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Binding of Transmissible Gastroenteritis Coronavirus to Brush Border Membrane Sialoglycoproteins

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Transmissible gastroenteritis coronavirus (TGEV) is a porcine pathogen causing enteric infections that are lethal for suckling piglets. The enterotropism of TGEV is connected with the sialic acid binding activity of the viral surface protein S. Here we show that, among porcine intestinal brush border membrane proteins, TGEV recognizes a mucin-type glycoprotein designated MGP in a sialic acid-dependent fashion. Virus binding assays with cryosections of the small intestine from a suckling piglet revealed the binding of TGEV to mucin-producing goblet cells. A nonenteropathogenic mutant virus that lacked a sialic acid binding activity was unable to bind to MGP and to attach to goblet cells. Our results suggest a role of MGP in the enteropathogenicity of TGEV.

Transmissible gastroenteritis coronavirus (TGEV) is an enteropathogenic coronavirus that causes diarrhea in pigs. The infection is correlated with high morbidity in animals of all ages and with high mortality in suckling piglets. TGEV is an enveloped virus with a positive-stranded RNA genome (3). Binding of the surface protein S to the cellular receptor porcine aminopeptidase N (pAPN) is required for the initiation of a TGEV infection (2). A second binding site, located on the N-terminal domain of the S protein, allows TGEV to interact with terminal sialic acid residues on glycoproteins or glycolipids and to agglutinate erythrocytes (12). Studies with TGEV mutants indicated that residues within a short stretch of amino acids (positions 145 to 209) are important for the recognition of sialic acids (5, 7). Point mutations that caused the loss of the hemagglutinating activity also resulted in the loss of the enteropathogenicity (7). These findings indicate that there is a connection between the sialic acid binding activity and the enteropathogenicity of TGEV.

The sialic acid binding activity of TGEV is dispensable for virus growth in cell culture (5, 7). However, we have demonstrated that TGEV binds to a high-molecular-mass sialoglycoprotein on cultured cells and that the sialic acid-dependent attachment of virus particles to the cell surface is more efficient than the binding via the pAPN interaction with APN (13).

In this study we analyzed the binding partners of TGEV on the porcine intestinal epithelium, the natural target of this virus. The Purdue strain of TGEV (PUR46-MAD) (10), used throughout this study, was propagated in swine testicular cells and harvested 20 to 24 h after infection. Sucrose gradient centrifugation and *Vibrio cholerae* neuraminidase (VCNA) treatment of virus were performed as described by Krempl and Herrler (6). Brush border membranes (BBM) were prepared from the jejunums of piglets as described by Schröder et al. (11). These preparations were tested for the activity of alkaline phosphatase as a marker for BBM and for the activity of Na^+/K^+-ATPase as a marker for basolateral membranes. The enzyme assays that indicated an adequate enrichment of BBM fractions were performed as described by Schröder et al. (11). BBM proteins from the total jejunum of a 3-day-old suckling piglet were treated with VCNA or mock treated, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (9), and blotted to nitrocellulose by a semidry-Western blotting method (8). A virus overlay binding assay as described by Schwegmann-Weßels et al. (13) was performed to compare the attachment properties of TGEV and a nonenteropathogenic mutant (Fig. 1). This mutant, m10, is impaired in its sialic acid binding activity, as evidenced by the lack of hemagglutinating activity (7). Both viruses bound to a protein migrating in a position where pAPN, the cellular receptor for TGEV, is expected (Fig. 1, lanes 1 to 4). This band was identified as pAPN by its reaction with the specific monoclonal antibody G43 (2) in a Western blot (Fig. 1, lanes 5 and 6). TGEV but not the mutant m10 recognized an additional band of high molecular mass designated MGP (Fig. 1, lane 1). Virus binding to this BBM component was abolished when VCNA treatment of the BBM proteins was performed prior to SDS-PAGE (Fig. 1, lane 2). This result indicates that the binding was mediated by sialic acid residues present on MGP. The enzymatic release of sialic acids did not affect the binding of TGEV, of the mutant m10, or of the monoclonal antibody to pAPN (Fig. 1, lanes 2, 4, and 6).

To find out if there are any age-dependent differences in the protein pattern recognized by TGEV, BBM from the total jejunums of four suckling piglets (1 to 3 days old) and from the middle jejunums of four weaned piglets (8 to 12 weeks old) were tested in the virus overlay binding assay (data not shown). MGP was detected in all four samples of BBM from suckling piglets. Among the samples from weaned piglets, the virus bound only in two of four samples to MGP whereas levels of binding to pAPN in all tested samples were similar. Taken together these results suggest that the sialoglycoprotein MGP

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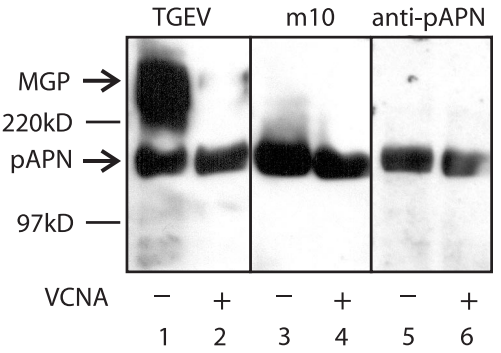

FIG. 1. Binding of TGEV and the mutant m10 to BBM. BBM were isolated from the small intestine of a 3-day-old piglet and either mock treated (−) or treated with VCNA (+). Following electrophoretic separation under nonreducing conditions, the proteins were transferred to nitrocellulose. The immobilized proteins were incubated with purified virus, and bound virus was detected by an enzyme-linked immunoassay. Lanes 5 and 6, Western blot with the anti-pAPN antibody. On the left the positions of molecular mass markers are indicated.

recognized by TGEV is more common in piglets than it is in older animals.

To analyze the carbohydrate content of the BBM proteins, samples from the 3-day-old piglet used for the experiment shown in Fig. 1 were treated with neuraminidase or mock treated, separated by SDS-PAGE, and blotted to a polyvinylidene difluoride membrane. The carbohydrates were oxidized with periodate as described by Zimmer et al. (14). After labeling of oxidized residues with biotin hydrazide and incubation with peroxidase-conjugated streptavidin, the bands were detected by chemiluminescence. As shown in Fig. 2, lane 1, the major band labeled by this procedure is MGP. A mild metaperiodate oxidation on ice selectively labels sialic acid residues. The major protein detectable by this procedure again is MGP. A second prominent band appeared at 200 kDa (Fig. 2, lane 3); this sialoglycoprotein, whose identity is not known, is not recognized by TGEV (Fig. 1), maybe because of a lower sialic acid content. After sialidase treatment, MGP changed its electrophoretic mobility and shifted to a position closer to the top of the gel (Fig. 2, lanes 2 and 4). This is a typical behavior of

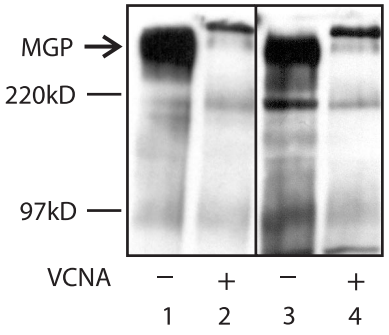

FIG. 2. Detection of major BBM glycoproteins. BBM from a 3-day-old piglet were either mock treated (−) or treated with VCNA (+). After electrophoretic separation, the proteins were transferred to a polyvinylidene difluoride membrane. Carbohydrate residues (lanes 1 and 2) and sialic acid residues (lanes 3 and 4) were detected by metaperiodate oxidation.

mucin-type sialoglycoproteins. After enzymatic release of sialic acids there still are some residues left for mild periodate oxidation (Fig. 2, lane 4). Obviously the enzymatic treatment with sialidase did not result in a complete release of sialic acids. Taken together, these results indicate that the BBM protein MGP that is recognized by TGEV is a carbohydrate-rich sialoglycoprotein, most likely a mucin-type glycoprotein. Therefore it is designated MGP. As mucins are highly O glycosylated, we analyzed whether MGP is recognized by jacalin (data not shown). This lectin binds to galactose-β(1-3)-*N*-acetylgalactosamine, a disaccharide present in O-glycosylated proteins. MGP was readily recognized by jacalin, suggesting that the sialoglycoprotein MGP is highly O glycosylated. The high carbohydrate content renders mucins rather resistant to degradation by proteolytic enzymes. For this reason, we have been

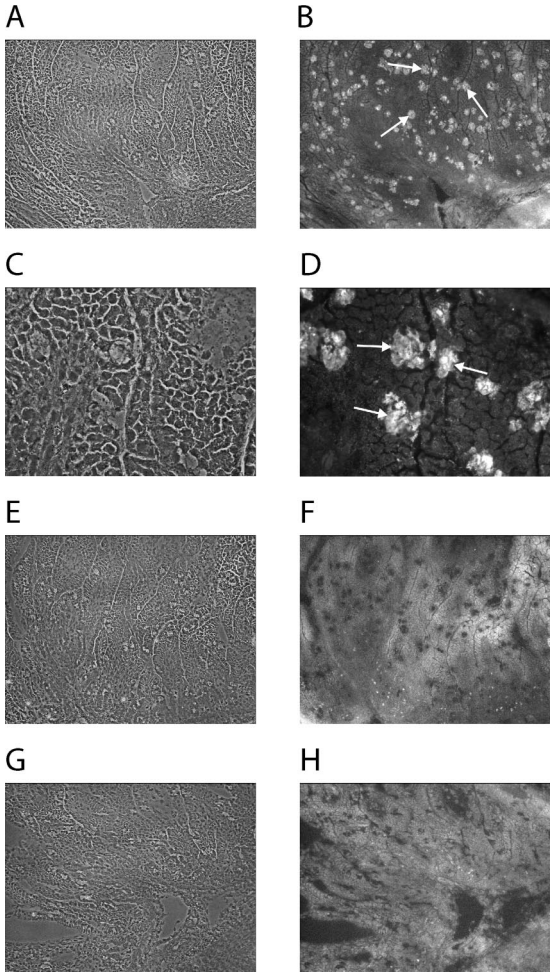

FIG. 3. Binding of TGEV and the mutant m10 to cryosections of the small intestine from a 3-day-old piglet. The cryosections were incubated with purified virions and with monoclonal antibody 6A.C3 (for detection of bound virus). The sections were examined by immunofluorescence microscopy (B, D, F, and H). The same parts of the sections are shown in phase contrast on the left (A, C, E, and G). The binding of TGEV is shown in an overview (B; arrows) and at a higher magnification (D; arrows). The binding of TGEV after VCNA treatment of the section (F) and the binding of the mutant m10 to the mock-treated intestine (H) are shown in an overview.

unable so far to determine the amino acid sequence of this mucin-type glycoprotein.

We were interested in knowing whether the binding of TGEV to BBM proteins in a virus overlay binding assay reflects virus binding to jejunal tissue. For this purpose, cryosections were prepared from the jejunal tissue of a suckling piglet (3 days old). After fixation the tissue was incubated with VCNA-treated purified TGEV or TGEV mutant m10. Bound virions were detected by incubation with a monoclonal antibody (6A.C3) against the viral S protein (4) and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Amersham Pharmacia). To see if virus binding is sialic acid dependent, some of the cryosections were incubated with VCNA or mock treated prior to incubation with TGEV virions. As shown in Fig. 3, TGEV bound to certain regions of the jejunal epithelium which have the characteristic appearance of goblet cells (Fig. 3A to D). Goblet cells are specialized epithelial cells which synthesize and secrete mucins. In VCNA-treated sections no binding of TGEV to goblet cells was detectable (Fig. 3F). Sections which were incubated with the nonenteropathogenic mutant m10 instead of TGEV also did not show any fluorescence in the area of goblet cells (Fig. 3H). These results indicate that TGEV attaches to mucin-producing goblet cells in a sialic acid-dependent fashion. As MGP is a mucin-type glycoprotein and is the only BBM component that interacts with TGEV in an overlay binding assay in a sialic acid-dependent fashion, it is likely that MGP mediates the binding of TGEV to goblet cells.

In our view, MGP may be involved in a TGEV infection as follows. After passage through the stomach, the virions reach the small intestine. There they bind via their sialic binding sites to mucins, such as MGP, synthesized in the jejunal goblet cells and present in the mucus layer. This binding prevents the loss of virions by the intestinal peristalsis. The interaction of TGEV with sialic acids is a dynamic process. The virus may detach from some sialic acids and attach to others. In this way the virus may pass the mucus layer, which is as thick as 100 μm (reviewed in reference 1), and reach the glycocalix covering the apical membrane of the intestinal cells (about 100 nm thick). Here the virus particles can again bind to mucin-type glycoproteins such as MGP. Finally the virions reach the cellular receptor pAPN present in the apical membranes of the epithelial cells. Binding to MGP may allow the virus to stay longer in the intestine and make it easier to find the pAPN receptor for initiating intestinal infection.

Taken together, our results indicate that the sialic acid binding activity of TGEV may help the virus to overcome the mucus barrier.

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