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RESEARCH ARTICLE

M.J. Caloca · S. Suárez · J. Soler

Identification and partial purification of K88ab *Escherichia coli* receptor proteins in porcine brush border membranes

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Abstract Six receptor proteins, with molecular masses ranging from 94 to 27 kDa, that bind to *Escherichia coli* K88ab fimbriae were recovered from brush border membranes and were detected after solubilization with Triton X-114. The recovery of these receptor proteins in the aqueous phase suggests their peripheral localization. The 63-, 60- and 33-kDa K88ab binding proteins were recovered using gel-filtration chromatography of the aqueous phase.

Keywords K88ab fimbriae · Receptor proteins · Brush border membranes

Introduction

K88-positive enterotoxigenic *Escherichia coli* (ETEC) strains are major causal agents of diarrhea in swine. In addition to producing toxins, these ETEC strains adhere to the wall of the small intestine by their K88 fimbriae. Binding to enterocytes facilitates colonization of the small intestine by preventing the removal of bacteria by intestinal peristalsis. The biochemical nature of pig intestinal brush border receptors for K88 fimbriae has been the subject of previous studies. Most reports have identified the receptors as glycoproteins of the intestinal mucus layer [20]. Payne et al. [17] demonstrated in vitro adherence of K88 fimbriae to glycosphingolipids, and Grange and Mouricout [12] demonstrated that transferrin associated with the porcine intestinal mucose is a K88ab receptor.

M.J. Caloca · J. Soler
Departamento de Bioquímica y Biología Molecular,
Universidad de León, 24007 León, Spain

S. Suárez (✉)
Departamento de Sanidad Animal,
Universidad de León, 24007 León, Spain
E-mail: dsamsr@unileon.es
Tel.: +34-987291295
Fax: +34-987291304

Other studies that also attempted to identify the porcine intestinal receptor for K88, both in the mucous layer and in the brush border, produced different results. Differences were probably due to the variants of K88 fimbriae tested and/or to different approaches [9, 10, 14, 15, 19,20]. We report here the identification and partial purification of the intestinal receptors, and propose their localization in the brush border membrane.

Materials and methods

Bacterial strain and purification of K88ab fimbriae

Escherichia coli K-12 W3110 harboring K88ab plasmid pRISS01 was provided by F.K. de Graaf, Department of Molecular Microbiology, Vrije Universiteit, Amsterdam, the Netherlands. Purification of K88ab fimbriae was performed essentially as described in [16]. Routine details are described in Caloca et al. [6,7].

Preparation of intestinal brush border membranes

Brush border membranes were obtained from 5-week-old pigs following the procedure by Atroshi et al. [1]. Control experiments were also carried out by using negative pig-phenotype enterocytes from stock of the Department of Animal Health (University of León, Spain). Scraped material was suspended at 50% in Hanks-dimethyl sulfoxide medium (34 ml Hanks solution, 16.7 ml lactoalbumin, 10 ml bovine fetal serum, 30 ml dimethyl sulfoxide, 20 ml glycerol). After 1 day at –20 °C, the mucosal suspension was stored at –70 °C. The study of the interaction between *E. coli* K88ab$^+$ (strain K-12 W3110) and the brush border of enterocytes by direct microscopy confirmed that the mucosal material was from pigs of the adhesive-susceptible phenotype [3]. As a control for the non-adhesive phenotype, frozen samples of pig erythrocytes provided by Gajecki et al. [11] were used. To prepare brush border membranes, the suspension was thawed at room temperature, washed twice with 2 mM Hepes buffer (pH 7.1) containing 50 mM mannitol, and suspended in the same buffer solution (13 g mucosa per 200 ml). Phenylmethylsulfonyl fluoride was added (25 μg per 100 g of mucosa), and the suspension was homogenized in a Sorvall Omnimixer for 1.5 min at the maximum speed and then cooled in an ice-bath. Powdered $MgCl_2$ was mixed with the homogenate to achieve a 10 mM final concentration. The homogenate was allowed to stand on ice for 20 min and centrifuged at 2,000 *g* for 10 min. The supernatant was centrifuged at 45,000 *g* for 30 min and the resulting pellet collected and resuspended in 50 mM Hepes buffer (pH 7.6).

Membrane material was briefly sonicated before being used in the adhesion assays. An analysis of bacterial adhesion onto isolated membranes was carried out as described [1]. Clumping of *E. coli* K88ab$^+$ at the brush border membranes was monitored by direct microscopy. The protein concentration of the membrane suspension was determined according to standard procedure.

Biotinylation of K88ab fimbriae

Fimbriae were biotinylated by reacting purified K88ab fimbriae with D-biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (Boehringer Mannheim, Mannheim, Germany) as described by Harlow and Lane [13].

Solid-phase binding and binding inhibition assay of isolated K88ab fimbriae

Binding of K88ab fimbriae was determined by the biotinylated adhesin assay essentially as described [9]. Specificity of binding was confirmed by binding inhibition assays with unlabeled K88ab fimbriae or anti-K88 antibodies. Details of the experimental procedures are given in [8].

Solubilization and phase separation of brush border membranes

Brush border membranes were suspended at a concentration of 1 mg ml^{-1} in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 1% Triton X-114 (Boehringer Mannheim, Mannheim, Germany). The mixture was incubated for 1 h on ice and centrifuged for 30 min at 10,000 *g* at 4 °C. Supernatant was layered over a cushion of 6% (w/v) sucrose, 10 mM Tris- HCl (pH 7.4), 150 mM NaCI, 0.06% Triton X-114 and was subjected to temperature-induced phase separation as described by Bordier [4]. Aqueous and detergent phases were collected for analysis. Triton X-114 was purified before use according to the method of Bordier[4]. Aqueous-phase proteins from Triton X-114 phase separation were fractionated by gel filtration on Sephacryl S-300. Fractions were collected and diluted to a protein concentration of 10 μg ml^{-1} before being immobilized to measure its binding activity using a solid-phase binding assay.

Gel filtration

The aqueous phase obtained from phase separation with Triton X-114 of solubilized brush border membranes was concentrated and then fractionated on a Sephacryl S-300 column (2.5×36.5 cm) equilibrated with phosphate buffered saline (PBS) solution (pH 7.4). A sample containing 3 mg of protein was applied to the column and eluted with PBS, and the protein concentration of each fraction was described according to the method of Bradford [5]. Fractions were diluted to a protein concentration of 10 μg ml^{-1}, immobilized on microtiter trays and assayed for biotinylated K88ab adhesion as described above.

SDS-PAGE

SDS-PAGE was carried out in Tris/glycine/SDS buffer (pH 8.3) according to the method of Laemmli. Aliquots of the solubilized membranes and of the aqueous and detergent phases were separated by SDS-PAGE.

Western blotting assays

Samples were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes (0.45-μm pore size) with a trans-blot semi-dry transfer cell (Bio-Rad, Richmond, California). Additional protein binding sites on the membranes were saturated by soaking in PBS solution containing 3% bovine serum albumin (BSA) for 1 h at room temperature. Receptor activity was detected by incubating the membranes with biotinylated K88ab fimbriae (0.6 μg ml in PBS-Tween containing 2% BSA). After 1 h at room temperature, membranes were washed with PBS-Tween (three washes of 5 min each) and then once with PBS. Bound biotinylated K88ab fimbriae were detected by incubating the filter with horseradish peroxidase-streptavidin (1:2,000 dilution in PBS-Tween, containing 2% BSA) for 1 h at room temperature. Membranes were washed three times with PBS-Tween (three washes of 5 min each) at room temperature and bound peroxidase was detected by using 4-chloro-1-naphtol as described by Harlow and Lane [13].

Results and discussion

Identification of K88ab binding proteins in brush border membranes

Brush border membrane proteins were solubilized to carry out a partial purification of K88ab receptors. Non-ionic detergents with polyoxyethylene head groups have been widely used to solubilize membrane proteins. During this process, detergents displace and replace many of the lipid molecules around hydrophobic domains of integral membrane proteins. As a result, the formation of soluble mixed micelles of detergent and integral proteins occurs, whereas hydrophilic proteins bind very small amounts of detergent.

Among the members of the Triton-X series of detergents, Triton X-114 has the advantage, compared with Triton X-100, that its micelles aggregate when the temperature exceeds 20 °C (the cloud point) (for review, see [17]). Aggregated micelles are then easily recollected by centrifugation, and this property allows integral and peripheral membrane proteins to be distinguished [4]. Under the conditions of our study, about 85% of the membrane proteins were solubilized, and, following a temperature-induced phase separation, 70% of the solubilized proteins in the aqueous phase were obtained. To detect the phase in which the K88 receptor was recovered, a solid-phase binding assay with biotinylated K88ab adhesin was carried out. Because the presence of detergents in the protein solution lowers the binding capacity of polystyrene plates [13], only the brush border membrane suspension and the aqueous phase were assayed. Most of the receptor activity was recovered in the aqueous phase. When the assay was carried out with the membrane suspension 0.83 ng of bound fimbriae were obtained, while with the aqueous phase 0.63 ng of bound fimbriae were recovered. In both cases, the amount of biotinylated fimbriae used in the assay was 11.25 ng.

By Western blot analysis, six proteins of 94, 70, 63, 60 33 and 27,5 kDa with affinity for biotinylated K88ab adhesin were detected (Fig. 1). These proteins were detected in all aliquots but in the detergent phase. Based on Coomassie brilliant blue R-250 stain sensitivity [8], the 94- and 33-kDa receptor proteins must we detected represent about 5 ng of the 15 mg of solubilized brush border membrane proteins. Each of the other three must

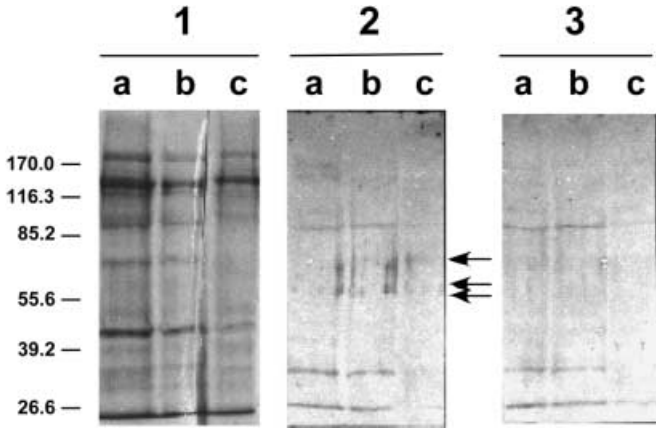

Fig. 1. Identification of K88ab fimbriae-binding proteins on Triton X-114 solubilized brush border membranes. Total solubilized brush border membranes (15 μg) (*a*), aqueous phase (10 μg) (*b*), and detergent phase (10 μg) (*c*). *Lanes 1a–c* SDS-PAGE with Coomassie-blue staining. *Lanes 2a–c* Detection of K88ab binding proteins with biotinylated K88ab fimbriae. *Lanes 3a–c* Detection of K88ab binding proteins with biotinylated K88ab fimbriae and a 50-fold excess of unlabeled K88ab fimbriae

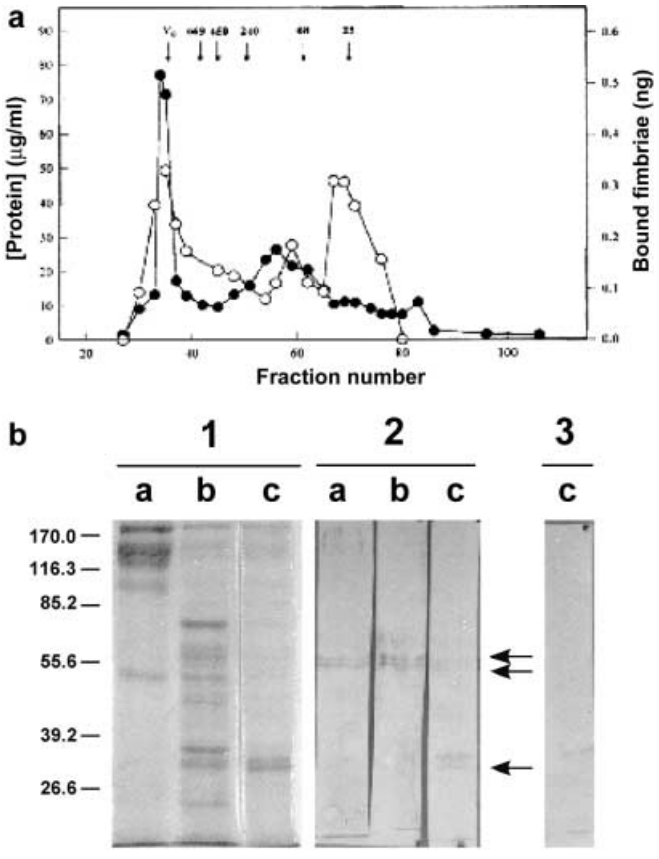

Fig. 2a, b. Identification of K88ab binding proteins on fractionated aqueous-phase proteins. **a** Aqueous-phase proteins (3 mg) were fractionated on a Sephacryl S-300 column (2.5×36.5 cm) equilibrated with PBS pH 8.4. Fractions of 2 ml were collected. ● Protein concentration (μg/ml), O Adhesion of biotinylated K88ab to immobilized fractions. V_0 Void volume. *Numbers and arrows at the top* Location of molecular mass standards (kDa). **b** Identification of K88ab binding proteins on fractionated aqueous-phase proteins. *1* SDS-PAGE with Coomassie-blue staining. *2* Detection of K88ab binding proteins with biotinylated K88ab adhesin. *3* Detection of K88ab binding proteins with biotinylated K88ab fimbriae and a 50-fold excess of unlabeled K88ab fimbriae. *a* V_0 fraction (9 μg), *b* fraction 59 (11 μg), *c* fractions 67–68 (8 μg)

represent less than this amount since they could not be visualized with this stain. When the experiments were carried out in the absence of biotinylated K88ab fimbriae [9], these proteins were not detected.

Binding of biotinylated K88ab adhesin to the 70-, 63- and 60-kDa proteins was blocked by a 50-fold excess of unlabeled K88ab adhesin, whereas with commercial K88 antiserum diluted 1:10 binding to the six proteins was blocked (data not shown). From these results we conclude that the six proteins are specific receptors for the K88ab adhesin, but only three were blocked by excess K88 fimbriae. Labeling of the 94- and 33-kDa proteins is not specific.

Based on the data obtained from both the solid-phase binding assay and the Western blot analysis, we concluded that these binding proteins from the brush border membranes were of the peripheral type. This is supported by the fact that the proteins, which were detected only in the aqueous phase, were obtained after phase-induced separation of solubilized Triton X-114 extracts. It cannot be ruled out, however, that several if not all of these binding proteins are integral, since anomalous behavior on induced-phase separation has been reported for glycoproteins [18]. Willemsen and de Graaf [20] described K88ab binding to a set of proteins with molecular masses ranging from 40 to 70 kDa, and to another 16-kDa protein. Biley et al. [2] showed that K88ab and K88ac adhesins bound to 210- and 240-kDa sialoglycoproteins. These findings agree with our results, except that we detected binding to a 94-kDa protein not described by these authors. Moreover binding to proteins smaller than the 27.5 kDa protein was not detected. Our results agree also with the previous reporting of K88ab binding proteins of 57, 64 and 91 kDa in mouse intestinal brush border preparations [14]. In addition, K88ab binding proteins ranging from 40 to 42 kDa [15] and of 26, 36 and 60 kDa [20] have been reported in porcine small intestinal mucus. Two K88 binding proteins from porcine intestinal brush border membranes of 23 and 32–35 kDa were also reported by Staley and Wilson [19]. There are no data about the possible relevance of certain K88 receptors. Receptors from porcine small intestine brush border are present at in pigs of all ages, whereas receptors on the mucus layer are age-dependent [20]. The importance of the specific receptors will need to be assigned using binding studies with isolated receptors.

Fractionation of aqueous-phase preparation by gel filtration

Aqueous-phase proteins eluted mainly in the void volume fractions and in fractions representing components in the range of 240–40 kDa. Binding of biotinylated

K88ab occurred over a broad range of fractions, with the greatest activity in the void volume fractions, in fractions representing components in the range of 100–75 kDa, and in fractions corresponding to a molecular mass ranging from 35 to 25 kDa. Fractions 35 (void volume), 59, and 67–68 were resuspended in distilled water and proteins were separated by SDS-PAGE. Binding of biotinylated K88ab adhesin to individual proteins was then analyzed by Western blot assay (Fig. 2a, b).

Four proteins, with molecular masses of 63, 60, 33 and 31 kDa, were bound by biotinylated K88ab fimbriae. The 63- and 60-kDa proteins were detected mostly in the fraction representing components of 89 kDa (fraction 59), although both proteins were also clearly detected in the void volume fraction. The 33- and 31-kDa proteins were detected in fractions representing components of 31–34 kDa (fractions 67–68). By comparing these results with those obtained before fractionation, we concluded that 63-, 60- and 33-kDa proteins are not subunits of the same protein, since they were obtained in different fractions. The 31-kDa protein, which was not detected before fractionation, may be a proteolytic product of any subunit of higher molecular mass.

In addition, the 33-kDa binding protein must be a unique subunit protein since the molecular mass determined by chromatography coincides with that calculated by Western blot assay. From our results it is not clear whether the 60- and 63-kDa binding proteins are two different subunits of the same protein or two different proteins. In any case, as these proteins were also detected in the void volume fraction, they may aggregate. This behavior has also been reported for K88 receptors on solubilized porcine brush border membranes [19] and on mouse intestinal mucus [14]. Our results agree with a previous report on the adherence of *E. coli* K-12 $K88^+$ to several fractions collected after gel filtration of pig ileal crude mucus, including the void volume ($Mr > 250{,}000$) [3]. Binding of biotinylated K88ab fimbriae to the 63-, 60- and 31-kDa proteins was completely blocked by a 50-fold excess of unlabeled K88ab fimbriae, whereas binding to the 33-kDa protein was only partially blocked. Diluted 1:10 commercial anti-K88 antiserum completely blocked binding to the four proteins (data not shown). From these results it was concluded that the 63-, 60-, 31- and 33-kDa proteins specifically bind purified K88ab adhesin.

In summary, six binding proteins with molecular masses ranging from 94 to 27.5 kDa were recovered. They belong to the brush border membranes and were detected after solubilization with Triton-X114. The recovery of these proteins in the aqueous phase suggests a peripheral localization.

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