Binding of Collagens to an Enterotoxigenic Strain of Escherichia coli

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An enterotoxigenic strain of Escherichia coli, B34289c, has been shown to bind the N-terminal region of fibronectin with high affinity (G. Fröman, L. M. Switalski, A. Faris, T. Wadström, and M. Höök, J. Biol. Chem. 259:14899–14905, 1984). We now report that this strain also binds collagen. The binding of 125I-labeled type II collagen to bacteria was time dependent and reversible. Bacteria expressed a limited number of collagen receptors (2.2 × 10^6 per cell) and bound collagen with a K_d of 20 nM. All collagen types tested (I to V) as well as all tested cyanogen bromide-generated peptides [xI(CB2, xI(CB3, xI(CB7, xI(CB8, and a2(I)CB4 were recognized by bacterial receptors, as demonstrated by the ability of these proteins to inhibit the binding of 125I-labeled collagen to bacteria. Of several unlabeled proteins tested in competition experiments, fibronectin and its N-terminal region strongly inhibited binding of the radiolabeled collagen to E. coli cells. Conversely, collagen competed with an 125I-labeled 28-kilodalton fibronectin fragment for bacterial binding. Collagen bound to bacteria could be displaced by excess amounts of either unlabeled fibronectin or its N-terminal fragment. Similarly, collagen could displace 125I-labeled N-terminal peptide of fibronectin bound to the bacterial cell surface. Bacteria grown at 41°C or in the presence of glucose did not express collagen or fibronectin receptors. These results indicate the presence of specific binding sites for collagen on the surface of E. coli cells and furthermore that the collagen and fibronectin binding sites are located in close proximity, possibly on the same structure.

The collagens constitute a heterogeneous family of fibrous proteins that represent major components of the extracellular matrix. In the connective tissues they mostly play a structural role; for instance, they give support to the bone and tensile strength to ligaments and tendons, and the arrangement of collagen fibrils confers transparency to the cornea.

Collagens may also affect biological activities of the cell, such as cell attachment (4, 6, 34), migration (33), and proliferation (1), as well as cell differentiation during hematopoiesis (26) and organogenesis (14). Eucaryotic cells express different cell surface receptors for collagen (5, 21). Membrane proteins that bind various types of collagen have been identified for a number of cell types (7, 22, 23, 29). Hence, collagen can be regarded as an adhesive protein and in this regard shares many properties with other adhesive proteins such as fibronectin and laminin.

Recent studies have shown that several pathogenic bacteria bind to extracellular matrix proteins such as fibronectin (10, 11, 24, 35–37), laminin (27, 38, 43, 48), and collagen (15, 16, 39, 40). For instance, streptococci (39) and strains of Staphylococcus aureus, particularly those isolated from septic arthritis, osteomyelitis, and infective endocarditis (16), express collagen receptors. Bacterial receptors for collagen and other connective tissue proteins have been proposed as potential virulence factors that may determine the ability of bacteria to adhere and colonize a tissue. Enterotoxigenic strains of Escherichia coli may cause severe diarrhea in humans and young animals (12). The pathogenesis of this infection involves the ability of bacteria to adhere to the small intestine epithelium and the production of nontoxic enterotoxins.

Enterotoxigenic E. coli strains produce heat-labile or heat-stable toxins (12, 13) or both (32). However, enterotoxin production alone does not appear sufficient to make an enterotoxigenic strain pathogenic. Several fimbrial adhesins differing in their serological properties and binding specificity have been identified in enterotoxigenic strains of E. coli isolated from humans (colonization factor antigens CFA/I, CFA/II, and CFA/III) (17).

Furthermore, some enterotoxigenic E. coli strains bind fibronectin, and it has been postulated that binding to fibronectin may represent a mechanism of tissue adherence (10). Along this line we have characterized the binding of collagen to the enterotoxigenic strain E. coli B34289c and compared it with the binding of fibronectin to the same strain.

In this report, we present data to show that collagen binding to E. coli is a receptor-mediated reaction and that collagen and fibronectin recognize the same structure or two closely spaced components on the surface of this microorganism. We propose that the binding of collagen and fibronectin to E. coli may represent a mechanism whereby these bacteria adhere to small intestine.

MATERIALS AND METHODS

Chemicals. Type II collagen was purified from bovine nasal septum as described by Strawich and Nimmi (41). Isolation of native collagen types I through V was as described previously (40). The cyanogen bromide peptides (CB peptides) were generated by treatment of alpha chains with cyanogen bromide and purified as described previously (2).

Carrier-free 125I (specific activity, 15 mCi/μg) was purchased from Radiochemical Centre, Amersham, England. Proteins were labeled with 125I by the chloramine T method (18). The specific activities of the radioactively labeled ligands were estimated to be 2.5 × 10^6 cpm/μg for collagen and 3 × 10^6 cpm/μg for the N-terminal fragment of fibronectin. More than 85% of the radioactivity in labeled samples could be precipitated with 10% trichloroacetic acid.

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Fibronectin was purified from human plasma as described previously (45). Thermolysin-generated fragments (3) were kindly provided by L. Zardi, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy. The purity of proteins and peptides was assessed by electrophoresis on a 5 to 20% gradient polyacrylamide gel in the presence of sodium dodecyl sulfate under reducing and nonreducing conditions, followed by Coomassie blue staining (25). Radioactive components were visualized by autoradiography of dried gels with X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) at −70°C. Electrophoretic analysis of collagen was carried out under reducing conditions on a 5 to 10% gradient gel. The protein was essentially pure as judged by Coomassie blue staining and autoradiography and migrated as a single 95,000-dalton band (Fig. 1), in agreement with previous findings (28).

Bovine serum albumin, protein A, gelatin, α-1-acid glycoprotein, fetuin, and Tween 80 were purchased from Sigma Chemical Co., St. Louis, Mo. Catalase was from Pharmacia Fine Chemicals, Uppsala, Sweden.

**Bacterial strain and growth conditions.** The strain used for this study, *E. coli* B34289c, was previously shown to bind fibronectin (10) and was obtained from T. Wadström (Department of Medical Microbiology, University of Lund, Sweden). This strain belongs to serotype O6:K2:M and was originally isolated from a child with diarrhea (9). Bacteria were maintained in deep brain heart infusion agar (Difco Laboratories, Detroit, Mich.) tubes at 4°C and grown on CFA agar medium (8) at 30°C (unless otherwise stated) for 24 h. Bacteria were scraped from the agar gel surface, suspended in phosphate-buffered saline (0.14 M sodium chloride, 0.02% sodium azide, 10 mM phosphate [pH 7.4]), washed, and finally suspended in phosphate-buffered saline to a density of 10^10 cells per ml. The concentration of bacteria was determined spectrophotometrically with a previously prepared standard curve relating A_600 to the cell number counted in a Petroff-Hauser chamber. Since the ability of bacterial cells to bind collagen was substantially reduced after prolonged storage at −70°C, fresh cells were routinely used.

**Binding assay.** The assay for collagen binding to bacteria was performed essentially as described previously (40). In brief, 5 × 10^8 cells were incubated with 5 × 10^4 cpm of

**RESULTS**

**Characterization of collagen binding to *E. coli*.** Incubation of *E. coli* B34289c with ^125^I-labeled collagen (20 ng, corresponding to 0.001 × the saturating concentration; see Fig. 5) resulted in a time-dependent binding of the protein to live bacteria (Fig. 2). The reaction was rapid and essentially completed within 60 min. Prolonged incubations resulted in little additional binding of ^125^I-labeled collagen. When heat-killed bacteria were incubated with collagen, binding kinetics were slower and the amount of ligand bound was 60% lower than those in live cells. Therefore, live bacteria were used throughout this study, and the cells were routinely incubated with the ligand for 60 min.

The collagen-binding potential of the bacterial cells varied with the temperature at which bacteria were cultured (Fig. 3). It was highest between 28 and 33°C and dramatically decreased when the cells were grown at 39 to 41°C. An identical relationship between cell growth temperature and binding of the N-terminal fibronectin fragment was observed
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FIG. 3. 125I-labeled collagen or 125I-labeled 28-kDa fibronectin fragment binding to E. coli cells grown at different temperatures. After bacteria were grown at temperatures ranging from 22 to 41°C for 24 h, the ability of cells to bind radiolabeled collagen (●) or the N-terminal domain of fibronectin (▲) was determined as described in the text. Data are expressed as percentages of the radioactivity added to the incubation mixture (5 x 10⁶ cpm).

FIG. 4. Reversibility of collagen binding to E. coli B34289c. Bacteria (5 x 10⁹ cells) were incubated with 20 ng of 125I-labeled collagen for 1 h. At the time point indicated by the arrow, a 10,000-fold excess of unlabeled collagen was added, and the cells were further incubated for the indicated times. The amount of bound 125I-labeled collagen was determined as described in Materials and Methods.

FIG. 5. Saturability of binding of 125I-labeled collagen to E. coli B34289c. Live bacteria (5 x 10⁹ cells) were incubated with increasing amounts of 125I-labeled collagen (specific activity, 2,000 cpm/µg) for 1 h. Background values were determined for each concentration of added collagen and subtracted for the incubation mixtures containing bacteria. Inset, Scatchard plot representation of the data.

collagen resulted in the displacement of two-thirds of the labeled ligand over a 3-h incubation (Fig. 4).

When the bacteria were incubated with increasing concentrations of 125I-labeled collagen, the amount of ligand bound to the bacteria increased to a saturation level of 5.50 µg of collagen bound per 5 x 10⁹ cells at a collagen concentration of 25 µg/0.6 ml (Fig. 5). If we assume a molecular weight of 2.85 x 10⁶ for collagen (28) and that all the 125I-collagen associated with bacteria represents receptor-bound ligand, the average number of available collagen-binding sites per cell is 2.2 x 10⁵. Scatchard plot analysis of the binding data revealed a straight line (Fig. 5, inset), which indicates the presence of one class of collagen receptor sites. From the slope of the curve a Kₐ of 20 nM was calculated.

Relationship between collagen and fibronectin binding to E. coli. The specificity of collagen binding to E. coli was analyzed by incubating bacterial cells with 125I-labeled type II collagen in the presence of excess amounts (250- to 2,500-fold) of unlabeled proteins as potential inhibitors (Table 1). The addition of unlabeled type II collagen to the incubation mixture effectively blocked the binding of radiolabeled ligand. A rabbit immunoglobulin G antibody to the collagen receptor on S. aureus (L. M. Switalski, P. Speziale, and M. Höök, manuscript in preparation), α-1-acid glycoprotein, and protein A did not affect the binding of 125I-labeled collagen to E. coli. In contrast, fibronectin significantly inhibited binding of collagen to bacteria. Also, fetuin showed inhibitory activity. There are at least two mechanisms whereby fibronectin could act as an inhibitor. Since the E. coli strain uses express fibronectin receptors (10), it is possible that the collagen and fibronectin receptors are located in close proximity and that receptor-bound fibronectin for sterical reasons interferes with binding of collagen to bacteria. Alternatively, since fibronectin also binds to collagen, the E. coli receptor and fibronectin could conceivably compete for the same domain in the collagen molecule. To differentiate between these two possibilities, different fragments of fibronectin were tested for their ability to inhibit
TABLE 1. Specificity of $^{125}$I-labeled collagen binding by E. coli B34289c
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<table>
<thead>
<tr>
<th>Competing protein</th>
<th>% Inhibition by protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 μg</td>
</tr>
<tr>
<td>None</td>
<td>0.0</td>
</tr>
<tr>
<td>Collagen type II</td>
<td>70.0 ± 3.1</td>
</tr>
<tr>
<td>Gelatin</td>
<td>62.0 ± 2.5</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>26.0 ± 2.2</td>
</tr>
<tr>
<td>Anti-S. aureus Cowan 1 immunoglobulin G</td>
<td>2.0 ± 2.8</td>
</tr>
<tr>
<td>Protein A</td>
<td>2.0 ± 1.5</td>
</tr>
<tr>
<td>α-1-Acid glycoprotein</td>
<td>2.5 ± 1.9</td>
</tr>
<tr>
<td>Fetuin</td>
<td>8.0 ± 3.0</td>
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</tbody>
</table>

* Bacteria ($5 \times 10^6$) were incubated with $5 \times 10^4$ cpm (20 ng) of $^{125}$I-labeled collagen in the presence of the indicated concentrations of competing proteins for 1 h. Inhibition is expressed as the percentage of $^{125}$I-labeled collagen bound to bacteria in the absence of unlabeled proteins. Values represent means ± standard errors of three separate experiments.

Collagen binding to bacteria. The results of these experiments (Table 2) showed that the inhibitory activity of fibronectin was located in the N-terminal domain, which was previously shown to bind to E. coli cells (10). The collagen-binding domain present in 40-kilodalton (kDa) fragment of fibronectin and 14-kDa peptide containing heparin- and DNA-binding activity did not interfere with the binding of collagen to bacteria. Furthermore, the fragment encompassing the Arg-Gly-Asp-containing cell-binding site (110 kDa) and the C-terminal domain (20 kDa) lacked inhibitory activity. The specificity of $^{125}$I-labeled collagen binding to E. coli cells was further examined by analyzing the inhibitory activity of different collagen types and catalase, a noncollagenous protein with an apparent molecular mass of 240 kDa (42) (Fig. 6).

All the collagen types tested, including types I to V, were potent inhibitors and showed essentially the same inhibitory potential when tested at different concentrations. On the contrary, the binding of $^{125}$I-labeled collagen to bacteria in the presence of catalase was not significantly reduced. When different collagen peptides isolated after degradation of type I collagen with cyanogen bromide were included in the incubation mixture (Fig. 7), they all inhibited the binding of $^{125}$I-labeled collagen to bacteria, although the extent of inhibition varied. In general, small fragments [α1(I)CB2 and α1(I)CB3] were less effective than large peptides [α2(I)CB4, α1(I)CB7, and α1(I)CB8]. The addition of increasing amounts of unlabeled 40-kDa α-1-acid glycoprotein (19) did not effectively inhibit binding of radiolabeled collagen to bacteria.

The fibronectin binding site of collagen has previously been located to a fragment corresponding to the α1(I)CB7 peptide (20). The observation that CB fragments mapping outside the fibronectin-binding site, such as α1(I)CB8, were effective inhibitors of collagen binding to bacteria suggests

![Collagen added (μg)](image)

FIG. 6. Inhibition of binding of $^{125}$I-labeled collagen to E. coli B34289c by different collagen types. Bacteria ($5 \times 10^6$ cells) were incubated for 1 h with $^{125}$I-labeled type II collagen in the presence of excess unlabeled type I (○), II (△), III (●), IV (●), or V (■) collagen or catalase (◇). The amount of bound collagen was determined as described in Materials and Methods. The data are expressed as percentages of inhibition of $^{125}$I-labeled collagen binding to bacteria in the absence of any potential inhibitor.

![Peptide added (μg)](image)

FIG. 7. Effect of CB collagen peptides on $^{125}$I-labeled collagen binding to E. coli B34289c. E. coli cells ($5 \times 10^6$) were incubated for 1 h with $^{125}$I-labeled collagen in the presence of increasing concentrations of α1(1)CB2 (●), α1(1)CB3 (■), α1(1)CB7 (△), α1(1)CB8 (◇), or α2(1)CB4 (▲) or α1-acid glycoprotein (○). The data are expressed as percentages of the control, in which binding to bacteria was performed in the absence of unlabeled peptides.

TABLE 2. Inhibition of binding of $^{125}$I-labeled collagen to E. coli B34289c by fibronectin and fibronectin fragments

<table>
<thead>
<tr>
<th>Competing protein</th>
<th>% Inhibition by fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 nmol</td>
</tr>
<tr>
<td>None</td>
<td>0.0</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>65.00 ± 4.2</td>
</tr>
<tr>
<td>28 kDa</td>
<td>51.60 ± 3.6</td>
</tr>
<tr>
<td>40 kDa</td>
<td>0.94 ± 2.0</td>
</tr>
<tr>
<td>14 kDa</td>
<td>1.67 ± 1.6</td>
</tr>
<tr>
<td>110 kDa</td>
<td>1.86 ± 0.2</td>
</tr>
<tr>
<td>20 kDa</td>
<td>4.33 ± 1.5</td>
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* E. coli B34289c (5 × 10^6 cells) was incubated for 1 h in the presence of 5 × 10^4 cpm of $^{125}$I-labeled collagen and 0.5 or 1.0 nmol of fibronectin (counted by the number of monomers of 220 kDa), or its thermolysin-generated fragments. The amount of radioactivity recovered in the tubes in the absence of competing proteins was set as 100% inhibition, and all data are expressed as percentages of this control. The values represent means ± standard errors of three separate experiments.

* ND, Not determined.
DISCUSSION

Data reported from several laboratories, including ours, have shown that both staphylococci and streptococci bind collagen. Collagen binds to a number of streptococcal strains belonging to serogroups A, B, C, D, and G (39) and to different species of staphylococci (47). Some gram-negative oral bacteria such as Bacterioides gingivalis bind type IV collagen (49). Westerlund et al. have also demonstrated that the O75X adhesin of uropathogenic E. coli interacts with type IV collagen and some other basement membrane glycoproteins (48). We now report on the binding of collagen to an enterotoxigenic strain of E. coli.

Binding of 125I-labeled collagen to E. coli B34289c was time dependent and involved a heat-labile component, since the binding to bacteria heated at 88°C for 10 min was significantly reduced. Bacterial cells could be saturated with the ligand, thus indicating the presence of a limited number of binding sites, estimated to be 2.2 × 10⁴ receptors per cell. The Kₐ for the binding of collagen to E. coli was approximately 20 nM and of the same order of magnitude as that of collagen for S. aureus Cowan 1 (40). However, in view of several assumptions made, e.g., the approximation in determining the specific activity of 125I-labeled collagen, these values should be regarded as rough estimates.

Bacterial receptors recognized all of the collagen types tested. Also, CB peptides generated by cyanogen bromide cleavage of type I collagen were efficient inhibitors. Large cyanogen bromide fragments had an apparent affinity higher than that of smaller ones. The fact that all collagen types and CB peptides inhibited the binding of 125I-labeled collagen to E. coli cells indicates that the E. coli collagen receptor recognizes a structure common to all collagen and that collagen contains multiple binding sites for bacteria along the molecule. A similar specificity was previously shown for a collagen receptor on S. aureus (40).

Binding of 125I-labeled collagen was specific; it was completely inhibited by an excess of either unlabeled collagen or
gelatin. Most other unrelated proteins did not affect the binding. The lack of inhibition by an immunoglobulin G antibody to the collagen receptor of *S. aureus*, which blocks collagen binding to these bacteria (data not shown), indicates that the collagen receptors from the two species are different. Fetuin reduced the binding of collagen to bacteria to some extent. The mechanism of this effect remains unclear. Binding of collagen to bacteria was reversible, and most cell-bound 125I-labeled collagen could be displaced by unlabeled ligand. Collagen was apparently not degraded during incubation with *E. coli*, as indicated by electrophoresis on polyacrylamide gel of 125I-labeled collagen before and after 1 h of incubation (data not shown). Furthermore, chloramine T labeling of collagen did not modify electrophoretic behavior of the protein (Fig. 1). Taken together, these results rule out the possibility that incomplete displacement of radiolabeled collagen bound to the *E. coli* cell surface by unlabeled collagen may reflect the presence of two or more types or sizes of collagen in the incubation mixture. The partial reversibility may indicate that, after initial binding of collagen to bacteria via a receptor, other interactions could occur that make the final binding tight and irreversible.

The expression of both collagen and fibronectin receptors in *E. coli* was dependent on the growth temperature in a parallel fashion and was prevented by the presence of 1% glucose in the growth medium. The effect of glucose is unclear, although a mechanism of cyclic AMP control appears to be excluded.

Several lines of evidence demonstrate that the collagen- and fibronectin-binding sites on *E. coli* are located in close proximity and possibly on the same structure or molecule. First, of the fibronectin fragments tested, only the N-terminal fragment strongly inhibited the binding of collagen to bacteria, whereas other fragments, including a collagen-binding fragment, were essentially inactive. Second, CB peptides that lacked affinity for fibronectin [i.e., α1(1CB8)] were more effective in inhibiting the binding of collagen to the bacterial cells than peptides containing the fibronectin binding site [i.e., α1(1CB7)]. Third, the binding of the 125I-labeled N-terminal fragment of fibronectin to bacteria was equally inhibited by both unlabeled collagen and by the N-terminal region of fibronectin. Finally, this conclusion is supported by displacement experiments, in which unlabeled fibronectin or its N-terminal fragment effectively displaced bound 125I-labeled collagen from *E. coli* cells or, conversely, unlabeled collagen was successfully used to reverse the binding of radiolabeled 28-kDa fragment to bacteria. Taken together, these observations highlight the possibility that the same cell surface structure may act as a receptor for collagen and fibronectin and perhaps other adhesive proteins. The bacterial receptor site may recognize the same active sequence in both collagen and fibronectin; alternatively, collagen and fibronectin may each have distinct domains that interact with different subsites that are close together on the receptor molecule.

In a recent study, Olsen et al. (30) identified in *E. coli* isolates a novel class of surface organelles, named curli, that mediate binding of bacteria to fibronectin. Curli were found to consist of a single type of protein, the curli, and their assembly occurred preferentially at growth temperatures below 37°C. It is not currently known whether curli are also involved in collagen binding.

*E. coli* B34289c, besides specifically interacting with collagen and fibronectin, also binds to laminin, the most abundant glycoprotein in basement membranes (data not shown). The biological significance of the specific binding of colla-

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**LITERATURE CITED**


