all IBV strains. This is unusual, since most IBV strains do not ordinarily hemagglutinate red blood cells unless the virions themselves are pretreated with NA (3,9,33), and IBV Beaudette does not hemagglutinate red blood cells at all (3). Unlike some other coronaviruses that use sialic acid receptors, IBV does not encode a hemagglutinin-esterase protein that might act to cleave the receptor moities and allow efficient virus release and spread (8). Ultimately it is likely that despite acting as low-affinity attachment factors, neither HS nor sialic acid comprise the critical receptor for IBV entry into cells. The identification of this receptor is currently unknown. Like Winter *et al.* (40), we have been unable to rescue IBV M41 infection in BHK cells by expression of feline or human aminopeptidase N (J. Aronson, V. Chu, and G. Whittaker, unpubl. data), suggesting that aminopeptidase N is not a functional IBV receptor. The entry and receptor binding of IBV is likely to be complex process, and the specific use of HS by IBV Beaudette clearly demonstrates one important piece of the complex picture for IBV entry—the initial use of heparin sulfate by IBV Beaudette, which may play a critical role of the pathogenesis and host range of the virus. It is presently unclear whether HS binding accounts for differential tissue tropism of IBV Beaudette within the infected embryo, in addition to its possible role in extended species tropism. Experiments to determine the role of HS for IBV Beaudette of the chick embryo are currently underway.

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