INFECTION AND IMMUNITY, May 1995, p. 1927–1932 Vol. 63, No. 5
0019-9567/95/$04.00+0

Use of Porcine Fibrinogen as a Model Glycoprotein To Study the Binding Specificity of the Three Variants of K88 Lectin

CORINE L'HÔTE,[1] SYLVIE BERGER,[1] SYLVAIN BOURGERIE,[1] YVONNE DUVAL-IFLAH,[2] RAYMOND JULIEN,[1] AND YANNIS KARAMANOS[1]*

Institut de Biotechnologie, Université de Limoges, 87060 Limoges cedex,[1] and Laboratoire d'Ecologie et de Physiologie du Système Digestif, Centre de Recherche de Jouy-en-Josas, Domaine de Vilvert, 78352 Jouy-en-Josas cedex,[2] France

Received 12 December 1994/Returned for modification 1 February 1995/Accepted 28 February 1995

Known glycoproteins were used to determine the differences occurring in the binding specificities of the three variants of the K88 lectin in an approach essentially based on lectin blotting. During the screening, it was demonstrated that each variant of the K88 lectin biotinylated via its amino groups (NbioK88) exhibited a characteristic binding to the three chains of porcine fibrinogen. NbioK88ab weakly bound to Aα chains, NbioK88ac bound to Bβ and γ chains, and NbioK88ad bound only to the γ chain. To validate this model, the oligosaccharide moieties of porcine fibrinogen were analyzed with glycosidases and by lectin blotting and sugar composition. Both the Bβ chain and γ chain carry biantennary N-glycans of the *N*-acetyllactosamine type that are not recognized by K88 lectins. Aα chains are substituted by sialylated T antigen. O-glycans were also detected on Bβ and γ chains of porcine fibrinogen and contribute to the recognition of these chains by K88ac and K88ad fimbriae.

The K88 adhesin of *Escherichia coli* occurs in three antigenic variants designated K88ab, K88ac, and K88ad (22). Each variant facilitates the attachment of enterotoxigenic *E. coli*, provided appropriate receptors are present on the porcine small intestine epithelium (7, 26, 48). The adhesion of K88-positive *E. coli* to the brush borders of piglets and to erythrocytes of different animal species is serotype specific (3, 7, 41). When the three variants of K88 are considered, five phenotypes can be distinguished with regard to brush border adhesiveness (7).

Pigs of each of the five phenotypes may possess either a unique receptor molecule that has undergone evolutionary changes in its adhesin-binding site (18) or completely different receptors. Several putative glycoprotein and glycolipid receptors have already been identified. By using glycoproteins or plant lectins to block either hemagglutination or adhesion of the K88 antigen to brush borders, it was concluded that fucose, galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine residues may be involved in the K88 lectin-receptor interaction (2, 19, 46). Later, it was demonstrated that the interaction of *E. coli* $K88^+$ with murine mucus and brush borders (34) or its receptors in the pig (38) was periodate sensitive. Glycoproteins of 16 to 70 (38, 51, 56) and 210 to 240 kDa (18, 45) have been reported as being recognized by K88, and Galβ1-3GalNAc and Fucα1-2Galβ1-3/4GlcNAc were proposed as the main sequences mediating the binding (45). A lectin specific for the Galα1-3Gal sequence inhibited the interaction of K88ab with isolated mucus proteins (56). The interactions of isolated K88 with such known glycoproteins as porcine and ovine submaxillary mucins, fetuin, and human α_1-acid glycoprotein have been described (2, 19, 46). Glycolipidic receptors were also reported (28), and recently it was shown that glycolipids with galactosyl residues like galactosyl ceramide were also recognized by the K88ab and K88ac lectins (8, 42). All these studies led to the general agreement that sugars are an important part of the structure of the receptor and that K88, like other fimbrial adhesins, could be considered a lectin.

From the observations discussed above, it appears that the differences in the composition of the oligosaccharide moieties of the receptor(s) probably reflect their complexity. To determine the structure of the oligosaccharide moieties from natural receptors, it is necessary to isolate them and use nuclear magnetic resonance spectroscopy and mass spectrometry, but this is not something that is easy to do, essentially because of the low amounts of the isolated receptors and also because of the question of their purity. We decided to use an indirect approach and investigate whether known glycoproteins could be used as models to understand the differences in the binding specificities of the three variants of the K88 lectin. During the screening, it was demonstrated that each variant of K88 lectin exhibited a characteristic binding to the three chains of porcine fibrinogen (pig Fbg). To validate the model, the oligosaccharide moieties of this glycoprotein were analyzed.

MATERIALS AND METHODS

Purification of the three variants of K88 fimbriae. The bacteria (*E. coli* O8 [$K88ab^+$ LT^+ ST^- Raf^+], P2200 [K88ac LT^+ ST^+ Raf^+], and O8 [$K88ad^+$ LT^+ $Raf^{\pm}$]) were grown for 16 h at 37°C on solid minimal casein medium (23) supplemented with 1 g of yeast extract per liter, harvested in phosphate-buffered saline (PBS) (10 mM sodium phosphate, pH 7.6, containing 0.12 M NaCl), centrifuged (8,600 × *g* for 25 min), and resuspended in 2 ml of the same buffer per g of fresh bacteria. The purification of the K88 fimbriae was carried out in the same manner as that described previously for K99 fimbriae (6), except that urea was not added to the buffers.

Biotinylation of the fimbriae via amino groups. The reaction was carried out with biotinyl-6-aminocaproyl *N*-hydroxysuccinimide ester (BcapNHS) at various BcapNHS/K88 molar (ϕ) ratios (6). Excess reagents were removed on a Trisacryl GF05 column (42 by 2.4 cm) equilibrated with PBS–0.05% NaN_3. Biotin was detected on the K88 subunits, as had been described previously for the K99 lectin (40). Hemagglutination was performed at 4°C with fresh citrated guinea pig (27) erythrocytes or with *Mesocricetus auratus* (3) erythrocytes. The titer was defined as the number of hemagglutinating units per 10 μg of protein. The amount of biotin linked to the lectin was determined with avidin-biotinylated alkaline phosphatase in a colorimetric assay (5) after digestion of the biotinylated K88 (NbioK88) with 1% (wt/wt) proteinase K.

De-N-glycosylation. De-N-glycosylation was carried out with peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase F (PNGase F; EC 3.5.1.52) prepared as described previously (10) and submitted to a final step on TSK-Butyl 650 S (43) to obtain a protease-free activity. Pig Fbg was dissolved (10 mg/ml) in 0.1 M

* Corresponding author. Mailing address: Institut de Biotechnologie, Université de Limoges, 123 avenue Albert Thomas, 87060 Limoges cedex, France. Phone: (33) 55 45 76 62. Fax: (33) 55 45 76 53. Electronic mail address: karamanos@unilim.fr.

TABLE 1. Specificity of the plant lectins used in this study

Lectin (abbreviation)	Control[a]				Specificity	Reference(s)
	F	AF	hL	bL		
Aleuria aurantia agglutinin (AAA)			+	−	L-Fuc	16
Amaranthus caudatus agglutinin (ACA)	+		−		Galβ1-3GalNAcα, even if substituted	44
Maackia amurensis leucoagglutinin (MAL)	+	−			NeuAcα2-3Galβ1-4GlcNAc NeuAcα2-3Galβ1-4Glc	29 and 53
Peanut agglutinin (PNA)	−	+			Galβ1-3GalNAc	36
Ricinus communis agglutinin (RCA)	−	+			Terminal Galβ1	21
Sambucus nigra agglutinin (SNA)	+	−			NeuAcα2-6Gal NeuAcα2-6GalNac	29 and 49

[a] Fetuin (F), asialofetuin (AF), human lactotransferrin (hL), and bovine lactotransferrin (bL) were used as positive (+) and negative (−) controls.

sodium citrate–0.3 M NaCl (pH 6.1) (citrate buffer). This was diluted with an equal volume of 0.1 M Tris-HCl–2% sodium dodecyl sulfate (SDS)–0.2 M β-mercaptoethanol (pH 8.6) and then boiled for 5 min, and Nonidet P-40 was added to give a final concentration of 1%. PNGase F was added at a concentration of 1 mU/mg of glycoprotein, and the mixture was incubated at 37°C.

De-O-glycosylation. Pig Fbg was dissolved in citrate buffer (see above), and then PBS (pH 7.6) containing 0.05% SDS was added to the solution. It was boiled for 5 min, and finally Triton X-100 was added to give a final concentration of 0.5%. O-glycopeptide endo-D-galactosyl-*N*-acetyl-α-galactosaminohydrolase (O-glycosidase; EC 3.2.1.97; Boehringer) was used at a concentration of 0.5 mU/mg of protein, and the mixture was incubated at 37°C.

β-Elimination. The alkali-borohydride cleavage of pig Fbg O-glycans was performed after electrophoresis and transfer of the proteins to nitrocellulose membranes with a solution of 2 M $NaBH_4$ in 0.1 M NaOH for 16 h at 45°C (11). After the treatment, the membrane was thoroughly washed with distilled water to remove unreacted regents and was incubated in the blocking solution before lectin blotting.

Desialylation. Desialylation of Fbg was carried out at 37°C with neuraminidase (EC 3.2.1.18) from *Arthrobacter urefaciens* (Boehringer) diluted in the citrate buffer (containing 10 mM EDTA) at a final concentration of 20 mU/mg of protein. For the desialylation of pig Fbg directly after electrotransfer, the membranes were first incubated in the blocking solution and then incubated with the enzyme (from *A. urefaciens*, 50 mU in 10 ml, or from Newcastle disease virus, 20 mU in 10 ml) in sodium acetate buffer (pH 5.2) containing 0.2% bovine serum albumin (BSA) and 0.1% Tween 20.

Affinity separation of pig Fbg chains. Pig Fbg was reduced and S-carboxamidomethylated, and the chains were separated by chromatography on a Sepharose 4B-concanavalin A column (1.4 by 16 cm) (9).

Lectin blotting. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) with 10% gels (32) and an SE 250 Mighty Small II (HSI, San Francisco, Calif.) electrophoresis apparatus. Proteins were stained with Coomassie blue or electrotransferred (40). The transferred proteins were stained with Ponceau S (0.05% solution in 1% acetic acid), and the positions of the standard molecular mass proteins were marked. The standard buffer for plant lectins was Tris-buffered saline (TBS) (50 mM Tris-HCl, 0.15 M NaCl [pH 7.6]), and that for the NbioK88 lectins was PBS or TBS. Membranes were incubated overnight at 4°C in a blocking solution (PBS or TBS containing 0.05% Tween 20, 2% BSA, and 0.05% NaN_3). The membranes were washed and incubated with the lectin for 1 h at 25°C or 4 h at 4°C. The NbioK88 variants (K88ab at 1 μg/ml, K88ac at 1.5 μg/ml, and K88ad at 2 μg/ml) were incubated in PBS or TBS containing 0.2% Tween 20 and 0.2% BSA. Plant lectins (Table 1) labelled with digoxigenin or biotin (Boehringer) were used as described previously (25), except that the TBS buffer contained 0.1% Tween 20 and 0.1% BSA. The sugars were also detected in the different pig Fbg chains by an enzyme immunoassay (24) with the glycan detection kit (Boehringer).

Controls. Fetuin, asialofetuin, human lactotransferrin (50), and bovine lactotransferrin were used as controls for the lectin blotting (Table 1). In all cases, the specificity of lectin blotting was checked by omission of the digoxigenin- or biotin-labelled lectin as previously suggested (35). The binding of each NbioK88 variant was inhibited by the addition of the corresponding unlabelled fimbriae. In addition, the lack of visualization of the molecular mass standards attested to the absence of nonspecific binding.

Analytical methods. Protein content was measured according to the Lowry method (37), with BSA as the standard. The monosaccharide composition was determined by gas-liquid chromatography after methanolysis (methanol–0.5 M HCl for 24 h at 80°C) and trimethylsilylation (39).

RESULTS

Purification and biotinylation of the K88 fimbriae. After the extraction by mechanical shearing, the ultracentrifugation, and the gel filtration chromatography step, the K88 fimbriae appeared homogeneous (Fig. 1). The silver-stained band at approximately 27 kDa corresponded to the molecular mass of the major K88 subunit. The final K88 fimbria preparation represented 30 to 50% of the total protein extracted by mechanical shearing. The biotin-streptavidin technology which had already proven itself useful, versatile, and sensitive in the case of the bacterial K99 lectin (6, 40) was also selected to label the K88 fimbriae. Biotinylation at high ϕ ratios resulted in the broadening of the 27-kDa band representing the K88 major subunit (Fig. 2A). Biotin was detected on this band in all cases (Fig. 2B). A linear relationship between the number of biotin residues linked per K88 subunit and the ϕ ratio was observed (correlation coefficient = 0.992). The effect of biotinylation on the activity of the fimbriae was measured by the modification of their hemagglutination titers (Table 2). A very weak hemagglutination with guinea pig erythrocytes was obtained with

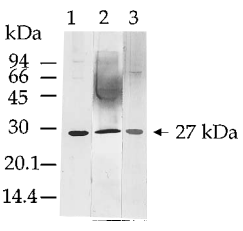

FIG. 1. Electrophoresis of purified K88 fimbriae. Samples were separated by SDS-PAGE on 20% gels, and proteins were silver stained. The molecular mass standards, indicated on the left, were phosphorylase *b*, 94 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.4 kDa. The molecular mass of the major K88 subunit is indicated on the right. Lane 1, K88ab; lane 2, K88ac; lane 3, K88ad.

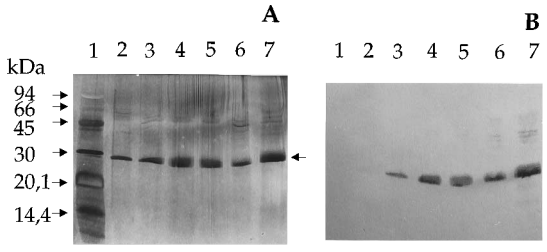

FIG. 2. Biotinylation of the K88 fimbriae. (A) SDS–20% polyacrylamide silver-stained gels. Lane 1, standard proteins (as described in the legend to Fig. 1); lane 2, native K88ac; lanes 3 to 7, biotinylated K88ac at ϕ ratios of 0.5, 1, 2, 5, and 10, respectively. The major K88 subunit is indicated on the right. (B) Visualization of the biotin linked to the K88 subunits by using streptavidin-alkaline phosphatase conjugate after the electroblotting of the samples. The lanes are as described for panel A.

TABLE 2. Effect of biotinylation on K88 activity

ϕ Ratio[a]	Hemagglutination titer[b]		
	K88ab	K88ac	K88ad
0	2^{13}	2^{6}	2^{12}
1	2^{13}	2^{4}	NR[c]
2	NR	2^{4}	2^{11}
2.5	2^{13}	NR	NR
5	2^{13}	2^{4}	NR
7.5	2^{10}	NR	NR
10	2^{10}	1	NR
20	2^{10}	NR	NR

[a] At the beginning of the reaction.
[b] The hemagglutination titer was measured with guinea pig erythrocytes.
[c] NR, not realized.

the K88ac lectin, a result not improved upon when *M. auratus* erythrocytes were used. A ϕ ratio of between 2 and 5 and a 4-h reaction appeared to be the compromise necessary to obtain sufficient biotin labelling and to maintain a maximum activity for the fimbriae.

Binding of the three NbioK88 variants to pig Fbg. Four major proteins (Fig. 3, lane 4) estimated at 69, 68, 57, and 51 kDa were seen after electrophoresis of pig Fbg. By comparison with the Fbg chains from other mammalian species (17, 54, 55), the 69- and 68-kDa proteins could be considered to correspond to the Aα chains and the 57- and 51-kDa proteins to the Bβ and γ chains, respectively. NbioK88ab preferentially bound to the Aα chains (Fig. 3, lane 1). This binding, much weaker than the one observed with NbioK88ac and NbioK88ad, could be due to low-affinity receptors (46, 47). NbioK88ac bound to the Bβ and γ chains (Fig. 3, lane 2), while NbioK88ad bound only to the γ chain (Fig. 3, lane 3).

Treatment of pig Fbg with protease-free PNGase F led to a decrease in the molecular mass (about 2 kDa) of the Bβ and γ chains of pig Fbg while that of the Aα chains remained unchanged (Fig. 4A, lane 2). The shift observed for the Bβ and γ chains is similar to those reported for the Bβ and γ chains of equine (12) and bovine (13, 15) Fbg and corresponds to the removal of one N-glycan per chain. No visible modification was observed by SDS-PAGE after desialylation (Fig. 4A, lane 1), but the presence of *N*-acetylneuraminic acid (NeuAc) was attested to by the use of *Sambucus nigra* agglutinin (SNA) lectin, which only bound to Bβ and γ chains (Fig. 4B, lane 3). No visualization was obtained after desialylation or de-N-glycosylation of pig Fbg (Fig. 4B, lanes 1 and 2). Binding of NbioK88ac and NbioK88ad to pig Fbg was not modified either by desialylation or by de-N-glycosylation (Fig. 4C and D).

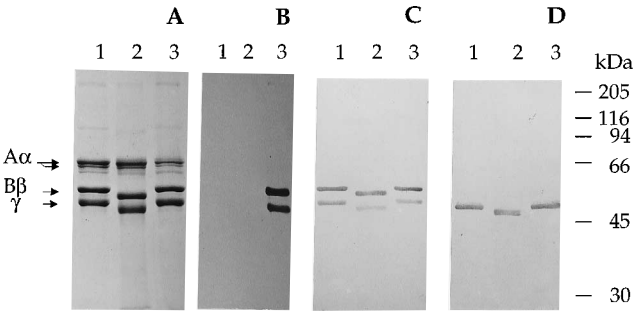

FIG. 4. Involvement of N-glycans and NeuAc in the binding of K88 to pig Fbg. The proteins were separated by SDS-PAGE on 10% gels and stained with Coomassie blue (A) or transferred to nitrocellulose (B to D) and detected with SNA (B), K88ac (C), and K88ad (D). Lane 1, desialylated Fbg; lane 2, de-N-glycosylated Fbg; lane 3, native Fbg. The positions of the molecular mass standards (as described in the legend to Fig. 3) are indicated on the right.

These results indicated that NbioK88ac and NbioK88ad do not recognize either NeuAc or N-glycans. A similar result was obtained for NbioK88ab, whose binding was not modified either by desialylation or by de-N-glycosylation. If N-glycans are not recognized by the lectins, it is probably because O-glycans also substitute the pig Fbg chains which are recognized by the K88ac and K88ad lectins. Otherwise, it must be concluded that K88 lectins display bifunctional properties, having a carbohydrate-binding site and a protein-binding site, as is found for other bifunctional lectins (4), and that the protein-binding site is at the origin of the interaction with pig Fbg. This observation prompts a reexamination of the carbohydrate content of pig Fbg.

Characterization of oligosaccharides linked to each pig Fbg chain. The N-linked oligosaccharides from pig Fbg were recently characterized, and their structure (see Fig. 6) was determined by nuclear magnetic resonance spectroscopy (14). However, no information was known about their distribution on the Bβ and γ chains that we studied here by lectin blotting (see Table 1 for the sugar specificity of the lectins).

(i) Oligosaccharides of the Aα chain. The peanut agglutinin (PNA) lectin recognized the Aα chains of pig Fbg only after desialylation (Fig. 5A, lane 1). This result suggested that the disaccharide Galβ1-3GalNAc substituted Aα chains and that it was sialylated since no binding of PNA was observed before desialylation (Fig. 5A, lane 2). The *Amaranthus caudatus* agglutinin (ACA) lectin recognized the Aα chains both prior to and after desialylation. Treatment of desialylated pig Fbg with O-glycosidase eliminated the PNA and ACA reactivities, thus

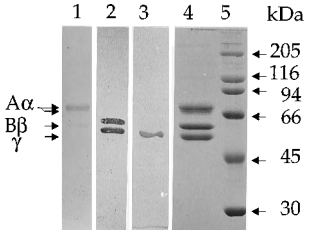

FIG. 3. Binding of biotinylated K88 lectins to the pig Fbg chains separated by SDS-PAGE. Lanes 1 to 3 represent nitrocellulose sheets after electrotransfer of pig Fbg from 10% gels. Lanes 4 and 5 were from a Coomassie blue-stained gel. Lane 1, K88ab; lane 2, K88ac; lane 3, K88ad; lane 4, pig Fbg; lane 5, molecular mass standards (myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 94 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; and carbonic anhydrase, 30 kDa).

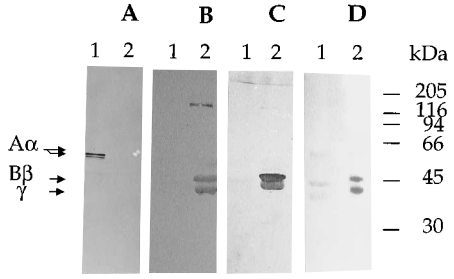

FIG. 5. Study of the pig Fbg glycans by lectin blotting. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, incubated with PNA lectin (A), RCA lectin (B), and AAA lectin (C), and visualized with the glycan detection kit (D). For panel A, lane 1 is desialylated Fbg and lane 2 is native Fbg. For panels B to D, lane 1 is de-N-glycosylated Fbg and lane 2 is native Fbg.

TABLE 3. Molar ratios of the monosaccharides from pig Fbg and the glycoproteins used as standards[a]

Glycoprotein	Monosaccharide					
	Fuc	Gal	Man	GalNAc	GlcNAc	NeuAc
Fetuin	ND[b]	3.61	3	1.06	3.67	3.46
Human serotransferrin	ND	2.08	3	ND	3.17	2.0
Pig Fbg	0.81	2.76	3	0.89	3.14	1.29
Pig Fbg (eluted fraction[c])	ND	0.94	ND	1	ND	0.79
Pig Fbg (retarded fraction[c])	0.79	2.45	3	0.54	3.19	1.18

[a] On the basis of three mannose residues for N-linked glycans and one GalNAc residue for O-linked glycans.
[b] ND, not detected.
[c] After affinity chromatography on Sepharose-concanavalin A.

confirming the presence of the structure Galβ1-3GalNAcα1-3Ser/Thr. Native Aα chains were not visualized with *Maackia amurensis* leucoagglutinin (MAL). NeuAc could be linked α2-3 to Gal without being recognized by this lectin (29). After desialylation by Newcastle disease virus neuraminidase, which specifically hydrolyzes the NeuAcα2-3 linkages, PNA did react with Aα chains. The composition of monosaccharides on Aα chains shows a Gal/GlcNAc/NeuAc molar ratio of 0.94/1/0.79 (Table 3), which is in agreement with the structure NeuAcα2-3Galβ1-3GalNAcα1-3Ser/Thr.

(ii) Oligosaccharides of the Bβ and γ chains. The *Ricinus communis* agglutinin (RCA) (Fig. 5B, lane 2) and the *Aleuria aurantia* agglutinin (AAA) (Fig. 5C, lane 2) lectins visualized Bβ and γ chains from native Fbg but not from de-N-glycosylated Fbg (Fig. 5B and C, lanes 1), showing that unsubstituted terminal nonreducing galactose and fucose occur on N-glycans of both chains. In addition, as suggested by the binding of the SNA lectin (Fig. 4B, lane 1), the N-glycans are sialylated.

In conclusion, Aα chains carry O-glycans with the structure NeuAcα2-3Galβ1-3GalNAcα1-3Ser/Thr (Fig. 6A). N-glycans were localized on both the Bβ chain and γ chain of pig Fbg and have structures corresponding to those recently characterized for glycopeptides from pig Fbg (Fig. 6B and C) (14). No conclusion as to the presence of O-glycans on the Bβ and γ chains could be reached from these results; therefore, it seemed obvious to study the monosaccharide composition of pig Fbg.

Monosaccharide composition of pig Fbg. The monosaccharide composition of pig Fbg (Table 3) was compared with the compositions of two reference glycoproteins: fetuin, containing both N- and O-glycans, and human serotransferrin, having only N-glycans. The GalNAc and Man contents characterize the O- and N-linked glycans, respectively. Pig Fbg seemed to be composed of both types of glycans. The composition indicated N-glycans of the *N*-acetyllactosamine type, which is in agreement with the determined structures (Fig. 6B and C) (14). *N*-acetylgalactosamine could have resulted from O-glycans whose presence was indicated by the lectin blotting described above. After affinity chromatography on Sepharose-concanavalin A of the reduced and alkylated pig Fbg, the Aα chains were unretarded and the Bβ and γ chains were eluted in the retarded fraction. This confirmed that both the Bβ chain and γ chain carry biantennary N-glycans of the *N*-acetyllactosamine type (39). In addition, since *N*-acetylgalactosamine was found in the retarded fraction, the presence of O-glycans on the Bβ and γ chains was further supported.

Demonstration of the presence of O-glycans on Bβ and γ chains. Sugars were visualized (with the glycan detection kit) on the three pig Fbg chains both before and after de-N-glycosylation. As expected, all the chains were visualized and the Aα chains displayed a lower sugar content (Fig. 5D, lane 2). In addition, the removal of N-glycans from the Bβ and γ chains resulted in the change in their molecular mass. The sugars detected on the Bβ and γ chains after the PNGase F action (Fig. 5D, lane 1, and Table 4) could correspond to O-glycans whose presence was confirmed by alkali-borohydride cleavage (β elimination). The reaction was done directly on the membranes, and it was verified (Ponceau red stain) that the treatment had not altered the protein pattern. After this treatment, the binding of the three NbioK88 variants to pig Fbg was eliminated (data not shown).

DISCUSSION

Fbg is composed of three different polypeptides (Aα, Bβ, and γ) and is arranged as a dimer with each half molecule containing a set of the chains. The two half molecules are linked together by three disulfide bonds. Carbohydrate moieties were found to substitute all the examined Fbg, but the

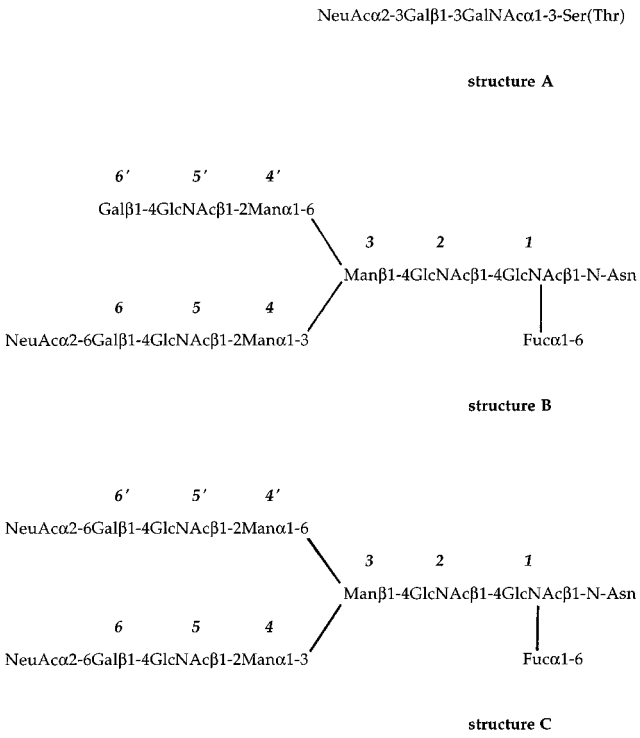

FIG. 6. The proposed structure for the O-glycans from Aα chains (structure A) and those for the N-glycans from the Bβ and γ chains of pig Fbg (structures B and C) were deduced with glycosidases and by lectin blotting and sugar composition.

TABLE 4. Structural particularities of the carbohydrate moieties of the three pig Fbg chains, deduced with glycosidases and by lectin blotting and sugar composition

Deduced structure	Method	Presence or absence on pig Fbg chain[a]:		
		Aα	Bβ	γ
N-glycans	PNGase F	−	+	+
NeuAc(α2-6)Gal	SNA lectin	− (−)	+ (−)	+ (−)
NeuAc(α2-3)Gal	MAL lectin	− (−)	− (−)	− (−)
Terminal D-Gal	RCA lectin	− (−)	+ (−)	+ (−)
Terminal L-Fuc	AAA lectin	− (−)	+ (−)	+ (−)
Gal(β1-3)GalNAc	ACA and PNA lectins	+ (+)	− (−)	− (−)
Gal(β1-3)GalNAc	O-glycosidase	+	−	−
GalNAc	Sugar composition	+	+	+
NeuAc	Neuraminidase	+	+	+

[a] The presence and absence of the structures are indicated by + and −, respectively. The results after PNGase F treatment are indicated in parentheses.

role of carbohydrate in its biological properties is unclear. Synthesis, secretion, and function of Fbg seems unaffected for polypeptides without the sugar moiety (20). Nevertheless, de-N-glycosylation of Fbg accelerated its polymerization and increased lateral aggregation of fibrin fibers (33). Like Fbg from other species, pig Fbg is composed of the three chains. Each variant of the biotinylated K88 lectin exhibited a characteristic binding to the pig Fbg chains. NbioK88ab weakly bound to the Aα chain, NbioK88ac lectin bound to the Bβ and γ chains, while NbioK88ad recognized only the γ chain. The structures of the N glycans from pig Fbg were recently established (14). The biantennary N-glycans contain a fucose linked α1-6 to *N*-acetylglucosamine and possess two sialylation patterns, with the major form (70%) having a single NeuAc residue linked α2-6 to galactose on only one antenna (Fig. 6B) and the minor form (30%) possessing two NeuAc residues linked α2-6 to both terminal galactose residues (Fig. 6C). The simultaneous reactivity with RCA and AAA lectins led us to propose that the Bβ and γ chains of pig Fbg are substituted by N-glycans having the two structures (Fig. 6B and C). Biantennary N-glycans were also found in human (52), bovine (13, 15), and equine (12) Fbg. Neuraminic acids were found to be acetylated (12) or glycolylated (13) and were also linked α2-6 to galactose, but fucose was not reported for Fbg from other species. N-glycans and NeuAc are not involved in the binding of NbioK88ac and NbioK88ad to the Bβ and γ chains. Our results provide evidence for the presence of sialylated T antigen on Aα chains (Fig. 6A), although no binding of SNA and MAL lectins to the Aα chain was observed. The Galβ(1-3)GalNAc sequence in human plasma Fbg has already been described by using the PNA lectin in a colorimetric assay (30), but it was not proved which chain was O glycosylated. The presence of O-linked glycans on the Bβ and γ chains of pig Fbg was indicated on the one hand by the fact that alkali-borohydride treatment abolished K88 binding and on the other hand by the fact that sugars were detected on the Bβ and γ chains after the quantitative enzymatic removal of N glycans. The recognition of these chains by K88ac and K88ad lectins might be due to O-glycans.

In conclusion, we demonstrated that pig Fbg could be used as a model for the study of the specificity of the three variants of the K88 lectin. It is obvious that each polypeptide chain of pig Fbg should be purified and that the O-glycans should be isolated. Fbg is structurally related to other extracellular proteins and exhibits RGD receptor sequence binding specificity for integrins (31). Therefore, Fbg and other proteins could be found associated with the extracellular matrix spontaneously or as a result of tissue damage. Whatever the mechanism of externalization, the carbohydrate moieties of these glycoproteins could then interact with bacterial lectins. The role of mucus in protecting intestine brush borders against the adhesion of pathogens like *E. coli* was emphasized (1), but when the mucus became defective, Fbg or analogous proteins could be specifically involved in bacterial infections by the K88-carrying *E. coli* strains.

Once the exact chemical structure of these glycans is established, the three biotinylated K88 lectins will be able to be used for the detection of receptors on the surfaces of cells by the methodology previously reported for the biotinylated K99 lectin (6, 40).

ACKNOWLEDGMENTS

We are indebted to Alastair Balloch for linguistic advice, to Florence Seguy for technical help, and to Henri Debray (Laboratoire de Chimie Biologique, Université des Sciences et Technologie de Lille) for helpful suggestions.

Corine L'Hôte was financed by the Institut National de la Recherche Agronomique and the Conseil Régional du Limousin.

REFERENCES

1. **Allen, A.** 1984. The structure and function of gastrointestinal mucus, p. 3–11. *In* E. C. Boedeker (ed.), Attachment of organisms to the gut mucosa, vol. 2. CRC Press, Inc., Boca Raton, Fla.
2. **Anderson, M. J., J. S. Whitehead, and Y. S. Kim.** 1980. Interaction of *Escherichia coli* K88 porcine intestinal brush border membranes. Infect. Immun. **29:**897–901.
3. **Bakker, D., P. T. J. Willemsen, L. H. Simons, F. G. van Zijdereld, and F. K. de Graaf.** 1992. Characterization of antigenic and adhesive properties of FaeG, the major subunit of K88 fimbriae. Mol. Microbiol. **6:**247–255.
4. **Barondes, S. H.** 1988. Bifunctional properties of lectins: lectins redefined. Trends Biochem. Sci. **13:**480–482.
5. **Bayer, E. A., H. Ben-Hur, and M. Wilchek.** 1986. A sensitive enzyme assay for biotin, avidin, and streptavidin. Anal. Biochem. **154:**67–70.
6. **Berger, S., Y. Karamanos, F. Schoentgen, and R. Julien.** 1994. Characterization and use of biotinylated *Escherichia coli* K99 lectin. Biochim. Biophys. Acta **1206:**197–202.
7. **Bijlsma, I. G. W., A. de Nijs, C. van der Meer, and J. F. Frik.** 1982. Different pig phenotypes affect adherence of *Escherichia coli* to jejunal brush borders by K88ab, K88ac, or K88ad antigen. Infect. Immun. **37:**891–894.
8. **Blomberg, L., H. C. Krivan, P. S. Cohen, and P. L. Conway.** 1993. Piglet ileal mucus contains protein and glycolipid (galactosylceramide) receptors specific for *Escherichia coli* K88 fimbriae. Infect. Immun. **61:**2526–2531.
9. **Bögli, C., A. Hofer, and M. Furlan.** 1988. Isolation of fibrinogen Aa-chain by affinity chromatography on concanavalin A-Sepharose. Thromb. Haemostasis **60:**308–310.
10. **Bourgerie, S., Y. Karamanos, S. Berger, and R. Julien.** 1992. Use of resorufin-labelled N-glycopeptide in a high-performance liquid chromatography assay to monitor endoglycosidase activities during cultivation of *Flavobacterium meningosepticum*. Glycoconj. J. **9:**162–167.
11. **Carlson, D. M.** 1968. Structures and immunochemical properties of oligosaccharides isolated from pig submaxillary mucins. J. Biol. Chem. **243:**616–625.
12. **Damm, J. B. L., A. A. Bergwerff, K. Hård, J. P. Kamerling, and J. F. G. Vliegenthart.** 1989. Sialic acid patterns in N-linked carbohydrate chains. Structural analysis of *N*-acetyl-/*N*-glycolyl-neuraminic-acid-containing N-linked carbohydrate chains of bovine fibrinogen. Recl. Trav. Chim. Pays-Bas Belg. **108:**351–359.
13. **Damm, J. B. L., H. Voshol, K. Hard, J. P. Kamerling, and J. F. G. Vliegenthart.** 1989. Analysis of N-acetyl-4-O-acetylneuraminic-acid-containing N-linked carbohydrate chains released by peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase F. Eur. J. Biochem. **180:**101–110.
14. **Da Silva, C. M. L., T. Toshiaki, T. McBroom, and K. G. Rice.** 1994. Tyrosine derivatization and preparative purification of the sialyl and asialyl N-linked oligosaccharides from porcine fibrinogen. Arch. Biochem. Biophys. **312:**151–157.
15. **Debeire, P., J. Montreuil, E. Moczar, H. van Halbeek, and J. F. G. Vliegenthart.** 1985. Primary structure of two major glycans of bovine fibrinogen. Eur. J. Biochem. **151:**607–611.
16. **Debray, H., and J. Montreuil.** 1989. Aleuria aurantia agglutinin. A new isolation procedure and further study of its specificity towards various glycopeptides and oligosaccharides. Carbohydr. Res. **185:**15–26.
17. **Doolittle, R. F., K. W. K. Watt, B. A. Cottrell, D. D. Strong, and M. Riley.** 1979. The amino acid sequence of the α-chain of human fibrinogen. Nature (London) **280:**464–468.

18. **Erickson, A. K., J. A. Willgohs, S. Y. McFarland, D. A. Benfield, and D. H. Francis.** 1992. Identification of two porcine brush border glycoproteins that bind the K88ac adhesin of *Escherichia coli* and correlation of these glycoproteins with the adhesive phenotype. Infect. Immun. **60:**983–988.
19. **Gibbons, R. A., G. W. Jones, and R. Sellwood.** 1975. An attempt to identify the intestinal receptor for the K88 adhesin by means of haemagglutination inhibition test using glycoproteins and fractions from sow colostrum. J. Gen. Microbiol. **86:**228–240.
20. **Gilman, P. B., P. Keane, and J. Martinez.** 1984. The role of the carbohydrate moiety in the biological properties of fibrinogen. J. Biol. Chem. **259:**3248–3253.
21. **Goldstein, I. J., and R. Poretz.** 1986. Isolation and characterization and carbohydrate binding specificity of lectins, p. 35–214. *In* I. E. Liener, N. Sharon, and I. Goldstein (ed.), The lectins, properties and physicochemical applications in biology and medicine. Academic Press, New York.
22. **Guinée, P. A. M., and W. H. Jansen.** 1979. Behavior of *Escherichia coli* K antigens K88ab, K88ac, and K88ad in immunoelectrophoresis, double diffusion, and hemagglutination. Infect. Immun. **23:**700–705.
23. **Guinée, P. A. M., W. H. Jansen, and C. M. Agterberg.** 1976. Detection of the K99 antigen by means of agglutination and immunoelectrophoresis in *Escherichia coli* isolates from calves and its correlation with enterotoxigenicity. Infect. Immun. **13:**1369–1377.
24. **Haselbeck, A., and W. Hösel.** 1990. Description and application of an immunological detection system for analysing glycoproteins on blots. Glycoconj. J. **7:**63–74.
25. **Haselbeck, A., E. Schickaneder, H. von der Eltz, and H. Wolfgang.** 1990. Structural characterization of glycoprotein carbohydrate chains by using digoxigenin-labeled lectins on blots. Anal. Biochem. **191:**25–30.
26. **Jones, G. W., and J. M. Rutter.** 1972. Role of K88 antigen in the pathogenesis of neonatal diarrhea caused by *Escherichia coli* in piglets. Infect. Immun. **6:**918–927.
27. **Jones, G. W., and J. M. Rutter.** 1974. The association of antigen with haemagglutinating activity in porcine strains of *Escherichia coli*. J. Gen. Microbiol. **84:**135–144.
28. **Kearns, M. J., and R. A. Gibbons.** 1979. The possible nature of the pig intestinal receptor for the K88 antigen of *Escherichia coli*. FEMS Microbiol. Lett. **6:**165–168.
29. **Knibbs, R. N., I. J. Goldstein, R. M. Ratcliffe, and N. Shibuya.** 1991. Characterization of the carbohydrate binding specificity of the leukoagglutinating lectin from *Maackia amurensis*. Comparison with other sialic acid-specific lectins. J. Biol. Chem. **266:**83–88.
30. **Köttgen, E., B. Hell, C. Müller, and R. Tauber.** 1988. Demonstration of glycosylation variants of human fibrinogen, using the new technique of glycoprotein lectin immunosorbent assay (GLIA). Biol. Chem. Hoppe-Seyler **369:**1157–1166.
31. **Kühn, K., and J. Eble.** 1994. The structural bases of integrin-ligand interactions. Trends Cell Biol. **4:**256–261.
32. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
33. **Langer, B. G., J. W. Weisel, P. A. Dinauer, C. Nagaswami, and W. R. Bell.** 1988. Deglycosylation of fibrinogen accelerates polymerisation and increases lateral aggregation of fibrin fibers. J. Biol. Chem. **263:**15056–15063.
34. **Laux, D. C., E. F. McSweegan, T. J. Williams, E. A. Wadolkowski, and P. S. Cohen.** 1986. Identification and characterization of mouse small intestine mucosal receptors for *Escherichia coli* K-12(K88ab). Infect. Immun. **52:**18–25.
35. **Li, W.-P., C. Zuber, and J. Roth.** 1993. Use of Phaseolus vulgaris leukoagglutinating lectin in histochemical and blotting techniques: a comparison of digoxigenin- and biotin-labelled lectins. Histochemistry **100:**347–356.
36. **Lotan, R., E. Skutelsky, D. Damon, and N. Sharon.** 1975. The purification, composition and specificity of the anti-T lectin from peanut (*Arachis hypogaea*). J. Biol. Chem. **250:**8518–8523.
37. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
38. **Metcalfe, J. W., K. A. Krogfelt, H. C. Krivan, P. S. Cohen, and D. C. Laux.** 1991. Characterization and identification of porcine small intestine mucus receptor for the K88ab fimbrial adhesin. Infect. Immun. **59:**91–96.
39. **Montreuil, J., S. Bouquelet, H. Debray, B. Fournet, G. Spik, and G. Strecker.** 1986. Glycoproteins, p. 143–228. *In* V. Chaplin and J. F. Kennedy (ed.), Carbohydrate analysis: a practical approach. IRL, Oxford.
40. **Ouadia, A., Y. Karamanos, and R. Julien.** 1992. Detection of ganglioside N-glycolyl-neuraminyl-lactosyl-ceramide by biotinylated *Escherichia coli* K99 lectin. Glycoconj. J. **9:**21–26.
41. **Parry, S. H., and P. Porter.** 1978. Immunological aspects of cell membrane adhesion demonstrated by porcine enteropathogenic *Escherichia coli*. Immunology **34:**41–49.
42. **Payne, D., M. O'Reilly, and D. Williamson.** 1993. The K88 fimbrial adhesin of enterotoxigenic *Escherichia coli* binds to β1-linked galactosyl residues in glycosphingolipids. Infect. Immun. **61:**3673–3677.
43. **Plummer, T. H., and A. L. Tarentino.** 1991. Purification of the oligosaccharide-cleaving enzyme of *Flavobacterium meningosepticum*. Glycobiology **1:**257–263.
44. **Rinderle, S. J., I. J. Goldstein, K. L. Matta, and R. M. Ratcliffe.** 1989. Isolation and characterization of amaranthin, a lectin present in the seeds of *Amaranthus caudatus*, that recognizes the T- (or cryptic T)-antigen. J. Biol. Chem. **264:**16123–16131.
45. **Seignole, D., P. Grange, Y. Duval-Iflah, and M. Mouricout.** 1994. Characterization of O-glycan moieties of the 210 and 240 kDa pig intestinal receptors for *Escherichia coli* K88ac fimbriae. Microbiology **140:**2467–2473.
46. **Sellwood, R.** 1980. The interaction of the K88 antigen with porcine intestinal epithelial cell brush borders. Biochim. Biophys. Acta **632:**326–335.
47. **Sellwood, R.** 1984. An intestinal receptor for the K88 antigen of porcine enterotoxigenic *Escherichia coli*, p. 167–175. *In* E. C. Boedeker (ed.), Attachment of organisms to the gut mucosa, vol. 2. CRC Press, Inc., Boca Raton, Fla.
48. **Sellwood, R., R. A. Gibbons, G. W. Jones, and J. M. Rutter.** 1975. Adhesion of enteropathogenic *Escherichia coli* to pig intestinal brush borders: the existence of two phenotypes. J. Med. Microbiol. **8:**405–411.
49. **Shibuya, N., I. J. Goldstein, W. F. Broeknaert, M. Nsimba-Lubaki, B. Peters, and W. J. Peumans.** 1987. Fractionation of sialylated oligosaccharides, glycopeptides, and glycoproteins on immobilized elderberry (*Sambucus nigra* L.) bark lectin. J. Biol. Chem. **263:**4576–4585.
50. **Spik, G., G. Strecker, B. Fournet, S. Bouquelet, J. Montreuil, L. Dorland, H. van Halbeek, and J. F. G. Vliegenthart.** 1982. Primary structure of the glycans from human lactotransferrin. Eur. J. Biochem. **121:**413–419.
51. **Staley, T. E., and I. B. Wilson.** 1983. Soluble pig intestinal cell membrane components with affinities for *E. coli* $K88^+$ antigen. Mol. Cell. Biochem. **52:**177–189.
52. **Townsend, R. R., E. Hilliker, Y.-T. Li, R. A. Laine, W. R. Bell, and Y. C. Lee.** 1982. Carbohydrate structure of human fibrinogen. Use of 300-MHz ^{1}H-NMR to characterize glycosidase-treated glycopeptides. J. Biol. Chem. **257:**9704–9710.
53. **Wang, W.-C., and R. D. Cummings.** 1988. The immobilized leukoagglutinin from the seed of Maackia amurensis binds with high affinity to complex-type Asn-linked oligosaccharides containing terminal sialic acid linked α-2,3 to penultimate galactose residues. J. Biol. Chem. **263:**4576–4585.
54. **Watt, K. W. K., B. A. Cottrell, D. D. Strong, and R. F. Doolittle.** 1979. Amino acid sequence studies on the α chain of human fibrinogen. Overlapping sequences providing the complete sequence. Biochemistry **18:**5410–5416.
55. **Watt, K. W. K., T. Tagaki, and R. F. Doolittle.** 1979. Amino acid sequence of the β chain of human fibrinogen. Biochemistry **18:**68–76.
56. **Willemsen, P. T. J., and F. K. de Graaf.** 1992. Age and serotype dependent binding of K88 fimbriae to porcine intestinal receptors. Microb. Pathog. **12:**367–375.