Identification and Characterization of S Fimbria-Binding Sialoglycoproteins on Brain Microvascular Endothelial Cells

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We have previously shown that S-fimbriated *Escherichia coli* binds brain microvascular endothelial cells (BMEC) via a lectin-like activity of SfaS adhesin specific for NeuAc α 2,3-galactose; however, BMEC molecules bearing these epitopes have not been identified. In the present study, we showed that the expression of S fimbriae conferred a three-fold increase in adhesion of *E. coli* to cow, human, and rat BMEC but did not enhance *E. coli* adhesion to systemic vascular endothelial cells such as human umbilical vein endothelial cells and human aortic arterial endothelial cells. Two BMEC-binding molecules for S fimbriae were identified as 65 (major)- and 130 (minor)-kDa sialoglycoproteins by S fimbria immunoblotting and were purified from bovine BMEC by wheat germ agglutinin and *Maackia amurensis* lectin (specific to NeuAc α 2,3-galactose) affinity chromatography. The 65-kDa BMEC glycoprotein showed effective inhibition of S fimbria-mediated binding of *E. coli* to BMEC. Polyclonal antibodies raised against the mixture of 65- and 130-kDa proteins reacted to 65-kDa protein present only on BMEC, not on systemic vascular endothelial cells. Immunoprecipitation of biotinylated BMEC membrane proteins and immunocytochemistry studies of BMEC with anti-S fimbria-binding protein antibodies revealed that the 65-kDa protein is a surface protein. The N-terminal amino acid sequence of 65- and 130-kDa proteins showed no significant sequence homology with any other known proteins. These findings suggest that 65- and 130-kDa proteins represent novel sialoglycoproteins involved in the binding of S-fimbriated *E. coli* to BMEC.

The mortality and morbidity associated with neonatal gram-negative bacillary meningitis have remained significant despite advances in antimicrobial chemotherapy and supportive care (8, 27). A major contributing factor is the incomplete understanding of the pathogenesis and pathophysiology of gram-negative bacillary meningitis. Although *Escherichia coli* is the most common gram-negative bacterium that causes meningitis during the neonatal period (8, 27) and most cases of neonatal *E. coli* meningitis develop as a result of hematogenous spread, it is not clear how circulating *E. coli* cells cross the blood-brain barrier, a lining comprised of brain microvascular endothelial cells (BMEC). Infections caused by pathogenic *E. coli* are often initiated by their adherence to host cell surfaces via specific bacterial adhesins associated with fimbriae. Fimbriae are defined as proteinaceous filaments protruding from the bacterial surface. Pathogenic *E. coli* isolates express many types of fimbrial adhesins responsible for hemagglutination, which can be divided into different groups by their affinity to specific receptor structures, such as α -D-Gal-1,4- β -D-Gal (P fimbrial adhesins [11]), α -D-mannosides (type 1 fimbrial adhesins [15]), NeuAc α 2,3-galactose (S fimbrial adhesins [12]), or glycophorin A (M fimbrial adhesins [28]).

S fimbriae, which bind to terminal NeuAc α 2,3-galactose sequences present on glycoproteins, have been implicated as one of the microbial factors involved in the pathogenesis of neonatal *E. coli* meningitis (12, 17, 18). Nine chromosomally encoded *sfa* genes are responsible for the production of the S fimbria adhesin complex (Sfa). Sfa is composed of four subunits, a major subunit, SfaA (16 kDa), and three minor subunits, SfaG (17 kDa), SfaS (14 kDa), and SfaH (29 kDa) (9, 10,

22). We have previously shown that the binding of S-fimbriated *E. coli* to BMEC is mediated by a lectin-like activity of the SfaS adhesin specific for NeuAc α 2,3-galactose-containing glycoproteins (24). We also have shown that binding of S-fimbriated *E. coli* to BMEC occurs in an animal model, whereas similar binding was not observed with nonfimbriated bacteria, indicating that our in vitro binding to BMEC is indeed biologically relevant (7). Furthermore, we showed that the binding of S-fimbriated *E. coli* to sulfated glycolipids on BMEC, but not to other gangliosides containing NeuAc α 2,3-galactose sequences, is mediated by the major fimbrellin protein SfaA (21). The SfaS adhesin is preferentially located at the tips of S fimbriae (22); thus, it appears that attachment to sialic acid-containing glycoprotein(s) is the first step in the binding of S-fimbriated *E. coli* to the brain vascular endothelium. In many cases, the binding specificity of bacterial adhesins to glycoproteins is well characterized at the oligosaccharide level, but the glycoproteins carrying the corresponding sugar chains are not known.

Here, we report that the binding of S-fimbriated *E. coli* is specific to endothelial cells of brain origin and not to endothelial cells of systemic origin. Two S fimbria-binding proteins (SBPs) on BMEC, a major 65-kDa sialoglycoprotein and a minor 130-kDa sialoglycoprotein, were identified. The major SBP, which was able to block the binding of S-fimbriated *E. coli* to BMEC, was purified to homogeneity. The partial N-terminal amino acid sequence of the 65- and 130-kDa glycoproteins showed no homology with any known proteins, suggesting that novel BMEC proteins are involved in the binding of S-fimbriated *E. coli*.

MATERIALS AND METHODS

Materials. *Maackia amurensis* (MA) lectin, wheat germ agglutinin (WGA), and concanavalin A (ConA) were obtained from Vector Laboratories (Burlingame, Calif.); WGA-Sepharose and peptide *N*-glycosidase F were purchased from Sigma (St. Louis, Mo.); protein A-Sepharose columns, the Fab fragment

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TABLE 1. Phenotypic and genotypic characteristics of *E. coli* strains

<i>E. coli</i> strain	Recombinant DNA ^a	S fimbria phenotype ^b	S fimbria genotype
HB101/13	pANN 801-13 (Ap ^r)	Fim ⁺	<i>sfaA sfaG sfaS sfaH</i>
HB101/322	pBR 322 (Ap ^r Tc ^r)	Fim ⁻	
HB101/1	pANN 801-1 (Tc ^r)	Fim ⁺	<i>sfaG sfaS sfaH</i>
HB101/1321	pANN 801-1321 (Ap ^r)	Fim ⁺	<i>sfaA sfaG sfaH</i>

^a Ap^r, resistant to ampicillin; Tc^r, resistant to tetracycline.

^b Fim, fimbriation by electron microscopy.

generation kit, and Sulfo-NHS-LC-biotin were obtained from Pierce Co. (Rockford, Ill.). Neuraminidase from *Vibrio cholera* and streptavidin peroxidase were from Boehringer-Mannheim (Indianapolis, Ind.). All other chemicals were obtained from Sigma unless otherwise stated.

Bacterial strains and labeling of the bacteria. The recombinant *E. coli* HB101 strains were grown in brain heart infusion broth (Difco Labs, Detroit, Mich.) containing appropriate antibiotics (22). *E. coli* HB101/13 carrying pANN 801-13 expressed Sfa, whereas nonfimbriated *E. coli* HB101/322 carried the vector plasmid pBR 322 (14, 16). *E. coli* HB101/1321 and HB101/1 contained plasmids pANN 801-1321 and pANN 801-1, respectively, lacking the minor subunit (*sfaS*) and the major subunit (*sfaA*) adhesin genes (14, 16) (Table 1). For biotin labeling, overnight cultures of bacteria were washed three times with Hank's balanced salt solution (HBSS) and resuspended in Dulbecco's phosphate-buffered saline (PBS) containing 0.1 mM CaCl₂ and 1 mM MgCl₂ (DPBS/C/M) to a final concentration of 10⁹ bacteria/ml. Bacterial surface proteins were biotinylated as previously described (21) by adding an equal volume of DPBS/C/M containing 1-mg/ml Sulfo-NHS-LC-biotin and incubating for 30 min on ice. The bacteria were collected by centrifugation, washed five times, resuspended in DPBS-20% glycerol, aliquoted, and stored at -70°C until use. The bacteria stored under these conditions remained viable for at least 1 month.

S-fimbriated *E. coli* adhesion to cultured BMEC. The isolation of bovine BMEC and adhesion assays were carried out as described previously (24). Briefly, the homogenates of bovine brain cortices were centrifuged in 25% bovine serum albumin (BSA), and the pellet containing crude microvessels was digested with collagenase-dispase. Microvascular capillaries were isolated by absorption to a glass bead column, plated on collagen-fibronectin-coated dishes, and cultured in growth medium containing D-valine (to inhibit the growth of nonendothelial cells). Human cerebral cortex fragments were obtained by surgical resection of the cortices of children (less than 7 years old) with seizure disorders at Childrens Hospital in Los Angeles. Human and rat BMEC were isolated as described above, except that 15% dextran instead of BSA was used for the centrifugation. Human BMEC were further purified by fluorescence-activated cell sorting and were >99% pure (25). Bovine, human, and rat BMEC were positive for factor VIII, fluorescently labeled acetylated low-density lipoprotein, carbonic anhydrase IV, and gamma-glutamyltranspeptidase, demonstrating their brain endothelial-cell characteristics. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, Calif.), and human aortic arterial endothelial cells (HAAEC) were obtained from Coriell Cell Repositories (Camden, N.J.). The binding assays were carried out at 4°C to prevent internalization of bacteria into BMEC as described previously (24). Briefly, the confluent endothelial cell monolayers containing approximately 10⁵ cells were incubated with bacteria (10⁷ CFU/well) in 500 µl of medium M 199-Ham's F-12 (1:1) containing 5% heat-inactivated normal calf serum at 4°C for 2 h. The monolayers were washed four times, and the endothelial cells with adherent bacteria were dissolved in 0.5% Triton X-100 in distilled water for 10 min. The number of bacteria was enumerated by plating on sheep blood agar. To examine the effect of temperature on binding of bacteria to BMEC, binding was also performed at 37°C. The cells were gently fixed with 0.2% glutaraldehyde in PBS for 5 min to prevent internalization of the bacteria. After washing of the BMEC monolayers, the binding assays were carried out for 2 h at 37°C as described above. Bacterial viability was not affected by 0.5% Triton X-100 under the conditions employed, and the results were expressed as CFU per well and/or percentage of bacteria inoculated unless otherwise stated. The numbers of endothelial cells did not differ significantly between different cell types. For inhibition studies, lectins (100 µg/well) were incubated with the monolayers for 1 to 12 h at either 4°C or 37°C before addition of the bacteria. The glycoproteins fetuin and thyroglobulin (100 to 400 µg) were incubated with bacteria for 1 h on ice before addition to the BMEC monolayers. The concentrations of glycoproteins were chosen to give similar quantities of sialic acid, which were estimated by thiobarbituric acid assay (1). The viability of bacteria in the presence of either lectins or glycoproteins was not affected.

Preparation of BMEC membrane proteins. BMEC were grown to confluency in T165 flasks (Costar, Cambridge, Mass.) and scraped from the flasks in the presence of media containing protease inhibitors, aprotinin, and phenylmethylsulfonyl fluoride (PMSF). All solutions contained protease inhibitors (aprotinin and PMSF) throughout all of the experiments hereafter. The cells were pelleted

by centrifugation at 4°C and resuspended in 50 mM Tris-HCl (pH 7.4) containing 0.1% Triton X-100 and 1 mM dithiothreitol (DTT). The cells were subjected to brief sonications (3 × 10 s) and were centrifuged at 4,000 × g to remove cellular debris. The supernatant was centrifuged at 105,000 × g at 4°C for 1 h. The membrane pellet was resuspended in Tris buffer containing 0.03% Triton X-100 and sonicated briefly to facilitate solubilization into the buffer, and the protein content was estimated by a modified Lowry method. For biotin-labeled BMEC surface proteins, confluent BMEC monolayers were washed three times with HBSS containing 0.1 mM CaCl₂ and 1 mM MgCl₂ (HBSS/C/M) and protease inhibitors. Then, the monolayers were incubated with 1-mg/ml Sulfo-NHS-LC-biotin in HBSS/C/M solution for 30 min, washed four times, and scraped from the flasks, and membrane proteins were prepared as described above.

DEAE-Sephadex and wheat germ agglutinin affinity chromatography. The BMEC membrane proteins (15 mg) were dialyzed against 50 mM Tris-HCl-0.03% Triton X-100 buffer (pH 8.0) for 24 h at 4°C and loaded onto a DEAE-Sephadex column (1 by 20 cm), which was pre-equilibrated with Tris buffer (pH 8.0). The column was washed with five bed volumes of loading buffer and then was eluted in a stepwise gradient with 0.1, 0.2, 0.3, and 0.4 M sodium chloride in the same buffer. The fractions were dialyzed against 50 mM Tris-HCl (pH 7.4) and concentrated, and the protein contents were estimated. All of the fractions were tested for reactivity with MA lectin by lectin blot analysis. Both 0.1 and 0.2 M sodium chloride fractions from a DEAE-Sephadex column containing most of the MA lectin-reactive proteins were further separated on a WGA-Sephadex column to eliminate the nonglycosylated proteins. The fractions were incubated with WGA affinity column for 1 h at 4°C and then washed with 10 bed volumes of Tris buffer containing 0.01% Triton X-100. The bound proteins were eluted with 150 mM N-acetylglucosamine in Tris-HCl (pH 7.4), and the eluted fractions were dialyzed against PBS extensively and concentrated.

MA lectin affinity chromatography. To prepare MA lectin-Sepharose, CNBr-activated Sepharose-6B (1 g of dry gel) was washed thoroughly with ice-cold coupling buffer containing 0.2 M NaHCO₃ and 50 mM GalNAc (pH 7.9) and added to MA lectin (10 mg) suspended in 2 ml of coupling buffer. The mixture was stirred gently for 24 h at 4°C, after which the Sepharose was centrifuged at 500 × g for 5 min and the supernatant containing uncoupled MA lectin was removed. Coupling efficiency was calculated by determining the protein concentrations before and after coupling and was estimated to be approximately 80%. The MA lectin-Sepharose was resuspended in the coupling buffer with 1 M ethanolamine (pH 7.9) and was incubated for 2 h at 4°C. Then, the MA lectin-Sepharose was packed into a column, washed with PBS extensively, and stored at 4°C in PBS containing 0.02% sodium azide. For chromatography on MA lectin-Sepharose, the BMEC glycoprotein fraction (6 mg) obtained from WGA-Sepharose column was applied to the prewashed column in 0.5 ml of PBS (pH 7.4) containing 0.03% Triton X-100, aprotinin, and PMSF. The column was incubated with the proteins for 4 h at 4°C and washed with 10 bed volumes of PBS containing 0.05% Triton X-100. The bound proteins were eluted with 5 bed volumes of glycine-HCl buffer (pH 2.5) and collected into a tube containing 1/20 volume of 1 M phosphate buffer (pH 7.9). The eluant was dialyzed against PBS for 48 h with several changes of buffer at 4°C.

Polyclonal antibody production and preparation of antibody affinity matrix. The WGA-bound proteins of the 0.2 M fraction (100 µg) were injected into rabbits for the production of polyclonal antisera by the standard procedures. The antiserum titers were determined by Western blot analysis of BMEC membrane proteins, and the immunoglobulin G (IgG) fraction was purified by protein A columns (Pierce Co.) and concentrated by Centricon-30 (Amicon).

The purified IgG (designated SBP antibody [3 mg of protein]) was extensively dialyzed against 0.5 M sodium phosphate buffer (pH 7.5), mixed with prewashed CNBr-activated Sepharose 4B beads, and gently rocked overnight at 4°C. After incubation, the beads were washed twice with 0.5 M sodium phosphate buffer and finally with 1 M NaCl in 0.05 M sodium phosphate buffer (pH 7.5). Then, the beads were incubated at room temperature overnight in 10 volumes of 100 mM ethanolamine (pH 7.5) with gentle mixing. Finally, the beads were washed four times with PBS and transferred to a small column (1 by 5 cm). BMEC membrane proteins (2 mg) were loaded onto an SBP antibody affinity column (pre-equilibrated with loading buffer) in 50 mM Tris-HCl (pH 7.5) and incubated for 1 h at 4°C. The column was washed with 20 bed volumes of binding buffer and then 2 bed volumes of preelution buffer-10 mM phosphate buffer (pH 6.8). The bound proteins were eluted with 100 mM glycine-HCl (pH 2.5), and the eluant was collected into 400 µl of 1 M phosphate buffer (pH 8.0). The eluted proteins were extensively dialyzed against PBS and concentrated, and the protein contents were estimated.

Preparation of Fab fragments of SBP antibodies. Fab fragments of SBP antibodies were isolated according to the instructions of the manufacturer (Pierce Co.). Briefly, SBP antibodies purified by protein A-Sepharose columns were dialyzed against 20 mM phosphate-10 mM EDTA buffer (pH 7.0) for 24 h at 4°C and lyophilized. The lyophilized IgG was dissolved in 1 ml of digestion buffer containing cysteine hydrochloride in phosphate buffer and incubated overnight with 0.5 ml of immobilized papain (prewashed three times with digestion buffer) at 37°C in a shaker water bath at high speed. The Fab and Fc fragments were separated from the immobilized papain and passed through an immobilized protein A column. The eluant-containing Fab fragments were collected and dialyzed against PBS extensively.

Immunoprecipitation of BMEC membrane proteins with SBP antibody. BMEC membrane proteins (500 μ g) were clarified by centrifugation at $4,000 \times g$ for 10 min and incubated with 50 μ l of normal rabbit serum for 1 h on ice. Then, 1 ml of 10% protein A-Sepharose was added, the mixture was incubated for 30 min on ice and centrifuged, and the supernatant was collected. The SBP antibody (100 μ g) was then added to the supernatant and incubated on ice for 1 h. The immune complex was purified by passage through a protein A column according to the instructions of the manufacturer (Pierce Co.). The eluted immune complex was electrophoresed on sodium dodecyl sulfate (SDS)-10% polyacrylamide gel and transferred to a nitrocellulose sheet (Immobilon P). Lectin blotting was carried out by using biotinylated MA lectin followed by streptavidin-peroxidase. Immunoprecipitation was also carried out with SBP antibody by using biotinylated BMEC membranes as described above.

Desialylation and deglycosylation of BMEC. The BMEC monolayers were treated with *V. cholera* neuraminidase (0.1 U/ml) in 50 mM sodium acetate buffer (pH 5.5) containing 5 mM CaCl_2 and 0.1% (wt/vol) BSA for 1 h at 37°C to desialylate the cellular surface structures. The monolayers were washed five times with PBS after incubation, and the membrane proteins were prepared as described above. Asparagine-linked oligosaccharides from the 65-kDa protein were cleaved with 5 U of peptide *N*-glycosidase F in 50 mM sodium phosphate buffer (pH 7.8) at 37°C overnight (23). The cleavage efficiency was monitored by WGA blotting.

Western and lectin blotting. The BMEC proteins were separated on SDS-10% polyacrylamide gels and transferred onto Immobilon P nitrocellulose by using Millipore electroblotting. The blot was incubated with either 2% (for antibodies) or 0.5% (for lectins) nonfat milk in PBS for 1 h and then incubated with SBP antibodies (1:1,000 dilution in blocking buffer) for 2 h followed by goat anti-rabbit IgG coupled to horseradish peroxidase and washed five times with PBS-0.05% Tween. For lectins, the blots were incubated with 10- μ g/ml biotinylated MA lectin or biotinylated *S. fimbriae* for 2 h, washed five times with PBS, and then incubated with streptavidin peroxidase (1:3,000 in blocking buffer) for 30 min. After washing, the blots were developed with diaminobenzidine and hydrogen peroxide as substrates. In some experiments, desialylation of BMEC membrane proteins was carried out as described above on nitrocellulose blot strips without serum albumin before immunoblotting.

Solid-phase binding assay. The binding of *E. coli* strains to purified 65-kDa protein immobilized on microtiter plates (Immunolon 3; Dynatech Laboratories) was determined by enzyme-linked immunosorbent assay. MA lectin-Sepharose-bound purified 65-kDa protein (1 μ g/well) was allowed to bind to the microtiter plate wells in PBS (50 μ l) overnight at 4°C. The solution containing the protein was aspirated, washed with PBS three times, and incubated with 5% BSA for 1 h at room temperature to block nonspecific binding sites. Either biotinylated bacteria (2×10^5 CFU in 10 μ l) or MA lectin (5 μ g/well) was added to the wells in 5% BSA, and the wells were incubated for 2 h at 4°C with intermittent shaking. The wells were washed four times with PBS and incubated with streptavidin peroxidase (1 to 10,000 in 5% BSA) for 30 min at room temperature. Bound bacteria were quantified by developing the color with orthophenylenediamine (0.4 mg/ml) in 0.1 M citrate phosphate buffer (pH 5.0) and 0.005% hydrogen peroxide. The brown reaction product was quantified at 490 nm on a Titertech multiscan spectrophotometer with the V_{max} computer program.

Immunocytochemical staining. The confluent BMEC monolayers, which were grown in an eight-well chamber slide (Lab-Tek), were fixed with ice-cold methanol for 5 min and dried under a hood. The monolayers were rehydrated with PBS and preincubated with normal goat serum for 1 h at room temperature. Then, the monolayers were incubated with either protein A-purified intact anti-SBP antibodies or the Fab fragments (1:300 dilution) for 2 h at 4°C. After washing with RPMI 1640 four times, the monolayers were incubated with peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad; 1:500 dilution) for 1 h at room temperature. The color was developed with diaminobenzidine and hydrogen peroxide as a substrate. In some experiments, the cells were permeabilized with 0.1% Triton X-100 and processed for the immunocytochemistry described above.

Electroelution of 65-kDa protein. The 65-kDa protein was electroeluted from both 0.1 and 0.2 M fractions for microsequencing with Hoefer Sixpac Gel Eluter GE 200. The WGA-bound proteins (10 μ g) were separated on an SDS-10% polyacrylamide gel (0.75-mm thick) and briefly stained with freshly prepared Coomassie brilliant blue solution. The band was excised and cut into small pieces. The gel slices were placed into the inner tubes containing 300 μ l of Laemmli buffer (25 mM Tris, 192 mM glycine, 0.1% SDS [pH 8.3]). The inner tube was placed in the outer tube containing 100 μ l of 4 $\times$ Laemmli buffer, and the electrodes were assembled according to the instruction manual. Elution was carried out for 1.5 h with a setting at +60 V and with reverse polarity for the last 5 s. After elution, the proteins were dialyzed against 0.2% ammonium bicarbonate solution for 24 h at 4°C, lyophilized, and submitted for sequencing.

Separation of WGA-bound proteins by HPLC. The 65- and 130-kDa *S. fimbriae*-binding proteins were separated by using a size exclusion column, SynChroapak 100 (SynChrom), on a high-performance liquid chromatography (HPLC) system (Applied Biosystems) from WGA-bound 0.2 M BMEC proteins. The BMEC glycoproteins (50 μ g in 50 μ l) were applied to the column, which was equilibrated with 0.1 M Tris-HCl buffer (pH 7.4) containing 0.1 M NaCl and 2 mM NaN_3 . The column was eluted at a flow rate of 0.5 ml/min, and 0.5-ml fractions were collected. A portion of separated 65- and 130-kDa proteins was subjected

TABLE 2. Binding of *S. fimbriated* and nonfimbriated *E. coli* to endothelial cells of different origins

Endothelial cell origin	Binding value (CFU/well) for ^a :	
	HB101/13	HB101/322
Bovine BMEC	$(5.1 \pm 1.2) \times 10^5$ (4.2) ^b	$(1.5 \pm 0.8) \times 10^5$ (1.1)
Rat BMEC	$(2.6 \pm 0.8) \times 10^5$ (2.1) ^b	$(1.1 \pm 0.1) \times 10^5$ (0.8)
Human BMEC	$(3.5 \pm 0.9) \times 10^5$ (3.0) ^b	$(1.0 \pm 0.3) \times 10^5$ (0.7)
HUVEC	$(2.0 \pm 1.0) \times 10^4$ (0.2)	$(1.9 \pm 0.2) \times 10^4$ (0.2)
HAAEC	$(9.6 \pm 0.5) \times 10^4$ (1.0)	$(1.1 \pm 0.3) \times 10^5$ (1.3)
HIVEC ^c	$(4.3 \pm 0.5) \times 10^4$ (0.4)	$(8.6 \pm 0.4) \times 10^4$ (0.9)

^a Values in parentheses are percentages of inoculum added.

^b $P < 0.05$ (significantly greater than value for HB101/322 by two-tailed *t* test).

^c HIVEC, human iliac vein endothelial cells.

to SDS-10% polyacrylamide gel electrophoresis (PAGE), transferred to a nitrocellulose sheet, and blotted with biotinylated MA lectin as described above. A duplicate gel was also transblotted and stained with India ink to assess the purity of the proteins. The proteins were then subjected to an Applied Biosystems solid-phase protein sequencer.

RESULTS

***S. fimbriated E. coli* binds to endothelial cells of brain origin.** We have previously shown that *S. fimbriated E. coli* binds NeuAc α 2,3-galactose-containing glycoproteins on brain endothelial cells via a lectin-like activity of SfaS adhesin (24). To verify whether the interaction of *S. fimbriated E. coli* is specific to endothelial cells of brain origin, binding assays were carried out with cow, human, and rat BMEC, as well as with systemic endothelial cells, e.g., human umbilical vein endothelial cells (HUVEC) and human aortic arterial endothelial cells (HAAEC). As shown in Table 2, the level of binding of *S. fimbriated E. coli* to endothelial cells originating from cow, rat, and human brains was significantly greater than that of nonfimbriated bacteria (approximately $3.5 \times 10^5 \pm 1.0 \times 10^5$ CFU/well for HB101/13 versus $1.2 \times 10^5 \pm 0.4 \times 10^5$ CFU/well for HB101/322 [$P < 0.05$]). In contrast, the level of binding of HB101/13 to non-brain endothelial cells was considerably less and did not differ from that of HB101/322. Comparable results were also obtained when the binding assays were carried out with fixed BMEC monolayers at 37°C (data not shown). Increased serum concentrations of as much as 20% did not affect the binding of *S. fimbriated E. coli* to BMEC (data not shown). These results suggest that the different levels of binding of *S. fimbriated* versus nonfimbriated *E. coli* to BMEC could be due to the specific presence or differential expression of *S. fimbriae*-binding epitopes on BMEC compared to systemic endothelial cells. Since MA lectin has the same carbohydrate specificity as *S. fimbriae*, i.e., NeuAc α 2,3-galactose (29), we examined the effect of MA lectin on the binding of *S. fimbriated E. coli* to BMEC (Fig. 1). We also included WGA, which is specific for GlcNAc β 1,4-GlcNAc (primary) and sialic acid (secondary [4]), along with ConA, which is specific for mannose, in binding assays as controls. Preincubation of BMEC for 1 h with MA lectin at either 4 or 37°C blocked the binding by approximately 30% at a concentration of 100 μ g/ml ($3.1 \times 10^5 \pm 0.4 \times 10^5$ CFU/well for HB101/13 with MA lectin versus $4.6 \times 10^5 \pm 0.6 \times 10^5$ CFU/well for the control). Further incubation with MA lectin for 6 to 12 h showed 65% inhibition of HB101/13 binding to BMEC ($1.6 \times 10^5 \pm 0.3 \times 10^5$ CFU/well for MA lectin versus $4.6 \times 10^5 \pm 0.6 \times 10^5$ CFU/well for control [$P < 0.05$]). In contrast, WGA and ConA did not show any blocking effect on the binding at any preincubation period. We have previously shown that either neuraminidase treatment of BMEC to remove sialic acids

FIG. 1. Inhibition of binding of S-fimbriated *E. coli* to bovine BMEC by various lectins and glycoproteins. The BMEC monolayers were incubated with 100- μ g/ml lectin for 6 h at 4°C before addition of the bacteria (10^7 CFU/well [13+MAL, 13+WGA, and 13+ConA]). In some experiments, the bacteria were incubated with 100 to 400 μ g of either fetuin, porcine thyroglobulin or bovine mucin for 1 h on ice, and the bacterial binding assays were carried out as described in Materials and Methods. The binding was expressed as numbers of CFU/well, and the bars indicate standard deviations from the means of triplicate wells. * $P < 0.01$ by two-tailed, unpaired *t* test.

from the cell surface or incubation of S-fimbriated *E. coli* with sialyl lactose (final concentration, 50 mM) completely blocked the binding of S-fimbriated *E. coli* to BMEC (24). Having demonstrated that S-fimbriated *E. coli* interacts with NeuAc α 2,3-galactose epitopes, we examined different sialylated glycoproteins for their abilities to block the S fimbria-mediated binding of *E. coli* to BMEC. The glycoproteins fetuin and bovine mucin containing NeuAc-linked α 2,3 or α 2,6 to galactose or *N*-acetylgalactosamine epitopes on its oligosaccharide portions did not show any significant inhibition on the binding of *E. coli* to BMEC at concentrations of 100 to 400 μ g of proteins. Porcine thyroglobulin, whose oligosaccharides contain NeuAc α 2,6-galactose moieties, was used as a control and also showed no inhibitory activity. Taken together, these results suggest that binding of S fimbriae to BMEC glycoproteins requires NeuAc α 2,3-galactose epitopes but that conformation of epitopes and/or other structures internal to the NeuAc α 2,3-Gal β 1-4GlcNAc trisaccharide may play a role in optimal binding.

S fimbriae bind to 65- and 130-kDa sialoglycoproteins on BMEC. To identify the sialoglycoproteins interacting with S fimbriae, biotinylated S fimbriae and MA lectin were used on proteins transferred to nitrocellulose blots. As shown in Fig. 2, the biotinylated S fimbriae reacted with two BMEC membrane proteins with approximate molecular masses of 65 and 130 kDa (Fig. 2A, lane 1), whereas no reactivity was observed in BMEC cytosolic fraction (lane 2). As predicted, desialylation of BMEC

FIG. 2. Binding of biotinylated S fimbriae and *M. amurensis* lectin to BMEC membrane proteins. BMEC membrane proteins (lane 1), BMEC soluble fraction (lane 2), and desialylated BMEC membrane proteins (lane 3) were separated by SDS-PAGE and blotted with either biotinylated S fimbriae (A) or MA lectin (B). Molecular mass markers are indicated on the left in kilodaltons.

FIG. 3. (A) Analysis of 0.2 M fraction after WGA-Sepharose chromatography. BMEC membrane proteins in the 0.2 M fraction after DEAE-Sephadex column chromatography were further separated with a WGA-Sepharose column and eluted with 150 mM *N*-acetylglucosamine solution as described in Materials and Methods. The proteins separated by SDS-PAGE were either stained with Coomassie (A) or blotted with biotinylated MA lectin (B). Lane 1, whole BMEC membrane proteins; lane 2, 0.2 M fraction; lane 3, runthrough of 0.2 M fraction.

membrane proteins with neuraminidase caused significant reduction in reactivity with S fimbriae (lane 3). The biotinylated MA lectin reacted with three BMEC proteins with apparent molecular masses of 180, 130, and 65 kDa (Fig. 2B, lane 1) and also did not show any reactivity with either cytosolic fraction or desialylated BMEC membranes. These data suggest that the BMEC proteins recognized by both S fimbriae and MA lectin are membrane-bound sialoglycoproteins. The reactivities of S fimbriae to only 65- and 130-kDa proteins compared to MA lectin's reactivity to an additional 180-kDa protein could be due to the requirement of additional structures on these proteins and/or the conformation of carbohydrate epitopes for the interaction with S fimbriae. Since isolation of large quantities of pure S fimbriae was laborious, the S fimbria-binding proteins were thereafter monitored with MA lectin.

Purification of SBPs from bovine BMEC. To isolate the SBPs on BMEC, endothelial cell membrane proteins were initially separated on DEAE-Sephadex based on charge. The eluted fractions showed a number of proteins by SDS-PAGE and Coomassie staining, whereas blotting with biotinylated MA lectin revealed that the 65-kDa protein was a major protein in 0.1 M NaCl fraction. The proteins with molecular masses of 180, 130, and 65 kDa were eluted in 0.2 M NaCl fraction, whereas the 180-kDa protein was eluted in 0.3 M NaCl fraction (blot not shown). Since S fimbriae reacted with only 65- and 130-kDa proteins, we focused our work on the 0.2 M fraction, which was further separated by WGA-Sepharose chromatography to eliminate nonglycosylated proteins. As shown in Fig. 3, both SDS-PAGE (Fig. 3A) and MA lectin blotting (Fig. 3B) revealed that the 65-kDa protein was a major protein in the 0.2 M NaCl-eluted fraction (designated WGA II). This fraction was effective in inhibiting S fimbria-mediated binding of HB101/13 to BMEC ($1.6 \times 10^5 \pm 0.3 \times 10^5$ CFU/well with WGA II versus $4.8 \times 10^5 \pm 0.6 \times 10^5$ CFU/well for the control [$P < 0.05$]). The 0.2 M fraction also showed a major 65-kDa protein under nonreducing conditions without β -mercaptoethanol (gel not shown).

WGA is known to exhibit strong interaction with the GlcNAc β 1-4GlcNAc moiety in the core portion of asparagine-linked oligosaccharides and has secondary specificity to sialic acid (4). Thus, it is possible that the WGA II fraction may also contain proteins lacking NeuAc α 2,3-galactose epitopes. To further separate the NeuAc α 2,3-galactose-containing proteins from nonsialoglycoproteins present in WGA II proteins, the fraction was further subjected to MA lectin-Sepharose chromatography. As shown in Fig. 4, the 65-kDa protein appeared to be the major protein reactive to MA lectin on blots in both

FIG. 4. A composite of MA lectin blots and SBP antibody Western blots of various BMEC membrane fractions and biotinylated BMEC proteins. Lanes: 1, BMEC membrane proteins; 2, WGA-bound 0.2 M fraction; 3, MA lectin-bound 0.2 M fraction after WGA-Sepharose column chromatography; and 4, SBP antibody affinity-purified proteins. These proteins were separated by SDS-PAGE and blotted either with biotinylated MA lectin (A) or with SBP antibody (B). Biotin-labeled surface membrane proteins immunoprecipitated with SBP antibody (lane 5) were separated by SDS-PAGE, transferred to a nitrocellulose sheet, and identified with streptavidin peroxidase followed by diaminobenzidine and hydrogen peroxide. Biotinylated whole BMEC membranes were loaded in lane 6.

the WGA II fraction (Fig. 4A, lane 2) and the MA lectin-bound WGA II fraction (Fig. 4A, lane 3). The reasons for the absence of the 130-kDa protein in the MA lectin-bound fraction are not clear. The MA lectin-bound WGA II fraction also showed inhibitory activity on the binding of S-fimbriated *E. coli* to BMEC by $>60\%$ at a concentration of $25 \mu\text{g/ml}$ ($1.6 \times 10^5 \pm 0.5 \times 10^5$ CFU/well versus $4.9 \times 10^5 \pm 0.6 \times 10^5$ CFU/well with buffer [$P < 0.05$]), whereas the deglycosylated protein showed a very slight blocking effect on binding (Fig. 5). These results suggest that the 65-kDa BMEC protein is a major SBP interactive with meningitis-causing *E. coli*.

Antibodies to 65-kDa SBP recognize the BMEC surface protein. Antiserum raised against the WGA II fraction reacted with the 65-kDa protein in both the WGA II fraction and the MA lectin-Sepharose-bound fraction (Fig. 4B, lanes 2 and 3) but not with the 130-kDa protein. These antibodies (designated SBP antibodies) were purified, and Fab fragments were prepared to eliminate nonspecific interaction of the Fc portion with BMEC in binding assays. Both the intact SBP antibodies and Fab fragments were tested whether they reacted to the surface protein on BMEC or not. SBP antibodies showed some reactivity to the apical surface membrane portion of BMEC (Fig. 6B), and this reactivity was considerably enhanced when the cells were permeabilized with Triton X-100 (Fig. 6C). MA lectin stained the BMEC membrane portion even in the absence of detergent (Fig. 6D). These results indicate that the antibody might recognize either the membrane portion or the intracellular portion of the protein, whereas MA lectin recognizes extracellular carbohydrate epitopes.

Antibodies to the 65-kDa SBP fail to inhibit binding of S-fimbriated *E. coli* to BMEC. The Fab fragments of the SBP antibody did not show any blocking effect on the binding of S-fimbriated *E. coli* to BMEC (Fig. 5), indicating that SBP antibodies might be directed against the protein portion of the glycoprotein, whereas the S fimbria-binding epitope is a carbohydrate moiety. To examine this possibility, nitrocellulose blots containing MA lectin-bound proteins were treated with neuraminidase and then blotted with either biotinylated MA lectin or SBP antibody. To confirm the specificity of this interaction, unbiotinylated MA lectin was incubated for some blots prior to the addition of biotinylated MA lectin as well as SBP antibodies. As shown in Fig. 7, both neuraminidase and unbiotinylated MA lectin treatments completely abolished the reactivity of MA lectin-bound proteins to MA lectin (Fig. 7A, lanes 2 and 3), whereas the SBP antibody reactivity was slightly affected by neuraminidase treatment (Fig. 7B, lane 2 and 3).

The altered binding of SBP antibodies to the neuraminidase-treated blot could be due either to the presence of small quantities of protease impurities in the neuraminidase preparation or to the altered binding site for the SBP antibody after removal of sialic acid by the enzyme. Similarly, 50 mM sialyl lactose (Fig. 7B, lane 4) blocked the reactivity to MA lectin significantly but did not affect antibody reactivity. In addition, to exclude the possibility that Fab fragments might have lost their activity due to cleavage, inhibition of S-fimbriated *E. coli* binding was also carried out with intact polyclonal antibodies. These results are similar to those for Fab fragments, i.e., no inhibition, indicating that the SBP antibody may be directed against the protein portion of S fimbria-binding sialoglycoprotein, probably away from the carbohydrate binding site. This possibility could also explain the results of the immunocytochemical analysis of SBP antibodies mentioned above.

To determine whether the inability of SBP antibodies to react with 130-kDa proteins is due to any denaturation of the proteins on Western blots, BMEC membrane proteins were passed through an SBP antibody affinity column, and the bound proteins were analyzed by MA lectin blotting. In addition, since bacteria are likely to interact with cell surface BMEC glycoproteins, we also assessed the surface localization of these proteins by immunoprecipitation of biotin-labeled surface membrane proteins with SBP antibody. As shown in Fig. 4, only the 65-kDa protein was identified in both cases (lanes 4 and 5), indicating that the 65-kDa protein is a surface protein and that the 65- and 130-kDa proteins represent different SBPs. WGA affinity chromatography of biotinylated BMEC surface proteins revealed that the 130-kDa protein was also a surface protein (data not shown). As mentioned earlier, the binding of S-fimbriated *E. coli* is specific to endothelial cells of brain origin, not to systemic endothelial cells, suggesting either the specific presence or differential expression of SBPs. To verify whether SBP antibody shows such differences, membrane proteins of different endothelial cells were separated and blotted with SBP antibody as well as with MA lectin. The results showed that human and rat BMEC contained only the 65-kDa SBP, whereas bovine BMEC contained both the 65-

FIG. 5. Inhibition of binding of S-fimbriated *E. coli* to bovine BMEC with purified 65-kDa protein. The S-fimbriated HB101/13 bacteria were incubated with $25 \mu\text{g}$ of purified 65-kDa protein in $50 \mu\text{l}$ of PBS (13+65 kDa), $50 \mu\text{l}$ of PBS (13+Buff.), BSA (13+BSA), or deglycosylated 65-kDa protein (13+Deglyc.) for 1 h on ice before addition to the BMEC monolayer, and the binding assays were carried out as described in Materials and Methods. For antibody inhibition, the anti-SBP antibodies (SBP-Ab) were incubated with the monolayers at 37°C for 1 h before addition of the bacteria. Binding was expressed as numbers of CFU/well, and the bars indicate standard deviations from the means of triplicate wells. * $P < 0.01$ by two-tailed unpaired *t* test.

FIG. 6. Immunocytochemical staining of BMEC with anti-SBP antibodies. The confluent BMEC monolayers grown in eight-well chamber slides were fixed with ice-cold methanol and processed for immunocytochemistry as described in Materials and Methods. The monolayers were stained with preimmune serum (A), anti-SBP antibodies (B), or MA lectin (D). In some experiments, the monolayers were permeabilized with Triton X-100 before incubation with anti-SBP antibodies (C). Magnification, $\times 200$.

and the 130-kDa SBPs, which were absent or undetectable in systemic endothelial cells (Fig. 8). Thus, the presence of the 65-kDa glycoprotein on BMEC might be responsible for significant binding of S-fimbriated *E. coli* to BMEC compared to systemic endothelial cells.

FIG. 7. Effects of neuraminidase, MA lectin, and sialyllactose on the reactivities of MA lectin and anti-SBP antibody. BMEC membrane proteins on nitrocellulose strips were either untreated (lane 1) or treated with *V. cholera* neuraminidase (lane 2) or with unlabeled MA lectin (50 $\mu\text{g}/\text{ml}$ [lane 3]) before immunoblotting was carried out with either biotinylated MA lectin (A) or anti-SBP antibody (B). For lane 4, 50 mM sialyllactose was incubated with either biotinylated MA lectin or anti-SBP antibody before being added to the blot.

Binding of S-fimbriated *E. coli* to purified 65-kDa protein.

As a direct measure to examine the interaction of 65-kDa SBP with S-fimbriated *E. coli*, solid-phase binding assays were carried out with MA lectin-affinity-purified 65-kDa protein and biotinylated bacteria in microtiter plates. Biotinylated MA lectin was used as a positive control. The binding levels of S-fimbriated *E. coli* HB101/13 and MA lectin were significantly greater than that of nonfimbriated *E. coli* HB101/322 (Fig. 9) (Table 1). A deletion mutant of *E. coli* (HB101/1321), which

FIG. 8. Blotting of different endothelial cell membrane proteins with MA lectin and anti-SBP antibodies. Bovine (lane 1), human (lane 2), rat (lane 3), HUVEC (lane 4), and HAAEC (lane 5) endothelial cell membrane proteins (8 μg each) were separated on 10% gels and blotted with either MA lectin (A) or anti-SBP antibodies (B). Molecular mass markers are indicated on the left in kilodaltons.

lacked the minor S-fimbria subunit gene *sfaS* (specific to NeuAc α 2,3-galactose epitopes), resulted in complete loss of binding to the 65-kDa protein. However, *E. coli* HB101/1, which had a deletion of the major subunit gene *sfaA*, did not show any loss in binding. These results indicate that the interaction between the 65-kDa protein and S-fimbriated *E. coli* is specific to the SfaS adhesin, which is in agreement with our previous results (24). Preincubation with anti-SBP antibodies did not abolish the binding of either S-fimbriated *E. coli* or MA lectin to the 65-kDa protein, indicating that the binding site of anti-SBP antibodies on the 65-kDa protein is far away from the S fimbria-binding site.

The SBPs are novel BMEC membrane glycoproteins. The N-terminal amino acid sequence of the 65-kDa protein was determined by electroeluting the protein from the MA lectin-bound WGA II fraction after SDS-PAGE. In addition, the N-terminal sequence for both the 65- and 130-kDa proteins was also determined after separation on an HPLC size exclusion column. The N-terminal amino acid sequences of the 65- and 130-kDa proteins are as follows: XLMSVAIVQLVFQAV FDLXE for the 65-kDa protein and DLMVIPSL for the 130-kDa protein, where X is an unidentified amino acid. A search of protein databases (PIR and Swiss-Prot [2]) revealed no significant sequence homology with any of the known proteins; thus, the 65- and 130-kDa proteins represent novel BMEC proteins.

DISCUSSION

Bacterial adhesion to host cell surfaces is thought to be an important first step in the pathogenesis of many infectious diseases, including meningitis. This pathogenic event in *E. coli* meningitis is most likely to take place through the binding of bacterial fimbriae to specific receptors on BMEC, which constitute the blood-brain barrier (6). We have previously shown that binding of S-fimbriated *E. coli* to BMEC is mediated by a lectin-like activity of the SfaS adhesin of S fimbriae, which is specific for NeuAc α 2,3-Gal-containing glycoproteins of BMEC (24). We have also shown that S-fimbriated *E. coli* binds to sulfated glycolipids via the SfaA subunit of S fimbriae (21). However, removal of sialic acid from cell surfaces by neuraminidase treatment completely blocked S-fimbriated *E. coli* binding (24). The SfaS adhesin is shown to be located at the tips of S fimbriae (22), thus suggesting that the initial interaction with NeuAc α 2,3-Gal epitopes of glycoproteins may be critical for binding to BMEC. In contrast, soluble sulfated glycolipid micelles blocked the binding by only 50%, indicating that the sulfated glycolipid-mediated adhesion may be important to ensure intimate contact of S-fimbriated *E. coli* to the host membrane to satisfy its nutritional requirements and subsequent crossing of the blood-brain barrier. In the present study, we have shown that this binding property of S-fimbriated *E. coli* is specific to endothelial cells originating and that binding from the brain to systemic endothelial cells such as HUVEC and HAAEC does not occur. This specific binding of S-fimbriated *E. coli* to BMEC could be due to the specific presence or differential expression of the receptor compared to non-brain endothelial cells.

To identify and isolate the NeuAc α 2,3-Gal-containing glycoproteins interacting with S fimbriae, we used MA lectin, which has the same specificity as S fimbriae. We found that despite having the same specificity as S fimbriae for NeuAc α 2,3-Gal, MA lectin inhibited the binding of S-fimbriated *E. coli* to BMEC by only 60%. In contrast, as shown previously, binding of S-fimbriated *E. coli* to BMEC was effectively blocked by small quantities of isolated S fimbria protein (>80% inhibition

by 20 μ g/ml [24]). The requirement of longer periods of preincubation of MA lectin with BMEC compared to those for S fimbriae to block S-fimbriated *E. coli* binding may relate to the possibility that the conformation of NeuAc α 2,3-Gal epitopes on BMEC may be more favorable to S fimbriae than MA lectin. A strong reactivity of MA lectin to BMEC glycoproteins observed with lectin blots, however, could be due to conformational changes. An alternative explanation is that S fimbriae may have greater affinity to BMEC glycoproteins due to their interaction with additional structures of BMEC and that S fimbriae may be able to competitively replace bound MA lectin from BMEC during inhibition studies. A similar phenomenon was observed in the interaction of MA lectin and S fimbriae with human erythrocytes (26, 30). Most of the erythrocyte sialic acids are contained in glycophorin oligosaccharides with terminal sequences, Asn-linked NeuAc α 2,3-Gal β 1-4GlcNAc β 1, 2Man-R and Ser/Thr-linked NeuAc α 2,3-Gal β 1-3GalNAc α 1-R. MA lectin interacts with a low affinity to these oligosaccharides, even though they contain sialic acid-linked α 2,3-galactose, and thus is a poor hemagglutinin (29). In contrast, the interaction of S fimbriae with glycophorin oligosaccharides exhibits a stronger hemagglutination (19). However, interactions of both MA lectin and S fimbriae with erythrocytes were blocked by an approximately 4 mM concentration of NeuAc α 2,3-Gal β 1-4Glc (19), whereas we showed previously that very high concentrations of NeuAc α 2,3-Gal β 1-4Glc (40 to 50 mM) were necessary to block binding of S-fimbriated *E. coli* to BMEC (24). Despite compelling evidence that NeuAc α 2,3-galactose is the binding epitope for S fimbriae, we were unable to inhibit the binding of S-fimbriated *E. coli* to BMEC by commercially available sialylated glycoproteins. Of interest, the 65-kDa S fimbria binding protein effectively blocked binding of S-fimbriated *E. coli* to BMEC (65% inhibition by 25 μ g/ml). Similarly, Liukkonen et al. have shown that *Streptococcus suis*, another pathogen causing meningitis in young piglets, recognizes poly-(N-acetylactosamine) sequences consisting of NeuAc α 2,3-Gal β 1-4GlcNAc β 1-3Gal β 1 on erythrocytes (13). These investigators showed that at least one galactose residue proximal to

FIG. 9. Binding of S-fimbriated *E. coli* to immobilized 65-kDa protein. MA lectin affinity-purified 65-kDa protein (1 μ g/well) was coated overnight in a 96-well microtiter plate. The wells were washed and incubated with biotinylated *E. coli* (2×10^5 CFU/well in 10 μ l) for 2 h at 4°C. The wells were washed and incubated with streptavidin peroxidase, and the color was developed with orthophenylenediamine and hydrogen peroxide. For antibody inhibition, the anti-SBP antibodies (SBP-Ab) were incubated with the coated proteins at 37°C for 1 h before addition of the bacteria. MA lectin was used as a positive control to verify the interaction. The optical density at 490 nm was measured, and the results are standard deviations from the means for triplicate wells. **P* < 0.05 by two-tailed, unpaired *t* test.

the *N*-acetylglucosamine residue is important for binding of *S. suis* to erythrocytes. This was based on the finding that sialoglycoproteins such as fetuin, which contains glycans with the same distal trisaccharide sequence, were a poor inhibitor of *S. suis*-mediated hemagglutination. Despite having the same carbohydrate specificity, MA lectin recognized three BMEC membrane proteins of 65, 130, and 180 kDa, whereas *S. suis* fimbriae reacted to only two of them, i.e., the 65- and 130-kDa proteins. Taken together, these findings suggest that the binding site of BMEC glycoproteins for *S. suis* fimbriae may be complex and may require, in addition to NeuAc α 2,3-Gal, other oligosaccharide sequences such as galactose and/or protein portion. This concept was further supported by the observation that *S. suis* fimbriae were unable to bind gangliosides which contained NeuAc α 2,3-Gal β 1-3GalNAc sequences (21). The precise carbohydrate structures involved in the interaction of BMEC glycoproteins with *S. suis*-fimbriated *E. coli* are currently under study.

The inability of anti-65-kDa SBP antibodies to block the binding of *S. suis*-fimbriated *E. coli* to BMEC is unexpected, since the MA lectin-bound 65-kDa protein showed good inhibition. This could reflect that binding epitopes of *S. suis* fimbriae and anti-SBP antibodies are far apart on the 65-kDa protein, as shown by inhibition studies with nitrocellulose blots as well as by solid-phase binding assays. Alternatively, the 65-kDa SBP could be located on the basolateral side of the BMEC, so that it cannot interfere with the binding of *S. suis*-fimbriated *E. coli* to the apical surfaces of BMEC (24). This possibility was eliminated because of the reactivity of anti-SBP antibodies to the BMEC membranes on the apical surface. In addition, no bacteria were found on the basal surface after binding experiments by transmission electron microscopy (unpublished results). The interaction of *S. suis*-fimbriated *E. coli* to purified 65-kDa protein compared to that of nonfimbriated bacteria is direct evidence that the 65-kDa protein is an *S. suis* fimbria-binding molecule receptor. The specificity was also verified by the demonstration that the transformants of *E. coli* carrying the *sfaS*-deleted gene did not show any binding to the 65-kDa protein, which is in agreement with our previous data (24). The role of the 130-kDa protein in the binding of *S. suis*-fimbriated *E. coli* to BMEC may be relevant to bovine BMEC, since the presence of the 130-kDa protein was undetectable in human and rat BMEC.

The amino acid sequence data indicate that the SBPs identified in this study represent two novel BMEC surface proteins which effectively blocked the binding of *S. suis*-fimbriated *E. coli* to BMEC. Interestingly, the 65-kDa SBP was found to be present on human and rat BMEC but not on systemic endothelial cells, suggesting the cellular basis for the neurotropic nature of *S. suis*-fimbriated *E. coli* causing meningitis. A similar specific presence of a 46-kDa protein on lateral membranes of the brain capillary endothelium but not on heart, liver, and kidney capillaries has been reported by Partridge et al. by immunocytochemistry (20). The localization of this protein was tentatively identified at the tight junction of brain endothelial cells. In contrast, we have shown that *S. suis*-fimbriated *E. coli* bind to the apical surfaces of the endothelial cells, not at intercellular junctions (24). Thus, despite the specific localization of the 46-kDa BMEC protein on the brain endothelium, this protein may not represent another SBP. Other investigators have also reported a WGA-bound 135-kDa glycoprotein specifically present on mouse BMEC by immunocytochemistry (3) and a 116-kDa transmembrane glycoprotein on bovine aortic endothelial cells (5). However, lack of structural details and antibodies to these glycoproteins did not permit us to compare our SBPs with these glycoproteins.

In summary, we demonstrated that the binding of *S. suis*-fimbriated *E. coli* is significantly greater to BMEC than to non-brain-

originating endothelial cells. In addition, the interaction of *S. suis* fimbriae with BMEC may differ from that of MA lectin by requiring internal sugar residues in addition to NeuAc α 2,3-galactose moieties. *S. suis* fimbriae and MA lectin recognize 65- and 130-kDa sialoglycoproteins on bovine BMEC but only the 65-kDa SBP on human and rat BMEC. The partial sequences of the 65- and 130-kDa proteins suggested that these are novel BMEC glycoproteins. Complete characterization of these SBPs would help to identify a specific binding epitope(s) for *S. suis* fimbriae and also to develop novel strategies for prevention of *E. coli* meningitis.

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