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Binding sites in fibronectin for an enterotoxigenic strain of *E. coli* B342289c

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The binding of fibronectin and fibronectin fragments to the enterotoxigenic strain *E. coli* B34289c was studied. *E. coli* cells bound to two distinct sites of fibronectin, one being the N-terminal domain, which also contains the binding sites for staphylococci and streptococci, and the other located within the central heparin binding region. In addition, the N-terminal and the heparin binding domain mediated the attachment of bacteria in a solid phase binding assay. *E. coli* cells expressed two classes of receptors, the first, a 17 kDa protein, recognized by the N-terminal fragment and the second, having a mol. mass of 55 kDa, which interacts with the internal heparin binding domain. Bacterial receptors, which bind the N-terminal end of fibronectin, may be structurally related.

Fibronectin; Fibronectin fragment; Bacterium; Receptor

1. INTRODUCTION

Fibronectin is a disulfide linked dimeric glycoprotein (M_r 550 000) which is present in soluble form in body fluids and in a fibrillar form in the extracellular matrix. Fibronectin has a wide range of functional activities. Its major function is probably related to the ability to mediate adhesion of eucariotic cells to the extracellular matrix [1]. Fibronectin can also bind specifically to a number of important macromolecules such as collagen, fibrin and glycosaminoglycans [2]. Moreover, fibronectin interacts with some pathogenic bacteria and it is believed that this binding may represent a mechanism of tissue adherence [3]. The main binding site for staphylococci and streptococci and some Gram-negative microorganisms has been located to the N-terminal domain of fibronectin, but, there are indications for a second binding site in the C-terminal region of the molecule [4]. However, this binding site has not yet been located to a specific domain in the fibronectin molecule.

Enterotoxigenic *E. coli* are the major causative enteropathogen in young piglets, lambs and calves and may induce severe diarrhea in humans. The pathogenesis of infection may involve the ability of bacteria to adhere to the adhesive proteins of subephitelial lamina propria and the production of non-cytotoxic enterotoxins.

In the present communication, we report on the binding of fibronectin and its preoteolytic fragments to the enterotoxigenic *E. coli* B34289c and show that there are two separate binding sites in fibronectin, which recognize two distinct structures on the bacterial cell surface. Furthermore, the relationship between fibronectin binding components from *E. coli* and those from staphylococci and streptococci is analyzed.

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2. MATERIALS AND METHODS

Fibronectin was purified from human plasma [5].

Thermolysin-generated fibronectin fragments (i.e. the N-terminal domain, the collagen binding fragment and the fragment containing eucaryotic cell binding region) were isolated as reported by Borsi et al. [6]. The internal heparin binding domain from bovine plasma fibronectin was purified following the protocol described by Skorstengaard et al. [7]. The COOH-terminal fragment containing a fibrin-binding site was isolated following the procedure by Garcia-Pardo et al. [8].

Carrier free ^{125}I (specific activity, 15 mCi/μg) was purchased from Amersham, UK. Proteins were labeled with ^{125}I by the Chloramine T method [9]. The specific activity of the radioactively labeled ligands was estimated to be 4.2×10^6 cpm/μg for fibronectin, 3×10^6 cpm/μg for the N-terminal fragment of fibronectin and 2.6×10^6 cpm/μg for the internal heparin binding site.

The strain used for this study, *E. coli* B34289c, belongs to serotype O6:K2:M and was previously shown to bind fibronectin [10] and collagen [11].

Bacteria were grown in CFA agar medium [12] at 30°C for 24 h. Cells were scraped from the agar gel surface, suspended in phosphate-buffered saline (PBS) (0.13 M sodium chloride, 0.02% sodium azide, 10 mM phosphate, pH 7.4), washed and used for binding experiments.

The assay of fibronectin binding to bacteria was carried out as reported previously [13].

Radiolabeling of bacteria was performed by the iodogen method following the procedure recommended by the manufacturer (Pierce). The specific activity of the bacterial preparation was about 5×10^4 cpm/6×10^6 cells.

Coating of microtiter plates (Immulon 2; Dynatech) with fibronectin and fibronectin fragments and the attachment assays of bacteria were carried out as previously reported [10].

The staphylococcal fusion protein, called ZZ-FR, containing two IgG-binding domains of protein A and a segment of fibronectin receptor containing three fibronectin binding repeats [5] was a gift from G. Raucci (Menarini Ricerche Sud, Italy). The method used for purifying fibronectin receptor from *S. dysgalactiae* S2 will be described in detail elsewhere.

Electrophoresis in 10% or 5–15% polyacrylamide gradient gels was performed according to Blobel and Dobberstein [16]. Blotting was performed essentially using the procedure of Towbin et al. [17]. Radioactive components were visualized by autoradiography of dried gels or nitrocellulose sheets using X-Omat AR film (Eastman Kodak).

3. RESULTS AND DISCUSSION

To identify the fibronectin sites involved in binding to *E. coli* fibronectin fragments obtained after trypsin digestion were labeled by ^{125}I-iodination, incubated with bacteria and cell bound peptides were then analyzed by gel electrophoresis, followed by autoradiography (Fig. 1).

Bacteria bound to two distinct sites of the molecule, one being an M_r = 28 kDa fragment and the other a large fragment with a molecular mass of 180–200 kDa. For comparison, fragments binding to *S. aureus* strain Newman or *Streptococcus dysgalactiae* S2 were analyzed and both these strains bound only a M_r = 28 000 fragment, presumably the amino-terminal domain [18]. To localize bacterial binding domains more precisely different fibronectin fragments were isolated (Fig. 2) and tested for their ability to inhibit [^{125}I]fibronectin binding to bacteria. The results of this experiment (Fig. 3) showed that the N-terminal region (28 kDa) and a fragment corresponding to the internal heparin binding domain (30 kDa) of fibronectin reduced the binding of

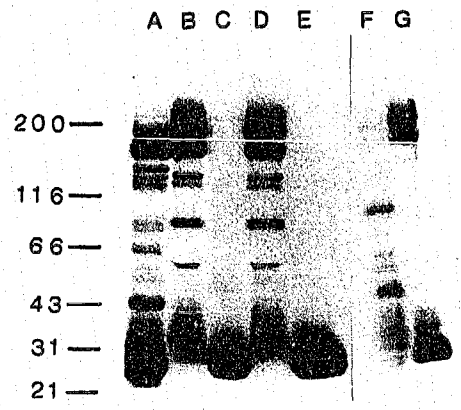

Fig. 1. Electrophoretic analysis of ^{125}I-labeled fibronectin fragments binding to bacteria. The ^{125}I-labeled fibronectin fragments were incubated with cells of *Staphylococcus aureus* Newman, *Streptococcus dysgalactiae* S2 and *Escherichia coli* B34289c and the cell-bound material was analyzed by electrophoresis under reducing conditions with a 5–15% gradient gel followed by autoradiography. Lanes C, E and G, peptides adsorbed on the surface of *S. aureus* Newman, *S. dysgalactiae* S2 and *E. coli* B34289c, respectively; lanes B, D and F, peptides remaining in the supernatant of the incubation mixtures; lane A, unfractionated fibronectin fragments. The arrows and numbers indicate the migration distances of standard proteins and their molecular weights.

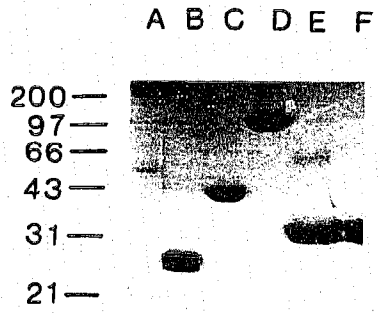

Fig. 2. Electrophoretic analysis of fibronectin fragments. Fibronectin fragments obtained as reported in section 2 were analyzed on SDS-polyacrylamide gel (10%) under unreduced conditions. Lane A, intact fibronectin; lane B, N-terminal 28 kDa fragment; lane C, the gelatin-binding domain; lane D, the cell binding fragment; lane E, internal heparin binding peptide; lane F, COOH-terminal binding fibrin domain. Positions of molecular-mass markers are indicated on the left.

[^{125}I]fibronectin to *E. coli* cells by 40% and 70%, respectively. Co-incubation of bacteria with equimolar concentrations of both peptides resulted in more than 90% inhibition of fibronectin binding to bacteria. In contrast, the collagen binding domain present in the 40 kDa fragment of fibronectin did not interfere with the binding. Furthermore, the fragment encompassing the RGD containing cell binding site (110 kDa) and C- terminal

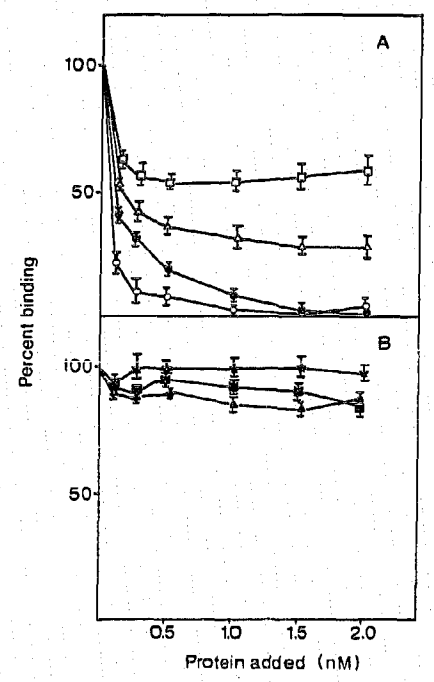

Fig. 3. Effect of fibronectin fragments on the binding of ^{125}I-labeled fibronectin to *E. coli* cells. Bacteria (5×10^8 cells) were incubated for 1 h with 12 ng (5×10^4 cpm) of ^{125}I-labeled fibronectin in the presence of increasing concentrations of unlabeled fibronectin (○), N-terminal fragment (□), internal heparin binding domain (△) or equimolar amounts of both peptides (●) (panel A). In panel B the effect of gelatin binding domain (★), cell binding fragment (▲), and C-terminal fibrin binding domain of fibronectin (■) is reported. Binding is expressed as percentage of control, i.e. incubations in the absence of unlabeled proteins.

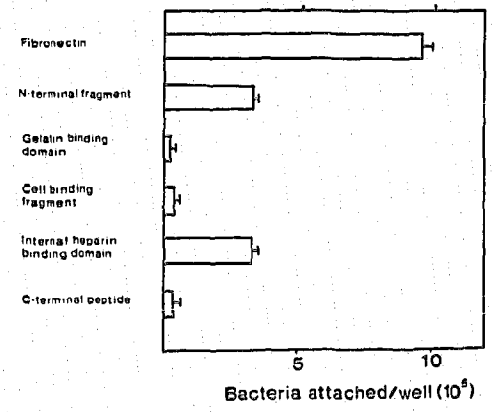

Fig. 4. Attachment of ^{125}I-labeled cells of *E. coli* B 34289c to fibronectin and fibronectin fragments. Radiolabeled bacteria (6×10^6) were incubated at 37°C in microtiter wells precoated with 5 μg of fibronectin or fibronectin fragments. Following 3 h incubation at 37°C, the wells were washed and bacterial adhesion was quantitated as described in section 2. Bars represent standard deviations from the means of three determinations.

fibrin binding domain (31 kDa) lacked inhibitory activity. Consistent with the presence of two binding sites, the *E. coli* cells bound both the ^{125}I-labeled N-terminal fragment or ^{125}I-labeled heparin binding domain of fibronectin in a time-dependent manner (unpublished results).

The role of the fibronectin fragments in substrate adherence of *E. coli* cells was determined by developing a solid-phase binding assay. Microtiter wells coated with intact fibronectin and with its different domains were used as substrate for the adherence of surface-labeled bacteria. The results (Fig. 4) indicate that bacteria attached to wells coated with fibronectin, with the N-terminal fragment and the C-terminal heparin binding peptide.

On the contrary, *E. coli* cells did not attach to the other fibronectin fragments. Taken together, these data indicate that two distinct domains of fibronectin can

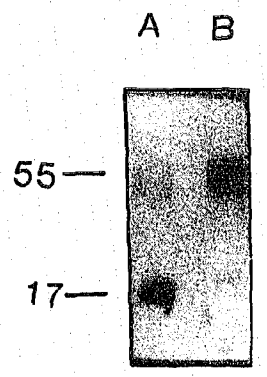

Fig. 5. Electroblotting of *E. coli* extracts separated by polyacrylamide gel electrophoresis. Bacteria (5×10^{10}/ml) were extracted at 65°C for 30 min in the presence of 0.5% SDS and fractionated by electrophoresis on 5–15% polyacrylamide gradient gel. The proteins in the gel were electroblotted onto nitrocellulose paper and probed with the ^{125}I-labeled 28 kDa fragment (lane A) or the ^{125}I-labeled 30 kDa peptide (lane B). The molecular masses of proteins are indicated by arrows and numbers on the left side.

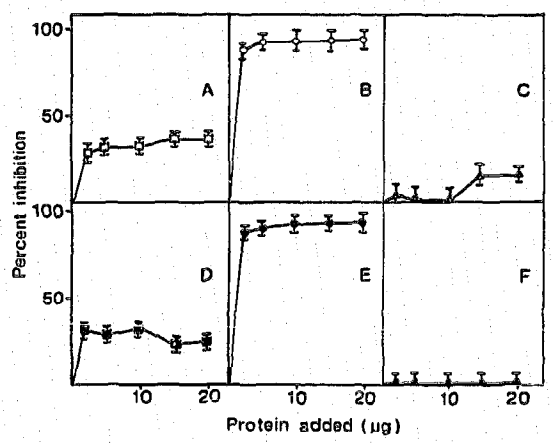

Fig. 6. Inhibition of fibronectin binding to *E. coli* B34289c by staphylococcal and streptococcal fibronectin receptors. Fusion protein ZZ-FR (panels A, B and C) and *Streptococcus dysgalactiae* S2 purified receptor (panels D, E and F) were tested as inhibitors of binding of [^{125}I]fibronectin (panels a and D), ^{125}I-labeled N-terminal fragments (panels B and E) and ^{125}I-labeled heparin binding peptide (panels C and F) to *E. coli* strain B34289c. Data are expressed as percentage of inhibition where binding to bacteria incubated in the absence of potential inhibitors was set as 0%.

mediate the adherence of the enterotoxigenic strain of *E. coli* B34289c.

To identify components that may act as bacterial cell surface receptors for fibronectin, extracts from *E. coli* cells were prepared, separated on SDS-PAGE and transferred to nitrocellulose paper. Nitrocellulose blots were incubated with ^{125}I-labeled 28 kDa or radiolabeled 30 kDa domains of fibronectin, rinsed and autoradiographed (Fig. 5). A single 17 kDa reactive band was positive with the ^{125}I-labeled 28 kDa peptide, whereas the radio- iodinated heparin binding peptide detected a protein with a molecular mass of 55 kDa. The 17 kDa peptide, recognized by the N-terminal fragment may represent curlin, a recently described structural subunit of organelles, named curli, having fibronectin binding activity [19]. The information we have concerning the 55 kDa protein, which binds to the internal 30 kDa peptide of fibronectin, is much more limited. It is possible that the expression and assembly of these two classes of receptors are under the same co-ordinated control. In fact, the binding of both the N-terminal domain and the heparin binding fragment to *E. coli* was maximal for bacteria grown at 30°C and was abolished when bacteria were cultured at 39–41°C (unpublished results).

The relationship between fibronectin binding components from *E. coli* and those from staphylococci and streptococci was analyzed by testing the chimeric protein ZZ-FR, which contains the active site of fibronectin binding protein from *S. aureus* and a fibronectin receptor isolated from *Streptococcus dysgalactiae* S2 as potential inhibitors of the binding of fibronectin and fibronectin fragments to *E. coli* cells. Our data demonstrate that staphylococcal and streptococcal receptors

were efficient inhibitors of the binding of the radioiodinated N-terminal fibronectin fragment to bacteria.

Moreover, they reduced the binding of intact fibronectin to *E. coli* B 34289c, but they did not interfere with that of internal heparin binding domain (Fig. 6). In view of these observations, it is reasonable to assume that bacterial receptors interacting with the N-terminal fragment recognize and bind to the same site in the domain and that they are somehow related, although competition between structurally different components cannot be ruled out. In addition, these results suggest that the N-terminal fragment binding receptors differ from the 55 kDa receptor protein.

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