INFECTION AND IMMUNITY, Feb. 1996, p. 606–610 Vol. 64, No. 2
0019-9567/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Transferrin Associated with the Porcine Intestinal Mucosa Is a Receptor Specific for K88ab Fimbriae of *Escherichia coli*

PHILIPPE A. GRANGE AND MICHELE A. MOURICOUT*

Biotechnologie, Faculté des Sciences, Limoges cedex, France

Received 19 July 1995/Returned for modification 5 October 1995/Accepted 30 November 1995

Putative receptors of *Escherichia coli* K88 fimbriae are either tightly membrane bound or an integral part of membranes. Thus, proteins associated with piglet small intestinal mucosae were solubilized by a detergent (deoxycholate). A 74-kDa glycoprotein (GP74) purified from enterocyte and brush border membrane preparations was specifically detected in vitro by K88ab fimbriae. GP74 was recognized only in the mucosae of phenotypically adhesive animals. Metaperiodate treatment abolished the recognition, indicating that K88ab fimbriae-GP74 binding required the carbohydrate moiety. This glycoprotein belongs to the transferrin family and differed from the serum transferrin of the same adhesive-phenotype piglets. Unlike intestinal transferrin, serum transferrin was recognized independently of the adhesion phenotype. The glycan moieties of intestinal and serum transferrins differed in their molar compositions. Transferrin GP74 contained one monosialylated and monofucosylated glycan chain of the *N*-acetyllactosamine type. Intestinal holotransferrin exhibited pI values of 5.2, 5.3, 5.5, and 5.6, whereas serum holotransferrin pI values ranged between 5.4 and 6.2. Since mucosal transferrin was found intimately entrapped on membranes, we hypothesize that a K88ab fimbriae-transferrin-cell transferrin receptor complex might allow the bacteria to adhere to specific sites of the mucosa.

The adhesion of enterotoxigenic *Escherichia coli* to receptors on small intestinal surfaces has been recognized as an early step in diarrheic pathogenesis. Among *E. coli* adhesins isolated from diseased animals, K88 (F4) and K99 (F5) as well as 987P (F6) and F41 fimbriae were often found (19, 20). Young piglets are commonly infected with K88-fimbriated *E. coli* (34). Since 1975, adhesive K88 has served as a model for the study of the relationship between fimbriated *E. coli* adhesion and infection incidence. Sellwood et al. (29) investigated the adhesion of $K88^{+}$ enterotoxigenic *E. coli* to brush borders and detected the existence of adhesive and nonadhesive piglets. Five phenotypes can be distinguished with regard to the adhesion of *E. coli* strains expressing ab, ac, or ad variants of K88 fimbriae (2, 26). The adhesion character (K88R) seems to be controlled by a single Mendelian locus with dominant *S* (susceptible) and recessive *s* alleles (9, 29). Studies of the segregation of different loci indicate a linkage between *S-s* and serum transferrin (*Tf*) loci, in the order TF-K88abR-K88acR (4, 9, 12), and also those of the ceruloplasmin (15).

Terminal β-galactose is involved in the binding of K88 fimbriae (3, 7, 22, 27, 32). It appears that each K88 variant binds to distinct glycolipids and glycoproteins (8, 27). Proteins with sizes of 25, 35, 40 to 42, and 60 kDa in intestinal mucus and 16, 23, 35, and 40 to 70 kDa in brush border membranes are recognized by K88ab fimbriae (18, 22). Furthermore, enterocyte receptors are independent of age, while mucus receptors decrease with age (33).

In order to identify glycoproteins specifically recognized by $K88ab^{+}$ enterotoxigenic *E. coli*, we purified and characterized a K88ab adhesin receptor from the intestinal mucosae of piglets. We report that K88ab fimbriae interact in vitro with a 74-kDa glycoprotein which is present at the surface of adhesive brush borders and belongs to the transferrin family.

* Corresponding author. Mailing address: Biotechnologie, Faculté des Sciences, 123 rue A. Thomas, Limoges cedex, France. Phone: (33) 55 45 76 61. Fax: (33) 55 45 76 53. Electronic mail address: mouricout@unilim.fr.

MATERIALS AND METHODS

Bacteria. *E. coli* C5148 (O117:K88ab; heat-labile enterotoxin positive [LT^{+}] and heat-stable enterotoxin positive [ST^{+}]), P2200 (O149:K91,K88ac [LT^{+} and ST^{+}]), and P423.1 (O8:K88ad [LT^{+} and ST^{-}]) (28) were grown on Minca agar at 37°C for 18 h. O8 (K88ab [LT^{-} and ST^{-}]), P2200, P423.1, and K88-negative strains were labelled with sodium [$^{14}C_2$]acetate (3.4 GBq/mol). They were harvested in Hanks' buffer (0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, 1.2 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.14 M NaCl, 5.4 mM KCl, 5.5 mM glucose [pH 7.2]). The presence of fimbriae was controlled as described in reference 27. The concentration for binding was 10^9 bacterial cells per ml.

Animals. Piglets (169 animals from 13 litters) were obtained from crossbreedings of Large White and Meishan breeds (Institut National de la Recherche Agronomique, Le Magneraud, France) from the European PiGMaP program. Piglets were weaned at 21 days and deprived of food for 24 h before being slaughtered at 60 days. Intestines were removed, washed with cold saline, and then processed. Brush borders were characterized as adhesive or nonadhesive with C5148, P2200, and P423.1 strains (28). Among 129 piglets assayed, 98 displayed adhesive phenotypes A (K88ab, -ac, and -ad variants bound) and B (only K88ab and -ac). Adhesive phenotypes corresponded to high adhesion of K88ab- and K88ac-positive *E. coli* (10).

Membrane protein extraction. Scrapings of intestine mucosa were harvested in ice-cold antiprotease buffer [1 mM KH_2PO_4, 25 mM $NaHCO_3$, 120 mM NaCl, 14 mM KCl [pH 7.4]) containing 5 mM EDTA and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). In some experiments, mucosa was recovered in phosphate-buffered saline (PBS) in the presence of 100 μg of Pefabloc SC per ml, 50 μg of EDTA per ml, 5 μg of leupeptin per ml, 5 mg of pepstatin per ml, and 1 μg of aprotinin per ml (Boehringer). Scrapings were homogenized in 1 mM Tris-HCl (pH 7.5)–100 mM mannitol, and the brush borders were purified by precipitation with $MgCl_2$ and $MgSO_4$ (21). Proteins were extracted with 1% (wt/vol) sodium deoxycholate as described in reference 27 or 1% (wt/vol) sodium dodecyl sulfate (SDS) (anionic denaturing detergent) in 10 mM Tris-HCl (pH 8)–1 mM PMSF or 1% (wt/vol) Triton X-100 (nondenaturing nonionic detergent) in 50 mM borate (pH 8.6)–1 mM PMSF. Preparations were incubated for 1 h at 4°C with stirring and centrifuged at 86,500 × *g* for 1 h. Alternatively, proteins were extracted with 88% (wt/vol) phenol in 100 mM NH_4CO_3 (pH 8.5)–0.5% (wt/vol) NaCl in 1-to-20 proportions (vol/vol). After the preparations were stirred for 10 min at 65°C and centrifuged at 1,000 × *g* for 2 h, the aqueous phase was collected. Supernatants were lyophilized after dialysis.

GP74 purification. Proteins dissolved in 100 mM NH_4HCO_3 (pH 8.5) were chromatographed on a Sephacryl S-200 HR column (1 by 100 cm) at a flow rate of 11 ml/h. Proteins were desalted on a BioGel P-30 column (1.5 by 30 cm) in 25 mM NH_4HCO_3, pH 8.5 (buffer B). Anion-exchange chromatography was performed on a DEAE-cellulose (DE-32) column (3 by 10 cm) equilibrated with buffer B. After washings with buffer B (150 ml), proteins were eluted stepwise with 25 to 50 (50 ml), 50 to 200 (200 ml), and 200 to 500 mM (100 ml) NH_4HCO_3, pH 8.5.

Porcine serum transferrin purification. Serum proteins were precipitated by saturation with between 55 to 95% $NH_4(SO_4)_2$ and dialyzed. The method of Baldwin et al. (1) was modified by an additional separation on a DE-32 column

(3 by 10 cm) with a linear gradient of from 50 to 500 mM NH_4HCO_3, pH 8.5, and a flow rate of 20 ml/h. Fractions containing transferrin were made up to 500 mM NaCl. They were loaded on a metal chelate-Sepharose column (1 by 5 cm), saturated with $CuSO_4$ (1 mg/ml), and equilibrated in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.6)–1 mM PMSF–500 mM NaCl (buffer C). Proteins were eluted with a linear pH gradient generated from buffer C (pH 4.0) and 50 mM sodium formate (pH 2.8)–500 mM NaCl.

Fe^{3+} ion removal. Intestinal and serum transferrin apoforms were prepared by dialysis against 20 mM sodium citrate, pH 5.0, containing 50 μg of desferrioxamine mesylate per ml and 100 μM EDTA for 24 h at 4°C (1).

Glycoprotein oxidation. Proteins were transferred on nitrocellulose sheets and treated overnight in darkness at 37°C with 200 mM sodium acetate (pH 4.5)–10 mM sodium metaperiodate. For the controls, the samples were incubated with sodium iodate. Washings were done with 15 mM KH_2PO_4–8 mM Na_2HPO_4–137 mM NaCl–2.6 mM KCl (pH 7.4).

Analytical methods. Proteins (50 μg) treated at 100°C for 5 min in 62.5 mM Tris-HCl (pH 6.8)–0.6 mM β-mercaptoethanol–2% (wt/vol) SDS were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized with 0.1% (wt/vol) Coomassie blue (27). The protein pI was determined with a PhastGel isoelectric focusing system (Pharmacia) covering a pH range of from 3 to 9. Proteins were assayed (17) with bovine serum albumin (BSA) being used as the standard and with neutral sugars as described in reference 25. Monosaccharides released by methanolysis were converted to trimethylsilyl derivatives and quantified (percent, weight to weight) by gas-liquid chromatography (DB-1 column [0.25 mm by 25 m]) with *meso*-inositol being used as the internal standard.

N-terminal sequence and amino acid composition. After SDS–10% PAGE and exposure to 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (pH 11.0)–0.5 mM dithiothreitol–10% methanol for 5 min, the proteins (30 pmol) were transferred to polyvinylidene difluoride membranes and stained with 0.1% (wt/vol) Coomassie blue in 50% methanol. The N-terminal sequence was determined by Edman degradation with a 470A Applied Biosystems protein sequencer. Phenylthiodantoin-amino acids were identified by reverse-phase (C_{18}) high-performance liquid chromatography (HPLC).

GP74 (200 μg) was hydrolyzed with 6 N HCl–0.5% (wt/vol) phenol–0.02% β-mercaptoethanol for 24 h at 110°C under a N_2 atmosphere. Amino acids were treated with ethanol-triethylamine-phenyl isothiocyanate (7:1:1 by volume) and separated by reverse-phase HPLC with 140 mM sodium acetate (pH 6.4)–5% (vol/vol) triethylamine–6.4% (vol/vol) acetonitrile–acetonitrile-water (6:4, vol/vol). The amino acid composition was assessed in triplicate with a Waters Pico-TAG amino acid analyzer.

Binding of K88-fimbriated *E. coli* to glycoproteins. Separated proteins (20 μg) were blotted to nitrocellulose sheets (0.45-μm pore size). Membranes were pretreated with 10 mM PBS (pH 7.2)–2% (wt/vol) BSA for 2 h at 20°C and overlaid with ^{14}C-labelled positive or negative K88 *E. coli* (10^9 cells per ml; specific activity, 8 to 10.10^{-3} Bq/cell) for 2 h at 20°C in the presence of 1% (wt/vol) D-mannose. Autoradiography was performed (Hyperfilm-MP film; Amersham) after washings with PBS–0.02% (wt/vol) Tween 20.

Binding of biotinylated fimbriae to glycoproteins. Purification and biotinylation of K88 fimbriae, determination of the specificity of binding, and lectin blotting assays were carried out as described in reference 16. Biotinylated fimbriae (K88ab, 1 μg/ml; K88ac, 1.5 μg/ml; and K88ad, 2 μg/ml) in PBS (pH 7.6)–0.1% (wt/vol) Tween 20–2% (wt/vol) BSA were incubated for 2 h at 4°C with crude proteins (20 μg) or pure GP74 (from 0.7 to 5 μg).

Immunoblotting assays. After the protein transfer, the nitrocellulose was incubated in 10 mM Tris-HCl (pH 7.5)–350 mM NaCl–1% (wt/vol) BSA for 1 h. Diluted (1:750) rabbit anti-pig transferrin polyclonal antibodies (5) or (1:400) anti-human ones (Dako) in 10 mM Tris-HCl (pH 7.5)–0.16 mM NaCl–0.1% (wt/vol) SDS–1% (wt/vol) Triton X-100–0.5% (wt/vol) sodium deoxycholate (buffer D) were applied for 1 h. The sheets were washed twice with buffer D for 20 min and incubated with 1:1,000-diluted goat anti-rabbit immunoglobulin G coupled to alkaline phosphatase. After the washings, the reaction was revealed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate toluidinium substrate (Gibco-BRL) and quantified by scanning (CD 60 Desaga).

RESULTS

Intestinal receptors for K88 fimbriae. Proteins from adhesive (Fig. 1a, lanes 1 and 2) and nonadhesive (Fig. 1a, lanes 3 to 5) piglet intestines were assayed for their binding to K88ab-fimbriated *E. coli*. Two labelling patterns were obtained (Fig. 1b, lanes 1 to 5). The 74-kDa band (GP74) was systematically observed in scrapings (Fig. 1b, lanes 1 and 2) and brush borders (data not shown) extracted from adhesive intestines. GP74 receptor was not detected in nonadhesive ones (Fig. 1b, lanes 3 and 4) even at fivefold protein concentrations (Fig. 1b, lane 5). Nonfimbriated *E. coli* did not recognize any proteins (Fig. 1c, lanes 1 and 3). Sodium metaperiodate directly applied to blotted proteins abolished GP74 binding to K88ab-fimbriated *E. coli* (Fig. 1d, lane 1). Hence, the GP74 carbohydrate moiety mediated binding. To further confirm that GP74 bound to isolated K88 fimbriae, Western blot (immunoblot) analyses with biotinylated fimbriae were performed (Fig. 2). GP74 was intensively recognized by K88ab fimbriae in adhesive extracts (Fig. 2, lanes 3 and 4), while it did not appear in nonadhesive ones (lanes 1 and 2). Both K88ac and K88ad fimbriae bound to intestinal proteins from adhesive pigs but not to GP74 (Fig. 2, lanes 5 and 6). K88ac recognized a 57-kDa protein (Fig. 2, lane 5), and K88ad bound to a 46-kDa protein (lane 6).

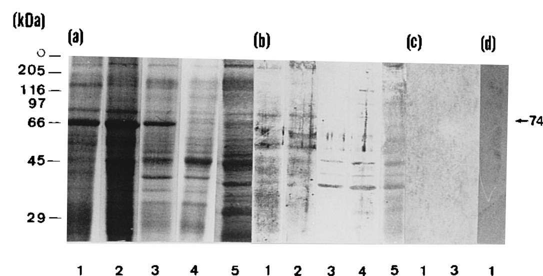

FIG. 1. Identification of K88ab$^+$ *E. coli* receptors in intestinal membrane proteins from adhesive and nonadhesive piglets (lanes 1 and 2 and 3 to 5, respectively, of panels a and b). (a) Solubilized proteins (50 μg, lanes 1 to 4, and 100 μg, lane 5) were separated by SDS–12.5% PAGE and stained with 0.1% Coomassie brilliant blue. (b) Separated proteins (20 μg, lanes 1 to 4, and 100 μg, lane 5) were transferred to nitrocellulose. K88ab$^+$ *E. coli* receptors were detected by incubation with ^{14}C-labelled (8.10^{-3} Bq per cell) K88ab-positive *E. coli* cells (10^9 cells per ml) and autoradiography. (c) In controls, ^{14}C-labelled K88ab-negative *E. coli* cells (10^9 cells per ml) did not bind to any components of adhesive (lane 1) and nonadhesive (lane 3) extracts. (d) Blotted glycoproteins extracted from adhesive intestines were treated with 10 mM sodium metaperiodate as described in Materials and Methods. The sheets were washed three times, and an overlay assay was performed. The arrow at 74 indicates the migration of the 74-kDa glycoprotein. Molecular mass standards are indicated at the left. O, origin of migration.

Since diffuse bands of 55- and 62-kDa proteins were revealed by K88ab-fimbriated *E. coli* (Fig. 1b, lanes 1 to 5) and K88ab fimbriae (Fig. 2, lanes 1 to 4) in both adhesive and nonadhesive preparations, the proteins were not responsible for the adhesive phenotype. They might be the result of postmortem proteolysis of intestinal extracts prior to the action of protease inhibitors.

Purification of GP74, a receptor for K88ab fimbriae. Intestinal crude glycoproteins (32.3 ± 0.3 mg of carbohydrate per g of protein [mean value ± standard deviation for 60-day-old piglets]) isolated from adhesive piglets were solubilized. After sodium deoxycholate anionic biliary salt treatment, 42% of the GP74 was recovered (13.6 mg). Phenol and Triton X-100

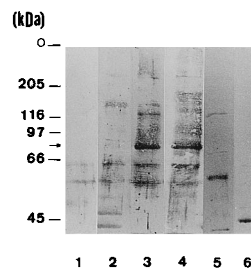

FIG. 2. Binding of K88 fimbriae (ab, ac, and ad) to membrane-bound proteins of nonadhesive (lanes 1 and 2) and adhesive (lanes 3 to 6) piglet intestine mucosae. Solubilized proteins (20 μg) were separated by SDS–7.5% PAGE and transferred to nitrocellulose. K88 fimbria-binding proteins were detected with biotinylated fimbriae as described in Materials and Methods. Lanes 1 to 4, K88ab (1 μg/ml); lane 5, K88ac (1.5 μg/ml); lane 6, K88ad (2 μg/ml). Molecular mass standards are indicated at the left. The arrow indicates the migration of 74-kDa glycoprotein.

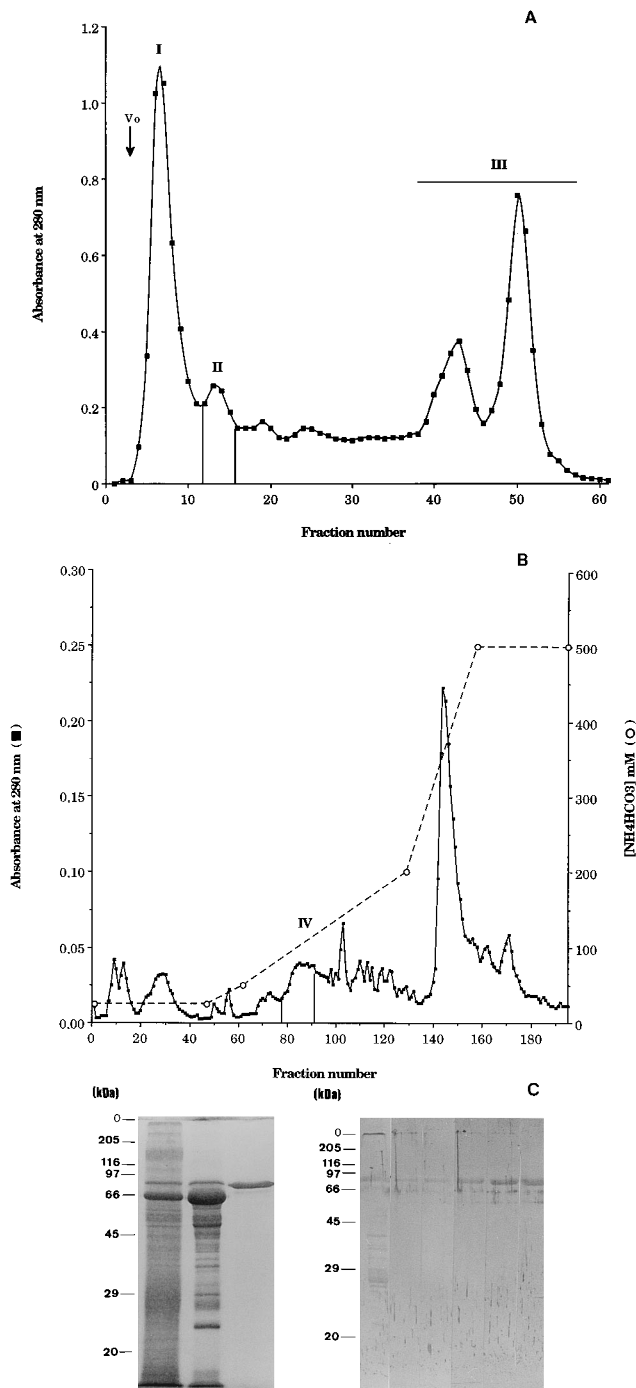

FIG. 3. Purification of GP74. (A) Membrane proteins (10 mg) extracted from the intestinal mucosae of adhesive piglets were loaded on a Sephacryl S-200 column. Elution was performed with 0.1 M NH_4HCO_3, pH 8.5, with a flow rate of 11 ml/h (fraction volume, 1.9 ml). The arrow indicates the void volume (V_0). Pooled fractions containing GP74 (peak II) were desalted and dissolved in 25 mM NH_4HCO_3, pH 8.5. (B) Proteins were separated on a DEAE-cellulose column at 20 ml/h with a linear gradient of 50 to 200 mM NH_4HCO_3, pH 8.5. Protein A_{280} was measured. (C) Proteins were separated by SDS–12.5% PAGE under reducing conditions. In the gel at the left, crude glycoproteins (50 μg) (left lane), GP74-containing fractions (35 μg) eluted in peak II (middle lane), and GP74 (3.0 μg) collected in peak IV (right lane) were stained with Coomassie brilliant blue. In the gel at the right, proteins of peak II (25 μg) (left lane) and peak IV (0.7, 1.4, 2.3, 3.0, and 5.0 μg) (remaining lanes) were blotted and probed with biotinylated K88ab fimbriae (1 μg/ml). In the controls, K88ac and K88ad biotinylated fimbriae did not recognize GP74 in adhesive and nonadhesive preparations. K88ab biotinylated fimbriae did not bind to the 74-kDa protein in nonadhesive extracts.

TABLE 1. N-terminal amino acid sequences and immunodetection of GP74[a]

Protein	N-terminal sequence[b]	Immunodetection with anti-transferrin antibodies[c]	
		Porcine	Human
GP74	V A Q K T V R F X T I X N[d,e]	++++	+
Serum transferrin	V A Q K T V R X X T I X X Q[e]	++++	++
Serum transferrin	V A Q K T V R X X T I X N Q[f]		
Gastric transferrin	V A Q K T V R W X T I X N Q[f]		
Lactoferrin	A R K R G V Q W C V I S T A[g]		

[a] Serum transferrin (3 μg) and GP74 (1 μg) from piglets were blotted and probed with transferrin-specific polyclonal antibodies. In the controls, human serum transferrin (750 ng) strongly reacted with human anti-transferrin antibodies and did not recognize porcine anti-transferrin antibodies. Serum transferrins from canine, ovine, bovine, and equine species (750 ng) did not react with porcine and human anti-transferrin antibodies.

[b] X indicates unidentified residues.

[c] ++++, high intensity (arbitrary index, of 100); ++, medium intensity (index range of 50 to 70); +, low intensity (index range of 10 to 20).

[d] The N-terminal sequence of GP74 was determined from the intestines of two phenotypically adhesive piglets as described in Materials and Methods.

[e] Intestinal and serum transferrins were purified from the same phenotypically adhesive piglets (this work).

[f] Obtained from reference 1.

[g] Obtained from reference 14.

yielded only 11.5 and 23% (3.6 and 7.1 mg), respectively. GP74 was not extracted with SDS.

Solubilized proteins were chromatographed with an S-200 HR column. GP74 (4.03 mg) was eluted in peak II (Fig. 3A and C). Mucins or mucin-type compounds (Fig. 3A, peak I) and degraded products (peak III) were removed. Fractions of peak II (61 mg of proteins) (Fig. 3A) were separated with a DE-32 column. GP74 was eluted in peak IV (Fig. 3B) at between 70 to 90 mM NH_4HCO_3 (Fig. 3C). A total of 3.4 mg of highly purified glycoprotein was obtained from 1 g of crude intestinal proteins which originally contained 31.2 mg of GP74.

GP74 and serum transferrin isolated from the same piglets presented total identity for their N-terminal sequences and were clearly distinct from porcine (Table 1) lactoferrin and the bovine one (23). Anti-pig transferrin antibodies reacted strongly with GP74, whereas anti-human ones did so only weakly (Table 1). No cross-reaction between anti-serum and serum transferrins of other species was observed.

Proteins with sizes of 55 and 62 kDa that coeluted in traces with GP74 were probably not degraded products issued from GP74, since no degradation was observed after GP74 was kept at −20°C for several months or at 18°C for several days.

Amino acid and monosaccharide compositions. The amino acid composition of GP74 differed slightly from those of gastric and serum transferrins (Table 2). GP74 contained 3% (wt/wt) sugars and was characterized by high amounts of mannose, galactose, and *N*-acetylglucosamine (Table 3). The sialic acid content was lower than that in other transferrins. The major difference between intestinal and serum transferrins was the high level of mannose (43%) and low level of fucose (2.6%) in the glycan of the serious form. Furthermore, K88ab fimbriae bound serum transferrins of adhesive and nonadhesive piglets (data not shown).

GP74 isoelectric point. GP74 was purified in its holoform and was separated into four components with pI values of 5.2, 5.3, 5.5, and 5.6. Small differences appeared with serum transferrin from piglets with the same phenotype. This serum transferrin exhibited several holoforms with pIs from 5.4 to 6.2. Human and bovine serum transferrins presented pIs in the

TABLE 2. The amino acid compositions of GP74 and gastric and serum porcine transferrins

Amino acid	Amino acid composition (residues per 100 amino acids)[a]		
	GP74	Serum transferrin[b]	Gastric transferrin[b]
Ala	10.5	9.4	8.4
Arg	5.0	4.2	3.8
AsX[c]	6.6	12.3	11.6
Cys	ND	4.8	4.7
GlX[d]	14.5	10.9	10.5
Gly	7.0	6.9	7.1
His	3.4	2.0	2.8
Ile	4.3	2.6	3.0
Leu	9.8	8.0	8.3
Lys	5.1	8.4	7.5
Met	ND	1.0	1.1
Phe	4.5	3.7	4.0
Pro	5.1	4.0	4.6
Ser	13.7	7.8	8.0
Thr	6.8	4.3	5.0
Trp	ND	ND	ND
Tyr	4.1	3.6	3.6
Val	6.5	6.1	6.3

[a] ND, not determined.
[b] Obtained from reference 1.
[c] AsX, Asn + Asp.
[d] GlX, Gln + Glu.

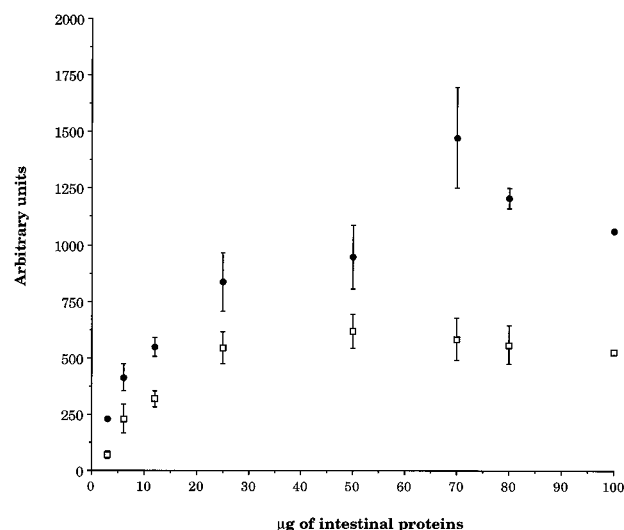

FIG. 4. Comparative levels of intestinal porcine transferrin in crude solubilized glycoproteins from intestines of adhesive (●) and nonadhesive (□) piglets. Intestinal proteins (from 3 to 100 µg) were separated by SDS–12.5% PAGE. The transferrin was revealed by immunoblotting, with anti-transferrin porcine antibodies being used, and quantified on eight phenotypically adhesive and eight nonadhesive individuals. Vertical bars represent mean values ± standard deviations.

range of 5.2 and 6.3. In the presence of iron chelator, apoforms of intestinal transferrin had pI values ranging from 5.7 to 6.1 and apoforms of serum transferrin had pI values ranging from 5.5 to 6.2. K88ab fimbriae adhesion to intestinal transferrin was not modified by the action of the chelator, indicating that adhesion did not require iron.

GP74 amounts in the intestines of two adhesion phenotype piglets. Transferrin was 3 to 5% of the total proteins in adhesive piglets (Fig. 1a, lanes 1 and 2). Conversely, in nonadhesive ones, intestinal transferrin was not detected or was detected only in traces (Fig. 1a, lanes 3 to 5). The immunodetection revealed that GP74 was 1.5 to 3.5 times more abundant in the intestinal mucosae of phenotypically adhesive piglets than in nonadhesive ones (Fig. 4).

DISCUSSION

The present work reports the first purification of a 74-kDa receptive glycoprotein (GP74) from the intestinal mucosa of a piglet and its characterization as a mucosal transferrin. GP74 was detected by K88ab fimbriae in all the 98 phenotypically adhesive piglets tested for brush border adhesiveness (10) and then for the presence of the binding proteins.

Occurrence of a transferrin intimately associated with the intestinal mucosa raises a question about its origin. GP74 could be attributed to plasma contamination. Nevertheless, the isoelectrophoretic mobilities and carbohydrate compositions of the mucosal and serum transferrins might allow us to distinguish the two forms. Confusion between GP74 and lactoferrin, which is synthesized locally and secreted by the mucosa of the gastrointestinal tract (14), can be discarded as a possibility because the respective N-terminal sequences were distinct.

Our results were obtained from experiments which involved the binding of fimbriae to GP74 in vitro. Therefore, we only hypothesize that the mucosal transferrin is a receptor for K88ab fimbriae in vivo. Intestinal transferrin has been implicated in the transcellular movement of iron from the intestinal lumen to plasma transferrin (6, 13, 24). Efforts by bacteria to obtain iron from their hosts underline the importance of iron in pathogenesis. Interactions between GP74 and *E. coli* cells are not needed to remove iron. Effectively, *E. coli* secretes low-molecular-mass, ferric ion-chelating siderophores (6). These siderophores successfully compete with transferrin for iron, contrary to the situations for *Neisseria meningitidis* and *Neisseria gonorrhoeae*, which require interactions between bacterial transferrin-binding proteins and mammalian transferrins (11). A suggested role for GP74 recognition could be that the binding of K88ab fimbriae to mucosal transferrin glycan gives access to particular sites on the epithelium surface. Interactions could lead to the formation of the bacterium-transferrin-mucosa cell receptor of transferrin complex.

Furthermore, we point out that specific glycosylation processes could exist in piglet intestines. N glycosylation of intestinal transferrin molecules differed for adhesive and nonadhesive piglets. The *N*-acetyllactosamine-type glycan of GP74 from adhesive intestines was monosialylated and monofucosylated. GP74 possessed one glycan per molecule, a characteris-

TABLE 3. Monosaccharide compositions of glycan moieties of GP74 and gastric and serum porcine transferrins[a]

Monosaccharide	Composition of glycan moiety (%, wt/wt)			
	GP74	Serum transferrin[b]	Serum transferrin[c]	Gastric transferrin
Fuc	7.1	2.6	5.0	5.3
Man	28.7	43	18.7	20.0
Gal	23.2	13	16.3	18.0
GalNAc			3.1	3.9
GlcNAc	30.4	28.4	28.7	26.5
NeuAc	10.6	12.4	28.4	26.4

[a] The glycosylation content and monosaccharide composition were assayed by gas-liquid chromatography.
[b] The carbohydrate content of GP74 was 3.0% (wt/wt), that of serum transferrin was 3.2 and 3.3% (wt/wt), and that of gastric transferrin was 4.7% (wt/wt). Transferrin was purified from the sera of phenotypically adhesive piglets.
[c] Obtained from reference 1.

tic similar to one of porcine serum transferrin, which possesses a single glycan chain linked to the C-terminal lobe (30). Even if all of the serum transferrins studied had possessed *N*-acetyllactosamine glycan types (31), we found that porcine serum transferrin could still contain a high level of mannose (43%), suggesting that a glycan of the oligomannoside type constituted at least one part of the glycoforms.

K88ac and K88ad fimbriae did not bind GP74, indicating the high specificity of glycan recognition. K88ac fimbriae recognized a 57-kDa protein in adhesive brush borders, but some variations in binding for intestines from different pigs were observed. Hence, this binding is probably not responsible for adhesive phenotypes. The 57-kDa protein might be a low-affinity receptor that may promote, in some animals, the attachment of bacteria to the intestine mucosa. Binding of K88ad fimbriae to a 46-kDa protein was detectable, but variations among piglets were also observed. However, this protein could belong to the set of proteins with sizes ranging from 45 to 70 kDa that were described in reference 33. All these differences in receptors for K88ab, K88ac, and K88ad fimbriae underlined the involvement of specific glycans in the binding of K88 family adhesins.

In this study, we distinguished at least two porcine populations on the basis of adhesion phenotype with respect to K88ab fimbriae and amounts of transferrin molecules tightly associated with the mucosa. A complete understanding of the cause of variations in transferrin levels requires further studies. Intestinal transferrin recognition by K88ab *E. coli* fimbriae was host specific and thus might be a marker for susceptibility and resistance to colibacillosis. Work to determine the structure of the glycan moieties of transferrins and the genetic basis of adhesion phenotypes is being undertaken.

ACKNOWLEDGMENTS

This work was supported by grants-in-aid (AIP 93/3/4924 and 94/4982) from INRA.

We are indebted to Daniela Cizova-Schroffelova (Immunogenetics Laboratory, Animal Breeding Institute, Hradistko, Czech Republic) for providing anti-pig transferrin antibodies and to Corine L'Hôte for giving us biotinylated K88 fimbriae. We thank Pascal Guillaume (INRA, CRJ) for the establishment of piglet phenotypes and Bernadette Coddeville (Chimie Biologique, Université Sciences et Techniques Lille) for monosaccharide analyses. We thank Yannis Karamanos for his critical reading and Alastair Balloch for linguistic advice.

REFERENCES

1. **Baldwin, G., T. Bacic, R. Chandler, B. Grego, J. Pedersen, R. Simpson, B. H. Toh, and J. Weinstock.** 1990. Isolation of transferrin from gastric mucosa: comparison with porcine serum transferrin. Comp. Biochem. Physiol. **95:**261–268.
2. **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.
3. **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.
4. **Bonneau, M., Y. Duval-Iflah, G. Guérin, L. Ollivier, C. Renard, and X. Renjifo.** 1990. Aspects génétiques et microbiologiques de la colibacillose K88 chez le porc. Ann. Rech. Vet. **43:**122–123.
5. **Cizova, D., A. Stratil, E. Müller, and S. Cepica.** 1993. A new, partially deficient transferrin variant in the pig. Anim. Genet. **24:**305–306.
6. **Crichton, R., and M. Charloteaux-Wauters.** 1987. Iron transport and storage. Eur. J. Biochem. **164:**485–506.
7. **Erickson, A. K., D. R. Baker, B. T. Bosworth, T. A. Casey, D. A. Benfield, and D. H. Francis.** 1994. Characterization of porcine intestinal receptors for the K88ac fimbrial adhesin of *Escherichia coli* as mucin-type sialoglycoproteins. Infect. Immun. **62:**5404–5410.
8. **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.
9. **Gibbons, R., R. Sellwood, M. Burrows, and P. Hunter.** 1977. Inheritance of resistance to neonatal diarrhea in the pig: examination of the genetic system. Theor. Appl. Genet. **51:**65–70.
10. **Grange, P., P. Guillaume, L. Hibrand, Y. Duval-Iflah, and M. Mouricout.** 1995. Modulation of the adhesion of *E. coli* K88ab, ac & ad in intestinal mucosa of piglets, p. 87–99. *In* M. N. Bellon-Fontaine and J. Fourniat (ed.), Adhésion des micro-organismes aux surfaces. TEC & DOC Lavoisier, Paris.
11. **Gray-Owen, S., and A. Schryvers.** 1993. The interaction of primate transferrins with receptors on bacteria pathogenic to humans. Microb. Pathog. **14:**389–398.
12. **Guérin, G., Y. Duval-Iflah, M. Bonneau, M. Bertaud, P. Guillaume, and L. Ollivier.** 1993. Evidence of linkage between K88ab, K88ac, intestinal receptors to *Escherichia coli* and transferrin loci in pigs. Anim. Genet. **24:**393–396.
13. **Huebers, H., E. Huebers, W. Rummel, and R. Crichton.** 1976. Isolation and characterization of iron-binding proteins from rat intestinal mucosa. Eur. J. Biochem. **66:**447–455.
14. **Hutchens, T., J. Magnuson, and T. Yiq.** 1989. Rapid purification of porcine colostral whey lactoferrin by affinity chromatography on single-stranded DNA-agarose. Characterisation, amino acid composition and terminal amino acid sequence. Biochim. Biophys. Acta **999:**323–329.
15. **Juneja, R. K., J. Kuryl, B. Gahne, and M. Zurkowski.** 1989. Linkage between the loci for transferrin and ceruloplasmin in pigs. Anim. Genet. **20:**307–311.
16. **L'Hote, C., S. Berger, S. Bourgerie, Y. Duval-Iflah, R. Julien, and Y. Karamanos.** 1995. Use of porcine fibrinogen as a model of glycoprotein to study the binding specificity of the three variants of K88 lectin. Infect. Immun. **63:**1927–1932.
17. **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.
18. **Metcalfe, J., K. Krogfelt, H. Krivan, P. Cohen, and D. Laux.** 1991. Characterization and identification of a porcine small intestine mucus receptor for the K88ab fimbrial adhesin. Infect. Immun. **59:**91–96.
19. **Morris, J., and W. Sojka.** 1985. *Escherichia coli* as a pathogen in animals. Spec. Publ. Soc. Gen. Microbiol. **13:**47–77.
20. **Mouricout, M.** 1991. Swine and cattle enterotoxigenic *Escherichia coli* mediated diarrhea. Development of therapies based on inhibition of bacteria-host interactions. Eur. J. Epidemiol. **7:**588–604.
21. **Muir, W. A., U. Hopfer, and M. King.** 1984. Iron transport across brush-border membranes from normal and iron-deficient mouse upper small intestine. J. Biol. Chem. **259:**4896–4903.
22. **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.
23. **Pierce, A., D. Colavizza, M. Benaissa, P. Maes, A. Tartar, J. Montreuil, and G. Spik.** 1991. Molecular cloning and sequence analysis of bovine lactoferrin. Eur. J. Biochem. **196:**177–184.
24. **Purves, L., M. Purves, N. Linton, W. Brandt, G. Johnson, and P. Jacobs.** 1988. Properties of the transferrin associated with rat intestinal mucosa. Biochim. Biophys. Acta **966:**318–327.
25. **Rao, P., and T. Pattabiraman.** 1989. Reevaluation of the phenol-sulfuric acid reaction for the estimation of hexoses and pentoses. Anal. Biochem. **181:**18–22.
26. **Rapacz, J., and H. Hasler-Rapacz.** 1986. Polymorphism and inheritance of swine small intestinal receptors mediating adhesion of three serological variants of *Escherichia coli* producing K88 pilus antigen. Anim. Genet. **17:**305–321.
27. **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.
28. **Seignole, D., M. Mouricout, Y. Duval-Iflah, B. Quintard, and R. Julien.** 1991. Adhesion of K99 fimbriated *Escherichia coli* to pig intestinal epithelium: correlation of adhesive and nonadhesive phenotypes with the sialoglycolipid content. J. Gen. Microbiol. **137:**1591–1601.
29. **Sellwood, R., G. Gibbons, W. Jones, and J. Rutter.** 1975. Adhesion of enteropathogenic *Escherichia coli* to pig intestinal brush borders: the existence of two pig phenotypes. J. Med. Microbiol. **8:**405–411.
30. **Sharma, N., R. Evans, K. Patel, B. Gorinsky, A. Mallet, and A. Aitken.** 1994. Evidence for the glycosylation of porcine serum transferrin at a single site located within the C-terminal lobe. Biochim. Biophys. Acta **1206:**286–288.
31. **Spik, G., B. Coddeville, and J. Montreuil.** 1988. Comparative study of the primary structures of sero-, lacto-, and ovotransferrin glycans from different species. Biochimie (Paris) **70:**1459–1469.
32. **Staley, T., and I. Wilson.** 1983. Soluble pig intestinal cell membrane components with affinities for *E. coli* K88^{+} antigen. Mol. Cell. Biochem. **52:**177–189.
33. **Willemsen, P., and F. K. De Graaf.** 1992. Age and serotype dependent binding of K88 fimbriae to porcine intestinal receptors. Microb. Pathog. **12:**367–375.
34. **Wilson, R. A., and D. H. Francis.** 1986. Fimbriae and enterotoxins associated with *Escherichia coli* serogroups isolated from pigs with colibacillosis. Am. J. Vet. Res. **47:**213–217.

Editor: B. I. Eisenstein