Identification of *Porphyromonas gingivalis* Components That Mediate Its Interactions with Fibronectin

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Porphyromonas (Bacteroides) gingivalis W12 binds and degrades human plasma fibronectin. In the presence of the protease inhibitor *N*- α -*p*-tosyl-L-lysyl chloromethyl ketone, *P. gingivalis* cells accumulated substantial amounts of ¹²⁵I-fibronectin as a function of incubation time. Fibronectin binding was specific, reversible, and saturable. The *K_d* for the reaction was estimated to be on the order of 100 nM, and there was an average of 3.5×10^3 fibronectin binding sites per cell. Unlabeled fibronectin inhibited the binding of ¹²⁵I-fibronectin to bacteria; however, fibrinogen was an even more efficient inhibitor of ¹²⁵I-fibronectin binding. Unrelated proteins were without effect on fibronectin binding. A fibronectin-binding component (*M_r*, 150,000) was identified in sodium dodecyl sulfate-solubilized *P. gingivalis*. Fibronectin was degraded into discrete peptides by *P. gingivalis* W12. The degradation of fibronectin was inhibited by *N*- α -*p*-tosyl-L-lysyl chloromethyl ketone. Two *P. gingivalis* components (*M_r*s, 120,000 and 150,000) degraded fibronectin in substrate-containing gels following sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In a previous study (M. S. Lantz, R. D. Allen, T. A. Vail, L. M. Switalski, and M. Hook, *J. Bacteriol.* 173:495–504, 1991), we found that the same strain of *P. gingivalis* bound and subsequently degraded human fibrinogen via apparently distinct cell surface components of molecular sizes similar to those of components now implicated in the binding and degradation of fibronectin. These results raise the possibility that the two ligands are recognized and modified by the same components on *P. gingivalis* W12. In support of this hypothesis, unlabeled fibrinogen effectively inhibited the binding of ¹²⁵I-fibronectin to bacteria and blocked ¹²⁵I-fibronectin binding to a *P. gingivalis* ligand-binding component (*M_r*, 150,000) immobilized on a nitrocellulose membrane.

Fibronectin is a multifunctional dimeric adhesive protein (molecular mass, ~440 kDa) found on cell surfaces, in extracellular matrices, and in body fluids, such as plasma (23). It is believed to play an important role in cell-matrix interactions by serving as a substrate for eukaryotic cell adhesion and migration. Fibronectin binds specifically to a number of extracellular matrix molecules, including collagens, glycosaminoglycans, proteoglycans, fibrinogen, and fibrin (see reference 25 for a recent review). Kuusela first reported that a bacterium, *Staphylococcus aureus*, can bind fibronectin (11), and it has since been proposed that the binding of bacterial cells to fibronectin may represent a mechanism of tissue adherence (32). Fibronectin has been reported to bind to a variety of gram-positive and gram-negative bacteria, including strains of *S. aureus* (2, 5, 11, 24, 26), coagulase-negative staphylococci (33), group A, C, and G streptococci (20, 29, 32), *Salmonella enteritidis* (1), *Escherichia coli* (4), and *Treponema pallidum* (22). In some cases, bacterial binding to fibronectin has been shown to be mediated by specific receptor proteins located on the surface of bacterial cells (5, 22, 28, 36). Some bacteria, for example, *S. aureus* (8), *Streptococcus sanguis* (17), *T. pallidum* (36), and *E. coli* (4), have been shown to adhere to fibronectin-coated surfaces.

Fibronectin is also degraded by bacteria. A number of gram-negative pathogenic bacteria, including *Pseudomonas aeruginosa* (40), *Serratia marcescens* (19), and *Vibrio cholerae* (3), produce fibronectin-degrading enzymes. Wikstrom and Linde (38) concluded that fibronectin-degrading ability is

often associated with bacteria implicated in soft tissue destruction. They demonstrated fibronectinolytic activity in strains of *S. aureus*, *Staphylococcus epidermidis*, *Peptococcus prevotii*, *Clostridium sporogenes*, *Bacteroides asaccharolyticus*, *Bacteroides fragilis*, *Propionibacterium acnes*, and oral bacteria, such as *Prevotella intermedia* (*Bacteroides intermedius*), *Prevotella (Bacteroides) loescheii*, and *Porphyromonas (Bacteroides) gingivalis*. *P. gingivalis* has been strongly associated with periodontitis lesions and has been implicated as an etiologic agent of periodontal diseases (18). In periodontal diseases, loss of connective tissue and bone from around the roots of the teeth results in the formation of a chronically inflamed soft tissue pocket (6). These periodontal pockets are colonized by bacteria which, together with root surfaces and the tissue lining the pocket wall, are bathed in a plasma filtrate from the local vasculature called gingival crevicular fluid (37). *P. gingivalis* produces potent proteases (18) that may degrade plasma proteins in gingival crevicular fluid or host tissue proteins in periodontitis lesions. Several groups have reported that *P. gingivalis* strains degrade human fibronectin. Fibronectin-degrading activity diffuses through agar during the growth of *P. gingivalis* (38). In addition, fibronectin-degrading activity has been detected in extracts of sonically disrupted *P. gingivalis* cells (16, 21), and both soluble and extracellular vesicle-associated fibronectinolytic proteases have been recovered from bacterial culture medium following the growth of *P. gingivalis* (30). While Imai et al. (10) failed to detect the binding of soluble ¹²⁵I-fibronectin to *P. gingivalis*, Winkler et al. (39) suggested that *P. gingivalis* may adhere to fibronectin present in a basement membrane-like matrix. The possible importance of these interactions in the pathogenesis of

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periodontitis, coupled with our recent findings (13), which suggest that protein ligands can be effectively degraded after they are bound to *P. gingivalis*, prompted us to examine further the interaction between soluble fibronectin and *P. gingivalis*.

MATERIALS AND METHODS

Chemicals. Human fibronectin (lyophilized with sucrose and phosphate buffer; New York Blood Center) was reconstituted with water at room temperature (10 mg/ml final concentration) according to the manufacturer's instructions, dialyzed to phosphate-buffered saline (PBS; 0.13 M sodium chloride, 10 mM phosphate buffer, 0.02% sodium azide [pH 7.4]), and used without further purification. Fibronectin was labeled with ^{125}I by the chloramine T method (9) as previously described (15). The specific activity of the labeled fibronectin was estimated to be 4.2×10^6 cpm/ μg . Human fibrinogen (Kabi, Stockholm, Sweden) was purified and labeled with ^{125}I as previously described (15). The specific activity of the ^{125}I -fibrinogen was estimated to be 1.7×10^6 cpm/ μg . D fragments of fibrinogen were prepared as previously described (12). Plasminogen-free human fibrinogen was purchased from Sigma and used in substrate gels (zymograms) without further purification. The purity and structural integrity of unlabeled and labeled proteins were determined by polyacrylamide gel electrophoresis (PAGE) in sodium dodecyl sulfate (SDS) under reducing and nonreducing conditions by the method of Studier (31). The resolving gel was 7.5% polyacrylamide and was overlaid with a 4.8% stacking gel. Samples were dissolved in sample buffer containing 4% (wt/vol) SDS. After electrophoresis, gels were stained with Coomassie brilliant blue R, dried, and subjected to autoradiography with either Kodak X-Omat AR or Eastman Diagnostic Blue Brand film (Eastman Kodak Co., Rochester, N.Y.).

Bovine serum albumin (BSA), chicken egg albumin (ovalbumin), human α -acid glycoprotein (orosomucoid), human immunoglobulin G, bovine fetuin, Triton X-100, *N*- α -p-tosyl-L-lysyl chloromethyl ketone (TLCK), and dithiothreitol (DTT) were purchased from Sigma. Na^{125}I (specific activity, 15 mCi/ μg) was purchased from Amersham Corp. (Arlington Heights, Ill.). All other chemicals were of reagent grade and were obtained from commercial sources.

Bacteria. *P. gingivalis* W12 was grown from stock strains maintained in the Anaerobic Core Facility, University of Alabama School of Dentistry. Bacteria used in the experiments were stored and maintained as previously described (13). Bacteria used in the experiments were grown in complete basal anaerobic broth (34) at 37°C under anaerobic conditions. After growth to the early logarithmic phase (approximately 10^8 cells per ml after 16 h of growth), bacteria were harvested by centrifugation ($13,000 \times g$; 20 min), washed three times with PBS, and resuspended in the same buffer. Bacteria were counted with a Petroff-Hauser chamber, and suspensions were adjusted to contain 1×10^{10} cells per ml (optical density at 540 nm, ≈ 2.0) or 2×10^{10} cells per ml (optical density at 540 nm, ≈ 4.0) and stored at 4°C as previously described (14).

Binding of ^{125}I -fibrinogen and ^{125}I -fibronectin to bacteria. Binding of ^{125}I -labeled protein to *P. gingivalis* W12 was determined as previously described (13, 14). In brief, bacteria (2.5×10^8 to 2.0×10^9 cells) were incubated with 4.0×10^4 to 5.0×10^4 cpm of radiolabeled fibrinogen or fibronectin in PBS containing 0.1% BSA (PBS-BSA) plus other additives as required in a total volume of 0.5 ml. Incubations

were performed at 4, 22, or 37°C for 15 min to 3 h. At the conclusion of the incubation period, 400 μl of the incubation mixtures was placed in a BSA-coated tube and rapidly diluted with 3 ml of ice-cold PBS containing 0.02% BSA. After centrifugation ($1,300 \times g$; 15 min), the supernatants were aspirated and the radioactivity associated with the pelleted bacteria was measured in a gamma counter (LKB-Wallac, Turku, Finland). Radioactivity recovered from incubation mixtures containing no bacteria was considered background and was subtracted from that obtained from incubation mixtures containing bacteria. All assays were performed in triplicate unless otherwise noted. In some experiments, a strain of *B. fragilis* that does not bind fibrinogen or fibronectin was used as a control for the nonspecific binding of these iodinated proteins to bacteria. Nonspecific binding never exceeded 2% of the radioactivity added.

Specificity of binding of ^{125}I -fibronectin to bacteria. Bacteria (10^9 cells) were incubated with competing protein (100 $\mu\text{g}/\text{ml}$ final concentration) for 1 h at 4°C in the presence of 30 mM TLCK, after which ^{125}I -fibronectin was added and incubation was continued for an additional 1 h. The amount of radioactivity bound to bacteria in the presence of competing protein was determined as described above.

Reversibility of binding of ^{125}I -fibronectin to bacteria. Bacteria were incubated with ^{125}I -fibronectin for 1 h at 4°C in the presence of 30 mM TLCK, after which various amounts of unlabeled fibronectin were added to incubation mixtures and incubation was continued for an additional 10 min. The amount of ^{125}I -fibronectin that remained bound to bacteria at the end of the incubation period was determined as described above.

Identification of a ^{125}I -fibronectin-binding component from *P. gingivalis* W12. *P. gingivalis* components that bind ^{125}I -labeled ligands were identified as previously described (13). In brief, SDS-solubilized *P. gingivalis* components separated in 3 to 9% polyacrylamide gradient gels were electrotransferred to nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.) as described by Towbin et al. (35). Additional protein binding sites on the nitrocellulose membranes were blocked by overnight incubation at 4°C in PBS-BSA. The membranes were subsequently incubated at 25°C for 2 h with ^{125}I -fibrinogen or ^{125}I -fibronectin (approximately 10^6 cpm) in PBS-BSA containing 0.1% Tween 80 and washed exhaustively (six changes) in Tris-buffered saline (0.15 M NaCl in 0.01 M Tris-HCl [pH 7.5]) containing 0.1% Tween 80. Membranes were dried and subjected to autoradiography with Eastman Diagnostic Blue Brand film. Transferred proteins were visualized by staining the nitrocellulose membranes with amido black (Bio-Rad).

Degradation of ^{125}I -fibronectin by *P. gingivalis* W12. The extent of degradation of ^{125}I -fibronectin by *P. gingivalis* and the relative molecular weights of the fibronectin fragments produced were determined by SDS-PAGE on linear 3 to 9% gradient polyacrylamide gels (13) and by autoradiography as described above. Bacteria (2.5×10^8 cells) were incubated with ^{125}I -fibronectin (5×10^5 cpm) in PBS-BSA in a final volume of 0.5 ml. In some incubation mixtures, the protease inhibitor TLCK (30 mM final concentration) was included. Incubations were performed at 4, 22, and 37°C for 15 min to 3 h. At the conclusion of the incubation period, bacteria were collected by centrifugation ($1,300 \times g$; 15 min) and the supernatant fractions were saved for analysis. Bacteria with bound iodinated material were resuspended in 0.5 ml of PBS-BSA containing unlabeled fibrinogen (100 $\mu\text{g}/\text{ml}$ final concentration) and incubated for an additional 30 min at 4°C

to displace bound iodinated fibronectin or fibronectin fragments. At the end of this second incubation period, which was performed to prevent further proteolysis of ^{125}I -fibronectin during preparation of the samples for electrophoresis (see below), the bacteria were removed from the incubation mixture by centrifugation and ^{125}I -labeled material displaced from the bacteria by fibrinogen, as well as any iodinated material still bound to the bacteria, was analyzed by SDS-PAGE and autoradiography.

Identification of fibronectin-degrading components of *P. gingivalis* W12 by substrate gel electrophoresis. Substrate gels were used as previously described (13) to identify components of *P. gingivalis* W12 that degrade human fibronectin or fibrinogen. In brief, plasminogen-free human fibronectin or fibrinogen (1 mg/ml final concentration) was added to 3 and 9% acrylamide solutions prior to casting of the gel, and the protein was copolymerized with the gel. *P. gingivalis* components solubilized in 4% SDS (wt/vol) were mixed with sample buffer and subjected to SDS-PAGE. After electrophoresis, the gel was washed first with 2.5% Triton X-100 in distilled water, then with 2.5% Triton X-100 in PBS, and finally with PBS. The washing procedure was performed over a 1-h period to remove SDS. For activation of proteolytic enzymes, the washed gel was incubated in PBS or Tris-buffered saline containing 10 mM DTT for 16 h at 37°C. Finally, the gel was fixed in 50% methanol–7% acetic acid and stained with 0.23% Coomassie brilliant blue R in 30% methanol–5.8% acetic acid. Proteolytic enzymes active in the gel were visualized as clear bands against the blue background of the gel after destaining.

RESULTS

Binding of ^{125}I -fibronectin to bacteria. We examined ^{125}I -fibronectin binding to *P. gingivalis* W12 as a function of time and temperature and found that bacteria accumulated iodinated fibronectin only in the presence of TLCK, a protease inhibitor. In the absence of protease inhibitors, only about 3% of the ^{125}I -fibronectin added became associated with the cells over a 3-h incubation period at 4°C (Fig. 1). In the presence of TLCK, significant amounts of ^{125}I -fibronectin bound to bacteria. At 4°C, a maximum of 20% of the added ligand bound to the bacterial cells after a 1-h incubation period. This amount corresponds to approximately 2.4 ng of fibronectin bound per 2.5×10^8 bacterial cells. In a previous study, we found that these bacteria accumulated ^{125}I -fibrinogen at 4°C whether or not a protease inhibitor was included in the incubation mixtures (13).

We used SDS-PAGE and autoradiography to examine the effects of temperature and the presence of TLCK on the degradation of ^{125}I -fibronectin associated with the cells or recovered from the supernatant after incubations similar to those described above. The results indicated that in the absence of TLCK, most of the labeled fibronectin present in the incubation mixtures after 3 h at 4°C was found intact in the supernatant fraction (Fig. 2A, lane 2). Small amounts of fibronectin degradation products were also present. Only a relatively small proportion of the total ^{125}I -fibronectin was associated with bacteria (Fig. 2A, lane 3), and a substantial portion of the cell-associated fibronectin occurred as degradation products. When bacteria were incubated with ^{125}I -fibronectin at 22°C for 1 h, intact fibronectin as well as significant amounts of discrete fibronectin fragments were found in the supernatant fraction (Fig. 2A, lane 5), and small amounts of fibronectin degradation products were associated with bacteria (Fig. 2A, lane 6). After 3 h of incubation of

FIG. 1. Time dependence of binding of ^{125}I -fibronectin to *P. gingivalis* W12 in the absence of protease inhibitors (○) or in the presence of 30 mM TLCK (●) at 4°C. Bacteria (2.5×10^8 cells) and ^{125}I -fibronectin (5.0×10^4 cpm) were incubated for the indicated times, and the radioactivity associated with bacteria was determined as described in the text. The error bars represent the standard deviations (SD) of triplicate determinations and are shown only when the SD exceeded $\pm 5\%$.

bacteria with ^{125}I -fibronectin at 37°C, all ^{125}I -fibronectin in the incubation mixtures was degraded and fibronectin fragments were found exclusively in the supernatant fraction; no fragments were cell associated. The largest fragment found in the incubation medium had an M_r of 97,000, and a number of smaller fragments could be detected (data not shown).

When 10 mM TLCK (Fig. 2B) was added to the incubation mixtures, bacteria accumulated substantial amounts of ^{125}I -fibronectin during incubations at both at 4 and 22°C (Fig. 2B, lanes 3 and 6), as well as at 37°C (data not shown). The cell-associated ^{125}I -fibronectin was primarily in the dimeric form, although some monomers were also present. Since the fibronectin preparation added to the bacteria contained similar amounts of dimers and monomers, it seems that both forms of fibronectin can bind to the cells. Degradation of fibronectin could not be detected after the incubation mixtures were incubated at 4, 22, or 37°C (data not shown), and no fibronectin degradation products were present either in the incubation mixtures or associated with the cells. The optimal concentration of TLCK required to block fibronectin degradation varied somewhat with the proteolytic activity of the bacteria, which in turn varied among cell harvests and decreased during storage of the bacteria at 4°C. In general, TLCK was an effective inhibitor of fibronectin degradation in the range of 1 to 40 mM. Higher concentrations of TLCK reduced the amounts of ^{125}I -fibronectin bound to bacteria. When TLCK in the incubation mixtures was replaced with other protease inhibitors, such as phenylmethylsulfonyl fluoride or EDTA, ^{125}I -fibronectin did not accumulate on bacteria, nor did these substances at any concentrations tested inhibit the degradation of ^{125}I -fibronectin by bacteria (data not shown).

Characterization of fibronectin binding to bacteria. We examined the specificity of binding of fibronectin to *P. gingivalis* W12 by incubating bacteria with ^{125}I -fibronectin in the presence of a large (10^4 -fold) molar excess of unlabeled proteins as potential inhibitors. The proteins tested included

FIG. 2. Distribution of ^{125}I -fibronectin and its degradation products between *P. gingivalis* W12 cells and the incubation medium as a function of time, temperature, and the presence of TLCK. Bacteria (2.5×10^8 cells) were incubated for 3 h at 4°C (lanes 2, 3, and 4) or for 1 h at 22°C (lanes 5, 6, and 7) with 5.0×10^5 cpm of ^{125}I -fibronectin in PBS-BSA with no protease inhibitor (A) or 10 mM TLCK (B) in the incubation medium. At the conclusion of the incubations, bacteria were removed from the incubation mixtures by centrifugation ($1,300 \times g$; 15 min) and the supernatant fractions were saved for analysis by SDS-PAGE and autoradiography (lanes 2 and 5). Bacteria were resuspended in 0.5 ml of PBS-BSA containing 100 μg of unlabeled fibrinogen per ml, and incubation was continued at the same temperatures for an additional 30 min to displace bound ^{125}I -labeled material. It was necessary to displace bound iodinated material from bacteria to prevent additional pro-

TABLE 1. Specificity of binding of ^{125}I -fibronectin to *P. gingivalis* W12^a

Competing protein	Relative ^{125}I -fibronectin bound to bacteria (%)
None	100.0 $\pm$ 1.4
Ovalbumin	101.9 $\pm$ 9.7
α -Acid glycoprotein	95.8 $\pm$ 10.6
Human immunoglobulin G	80.6 $\pm$ 7.1
Fetuin	84.6 $\pm$ 9.9
Fibrinogen D fragments	103.8 $\pm$ 11.7
Fibrinogen	5.8 $\pm$ 1.1
Fibronectin	45.1 $\pm$ 7.1

^a Bacteria (10^9 cells) were incubated in the presence of competing protein (100 $\mu\text{g}/\text{ml}$) for 1 h, after which ^{125}I -fibronectin (5×10^4 cpm) was added to the incubation mixture. The amount of ^{125}I -fibronectin that bound to the bacteria was determined as described in the text. Data are presented as mean percentages $\pm$ standard deviations for four replicate samples.

ovalbumin, α -acid glycoprotein, immunoglobulin G, fetuin, fibrinogen D fragments, fibrinogen, and fibronectin. Unlabeled fibronectin as well as unlabeled fibrinogen inhibited the binding of ^{125}I -fibronectin to bacteria, whereas other proteins were without effect (Table 1). In fact, unlabeled fibrinogen appeared to be a more effective inhibitor of ^{125}I -fibronectin binding than did unlabeled fibronectin. The effect of fibrinogen on ^{125}I -fibronectin binding to *P. gingivalis* could be the consequence of competition between the two proteins for the same or closely spaced cellular binding sites. Alternatively, the bacterial binding site on fibronectin for *P. gingivalis* could overlap with the fibrinogen binding site on fibronectin or be influenced by fibrinogen binding to fibronectin. The effect of unlabeled proteins on the binding of ^{125}I -fibrinogen to *P. gingivalis* W12 at 4°C is shown in Table 2. Only unlabeled fibrinogen, not fibronectin or any of the other tested proteins, significantly reduced ^{125}I -fibrinogen binding to bacteria.

Binding of ^{125}I -fibronectin to *P. gingivalis* W12 was at least partially reversible, since greater than 60% of bound iodinated fibronectin was rapidly displaced by unlabeled fibronectin (Fig. 3). Unlabeled fibrinogen could also displace bound ^{125}I -fibronectin and its degradation products from *P. gingivalis* W12 (Fig. 2A and B, lanes 3, 4, 6, and 7). When increasing amounts of ^{125}I -fibronectin were added to bacteria, the amount of bound ^{125}I -fibronectin increased to a maximum of 0.97 μg per 2.5×10^8 bacterial cells at a fibronectin concentration of 50 μg per incubation. Further increases in the ^{125}I -fibronectin concentration did not result in increased binding of fibronectin to bacteria (Fig. 4A). These data suggest that bacteria possess a limited number of fibronectin binding sites. Analysis of the binding data by the Scatchard method (27) (Fig. 4A, inset) suggested a single binding site and an apparent K_d for the reaction of 98 nM. For comparison, the concentration-dependent binding of

teolytic processing of the ligand during preparation of samples for electrophoresis. At the end of this final incubation, bacteria were again collected by centrifugation and ^{125}I -labeled material displaced from bacteria by fibrinogen (lanes 3 and 6), as well as ^{125}I -labeled material still remaining with bacteria (lanes 4 and 7), was analyzed by SDS-PAGE and autoradiography. Electrophoresis was performed at 4°C , and ^{125}I -fibronectin (5.0×10^4 cpm) starting material is shown in lane 1. Arrows and numbers on the left side indicate the migration distances and molecular sizes (in daltons) of standard proteins.

TABLE 2. Specificity of binding of ^{125}I -fibrinogen to *P. gingivalis* W12^a

Competing protein	Relative ^{125}I -fibrinogen bound to bacteria (%)
None	100.0 $\pm$ 7.5
Ovalbumin	109.6 $\pm$ 4.7
α -Acid glycoprotein	92.5 $\pm$ 14.1
Human immunoglobulin G	114.1 $\pm$ 10.4
Fetuin	115.2 $\pm$ 15.1
Fibrinogen D fragments	121.1 $\pm$ 2.5
Fibrinogen	14.5 $\pm$ 3.4
Fibronectin	112.7 $\pm$ 9.1

^a Bacteria (10^9 cells) were incubated in the presence of competing protein (100 $\mu\text{g}/\text{ml}$) for 1 h, after which ^{125}I -fibrinogen (5×10^4 cpm) was added to the incubation mixture. The amount of ^{125}I -fibrinogen that bound to the bacteria was determined as described in the text. Data are presented as mean percentages $\pm$ standard deviations for four replicate samples.

^{125}I -fibrinogen to *P. gingivalis* was examined under the same conditions (4°C, incubation for 1 h) with the same batch of bacteria (Fig. 4B). A maximum of 0.75 μg of fibrinogen bound to 2.5×10^8 bacterial cells at a fibrinogen concentration of 30 μg per incubation. Analysis by the Scatchard method (27) (Fig. 4B, inset) suggested a single binding site and an apparent K_d of 10 nM, in good agreement with the K_d value calculated for this interaction with a different strain of *P. gingivalis* (14). At saturation, 2.5×10^8 bacteria bound 2.2 pmol of each protein, corresponding to approximately 3,500 fibronectin or fibrinogen binding sites per cell, assuming that all sites are occupied at saturation.

Identification of a fibronectin-binding component of *P. gingivalis* W12. In attempts to identify fibronectin-binding components in *P. gingivalis*, bacterial components were solubilized in SDS and fractionated by PAGE in SDS. *P. gingivalis* components in the gel were electroblotted onto a nitrocellulose membrane, unoccupied protein binding sites

FIG. 3. Reversibility of binding of ^{125}I -fibronectin to *P. gingivalis* W12. Bacteria (2.5×10^8 cells) were incubated with ^{125}I -fibronectin (5.0×10^4 cpm) in the presence of 30 mM TLCK for 1 h at 4°C, after which a large excess of unlabeled fibronectin (up to 100 $\mu\text{g}/\text{ml}$ final concentration) was added and incubation was continued for an additional 10 min. ^{125}I -fibronectin associated with bacteria was determined as described in the text. The error bars represent the standard deviations (SD) of triplicate determinations and are shown only when the SD exceeded $\pm 5\%$.

FIG. 4. Saturation kinetics for binding of fibronectin (A) and fibrinogen (B) to *P. gingivalis* W12. Bacteria (2.5×10^8 cells) were incubated with increasing amounts of ^{125}I -protein (specific activity, 2.0×10^4 cpm/ μg) at 4°C for 1 h (20 mM TLCK). The amount of ^{125}I -protein bound to bacteria was determined as described in the text. The insets show Scatchard analyses of the binding data (B/F, bound protein/free protein). Each value is the average of three determinations.

were blocked by exposure to BSA, and the membrane was incubated in a solution containing ^{125}I -fibronectin. After being extensively washed, the membrane was dried and ^{125}I -fibronectin bound to the membrane was detected by autoradiography. A fibronectin-binding component (M_r , 150,000) was identified by this technique (Fig. 5). A fibrinogen-binding component of the same relative size was demonstrated when a nitrocellulose membrane containing electroblotted *P. gingivalis* components was probed with ^{125}I -fibrinogen (Fig. 5), consistent with our previous data (13).

To study the binding specificity of the M_r -150,000 *P. gingivalis* component(s), we conducted a series of experiments in which strips of nitrocellulose membrane containing *P. gingivalis* components were incubated with various unlabeled proteins and washed extensively prior to exposure to ^{125}I -labeled ligand (Fig. 6). An unrelated protein, ovalbumin,

FIG. 5. Identification of a fibronectin-binding component of *P. gingivalis* W12 by autoradiography. Bacteria (10^9 cells) were solubilized in 4% SDS, and bacterial components were fractionated by SDS-PAGE on 3 to 9% gradient gels under nonreducing conditions. After electrophoresis, separated components were transferred to nitrocellulose membranes as described in the text. Nitrocellulose membranes were incubated overnight in PBS-BSA to block additional binding sites and then incubated with either ^{125}I -fibronectin (HFn) or ^{125}I -fibrinogen (HFbg) (10^6 cpm) for 2 h at 22°C as described in the text. ^{125}I -protein-binding components were visualized by autoradiography. Arrows and numbers on the left indicate the migration distances and molecular sizes (in daltons) of standard proteins. The arrow and number on the right indicate the migration distance and the calculated relative molecular weight of the ligand-binding component.

did not interfere with the binding of ^{125}I -fibronectin or ^{125}I -fibrinogen to the binding component(s). On the other hand, both unlabeled fibronectin and fibrinogen effectively blocked the binding of ^{125}I -fibronectin to an M_r -150,000 component (Fig. 6B). Unlabeled fibrinogen effectively inhibited the binding of ^{125}I -fibrinogen to the nitrocellulose strips;

however, a large excess of unlabeled fibronectin was unable to block the binding of ^{125}I -fibrinogen to an M_r -150,000 component (Fig. 6A).

Identification of fibronectin-degrading components of *P. gingivalis* W12. Zymography was tried in attempts to identify fibronectin-degrading *P. gingivalis* components. In this approach, bacterial components solubilized by SDS are fractionated in a polyacrylamide gel containing copolymerized fibronectin. After the SDS is removed, the gel is incubated in a buffered DTT solution to activate proteases. The gel is finally fixed and stained. The presence of an active fibronectin-degrading component is indicated by a clearing zone in the gel. Two fibronectin-degrading components (M_r s, 150,000 and 120,000) were identified by this technique (Fig. 7). Activation of these enzymes was absolutely dependent on the treatment of the gel with a reducing agent, since no

FIG. 6. Competition of fibronectin and fibrinogen for an M_r -150,000 *P. gingivalis* component. Conditions of the experiment were essentially the same as those used for the experiment shown in Fig. 5, except that the nitrocellulose membranes were incubated for 2 h at 22°C with PBS-BSA [(-)], 100 μg of human fibrinogen per ml (Fbg), 100 μg of human fibronectin per ml (Fn), or 100 μg of ovalbumin per ml (Ov) and washed exhaustively (five changes) with Tris-buffered saline containing 0.1% Tween 80 prior to being probed with ^{125}I -fibrinogen (^{125}I -HFbg) (A) or ^{125}I -fibronectin (^{125}I -HFn) (B). Arrows and numbers on the left indicate the migration distances and molecular sizes (in daltons) of standard proteins.

FIG. 7. Identification of fibronectin-degrading components of *P. gingivalis* W12 by SDS-PAGE in substrate gels. *P. gingivalis* W12 cells (5×10^8) were solubilized in 4% SDS and fractionated by electrophoresis in fibrinogen (HFbg)- or fibronectin (HFn)-containing polyacrylamide gradient gels as described in the text. Washed gels were incubated for 16 h in the presence (left lanes) or absence (right lanes) of 10 mM DTT, stained with Coomassie brilliant blue R, and destained to reveal bands in which proteolysis occurred. Arrows and numbers on the left indicate the migration distances and molecular sizes (in daltons) of standard proteins. Arrows and numbers on the right indicate the migration distances and calculated relative molecular weights of the two major ligand-degrading components.

fibronectin degradation was detected when the reducing agent was omitted during the development of the zymogram. Proteolytic enzymes of similar sizes degraded fibrinogen copolymerized in polyacrylamide gels (Fig. 7), as previously described (13).

DISCUSSION

We examined the ability of *P. gingivalis* W12 to bind soluble fibronectin and found that substantial amounts of fibronectin did not accumulate on *P. gingivalis* cells in the absence of TLCK, a protease inhibitor. Fibronectin binding by these bacteria in the presence of TLCK occurred at 4, 22, and 37°C. Further characterization of fibronectin binding was performed at 4°C in the presence of TLCK and was found to be specific, at least partially reversible, and saturable. The apparent K_d for the reaction was determined to about 100 nM, similar to the K_d for the binding of fibronectin to *T. pallidum* (36). Fibronectin and fibrinogen, but no other unrelated proteins, were able to inhibit the binding of fibronectin to *P. gingivalis*, and unlabeled fibrinogen was a more effective inhibitor of ^{125}I -fibronectin binding to bacteria than was unlabeled fibronectin. Fibrinogen was also able to displace cell-associated fibronectin and fibronectin degradation products from *P. gingivalis* W12. A fibronectin-binding component (M_r , 150,000) was identified on nitrocellulose

membranes containing SDS-PAGE-fractionated *P. gingivalis* components after incubation of the membranes with ^{125}I -fibronectin and autoradiography. Both unlabeled fibronectin and unlabeled fibrinogen were able to block the binding of ^{125}I -fibronectin to an M_r -150,000 *P. gingivalis* component.

We found that *P. gingivalis* produced enzymes capable of degrading fibronectin, in agreement with previous reports (16, 21, 30, 38). Fibronectin degradation was time and temperature dependent, and some fibronectin lysis was observed even at 4°C. Fibronectin was degraded into discrete peptides by *P. gingivalis* W12, and fibronectin degradation was completely inhibited by TLCK. Fibronectin lysis appeared to be mediated at least in part by thiol-dependent proteases with M_r s of 150,000 and 120,000. We cannot rule out the possibility that other *P. gingivalis* proteases are also involved in fibronectin degradation, since zymography will only permit the detection of enzymes that can be activated after SDS treatment (7). When TLCK was added to zymograms along with DTT, the degradation of fibronectin was inhibited (data not shown).

Previous studies in our laboratory showed that *P. gingivalis* W12 binds and degrades human fibrinogen (13, 14) and that these interactions appear to occur at the cell surface. Fibrinogen binding, like fibronectin binding, was found to be rapid, reversible, and specific. However, the affinity of *P. gingivalis* W12 for fibrinogen appears to be approximately 10-fold higher than that for fibronectin (apparent K_d s of 10 and 98 nM, respectively). Fibrinogen and fibronectin both bind to a *P. gingivalis* component (M_r , 150,000). Fibronectin lysis was observed at 4°C, but fibrinogen lysis was not (13). In this sense, fibronectin appears to be a somewhat better substrate for the *P. gingivalis* proteases than is fibrinogen. Fibronectin degradation and fibrinogen degradation appear to be mediated at least in part by thiol-dependent proteases with M_r s of 120,000 and 150,000. TLCK inhibits the degradation of both ligands by these proteases in zymograms. In addition, TLCK blocks the degradation of both protein ligands by bacteria, resulting in the enhanced accumulation of nondegraded ligands by the cells.

The results of this study and a previous study (13) suggest that fibrinogen and fibronectin may be bound and degraded by the same components on *P. gingivalis*. This hypothesis is supported by the observations that fibrinogen blocks the binding of fibronectin to bacteria, that fibrinogen can displace fibronectin and fibronectin fragments bound to *P. gingivalis*, and that fibrinogen and fibronectin can block the binding of ^{125}I -fibronectin to an M_r -150,000 *P. gingivalis* component. The failure of unlabeled fibronectin to inhibit the binding of fibrinogen to intact bacteria or to an immobilized *P. gingivalis* fibrinogen-binding component (M_r , 150,000) was unexpected. The approximately 10-fold-higher affinity of fibrinogen for the bacteria may be a contributing factor; however, these observations may indicate a complex set of interactions of these ligands with *P. gingivalis*. For example, fibrinogen binding to the bacterial binding component may structurally or conformationally alter the binding component so that fibronectin is no longer recognized. In this model, fibronectin binding would not prevent the subsequent recognition of fibrinogen by the binding component. Further studies will be needed to sort out these possibilities.

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