Plasmid-Encoded Outer Membrane Protein YadA Mediates Specific Binding of Enteropathogenic Yersiniae to Various Types of Collagen

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The plasmid-encoded outer membrane protein YadA of enteropathogenic yersiniae is associated with pathogenicity. Recently, collagen binding of YadA-positive yersiniae was reported without detailed characterization (L. Emödy, J. Heesemann, H. Wolf-Watz, M. Skurnik, G. Kapperud, P. O'Toole, and T. Wadström, J. Bacteriol. 171:6674–6679, 1989). To elucidate the nature of collagen binding to YadA, we used a recombinant *Yersinia* strain expressing the cloned YadA gene. Direct binding of YadA-positive yersiniae to collagens was demonstrated in affinity blot experiments on nitrocellulose filters. A spectrum of collagen types in a wide concentration range were tested for their ability to block binding of ^{125}I-labeled collagen type II to YadA-positive yersiniae. The results indicate a specific binding site(s) for YadA in collagen types I, II, III, IV, V, and XI. In contrast, collagen type VI did not bind to YadA. To characterize the binding site(s) more precisely, isolated collagen chains and cyanogen bromide fragments were investigated. These studies revealed that binding of YadA to collagen type I is confined to the α1(I) chain, whereas the binding site within collagen type XI is localized in the α3(XI) chain. α2(I), α1(XI), and α2(XI) did not bind to YadA. Most interestingly, in the α1(II) chain the specific binding site for YadA resides in the cyanogen bromide fragment CB10. The latter might indicate a binding site that does not depend on conformation. Based on these findings, further fragmentation and the synthesis of peptides may allow definition of the peptide sequence(s) relevant for YadA binding.

Yersinia enterocolitica and *Yersinia pseudotuberculosis* are enteropathogenic for humans (4, 7). The disease spectrum comprises both intestinal and extraintestinal manifestations involving the connective tissue, such as reactive arthritis, erythema nodosum, and different types of vasculitis (4, 7, 38). Recently, it was demonstrated that a plasmid of about 70 kb is necessary for full virulence expression in yersiniae (for reviews, see references 6, 15, 29, and 41). Different plasmid-encoded proteins, including outer membrane proteins with pathogenic functions, have been described (2, 3, 6, 13, 14). One of the most prominent plasmid-encoded outer membrane proteins is the *Yersinia* adhesin YadA. It forms multimeric fibrillae on the surface of enteropathogenic yersiniae (25). There is plenty of evidence that YadA is involved in resistance to complement lysis, phagocytosis, and adherence to mammalian cells (14, 16). Binding of collagen types I, II, and IV to YadA-positive yersiniae has been reported (8). This property might be relevant for the pathogenesis of diseases induced by enteropathogenic yersiniae. However, the specificity of the collagen-YadA interaction still remains obscure, since the reported competition experiments were performed at a 400- to 1,000-fold molar excess of the unlabeled inhibitor. This prompted us to investigate the nature of collagen binding by YadA in more detail. In a first series of experiments we tested direct binding of YadA-positive yersinia to a broad spectrum of collagens immobilized on nitrocellulose filters. We demonstrate here that YadA expression mediates binding of yersiniae to all collagens investigated except collagen type VI. In blocking experiments, different collagens in a wide concen-

tration range were tested for their ability to inhibit binding of the main cartilage collagen, collagen type II, to YadA-positive yersiniae; blocking occurred at picomolar concentrations. The experiments indicate a specific high-affinity binding site(s) for YadA on many collagen types. Consequently, the analysis was extended to assign the putative binding site to either isolated collagen chains or cyanogen bromide fragments.

MATERIALS AND METHODS

Bacterial strains. Three *Y. enterocolitica* strains of serotype O:5 were used in this study. Strain NFO is of environmental origin, plasmid free, and YadA negative. Strain NF-pCB9::Tn5 harbors the virulence plasmid of *Y. enterocolitica* serotype O:9 with an inactivated YadA gene. Strain NF-pRK290B9-4 harbors a hybrid plasmid consisting of the mobilizable vector pRK290B (16) and a *Bam*HI fragment of the virulence plasmid of *Y. enterocolitica* O:9 encoding YadA and is otherwise homologous to NFO; it has been demonstrated that the vector pRK290B does not encode collagen binding factors (8). All strains were described previously (8, 14, 16, 17). Bacteria were grown in Luria-Bertani bouillon at 37°C for 24 h, heat inactivated at 60°C for 45 min, and stored in phosphate-buffered saline (PBS)–0.01% NaN₃ (pH 7.3) at 4°C.

Collagen preparation. Human type I and V collagens from placental tissue were prepared as described by Bentz et al. (1) and kindly provided by K. Kühn, Martinsried, Germany. Type II, IX, and XI collagens were prepared from the chicken xyphoid process by extraction with guanidinium hydrochloride for removal of proteoglycans, digestion with pepsin, and fractional salt precipitation as described by von

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der Mark et al. (40). The four-step procedure of collagen type IV preparation from human placenta was performed as described by Glanville and Rauter (12) and included pepsin solubilization of tissue, fractional salt precipitation of solubilized collagens, native reduction and carboxymethylation, a second pepsin treatment, and precipitation of high-molecular-weight materials out of the denatured sample with 3 M NaCl. Type III collagen was prepared from fetal bovine skin. Extraction of collagen from the skin, precipitation by ammonium sulfate and NaCl, dialysis against 2 M urea containing NaCl-Tris buffer, and separation of collagen type III with DEAE-cellulose columns were performed as described by Timpl et al. (37). Pepsin-solubilized collagen type VI from human placenta was purified as described by von der Mark et al. (39).

Proteoglycans. Proteoglycans were prepared by extraction with 1 M NaCl of human fetal cartilage followed by DEAE chromatography (23). They were kindly provided by T. Kirsch, MPG-Arbeitsgruppen, Erlangen, Germany.

CNBr cleavage and CB fragment purification. Samples of 50 mg of collagen type II were cleaved with CNBr, and the cyanogen bromide fragments (CB fragments) were purified as recently described by Burkhardt et al. (5). Briefly, collagen type II was dissolved in 70% HCOOH (10 ml), warmed to 50°C to ensure denaturation, and flushed with nitrogen. Then CNBr was added in a 100-fold molar excess over methionine residues (1.5 mg/mg of collagen) and allowed to react for 4 h at 37°C. For further purification the CB fragments were subjected to fast protein liquid chromatography in 0.1 M ammonium acetate (pH 5) on a Superose 12 column. Homogeneity of the peptides was controlled by sodium dodecyl sulfate (SDS)-gel electrophoresis in 18% acrylamide minigels with the buffer system of Laemmli (26).

Isolation of α1(I) and α2(I). α1(I) and α2(I) collagen chains were separated by chromatography on carboxymethyl cellulose at 42°C in 0.04 M sodium acetate (pH 4.8) containing 4 M urea as described previously (40). The purity of the chains was controlled by SDS-polyacrylamide gel electrophoresis (PAGE) in 7% acrylamide minigels with the buffer system of Laemmli (26).

Radiolabeling of collagen type II. Collagen type II was radiolabeled by the iodogen method (10), and a specific activity of 2×10^6 to 3×10^6 cpm/μg was determined.

Binding of radiolabeled collagen type II to yersiniae and blocking experiments. Increasing amounts of ^{125}I-labeled collagen type II (2.7×10^6 cpm/μg) were incubated with 100 μl of particulate bacterial suspension (2×10^9 bacteria) for 75 min at room temperature under gentle agitation. Then 1 ml of PBS-0.01% Tween 20 was added, and the suspension was centrifuged (30 min, 10°C, $7,500 \times g$). The supernatant was aspirated, and the radioactivity associated with the pellet was determined in a gamma counter (COBRA 5005; Canberra and Packard Instruments). YadA-negative yersiniae were used as a control for unspecific binding of collagen type II to yersiniae. In the blocking assays, 100 ng of radiolabeled collagen type II was used; without blocking proteins, this gave signals of about 2.2×10^4 cpm with YadA-positive yersiniae and about 3.5×10^3 cpm with YadA-negative yersiniae after the washing procedure. For blocking experiments, the desired amount of protein in 10 μl of PBS was incubated with 100 μl of the particulate bacterial suspension for 75 min at room temperature unless otherwise stated. Samples of 100 ng of ^{125}I-collagen type II in 10 μl of PBS were added and allowed to react for 75 min at room temperature unless otherwise stated. Washing and determination of radioactivity were performed as described above.

All samples were analyzed in at least two independent experiments in duplicate assays. The plastic tubes used for the assays were precoated with bovine serum albumin (BSA) to minimize nonspecific binding of collagen and bacteria to the walls of the tubes.

Affinity blots. SDS-PAGE was performed on collagen and collagen fragments with 18 and 10% acrylamide minigels, respectively. The proteins were electrophoretically transferred to nitrocellulose filters. After nonspecific protein binding sites were blocked with 1% BSA in PBS for 2 h at room temperature, the filter was incubated with a particulate *Yersinia* suspension (3×10^9 bacteria per ml in PBS) for 2 h at room temperature. A rabbit anti-*Y. enterocolitica* O:5 antibody was used to detect filter-bound yersiniae, and peroxidase-conjugated goat anti-rabbit immunoglobulin G was used as the detection system.

ELISA. For the enzyme-linked immunosorbent assay (ELISA), samples (10 μl) of a collagen solution (various concentrations in PBS) were incubated with 100 μl of the bacterial suspension (2×10^9 bacteria) in V microtiter plates for 90 min at room temperature. The plates were centrifuged (20 min, 15°C, $550 \times g$), and the pellets were washed with 100 μl of PBS-0.1% Tween by resuspension. Centrifugation and washing were repeated. The bacteria were incubated with an anti-collagen type II-specific antibody (in 100 μl of PBS-1% BSA, 4°C, overnight) and then washed as described above. The pellets were incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Bio-Rad Laboratories) diluted 1:5,000 in PBS-1% BSA for 2 h at room temperature. Bacteria were washed again, and the substrate (3,3'-5,5'-tetramethylbenzidine [50 ml], 0.01% in 100 mM citrate-phosphate (pH 6) plus 5 μl of 30% H_2O_2) was added. The A_{450} was determined for the supernatant after centrifugation. The monoclonal anti-chicken collagen type II antibodies used in this study (B1, C1, D3) were kindly provided by R. Holmdahl, Uppsala, Sweden. They were described previously (5, 19). The epitopes of these antibodies on chicken collagen type II are located on specific CB fragments: CB8 (B1), CB10 (D3), and CB11 (C1). The curves for binding of all three antibodies to their corresponding fragments were similar, and the antibody concentrations were adjusted to similar signal intensities.

RESULTS

Binding of YadA-positive yersiniae to immobilized collagen. To test for direct binding of yersiniae to a spectrum of collagens, we performed affinity blot experiments with immobilized collagen. After SDS-PAGE and electroblotting of the proteins to a nitrocellulose filter, yersiniae were added; the bacteria bound to the filter were detected by using an anti-*Y. enterocolitica* O:5 antibody.

YadA-positive yersiniae bound to collagen types II, V, and XI, whereas no binding was detectable to type VI collagen (Fig. 1). Within the heterodimeric collagen type V, YadA-positive yersiniae bound to both chains, whereas, within the heterotrimeric collagen type XI, YadA-mediated binding was confined to the α3(XI) chain. Collagen types III and IV also bound YadA-positive yersiniae (data not shown). YadA-negative yersiniae did not bind to any of the collagens (Fig. 1).

The most abundant collagen is type I collagen, a heterotrimeric collagen consisting of two α1(I) chains and one α2(I) chain. We were interested in determining whether binding of YadA to collagen type I is restricted to both chains, which show 65% homology at the protein level. The chains were
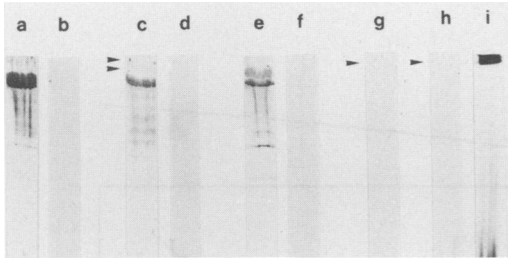
FIG. 1. Binding of YadA-positive and YadA-negative yersiniae to immobilized collagen. Collagen types II (a, b), XI (c, d), V (e, f), and VI (g, h) were electroblotted to nitrocellulose filters after SDS-PAGE. YadA-positive (a, c, e, g) or YadA-negative (strain NFpCB9::Tn5) (b, d, f, h) yersiniae were added, and filter-bound bacteria were assessed by using a rabbit anti-*Y. enterocolitica* O:5 antibody. An anti-rabbit peroxidase conjugate was used as the detection system. The positions of the proteins not bound by yersiniae [α1(XI) and α2(XI) in lane c, collagen type VI in lanes g and h] are indicated according to their localization by Ponceau red staining. Coomassie-stained SDS-PAGE of collagen type VI is also shown (i).

isolated by ion-exchange chromatography and further purified by reverse-phase high-pressure liquid chromatography. The affinity blot experiment with the isolated chains of collagen type I showed that binding to YadA was limited to the α1(I) chain (Fig. 2).

Binding of ^{125}I-labeled collagen type II to YadA. The main cartilage collagen, collagen type II, was radiolabeled to further characterize binding of YadA. Increasing amounts of ^{125}I-labeled collagen type II (2.7×10^6 cpm/µg) were added to 2×10^9 YadA-positive and YadA-negative yersiniae. Binding of collagen type II to YadA-negative yersiniae was used to detect nonspecific binding. The result indicates saturation of specific binding of collagen type II to YadA at higher concentrations (Fig. 3). Based on these findings, 100 ng of ^{125}I-labeled collagen type II was chosen as reference for further binding and blocking experiments. In the assay, after incubation and washing, 100 ng of radiolabeled collagen type II (2×10^5 to 3×10^5 cpm) gave signals of about 22,000 cpm with YadA-positive yersiniae and about 3,500 cpm with YadA-negative yersiniae.

Blocking of collagen type II binding to YadA by various collagens. Various collagens and collagen preparations were
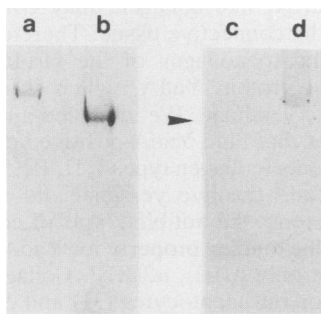

FIG. 2. Binding of YadA-positive yersiniae to immobilized collagen type I chains. Isolated chains α1(I) and α2(I) were subjected to SDS-PAGE (a and b, respectively) and then electroblotted to nitrocellulose filters (d and c, respectively). The arrowhead marks the position of the α2(I) chain, according to its localization by Ponceau red staining. The experiments were performed as described in the legend to Fig. 1.

used to inhibit binding of radiolabeled collagen type II to YadA-positive yersiniae. Heat-denatured and native type II collagens were equally effective in preventing binding of subsequently added native radioactive collagen type II (Fig. 4). For this experiment collagen type II was heat denatured (42°C, 60 min) and the blocking assay was performed at 37°C to prevent spontaneous renaturation. Collagen types II, III, IV, V, and XI showed very similar inhibition curves (Fig. 4 and 5). The data suggest high-affinity binding of all five collagens to YadA. About 60% inhibition was observed already with a fivefold molar excess (500 ng) of the inhibitor. The control proteins BSA and cartilage proteoglycans did not show any inhibition of collagen type II binding to YadA (Fig. 5A).

However, the ability to block collagen type II-YadA binding is not simply a feature of all triple-helical collagens. Collagen type VI at up to a 20-fold molar excess over radiolabeled collagen type II did not block collagen type II-YadA binding. Only at a higher concentration (50-fold molar excess) was inhibition observed. The collagen type IX inhibition curve showed greater inhibition compared with that of collagen type VI and did not show the typical high efficient blocking of collagen types II, III, IV, V, and XI in the low concentration range.

Blocking of collagen type II-YadA binding by collagen type I and isolated collagen type I chains. Type I collagen did not block collagen type II-YadA binding as efficiently as the other fibril-forming collagens, e.g., types II, III, V, and XI, did (Fig. 5 and 6). Since the affinity blot experiments with the isolated collagen type I chains revealed that the binding site for YadA was confined to the α1(I) chain only (Fig. 2) and binding of collagen type II to YadA was independent of the triple-helical conformation (Fig. 4), we performed blocking experiments with the isolated chains of collagen type I. The blocking capacity of collagen type I at low molar concentrations resided completely in the α1(I) chain (58% inhibition at 500 ng) (Fig. 6). The blocking potential of α1(I) was comparable to that of collagen type II in native and denatured forms (Fig. 4). The α2(I) chain failed to exhibit significant blocking capacity (9% inhibition at 500 ng).

Blocking of collagen type II-YadA binding by cyanogen bromide fragments of collagen type II. To further characterize the binding site for YadA on collagen type II, CNBr cleavage at methionine sites of collagen type II was performed, and then the fragments were purified by fast protein liquid chromatography. The modified solid-phase ELISA with the purified fragments and fragment-specific monoclonal antibodies demonstrated YadA-mediated binding for CB10 only (data not shown). Therefore, CB10 (346 amino acids) and the approximately equal sized CB11 (279 amino acids) were used in the blocking experiments shown in Fig. 7. The amounts of the three inhibitor proteins shown in Fig. 7 are indicated in molarities for better comparison [α1(II), ~95 kDa; α1(II)CB10, ~31 kDa; α1(II)CB11, ~25 kDa]. Only CB10 was efficient in blocking collagen type II-YadA binding. Up to a concentration of 3 pM, the blocking capacities of CB10 and the whole collagen type II molecule were similar. At this concentration the maximum inhibition (55%) was reached for the inhibitor CB10. Further rise of inhibitor concentration up to a 150-fold molar excess (corresponding to 5 µg) did not increase the blocking potential for the collagen type II-YadA interaction. CB11 did not block collagen type II binding to YadA. Interactions at high inhibitor concentrations such as those observed with the complete polypeptide chains could not be detected with the CB fragments used for the binding studies.
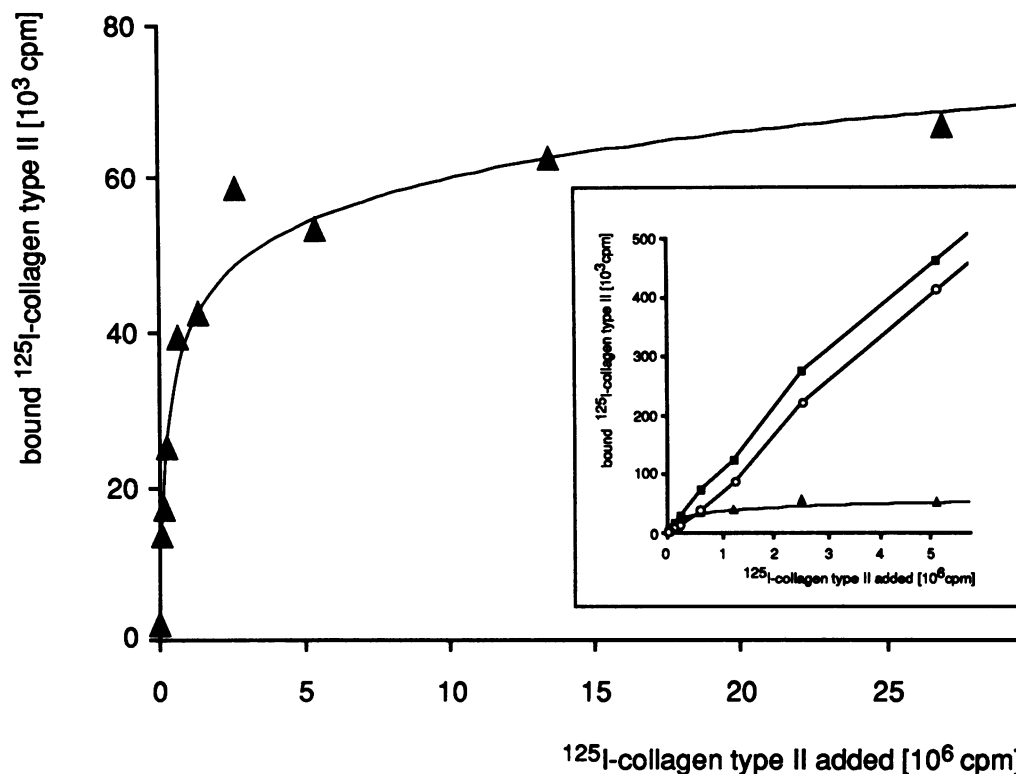
FIG. 3. Specific binding of radiolabeled collagen type II to YadA-positive yersiniae. Increasing amounts of ^{125}I-labeled collagen type II (2.7 × 10^{6} cpm/μg) were incubated with 2 × 10^{9} bacteria. Unspecific binding to YadA-negative yersiniae was determined for each concentration and subtracted. The insert demonstrates total counts obtained with YadA-positive (■) and YadA-negative (O) yersiniae as well as the resulting specific binding (▲). Each value represents the average of duplicate assays. The variation of each individual value from the mean never exceeded 10%.
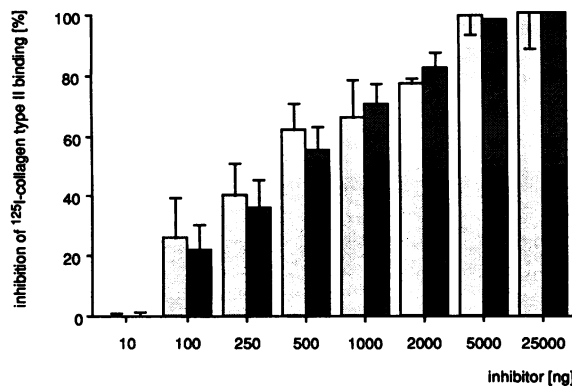

FIG. 4. Inhibition of the binding of radiolabeled collagen type II to YadA by native (light stippling) and heat-denatured (42°C, 60 min) (dark stippling) collagen type II. YadA-positive yersiniae (2 × 10^{9} bacteria) were incubated with different concentrations of the collagen preparations, and then radiolabeled native collagen type II (2 × 10^{5} to 3 × 10^{5} cpm) was added. The assays were performed at 37°C to prevent spontaneous renaturation. Radioactivity associated with the pellet was measured in a gamma counter. Radioactivity obtained with radiolabeled collagen type II and YadA-positive yersiniae without preincubation with other proteins was defined as 100% ^{125}I-labeled collagen type II binding. Each value represents the mean and standard deviation of at least two independent duplicate assays.

DISCUSSION

Attachment of bacteria or bacterial products to host cells and host tissue is crucial for infectious diseases caused by enteropathogens like *Y. enterocolitica*. Previous studies have reported on binding of different bacteria to extracellular matrix proteins like laminin (9, 27, 32, 34, 35), fibronectin (24; for a review, see reference 20), and collagen (8, 9, 18, 33) and have moreover shown the importance of these interactions for tissue adhesion (21, 22). The interaction of proteins expressed by *Y. enterocolitica* with components of the extracellular matrix, like collagen, may contribute to diseases affecting the connective tissue. Therefore, we investigated the binding to collagen of the virulence-associated outer membrane protein YadA, which is common to all enteropathogenic yersiniae. We identified a broad spectrum of collagen types that bind YadA-positive yersiniae in affinity blot experiments (collagen types I, II, III, IV, V, and XI). Interestingly, YadA-positive yersiniae did not bind to all collagens tested (e.g., did not bind type VI collagen), and in some collagens the binding property for YadA was restricted to a single chain only [α1(I), α3(XI)]. Collagen binding has been reported for rat hapatocytes (31) and *Staphylococcus aureus* (33), both of which bound to all collagens tested (collagen types I, II, III, IV, and V for rat hepatocytes and collagen types I, II, III, IV, V, and VI for *S. aureus*). Different CNBr-generated peptides of the α1(I) chain and synthetic peptides with collagenlike structures [e.g., (Pro-Gly-Pro)$_n$] were also bound indiscriminately. In contrast to these findings, our results argue in favor of a YadA collagen
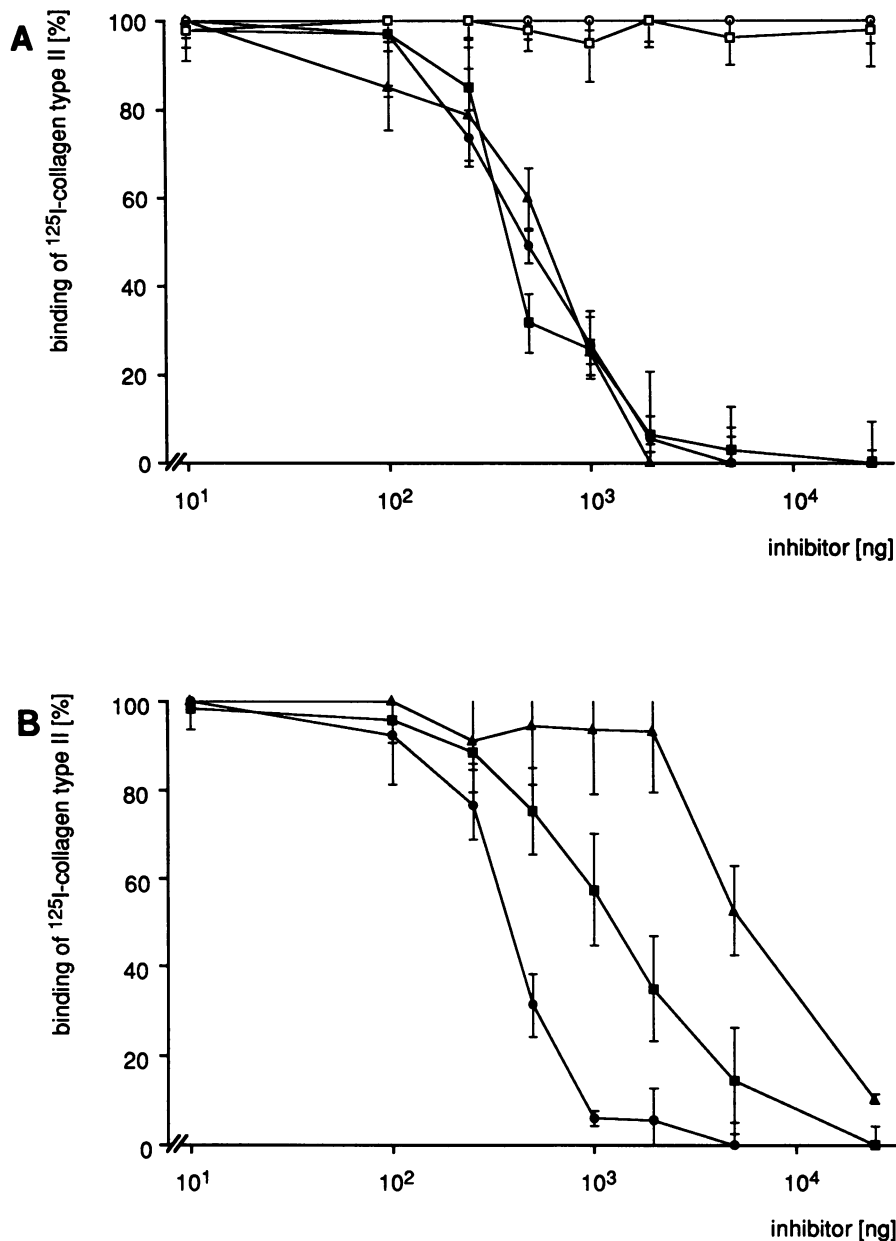
FIG. 5. Inhibition of the binding of radiolabeled collagen type II to YadA by different collagens and other peptides. Collagen types IV (▲), V (■), and XI (●) (A) and types III (●), VI (▲), and IX (■) (B) were investigated. The experiments were performed as described in the legend to Fig. 4, except that the temperature was room temperature. Cartilage proteoglycans (□) and bovine serum albumin (○) were used as controls (A). Each value represents the mean and standard deviation of at least two independent duplicate assays.

binding site that is not determined by the simple repetitive sequence of collagen (Gly-X-Y) but rather by a specific amino acid motif. Studies with YadA-positive and YadA-negative yersiniae demonstrated that binding of collagen type II to YadA is saturable, which is further evidence for a specific interaction.

Preincubation of YadA-positive yersiniae with unlabeled collagen type II prevented binding of subsequently added ^{125}I-collagen type II in a concentration-dependent manner. Moreover, native and heat-denatured type II collagens inhibited the collagen type II-YadA interaction to similar degrees. Furthermore, our experiments revealed that, in contrast to the α2(I) chain, the α1(I) chain and a 346-amino-

acid CB fragment (CB10) of collagen type II harbor a specific binding site for YadA. Therefore, YadA seems to interact with a locally restricted peptide sequence, whereas binding to a conformationally dependent epitope formed by scattered amino acids and dependent on the triple helical structure of collagen seems rather unlikely.

It remains to be shown whether the blocking capacities of collagen type VI and of the α2(I) chain at vast molar excesses (Fig. 5B and 6) were due to (i) minute contaminations (5%) of the preparations with other collagens not visible in Coomassie-stained SDS-PAGE, (ii) a specific binding site with lower affinity, or (iii) simply a stickiness because of weak noncovalent forces along the whole mole-
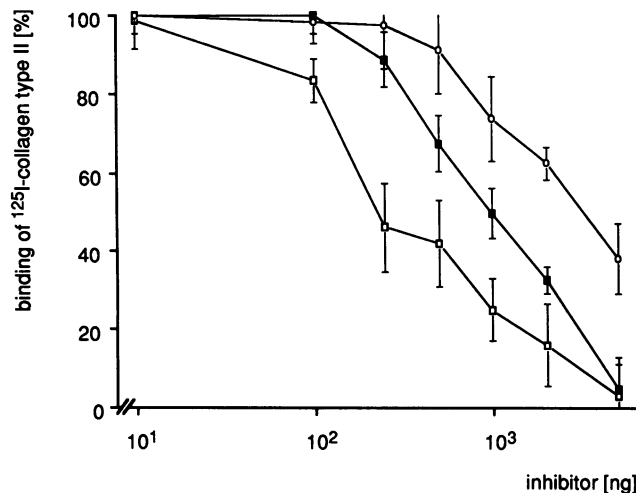
FIG. 6. Inhibition of the binding of radiolabeled collagen type II to YadA by collagen type I (■) and isolated collagen type I chains α1(I) (□) and α2(I) (○). The experiments were performed as described in the legend to Fig. 4, except that the temperature was room temperature. Each value represents the mean and standard deviation of at least two independent duplicate assays.

cule. Considering one of the last two options, the failure of the CB fragments investigated in this study to further block collagen type II-YadA binding at high inhibitor concentrations might be due to (i) an additional binding site for YadA on collagen type II, localized on a CB fragment not tested in the blocking assays, or (ii) destruction of low-affinity binding to YadA by the CNBr cleavage.

The capacities of the collagens tested in this study to bind YadA are different. However, some collagens had similar reaction patterns. Collagen types II, III, IV, V, and XI but not types VI and IX bound to YadA with high affinity and were able to inhibit collagen type II-YadA binding in blocking experiments at low inhibitor concentrations. All fibril-forming collagens (28) except type I collagen (i.e., collagen
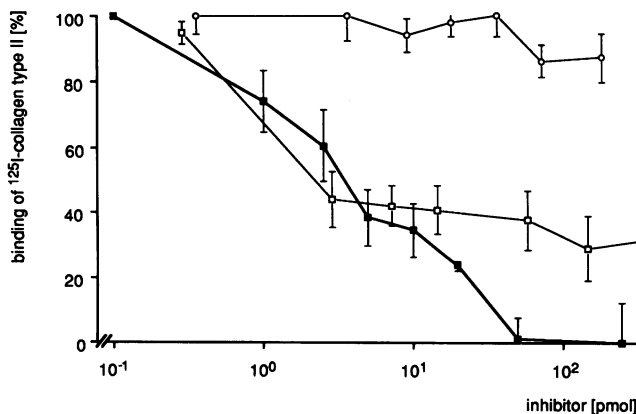

FIG. 7. Inhibition of the binding of radiolabeled collagen type II to YadA by collagen type II (■) and the collagen type II fragments CB10 (□) and CB11 (○). The amounts of the inhibitor proteins are indicated in molarities [α1(II), ~95 kDa; α1(II)CB10, ~31 kDa; α1(II)CB11, ~25 kDa). The experiments were performed as described in the legend to Fig. 4, except that the temperature was room temperature. Each value represents the mean and standard deviation of at least two independent duplicate assays.

types II, III, V, XI) exhibited high relative affinities for YadA. Within collagen type I, the α1(I) chain also bound to YadA to a degree comparable to other fibril-forming collagens. Moreover, collagen type II is a homotrimer of three α1(II) chains, whereas the type XI molecule consists of three different chains (α1, α2, α3), of which only the α3(XI) chain was shown to bind YadA in the affinity blot experiments. There is some evidence that the α3(XI) chain and the α1(II) chain are encoded by the same gene, with minor differences due to posttranslational modifications (11, 36). Therefore, it could well be that collagen types II and XI interact with YadA via the same binding site, which is conserved in both evolutionarily closely related molecules. It might be speculated that the interaction site remained conserved during the evolution of the collagen family from an ancestral 54-bp unit (30) in collagens exhibiting YadA binding. Whether the binding site for YadA on collagen type IV is homologous to the YadA binding site on collagen type II is currently under investigation. Collagen types VI and IX, which bound considerably less well to YadA, belong to distinct classes of the collagen supergene family (28).

Further experiments are required to localize precisely the interaction sites on collagens and on YadA to answer the question of whether collagen binding by YadA contributes to the pathogenicity of yersiniae. Binding of YadA immuno-complexes to collagen type I, a major component of the extracellular matrix of the skin, could be implicated in the induction of erythema nodosum, a skin affliction that is frequently caused by *Y. enterocolitica*. In this study we have demonstrated that the cartilage collagen types II and XI specifically bind YadA. Whether binding to cartilage-restricted collagens may be linked to the arthritogenic potential of enteropathogenic yersiniae remains to be investigated.

We have provided structural data on collagen-YadA interactions that may stimulate further studies, including in vivo studies with collagen peptides for modulation of *Yersinia*-induced experimental animal diseases.

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