Inhibition of Adhesive Activity of K88 Fibrillae by Peptides Derived from the K88 Adhesin

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A cyanogen bromide fragment derived from the K88ab adhesin inhibited the hemagglutinating activity of K88 fibrillae. Smaller fragments which inhibited the adherence of K88 fibrillae to erythrocytes or to intestinal epithelial cells were obtained by digestion of K88ab fibrillae with α-chymotrypsin. Active peptides were isolated from the digestion mixture and identified as Ser-Leu-Phe and Ala-Ile-Phe. Both tripeptides correspond to the peptide stretches Ser-148–Leu–Phe-150 and Ala-156–Ile–Phe-158, respectively, which are part of conserved regions in the primary structure of the K88 variants ab, ac, and ad. The isolated tripeptides inhibited the hemagglutinating activity of purified K88 fibrillae in the 1 to 5 μM range, while adherence of the fibrillae to intestinal epithelial cell brush borders was inhibited in the 10 to 50 μM range. Furthermore, the tripeptides were capable of eluting attached bacteria from agglutinated erythrocytes. The inhibitory activity of the isolated peptides was confirmed by testing various synthetic peptides for their ability to inhibit the interaction of the different K88 variants with various species of erythrocytes. The significance of these findings for the localization of the receptor-binding domain is discussed.

K88 fibrillae are nonflagellar, filamentous adhesins found on many enterotoxigenic *Escherichia coli* strains that cause neonatal diarrhea in pigs (4). They enable the bacteria to colonize the small intestinal epithelium, which is considered to be a prerequisite for the establishment of diarrheal disease. The K88 fibrillae consist of multimers of the K88 adhesin subunit with a molecular weight of 27,500 (4). Three serological variants of K88 fibrillae have been described and designated K88ab, K88ac, and K88ad (7). The primary structure of all three proteins has been determined, showing both conserved and variable regions (5, 6, 12). The conserved regions are supposed to be involved in common features such as subunit-subunit interaction and receptor binding, while the variable regions might have evolved to evade the immune response of the host (16).

The receptor-binding domain of the K88 fibrillae and the corresponding ligand(s) on susceptible erythrocytes and pig epithelial cell brush borders are not known. Recent biochemical studies indicated a possible role for arginine residues in the receptor-binding domain of the K88 adhesin, but the positions of the modified residues in the primary structure are not identified (10).

To localize the receptor-binding site(s) on the fibrillar adhesin, we isolated cyanogen bromide and α-chymotryptic fragments which were able to inhibit the interaction between native K88 fibrillae and their receptors on erythrocytes and epithelial cell brush borders.

MATERIALS AND METHODS

Materials. Acetonitrile and HCl (supra pur) were purchased from E. Merck AG (Darmstadt, Federal Republic of Germany); CNBr and trifluoroacetic acid were from Janssen Chimica; carboxypeptidase Y and aminopeptidase M were from Boehringer GmbH (Mannheim, Federal Republic of Germany); and α-chymotrypsin and *ortho*-phenylenediamine were obtained from Sigma Chemical Co. (St. Louis, Mo.). All other reagents were of analytical grade.

Purification of fibrillae. K88ab, K88ac, K88ad, F41, and K99 fibrillae were isolated and purified as described previously (10).

Isolation of brush borders. Brush borders were prepared from the pig intestine as described by Middeldorp and Witholt (15) and stored in 50% glycerol at −20°C.

Hemagglutination inhibition test. Suspensions of washed erythrocytes (2%) in PBSM (50 mM sodium phosphate [pH 7.3] containing 0.9% NaCl and 0.5% mannose) were mixed 1:1 with various amounts of peptide fractions in the same buffer. Subsequently, 50-μl portions of these suspensions were added to serial twofold dilutions (50 μl) of purified fibrillae in PBSM, using polystyrene microtiter trays with V-shaped cups. After 2 h of incubation at 4°C, the trays were examined. The initial concentrations of K88ab, -ac, and -ad fibrillae used were 50, 500, and 25 μg/ml, respectively. These suspensions have a hemagglutination titer of 2^{10} in the control experiments.

Brush border-binding inhibition test. An enzyme-linked immunosorbent assay was used to determine the inhibitory activity of peptides on the interaction between fibrillae and their receptors on epithelial cell brush borders. Polystyrene microtiter wells (Cooke) were coated with 100 μl of a brush border suspension (diluted in phosphate-buffered saline) that gave a final absorption of 1.5 at 492 nm in the control experiments. After overnight incubation at 37°C, the wells were washed in running tap water and filled with serial twofold dilutions (50 μl) of the peptide fractions in BST (50 mM sodium phosphate [pH 7.3] containing 0.2% bovine serum albumin and 0.01% Tween 80). Subsequently, 50 μl of a solution of fibrillae in BST (0.25 μg/ml) was added to each well. After 2 h of incubation at 37°C and subsequent washing, the bound fibrillae were quantitated colorimetrically with anti-K88 immunoglobulin G conjugated to horseradish

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TABLE 1. Amino acid composition of the inhibitory cyanogen bromide fragment isolated from K88ab fibrillae[a]

Amino acid	Calculated from amino acid analysis	Theoretical value for AA-114 to AA-217[b]	Theoretical value for AA-3 to AA-113
Asx	10.5	10	16
Thr	7.7	8	13
Ser	10.1	11	4
Glx	6.4	6	6
Pro	0.3	0	4
Gly	15.3	15	18
Ala	14.2	14	6
Val	9.1	9	8
Met	0.1	1	1
Ile	4.3	4	6
Leu	8.8	9	9
Tyr	2.9	3	3
Phe	5.0	5	5
Lys	4.1	4	7
Arg	5.2	5	3
Trp	ND[c]	0	2

[a] Data were normalized assuming 104 nmol of residues for the active cyanogen bromide fragment. The K88ab adhesin contains no cysteine residues, and tryptophan was not determined. The amino acid compositions of two different theoretical cyanogen bromide fragments as deduced from DNA sequence of the K88ab adhesin gene are shown for comparison.

[b] AA, Amino acid.

[c] ND, Not determined.

peroxidase and ortho-phenylenediamine as the substrate. The A_{492} was determined with a Titertek Multiscan micro-ELISA reader (Organon Teknika).

Cyanogen bromide cleavage. Chemical cleavage of the K88 protein at methionine residues was performed in 70% trifluoroacetic acid with a molar excess of 250:1 cyanogen bromide over methionine. After 18 h of incubation at room temperature in the dark, the mixture was diluted 10-fold and lyophilized.

Purification of cyanogen bromide fragments. The cyanogen bromide-treated lyophilized protein was dissolved in 10% acetic acid containing 6 M guanidine hydrochloride and consecutively dialyzed against 10% acetic acid containing 5, 4, and 3 M guanidine hydrochloride. Subsequently, the mixture was centrifuged to remove any precipitated material, and the soluble fraction was fractionated by high-pressure liquid chromatography (HPLC)-gel filtration with a TSK G-2000SW column. The material was eluted in 10% acetic acid containing 3 M guanidine hydrochloride at a flow rate of 0.6 ml/min. The eluate was monitored at 280 nm. Peak fractions were collected manually, dialyzed against PBSM, and stored at -18°C until further use.

Enzymatic digestion. Heat-denatured K88 fibrillae in 100 mM sodium phosphate (pH 8.0) were digested with α-chymotrypsin (1% by weight per addition). Proteolysis was carried out for 16 h with a second addition of enzyme 3 h after the initial addition.

Purification of α-chymotryptic peptides. The soluble α-chymotryptic peptides were separated by reverse-phase HPLC on a Bio-Rad Hi-Pore RP-318 column equipped with an RP-300 guard column, using a microprocessor-controlled Gilson model 303 HPLC system. An increasing gradient of acetonitrile in 0.075% trifluoroacetic acid (pH 2.0) or an increasing gradient of acetonitrile in 5 mM sodium phosphate (pH 6.8) was used to elute the peptides. The A_{220} was monitored to detect the peptides. Fractions of 1 ml or peak fractions were collected manually and lyophilized.

Peptide synthesis. Peptides were synthesized by the solid-

phase method (1). The composition of each peptide was confirmed by amino acid analysis.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10 to 18% linear gradient gels containing 7 M urea (with an acrylamide-bisacrylamide ratio of 20:1) and sample preparations were performed as described by Hashimoto et al. (9).

Amino acid analysis. Samples were hydrolyzed in 6 M HCl (supra pur) at 105°C for 24 h under nitrogen. Analyses were performed on a Beckman 119 CL automatic amino acid analyzer.

N- and C-terminal amino acid sequence determination. About 5 nmol of purified peptide in 20 mM sodium phosphate (pH 6.8) was incubated with aminopeptidase M (2%, wt/wt) or with carboxypeptidase Y (5%, wt/wt) at room temperature for various incubation times. The reaction was stopped by freezing the solution in liquid nitrogen. After lyophilization the amino acids were determined by HPLC as described by Ebberink et al. (2).

RESULTS

Cyanogen bromide fragments of K88ab fibrillae. To determine the location of the receptor-binding domain on the K88 fibrillar adhesin, protein fragments resulting from the cyanogen bromide cleavage of K88ab fibrillae were fractionated by HPLC-gel filtration and tested for their ability to inhibit the interaction between native K88ab fibrillae and cavia erythrocytes. For this purpose, peak fractions were dialyzed into 50 mM sodium phosphate (pH 7.3) containing 0.9% NaCl and 0.5% D-mannose. After dialysis the fractions were boiled to resuspend precipitated material. None of the fractions exhibited hemagglutinating activity, but one fraction was able to inhibit the interaction between intact K88ab fibrillae and cavia erythrocytes. Upon sodium dodecyl sulfate-gel electrophoresis this fraction resolved as one major protein band with an apparent molecular weight of 10,000 (data not shown). Amino acid analysis showed an amino acid composition corresponding to the peptide stretch Lys-114-Met-217 in the primary structure of the K88ab adhesin (Table 1). The lowest concentration of this fragment which still inhibited the hemagglutinating activity of native K88ab fibrillae by more than 95% was 0.4 mM. The hemagglutination of cavia or horse erythrocytes by F41 fibrillae and of horse erythrocytes by K99 fibrillae was not inhibited by this fragment.

Purification and sequencing of α-chymotryptic peptides derived from K88ab fibrillae. To investigate whether smaller fragments could also inhibit the hemagglutinating activity of the K88ab fibrillae, the large cyanogen bromide fragment was digested with α-chymotrypsin. This digestion resulted in a 500-fold increase in hemagglutination-inhibiting activity. To isolate and purify the active peptides, heat-denatured K88ab fibrillae were digested with α-chymotrypsin, and the resulting peptides were separated by reverse-phase HPLC with an increasing gradient of acetonitrile in 0.075% trifluoroacetic acid (pH 2.0) (Fig. 1). Fractions (1 ml) were collected, lyophilized, and tested for hemagglutination-inhibiting activity. Only fractions 23 and 24 inhibited the interaction between native K88ab fibrillae and cavia erythrocytes. Both fractions were pooled and subjected to a second chromatography on the same column, using an increasing gradient of acetonitrile in 5 mM sodium phosphate (pH 6.8) to elute the peptides. At neutral pH the combined fractions 23 and 24 eluted as seven major peaks (Fig. 2). The peak fractions were collected, lyophilized, and tested for

hemagglutination-inhibiting activity. The material in peaks 5 and 6 appeared to inhibit the hemagglutinating activity of K88ab fibrillae with cavia erythrocytes. Amino acid analysis of the peptide fractions 5 and 6 revealed equimolar concentrations of Ala, Ile, Phe and Ser, Leu, Phe, respectively. The amino acid composition of peptide fraction 6 corresponds to the peptide stretch Ser-148–Leu–Phe-150 in the primary structure of the K88ab adhesin, while peptide fraction 5 could correspond to either the sequence Ile-83–Ala–Phe-85 or the sequence Ala-156–Ile–Phe-158 (Fig. 3). After 1 h of incubation with carboxypeptidase Y, the inhibitory activity of both peptide fractions disappeared, and Phe was the only amino acid released. After 5 min of incubation of peptide fractions 5 and 6 with aminopeptidase M, the only amino acids detectable were Ala and Ser, respectively. Thereafter, equimolar concentrations of Ile and Phe or of Leu and Phe were found in addition to Ala and Ser. Thus, the sequence of the peptides in fraction 5 and 6 could be established as Ala-156–Ile–Phe-158 and Ser-148–Leu–Phe-150, respectively. Both peptides are contained in the sequence of the active cyanogen bromide fragment from Lys-114 to Met-217 (Fig. 3). Furthermore, both peptides are conserved in all three K88 variants. The lowest concentrations of Ser-Leu-Phe and Ala-Ile-Phe which still inhibited the interaction between native K88ab and cavia erythrocytes by more than 95% were 1.5 and 0.5 μM, respectively. Based on the concentration of adhesin molecules used in the hemagglutination inhibition test, it can be calculated that 0.5 μM Ala-Ile-Phe or 1.5 μM Ser-Leu-Phe was required to block the hemagglutinating activity of 0.5 μM adhesin, respectively. Apparently, both tripeptides possess a high affinity for the K88ab receptors on cavia erythrocytes. A twofold-higher concentration of the respective peptides abolished the hemagglutinating activity of 10 μM adhesin, indicating that hemagglutination inhibition is a saturable process.

To test for the binding of the peptides to erythrocytes, an equal volume of a solution of 8 μM Ser-Leu-Phe or 2 μM Ale-Ile-Phe was mixed with a 50% suspension of cavia or horse erythrocytes and incubated for 2 h at 4°C. Subsequently, the erythrocytes were spun down, and the hemagglutination-inhibiting activity in the supernatant was determined. Incubation with cavia erythrocytes reduced the hemagglutination-inhibiting activity of the peptide solutions
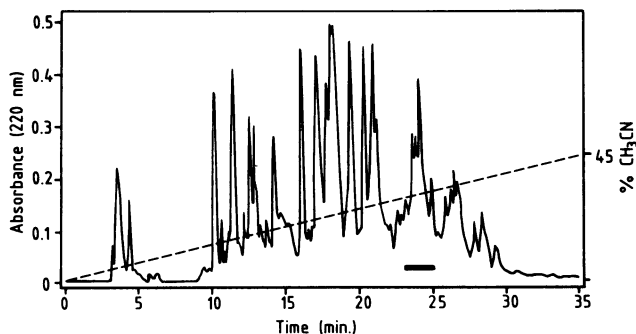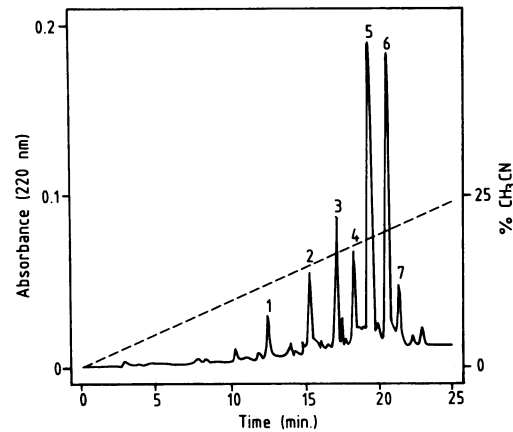

FIG. 2. Elution profile of the pooled inhibitory fractions, as indicated in Fig. 1, by reverse-phase HPLC. The peptides were separated on an RP-318 column by using an increasing gradient of acetonitrile in 5 mM sodium phosphate (pH 6.8) and a flow rate of 1 ml/min. The absorbance was monitored at 220 nm.

by more than 95%, while incubation with horse erythrocytes had no effect.

Peptides derived from K88ac and K88ad fibrillae. The digestion of both K88ac and K88ad fibrillae with α-chymotrypsin yielded one chymotryptic peptide that possessed hemagglutination-inhibiting activity. Amino acid analysis of the K88ac-derived peptide revealed the presence of Ala, Ile, and Phe in equimolar concentrations. This peptide isolated from K88ac was about 10 times less inhibitory than the peptide Ala-Ile-Phe isolated from K88ab. Theoretically, this peptide could correspond to the peptide stretch Ile-85–Ala–Phe-87 or to Ala-156–Ile–Phe-158 in the primary structure of the K88ac adhesin. Amino acid analysis of the K88ad-derived peptide showed equimolar concentrations of Ser, Leu, and Phe, corresponding to the peptide stretch Ser-148–Leu–Phe-150 in the primary structure of the K88ad adhesin. Apparently, digestion of the various types of K88 fibrillae with α-chymotrypsin resulted in the generation of a number of different tripeptides that possessed a very efficient hemagglutination-inhibiting activity. The amino acid composition of all isolated tripeptides corresponded to conserved peptide stretches in the primary structure of the various K88 adhesins.

Synthetic peptides. To confirm the activity of the isolated peptides, the tripeptides Ile-Ala-Phe, Ser-Leu-Phe, and Ala-Ile-Phe were chemically synthesized and tested for their ability to inhibit the interaction between the three K88 variants and their receptors on cavia, chicken, and pig erythrocytes (Table 2). K88ab agglutinated both cavia and chicken erythrocytes (50 μg/ml gave a titer of 2^{10} with both types of erythrocytes). K88ac only agglutinated cavia erythrocytes (a concentration of 500 μg/ml gave a titer of 2^{10}), and K88ad agglutinated both cavia and pig erythrocytes (25 μg/ml gave a titer of 2^{10} with both typs of erythrocytes). The synthetic peptides Ser-Leu-Phe and Ala-Ile-Phe inhibited hemagglutinating activity at concentrations similar to those of the corresponding peptides isolated from the fibrillae. With respect to hemagglutination inhibition of K88ab and K88ac, Ala-Ile-Phe was three to five times as active as Ser-Leu-Phe and 10 to 30 times more active than Ile-Ala-Phe. K88ad was poorly inhibited when compared with K88ab and K88ac.

All active tripeptides possessed a phenylalanine residue at

FIG. 1. Elution profile of an α-chymotryptic digest of K88ab fibrillae on reverse-phase HPLC. The peptide mixture was separated on an RP-318 column by using an increasing gradient of acetonitrile in 0.075% trifluoroacetic acid (pH 2.0) and a flow rate of 1 ml/min. The absorbance of the effluent was monitored at 220 nm. Fractions (1 ml) were collected manually, lyophilized, and tested for hemagglutination-inhibiting activity. The inhibitory fractions are indicated by a bar.

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           1                   5                  10                  15                  20                  25
K88ab Trp Met Thr Gly Asp Phe Asn Gly Ser Val Asp Ile Gly Gly Ser Ile Thr Ala Asp Asp Tyr Arg Gln Lys Trp
K88ac
K88ad

                              30                  35                  40                  45                  50
K88ab Glu Trp Lys Val Gly Thr Gly Leu Asn Gly Phe Gly Asn Val Leu Asn Asp Leu Thr Asn Gly Gly Thr Lys Leu
K88ac
K88ad                                           Ser                                             Glu

                              55                  60                  65                  70                  75
K88ab Thr Ile Thr Val Thr Gly Asn Lys Pro Ile Leu Leu Gly Arg Thr Lys Glu Ala Phe Ala Thr Pro Val Ser Gly
K88ac                                                                                         Thr
K88ad                                       Ser                 Arg                           Val

                              80                  85                  90                  95                 100
K88ab Gly Val Asp Gly Ile Pro Gln Ile Ala Phe Thr Asp Tyr Glu Gly Ala Ser Val Lys Leu Arg Asn Thr Asp Gly
K88ac                             His                                     Val             Pro
K88ad                             His                                     Glu             Pro

                             105                 110                 115                 120                 125
K88ab Glu Thr Asn Lys --- Gly Leu Ala Tyr Phe Val Leu Pro Met Lys Asn Ala Glu Gly Thr Lys Val Gly Ser Val Lys
K88ac                  Lys
K88ad         Ile Glu

                             130                 135                 140                 145                 150
K88ab Val Asn Ala Ser Tyr Ala Gly Val Phe Gly Lys Gly Gly Val Thr Ser Ala Asp Gly Glu Leu Phe Ser Leu Phe
K88ac                             Leu     Arg                                     Leu
K88ad                         Ala Leu     Arg                                     Met

                             155                 160                 165                 170                 175
K88ab Ala Asp Gly Leu Arg Ala Ile Phe Tyr Gly Gly Leu Thr Thr Thr Val Ser Gly Ala Ala Leu Thr Ser Gly Ser
K88ac               Ser                          Pro Arg --- --- ---     Ser Glu Ser     Ser Ala
K88ad         Glu       Ser His                  Pro     Asn     Gln Asn Ser         Pro Gly

                             180                 185                 190                 195                 200
K88ab Ala Ala Ala Ala Arg Thr Glu Leu Phe Gly Ser Leu Ser Arg Asn Asp Ile Leu Gly Gln Ile Gln Arg Val Asn
K88ac                         Lys
K88ad                                               Lys

                             205                 210                 215                 220                 225
K88ab Ala Asn Ile Thr Ser Leu Val Asp Val Ala Gly Ser Tyr Arg Glu Asp Met Glu Tyr Thr Asp Gly Thr Val Val
K88ac                                                       Asn
K88ad                         Asn         Pro             Phe Asn     Asn     Ala             Ser

                             230                 235                 240                 245                 250
K88ab Ser Ala Ala Tyr Ala Leu Gly Ile Ala Asn Gly Gln Thr Ile Glu Ala Thr Phe Asn Gln Ala Val Thr Thr Ser
K88ac
K88ad         Val                         Arg

                             255                 260
K88ab Thr Gln Trp Ser Ala Pro Leu Asn Val Ala Ile Thr Tyr Tyr
K88ac
K88ad
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FIG. 3. Primary structures of the K88ab, K88ac, and K88ad subunits (5, 6, 12). The K88ac and K88ad sequences are the same as shown for K88ab except where indicated. Numbering refers to the K88ab sequence. Amino acid sequences corresponding to the amino acid composition of isolated active peptides are underlined.

their carboxy-terminal end. The relevance of phenylalanine at this particular position with respect to the hemagglutination-inhibiting activity was tested with two other synthetic peptides: Ser-Leu-Phe-Ala and Leu-Phe-Ala (Ala-152 is conserved in all three variants). The tetrapeptide was about 40 times less inhibitory than Ser-Leu-Phe. The tripeptide Leu-Phe-Ala had no activity at all except for K88ac for which it was as active as the tetrapeptide.

Hemagglutination inhibition experiments with whole cells of K88-positive E. coli gave results comparable to those found for purified fibrillae. The hemagglutination of cavia and horse erythrocytes by F41 and of horse erythrocytes by K99 was not inhibited by the synthetic peptides.

Reversibility of the hemagglutination reaction. The addition of small amounts of peptides to a suspension of agglutinated erythrocytes resulted in an elution of the fibrillae from the erythrocytes. The clumps dispersed, and the erythrocytes formed a pit at the bottom of the microtiter tray within 5 to 10 min. Peptide concentrations were comparable to those used in the hemagglutination inhibition test.

Inhibition of binding to epithelial cell brush borders. The nature of the K88ab, -ac, and -ad receptor(s) on erythrocytes and epithelial cells is unknown. Theoretically, small structural differences might exist between the receptors on both types of eucaryotic cells. Therefore, we tested the ability of the peptides to inhibit the interaction between K88ab fibrillae and their receptors on the epithelial cell brush borders (Fig. 4). All three peptides tested showed a saturable inhibition. Again Ala-Ile-Phe was the most potent inhibitor; 50% inhibition occurred at a concentration of 15 µM. Ser-

Leu-Phe and Ile-Ala-Phe gave 50% inhibition at concentrations of 40 and 260 µM, respectively.

DISCUSSION

Attachment of pathogenic *E. coli* cells to epithelial cells of the intestinal or the urinary tract is mediated by specific adhesins present on the bacterial cell surface. In general, different types of bacterial fimbriae have been identified as the carriers of this adhesive capacity. Recent genetic evidence obtained with Pap, type 1, and X fimbriae suggests that although these fimbriae function as carriers of adhesiveness, the adhesive capacity is not represented by the major fimbrial subunit but by specific adhesin molecules which constitute a minor component of the respective fimbriae (8, 13, 14, 18). The major fimbrial subunits and the adhesin molecules are encoded by different cistrons in the respective operons. The genetic organization of the K88 determinant is, in principle, comparable to the operons encoding Pap, type 1, or X fimbriae, but in this case, the gene encoding the fimbrial subunits is expressed at a very low level, while the gene encoding the adhesive protein is expressed at a high level, and purified K88 fibrillae consist of adhesin molecules as major component (17). Therefore, K88 fibrillae are very suitable structures with which to study structure-function relationships in vitro.

In searching for the location of the receptor-binding domain on the K88 adhesin, we investigated the inhibitory activity of adhesin fragments on hemagglutination and binding of K88 fibrillae to intestinal epithelial cells. Three tripeptide fragments derived from conserved sequences in the primary structure of the different K88 variants were found to possess adhesion-inhibiting activity. A larger cyanogen bromide fragment containing the active tripeptide fragments Ser-Leu-Phe and Ala-Ile-Phe showed much less inhibitory activity, probably owing to its poor solubility or to a less favorable conformation of the receptor-binding site, or both.

It should be mentioned that any possibility that the isolated tripeptides are derived from the fimbrial subunit instead of the K88 adhesin could be excluded because (i) the yield of tripeptide isolated from K88 fibrillae is far too high to be derived from fimbrial subunits which represent a minor component of K88 fibrillae, and (ii) no such tripeptide sequences occur in the primary structure of the fimbrial subunits (unpublished data).

The possibility of isolating the various active tripeptides by digestion of the different K88 variants with α-chymo-
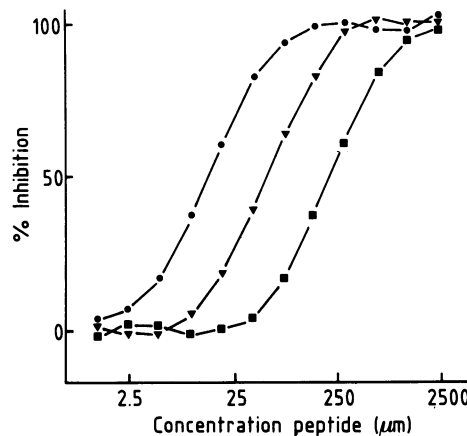

FIG. 4. Inhibitory activity of various peptides on the interaction between native K88ab fibrillae and their receptors on epithelial cell brush borders. The test was performed as described in Materials and Methods. The inhibitory activities of Ile-Ala-Phe (■), Ser-Leu-Phe (▼), and Ala-Ile-Phe (●) are presented graphically as percent inhibition versus peptide concentration.

trypsin depends on the specificity of the chymotrypsin-induced hydrolysis. Digestion of K88ad fibrillae, for instance, resulted in the isolation of only one active tripeptide composed of equimolar concentrations of Ser, Leu, and Phe and corresponding to the sequence Ser-148–Leu–Phe-150 in the primary structure of the K88ad adhesin. Digestion of K88ac fibrillae resulted in the isolation of an active tripeptide composed of equimolar amounts of Ala, Ile, and Phe. The inhibitory activity of this tripeptide was about 10 times less than observed with the tripeptide Ala-Ile-Phe but was comparable to the activity of the synthetic tripeptide Ile-Ala-Phe. Therefore, we presume that the K88ac-derived tripeptide corresponds to the sequence Ile-83–Ala–Phe-85 in the primary structure of the K88ac adhesin. Apparently, larger chymotrypic fragments containing the isolated tripeptides possess no significant inhibitory activity. Comparable results were obtained by trypsinolytic digestion of the various K88 fibrillae. These digestion mixtures possessed a detectable but rather weak inhibitory activity on the hemagglutination of cavia erythrocytes (data not shown). In addition, it should be noted that α-chymotrypsin might hydrolyze the various fibrillar adhesins within the sequence of other possible inhibitory peptide fragments. All isolated active peptides, however, possess a carboxyl-terminal phenylalanine residue possibly indicating an essential common feature of inhibiting peptide fragments.

Inhibition of adherence appeared to be a saturable process as observed in the hemagglutination and epithelial cell brush border binding test. The specificity of this process was shown by the observation that incubation with cavia erythrocytes significantly reduced the amount of peptides in solution, whereas incubation with horse erythrocytes did not. Furthermore, the hemagglutination reaction was shown to be reversible since addition of small amounts of the tripeptides eluted the fibrillae from the erythrocytes.

Although the K88 fibrillar adhesins were shown to contain small conserved amino acid sequences which appeared to be recognized by the K88 receptors present on eucaryotic cells, the question remains whether one or more of these sequences possess the same activity when contained within the intact fibrillar adhesin, i.e., constitute an essential part of the receptor-binding domain. In all three K88 variants the se-

TABLE 2. Hemagglutination inhibition by synthetic peptides[a]

K88 variant	Erythrocytes	Synthetic peptide (µM)				
		SLF	AIF	IAF	SLFA	LFA
ab	Cavia	2	0.4	15	80	>400
ab	Chicken	2	1.0	15	150	>400
ac	Cavia	2	0.8	8	80	80
ad	Cavia	100	100	200	400	>400
ad	Pig	>400	>400	>400	>400	>400

[a] The ability of various synthetic peptides to inhibit the interaction between K88ab, -ac, and -ad fibrillae and their receptors on cavia, chicken, and pig erythrocytes was tested as described in Materials and Methods. The lowest concentrations of the peptides that reduced the hemagglutination titer of native fibrillae from 2^{10} to 2^5 (more than 95% inhibition) are shown in the table. No significant residual hemagglutination was detectable with twofold-higher concentrations of the peptides. K88ab did not agglutinate pig erythrocytes, K88ac did not agglutinate chicken and pig erythrocytes, and K88ad did not agglutinate chicken erythrocytes. The peptides are given in the single-letter code.
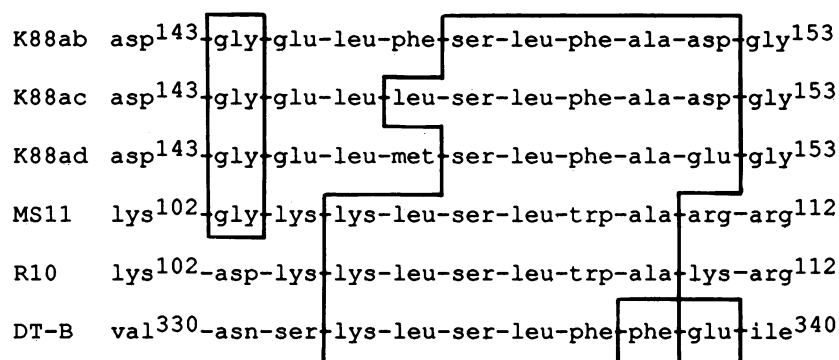
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K88ab   asp143 gly glu-leu-phe ser-leu-phe-ala-asp gly153

K88ac   asp143 gly glu-leu leu-ser-leu-phe-ala-asp gly153

K88ad   asp143 gly glu-leu-met ser-leu-phe-ala-glu gly153

MS11    lys102 gly lys lys-leu-ser-leu-trp-ala arg-arg112

R10     lys102-asp-lys lys-leu-ser-leu-trp-ala lys-arg112

DT-B    val330-asn-ser lys-leu-ser-leu-phe phe glu ile340
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FIG. 5. Comparison of a part of the primary structure of six lectins: K88ab, K88ac, and K88ad adhesin (5, 6, 12), gonococcal MS11 and R10 fimbrial subunits (20), and fragment B of diphtheria toxin (3). Identical or functionally identical residues are boxed. The amino acids Trp, Phe and Asp, Glu were assumed to be functionally identical. Homologous residues which occur only in the K88 or gonococcal variants are not considered.

quence Ser-Leu-Phe is followed by Ala-151. The tetrapeptide Ser-Leu-Phe-Ala was about 40 times less inhibitory than the tripeptide. This observation might be explained by a decreased flexibility of the aromatic phenylalanine residue in the tetrapeptide. The tripeptide Leu-Phe-Ala had no inhibitory effect on the hemagglutinating activity of the K88ab and -ad fibrillae but inhibited the activity of K88ac at the same concentrations as Ser-Leu-Phe-Ala. Thus, the serine residue seems to be critical for K88ab and -ad, but not for K88ac. These results might be explained by small differences in the receptor molecules or by differences in the three-dimensional folding of the receptor-binding domain. In general, the results suggest that the receptor binding event, at least in part, involves a hydrophobic interaction between particular sites on the K88 adhesin and the receptors on epithelial cell brush borders or erythrocytes. The receptor-binding domain of the K88 variants could be a hydrophobic cleft in the molecule which encompasses the conserved amino acid sequences Ser-148–Leu–Phe-150 or Ala-156–Ile–Phe-158 or both and which is complementary to the ligand molecules. The surrounding variable amino acid sequences in the three K88 variants might be responsible for the observed differences in hemagglutination spectrum and activity, implicating that the receptor-binding domain of the K88 adhesin encompasses (in part) the variable sequences of the respective adhesin. In addition, the relatively high concentrations of tripeptide required to inhibit the hemagglutinating activity of K88ad fibrillae suggests that differences in receptor molecules exist in particular between the receptors for K88ad and for K88ab or -ac.

Recent biochemical studies showed that modification of two arginine residues on an average of one adhesin molecule results in the loss of hemagglutinating activity of the K88ab fibrillae (11). This indicates that besides hydrophobic amino acid side chains charged residues also contribute to receptor binding. However, in the present study we cannot attribute a function of arginine residues in the receptor binding event. It remains to be investigated whether arginine residues are directly involved in the subunit-receptor interaction or are essential for the proper conformation of the receptor-binding domain.

Comparison of the primary structure of the K88ab, K88ac, and K88ad fibrillar adhesins with the primary structure of the fimbrial subunits of several other fimbriae or bacterial toxins that bind to eucaryotic cells (Fig. 5) revealed a segment of about 10 amino acid residues, encompassing the sequence Ser-Leu-Phe, which is fairly homologous with segments of

the gonococcal MS11 and R10 fimbrial subunits (20) and fragment B of diphtheria toxin (3). This homology suggests that this particular segment has a common function in these proteins, irrespective of the differences in binding specificity. Segment Lys-102–Arg-111 of the MS11 gonococcal fimbrial subunit is part of a larger fragment, Ala-31–Arg-111, derived from this subunit which was shown to bind to human endocervical epithelial cells (20). Furthermore, the segment Leu-106–Ser–Leu–Trp–Ala-110 is conserved among all gonococcal fimbrial subunit variants that have been analyzed (21). Segment Val-330–Ile-340 of the B fragment of diphtheria toxin is part of the C-terminal cyanogen bromide fragment Arg-267–Ser-342 derived from this protein, which was implicated in cell surface receptor binding (19). A common function for the homologous sequences was supported by preliminary experiments which have shown that diphtheria toxin is able to inhibit the agglutination of cavia erythrocytes by K88 fibrillae. Agglutination of human erythrocytes by Neisseria gonorrhoea, however, was not inhibited by the tripeptide Ser-Leu-Phe. This might be due to either the substitution of the phenylalanine residue for a tryptophan residue or the absence of additional sequences involved in the binding of gonococci to human erythrocytes.

Future experiments with other synthetic peptides, an analysis of the role of the amino- or carboxyl-terminal charges of the peptides, and the use of site-specific in vitro mutagenesis of various phenylalanine and arginine residues will be necessary to confirm the role of particular amino acid residues in receptor-binding activity.

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