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Characterization of an Adherence and Antigenic Determinant of the ArgI Protease of *Porphyromonas gingivalis* Which Is Present on Multiple Gene Products

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This study was performed to characterize the antigen(s) recognized by a panel of monoclonal antibodies (MAbs) produced to be specific for *Porphyromonas gingivalis* whole cells which we had previously shown to bind to epitopes recognized by sera from periodontitis patients. Preliminary data had suggested that the arginine-specific proteases of *P. gingivalis* (ArgI, ArgIA, and ArgIB) contained the antigenic determinants of four of these antibodies (MAbs 1A1, 2B/H9, 7D5, and 3B1). The location of the binding sites was examined with purified *P. gingivalis* enzymes and recombinant regions of the ArgI polyprotein expressed by subclones of the *prpR1* gene in *Escherichia coli* XL-1 Blue cells. All four antibodies were reactive with protein determinants within the β subunit, a hemagglutinin and/or adhesin component, of the ArgI dimer. MAb 1A1 strongly inhibited the agglutination of human erythrocytes by *P. gingivalis* W50 culture supernatant, suggesting that the binding site for this antibody contains residues which are critical for the interaction with the erythrocyte surface. The determinant for MAb 1A1 was examined further by construction of a set of truncated forms of the β component expressed as fusion proteins with glutathione *S*-transferase at the N terminus. Analysis of these constructs mapped the binding site for MAb 1A1 to PrpRI residues G-907 to T-931, GVSPKVCKDV TVEGSNEFAP VQNLT. Western blot (immunoblot) analysis of *P. gingivalis* whole-cell proteins demonstrated that MAb 1A1 reacts with several proteins in the M_r range of 20,000 to 120,000. Furthermore, an oligonucleotide probe corresponding to the coding sequence for the region of the ArgI β component containing the MAb 1A1 binding site hybridized to multiple bands on genomic digests of *P. gingivalis* DNA. These data indicate that the MAb 1A1 epitope may be a component of a binding domain common to multiple gene products of this organism and may thus represent a functionally important target of the host's specific immune response to *P. gingivalis* in periodontal disease.

Porphyromonas gingivalis is now recognized as an important etiological agent in adult periodontal disease—one of a group of chronic inflammatory conditions of the tooth-supporting tissues (35). Patients with a history of destructive disease frequently demonstrate an elevated immunoglobulin G (IgG) antibody response to this organism in assays of both serum and the inflammatory exudate at the site of the disease (10, 15, 40). Although specific IgG antibody is likely to be important in the control of the products of this and other organisms in the subgingival environment, the consequences of an IgG-mediated defensive strategy at this mucosal surface may also lead to host tissue damage (9, 21). Hence, the precise role of this antibody in the etiology of the disease is still uncertain. Studies in this area would be facilitated by a better understanding of the nature of the challenge presented to the host by the extracellular antigens of plaque microorganisms in order to estimate the efficacy of the specific immune response in their control.

Identification of the major surface and extracellular antigens of *P. gingivalis* has been pursued by several investigators. Usually this has involved the estimation of the IgG antibody response of periodontal patients' sera to individual components of complex mixtures of cell surface and extracellular proteins resolved by gel electrophoresis (7, 38). An alternative strategy was employed as part of a recent study by Cridland et al. (5) in which the ability of periodontal patients' sera to block the binding of a panel of monoclonal antibodies (MAbs) to *P. gingivalis* whole cells was examined. Five antibodies were found to bind to antigenic determinants recognized by periodontal patients' sera. Preliminary characterization studies demonstrated that four of these MAbs recognized an extracellular protease preparation which had been fractionated by arginine affinity chromatography.

Biochemical investigations of the extracellular and cell-associated proteases of *P. gingivalis* suggest that in addition to putative roles in nutrition, these activities may have other diverse functions in the survival strategy of the organism. For example, cell-bound protease activity of *P. gingivalis* is thought to play a significant role in evasion of the inflammatory cell-based host defenses in the periodontal pocket via the proteolytic elimination of opsonins at the bacterial cell surface (8, 33). Furthermore, it has been proposed that the adherence of *P. gingivalis* to erythrocytes (6, 25, 29), fibrinogen (19), fibronectin (18), collagenous substrata (24), and other bacteria (11) is mediated, at least in part, by proteases at the cell surface. Moreover, there is now increasing evidence to suggest that the proteases of this organism, particularly those with specificity

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TABLE 1. Characteristics of plasmids used in this study

Plasmid	Characteristics	Source
pJM2	5.2-kb *Sau*3A fragment of *P. gingivalis* W50 DNA in *Bam*HI site of pUC18, including incomplete *prpR1* gene coding for residues M-1 to N-1526 of PrpRI	Aduse-Opoku et al. (1)
pJM7	6.0-kb *Sau*3A fragment of *P. gingivalis* W50 DNA in *Bam*HI site of pUC18 including *tla* gene (3.29-kb open reading frame)	Aduse-Opoku et al. (1)
pSpL	5′ truncation of pJM2 to nucleotide 2067 coding for residues M-488–N-1526 of PrpRI	Aduse-Opoku et al. (1)
pKpL	3′ truncation of pJM2 from nucleotides 3473–5205 coding for residues M-1–T-949	Aduse-Opoku et al. (1)
pJM11	1.1-kb *Sau*3A fragment of *P. gingivalis* W50 DNA in *Bam*HI site of pUC18; an internal fragment of *prpR1* gene coding for PrpRI residues D-784–V-1130 under *lac* promoter	Aduse-Opoku et al. (1)
pMC11	1.1-kb *P. gingivalis* W50 DNA insert from pJM11 in pGEX-3X expressing PrpRI residues D-784–V-1130 as a fusion protein with GST under *tac* promoter	This study
pMC11-K	3′ truncation of pMC11 to *Kpn*I site expressing PrpRI residues D-784–T-949 as a GST fusion protein	This study
pMC11-A	3′ truncation of pMC11 to *Age*I site; PrpRI residues D-784–T-931 as a GST fusion protein	This study
pMC11-N	3′ truncation of pMC11 to *Ngo*MI site; PrpRI residues D-784–G-907 as a GST fusion protein	This study
pMC11-E	3′ truncation of pMC11 to *Eag*I site; PrpRI residues D-784–P-803 as a GST fusion protein	This study
pUC18	Cloning vector, Amp^r *lacI*	Yanisch-Perron et al. (42)
pGEX-3X	Expression vector, Amp^r $lacI^q$	Pharmacia

for arginine- and lysine-containing peptide bonds have the potential to contribute directly to the inflammatory disease process through direct complement activation and C5a generation (41), high-molecular-weight kininogen (34) and prekallikrein activation, and bradykinin release (12). Hence, the efficiency of the specific immune response to these activities may have a critical role in determining the severity of disease associated with this organism.

Recently, the major components of the extracellular protease activity of *P. gingivalis* W50 with specificity for arginine-containing peptide bonds have been characterized by biochemical analysis (32) and shown to comprise three interrelated forms: ArgI, ArgIA, and ArgIB. ArgI is a heterodimeric species composed of α and β components which have similar molecular weights (~50,000) and migrate to the same position by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). ArgIA is a monomer of the free α chain and is indistinguishable from the dimeric form by SDS-PAGE. In contrast, ArgIB is a highly posttranslationally modified form of the α chain which migrates in the M_r 70,000-to-80,000 region of SDS-PAGE. The gene for ArgI (*prpR1* [protease polyprotein for ArgI]) has been cloned from *P. gingivalis* W50 (1), and sequence analysis demonstrates that the α and β components are contiguous on the initial translation product and are flanked by large N- and C-terminal extensions. Similar data have been reported for the *rgp-1* gene of *P. gingivalis* H66 (28) and 381 (26) and the *prtR* gene of W50 (16). These molecular characterization studies have enabled a more detailed analysis of the identity of the antigenic determinants recognized by the MAbs described above. In the present report, we describe the localization of these antibody binding sites and the characterization of one epitope which plays a significant role in the agglutination of human erythrocytes by *P. gingivalis* and which appears to be a common motif of multiple gene products of this organism.

MATERIALS AND METHODS

Bacteria and culture conditions. *P. gingivalis* and *Porphyromonas asaccharolytica* were maintained by subculture on blood agar. The following strains were employed: *P. gingivalis* W50, W50Br1, W50Be1, W83, LB13D3, A7436, 381, 33277, and 11834 and *P. asaccharolytica* ATCC 25260. Broth cultures for enzyme purification were grown in brain heart infusion medium supplemented with hemin (5 mg $liter^{-1}$) in an atmosphere of 80% N_2, 10% CO_2, and 10% H_2 at 37°C for 48 h. Cultures for whole-cell protein immunoblots were harvested in mid-log phase and either acidified with formic acid (final concentration, 5% [vol/vol]) or treated with 10 mM EDTA at 60°C for 30 min (7) to prevent proteolytic degradation of cellular proteins and sonicated with an MSE Soniprep 150. After centrifugation at 14,000 × *g*, the supernatants were freeze-dried and stored at −20°C prior to solubilization in SDS-PAGE reducing sample buffer for electrophoresis. *Escherichia coli* XL-1 Blue cells were grown on LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) supplemented with 20 mg of tetracycline ml^{-1}. Ampicillin was added to 50 mg ml^{-1} for plasmid selection.

Protease purification of ArgI. ArgI, ArgIA, and ArgIB were prepared from *P. gingivalis* W50 culture supernatants after precipitation with 85% ammonium sulfate by a combination of gel filtration, affinity chromatography on arginine agarose, and ion exchange as described elsewhere (1, 32). Purified enzyme preparations were stored in 50 mM sodium acetate buffer (pH 5.3). In the case of ArgIB, 0.05% detergent (Zwitterionic 3-14) was included in all solutions throughout the purification and storage in order to maintain solubility.

Recombinant protein expression. The location of the antibody binding sites was determined by immunochemical analysis of different regions of the ArgI gene (*prpR1*) expressed in *E. coli* XL-1 Blue cells. The plasmids used in this study are shown in Table 1. pJM2, pJM11, and pJM7 were isolated during the original cloning of the gene from *Sau*3A digests of *P. gingivalis* W50 DNA in pUC18. pJM2 contains a 5.2-kb insert which incorporates a 4.6-kb incomplete open reading frame corresponding to PrpRI residues M-1 to N-1526. The α and β components of ArgI are located within this sequence at PrpRI residues Y-228 to R-1260. pJM11 contains a fragment of the *prpR1* gene corresponding to an internal region of the β component (approximately 40 kDa) at PrpRI residues D-784 to V-1130 in frame with the *lacZ a* peptide. pJM7 contains a 6.5-kb *P. gingivalis* DNA insert incorporating the TonB-like adhesin gene (*tla*) (2). The deduced translation of *tla* shares a region of sequence identity (Tla protein residues V-304 to N-768) with the β component of ArgI between PrpRI residues T-917 to K-1384. Protein expression in both JM2 and JM7 is under the control of *P. gingivalis* promoters within the cloned insert, whereas expression in JM11 is under vector control and is inducible by addition of IPTG (isopropyl-β-D-thiogalactopyranoside). JM2 subclones KpL and SpL were generated by 3′ truncation of the pJM2 insert with the *Kpn*I and *Sph*I restriction digests, respectively.

Finer mapping of the binding site for MAb 1A1 was examined by expression of JM11 as a fusion protein with glutathione *S*-transferase (GST). The pJM11 insert was transferred from the pUC18 cloning vector into pGEX-3X by restriction digestion with *Xba*I and *Sma*I, followed by filling in of the *Xba*I site with Klenow fragment and deoxy-nucleoside triphosphate (dNTP) and ligation into the *Sma*I site of the pGEX-3X multiple cloning site to generate in-frame constructs. Transformed *E. coli* cells were screened for expression of the fusion protein by dot blotting of lysed cells onto nitrocellulose and immunochemical staining with rabbit polyclonal antisera specific for GST (Sigma Chemical Co.) and the PrpRI β component. Clone MC11 produced a recombinant protein with a size of approximately 66 kDa which was reactive with both antisera, and this clone was selected for the production of a set of 3′ truncation derivatives with unique restriction sites within the JM11 insert. pMC11 was initially digested with either *Kpn*I, *Age*I, *Ngo*MI, or *Eag*I and then with *Eco*RI at the residual multiple cloning site at the 3′ end of the insert prior to filling-in reactions with Klenow fragment and dNTP. Ion-exchange chromatography (Wizard Matrix; Promega) was used to purify the DNA samples after each reaction. The blunt-ended pMC11 derivatives were gel purified and self-ligated (Ready-To-Go; Pharmacia). Transformed *E. coli* XL-1 Blue cells were screened initially by dot blotting with anti-GST antiserum, and the predicted size of the recombinant protein was confirmed by SDS-PAGE and Western blotting (immunoblotting). All restriction and modification enzymes were purchased from Pharmacia, except for *Age*I and *Ngo*MI, which were obtained from New England Biolabs.

Antibodies and immunochemistry. *E. coli* cells used for the immunochemical

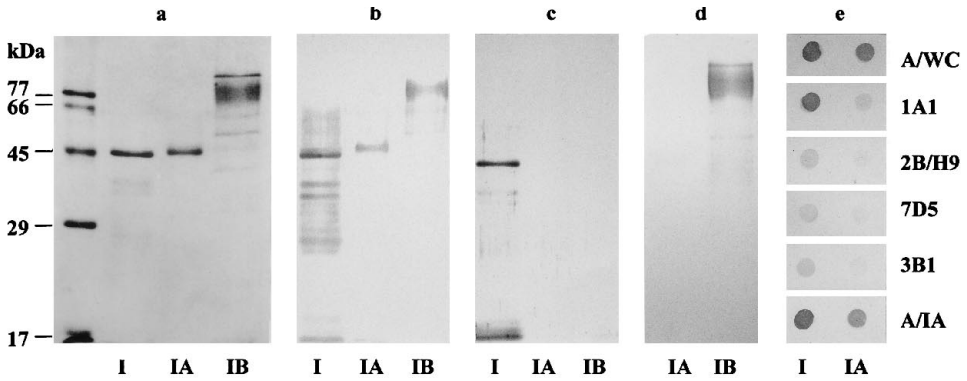

FIG. 1. Immunoreactivity of ArgI (I), ArgIA (IA), and ArgIB (IB) of *P. gingivalis* W50. (a) Coomassie blue-stained SDS-PAGE (12.5% polyacrylamide) gels with 2 mg of protein for each enzyme. (b to d) Western blots of the enzymes with rabbit (Rb) anti-ArgIA (b), MAb 1A1 (c), and MAb 6B3 (d). (e) Dot blots of ArgI and ArgIA with Rb anti-*P. gingivalis* W50 whole cells (A/WC), Rb anti-IA, and MAb 1A1, 2B/H9, 7D5, and 3B1. Numbers on the left-hand axis indicate migration positions of standard molecular mass markers.

detection of recombinant protein were harvested by centrifugation and lysed in 1/10 the original culture volume in 3% SDS. Dot blots on nitrocellulose were prepared with a Biodot apparatus (Bio-Rad). Cell lysates corresponding to 2 ml of the original culture were applied to each well.

For the analysis of purified enzymes from *P. gingivalis*, equivalent amounts of each preparation (approximately 0.2 mg on the basis of optical density at 280 nm and staining intensity with Coomassie blue on SDS-PAGE gels) were applied. The membranes were blocked in 5% bovine serum albumin in phosphate-buffered saline (BSA-PBS) for 30 min, and the primary antibodies were applied at the appropriate dilution in 1% BSA-PBS for a minimum of 2 h. After a wash in PBS, antibody binding was detected with a horseradish peroxidase-conjugated anti-species antiserum (DAKO) at 1:500 and color developed with diaminobenzidine. Cell lysates of *E. coli* XL-1 Blue cells transformed with either pUC18 or pGEX-3X were used as the control antigen. SDS-PAGE was performed as described by Laemmli (17) with 10 or 12.5% acrylamide-bisacrylamide gels. Western blots were prepared by electroblotting of samples onto nitrocellulose for 2 h at 400 mA in pH 9.9 carbonate-bicarbonate transfer buffer. In general, the sample preparation, loadings, and immunochemical detection used for Western blotting were identical to those described above for the dot blotting procedures. For Coomassie blue-stained gels, the amount of sample applied to each lane was increased by 10-fold.

The production of the MAbs used in this study was described previously (5). MAb detection of antigens was performed with dilutions of the hybridoma supernatants in 1% BSA-PBS. However, the ability of individual MAbs to block *P. gingivalis* culture supernatant-mediated hemagglutination was examined with purified Ig after chromatography of the hybridoma supernatants on protein A. Polyclonal antisera specific for *P. gingivalis* W50 whole cells (A/WC) and ArgIA (A/IA) were prepared in hybrid-lop Dutch rabbits as previously described (1).

Analysis of synthetic peptides. The location of the determinants of the antibodies was also investigated by examining their binding to synthetic peptides corresponding to different regions of the PrpRI. Overlapping peptides were synthesized on membranes in a 96-well microtiter plate format with a SPOTS kit (Genosys, Cambridge, United Kingdom), which employs fluorenylmethoxycarbonyl amino acid chemistry. The membrane was then probed with the individual MAbs by the same immunochemical procedure described above for the dot blot analyses.

Hemagglutination. The hemagglutinating activity of the culture supernatant of *P. gingivalis* W50 was assayed with washed human erythrocytes in complement fixation diluent (CFD [Oxoid]). Doubling dilutions of the culture supernatant were made in CFD from 1/2 to 1,024 in a total volume of 50 μl in microtiter plates. To each well, a further 50 μl of CFD and 50 μl of 0.5% erythrocytes were added, and the plates were incubated for 2 h at room temperature. When an agglutinating titer had been established (1/240 dilution), the effect of the purified MAbs was examined. The antibodies were double diluted from 100 μg/ml and incubated with four times the minimum hemagglutinating dose of culture supernatant for 1 h at room temperature prior to the addition of the erythrocytes and then incubated for a further 2 h. Hemagglutination inhibitory titers were then expressed as the minimum concentration of antibody required for inhibition. A purified mouse IgG1 MAb (Sigma) was used as the control.

Southern hybridization. Southern blot analyses were performed to determine whether the nucleotide sequence coding for the region within the β component recognized by MAb 1A1 is present elsewhere on the chromosome in addition to the *prpR1*. Bacterial chromosomal DNA was digested to completion and after fractionation in agarose was depurinated in 0.25 M HCl. The fragments were then transferred to Hybond N^+ membrane (Amersham) under alkaline conditions (0.4 M NaOH) in accordance with the manufacturer's instructions with a vacuum blotter (Hybaid). The membrane was prehybridized for 1 h at 42°C in Rapid Hyb (Amersham) prior to hybridization for 2 h in the same solution with ^{32}P-labelled oligonucleotide 1A1C (5′-CGGTAGAAGGATCCAATGAATTT GCTCCTGTACAGAACC-3′) corresponding to PrpRI residues T-917 to N-929. Membranes were then washed twice for 15 min in 2× standard saline citrate containing 0.1% SDS at 42°C. 1A1C was end labelled with T4 polynucleotide kinase and Redivue [γ-^{32}P]dATP (3,000 mCi/mmol [Amersham]) in One Phor All Plus buffer (Pharmacia). The reaction was stopped by addition of EDTA (pH 8) to 25 mM, and unincorporated dATP was removed by passage over a NAP-5 column (Pharmacia). Autoradiography was performed with Kodak X-Omat-LS films at −70°C with intensifying screens for 1 to 18 h.

RESULTS

MAb recognition of *P. gingivalis* proteases. The ArgI, ArgIA, and ArgIB preparations from *P. gingivalis* W50 which were used throughout this investigation are shown in Fig. 1a. The immune recognition of these enzymes was assessed by Western and dot blotting. Polyvalent rabbit antiserum raised against ArgIA recognized all three proteases, indicating that the α chains of each enzyme are immunochemically related (see Fig. 6). However, MAbs 1A1, 2B/H9, 7D5, and 3B1 recognized only the dimeric ArgI (Fig. 1c and e), suggesting that they are specific to determinants present on the β chain of this enzyme. In addition, MAb 6B3, which binds to *P. gingivalis* lipopolysaccharide (LPS) preparations (5), recognized only ArgIB (Fig. 1d), indicating that the posttranslational modifications to this form include structural elements common to LPS.

Periodate sensitivity of MAb binding sites. The binding of MAbs 1A1 and 2B/H9 to multiple bands of *P. gingivalis* cell sonicates on Western immunoblots (e.g., see Fig. 5) led us to examine whether the binding sites for these antibodies corresponded to epitopes on either noncovalently associated carbohydrate-lipid or common posttranslational modifications rather than peptide determinants. Treatment of *P. gingivalis* W50 sonicates on nitrocellulose dot blots with either 0.2 or 1% sodium periodate in acetic acid failed to reduce the binding of MAbs 1A1, 2B/H9, 7D5, and 3B1. In contrast, a substantial reduction in the binding of MAb 6B3—anti-LPS—to periodate-treated cell sonicates was observed (data not shown). These data were therefore consistent with the recognition of antigenic determinants within the protein sequence of the β chain of ArgI, and this was confirmed by analysis of the recognition of recombinant PrpRI expressed in *E. coli.*

Immunochemical analysis of recombinant products of the *prpR1* gene of *P. gingivalis*. The immune recognition of recombinant protein derived from *E. coli* expression of different regions of *prpR1* was examined by dot blot analysis (Fig. 2). All four MAbs recognized the recombinant product of pJM2

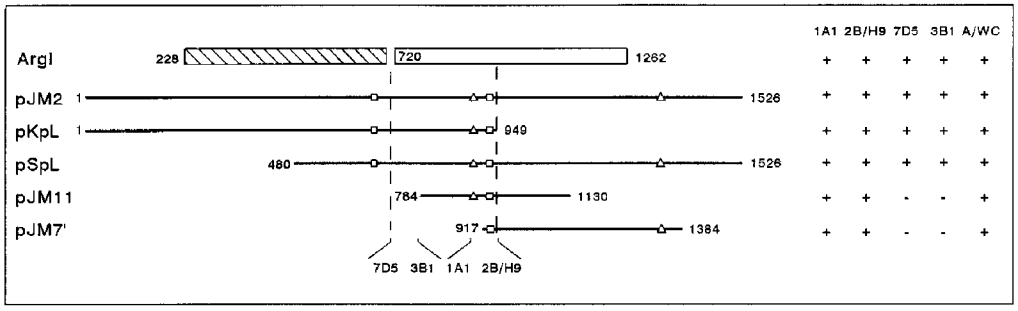

FIG. 2. Immune recognition of ArgI and recombinant regions of the ArgI polyprotein expressed by subclones of the *prpR1* gene in *E. coli* XL-1 Blue cells. Solid horizontal lines represent regions of PrpRI expressed by each clone, and numbers refer to PrpRI residues. pJM7′ represents a region of Tla which is homologous to PrpRI T-917 to K-1384. Peptide repeats are shown as open boxes (G-676 to P-686 and G-936 to P-946) and open triangles (D-865 to V-918 and D-1322 to V-1375). Vertical dashed lines indicate the region of PrpRI which contains determinants for all four MAbs on the basis of the immunoreactivity of ArgI (but not ArgIA) and the product of pKpL. Brackets at the bottom of the figure indicate mapped positions of the 7D5-3B1 and 1A1-2B/H9 binding sites. The table to the right shows the presence (+) or absence (−) of immunoreactivity of each clone with each antibody.

(PrpRI residues M-1 to N-1526), which contains a cloned *P. gingivalis* DNA insert including the coding region for the α and β chains of PrpRI (PrpRI residues Y-228 to R-1262). Similarly, the product of pKpL (residues M-1 to T-949), which was generated by deletion of 1.9 kb from the 3′ end of pJM2 via *Kpn*I digestion, was recognized by all four MAbs. In addition, the product of pSpL, which was generated by deletion of a 2.2-kb *Sph*I(multiple cloning site)-*Sph*I(insert) fragment from the 5′ end of the JM2 sequence was reactive with these MAbs. We have previously reported that transcription from this plasmid is from an internal transcription site on the insert which is recognized by the *E. coli* transcription machinery (1). The precise translation start site in pSpL has not been determined to date, but for the purposes of these mapping studies, we have used the predicted product of the entire insert (PrpRI residues M-480 to N-1526). On the basis of these subcloning experiments, we were able to locate the antibody binding sites of 1A1, 2B/H9, 7D5, and 3B1 at residues M-480 to T-949 on the polyprotein. Since all four MAbs appear to recognize the β component of ArgI, the antigenic determinants must lie downstream of the α component on the polyprotein at S-720 to T-949.

Further clarification of the binding sites was obtained with JM11, the recombinant product of which corresponds to an internal region of the PrpRI β chain (residues D-784 to V-1130). JM11 was only reactive with MAbs 1A1 and 2B/H9. Therefore, the binding sites for these MAbs are located within D-784 to T-949, and the 7D5 and 3B1 epitopes are N terminal to this region between S-720 and D-784 on the β chain (Fig. 2).

A final description of the binding sites of 1A1 and 2B/H9 in these experiments was obtained by examination of the immunoreactivity of a related *P. gingivalis* gene product, Tla (TonB-linked adhesin) in JM7. An internal region of Tla displays 99% identity to the PrpRI between residues T-917 and K-1384, and this is aligned with the PrpRI derivatives in Fig. 2. Only 1A1 and 2B/H9 were immunoreactive with JM7. Since the immunoreactivity of these MAbs with KpL indicates that they recognize sites upstream of PrpRI T-949, these data suggest that the determinants for 1A1 and 2B/H9 are within PrpRI T-917 to T-949. However, a 53-residue sequence upstream from here at D-865 to V-918, which forms part of the peptide repeat on PrpRI (shown as a triangle in Fig. 2), is also present in the Tla sequence toward the C terminus of the region of identity. Hence, recognition of determinants within this repeat sequence would also explain the immunoreactivity of 1A1 and 2B/H9 with JM7. Consequently, on the basis of these data, the binding sites for these two antibodies were mapped to within

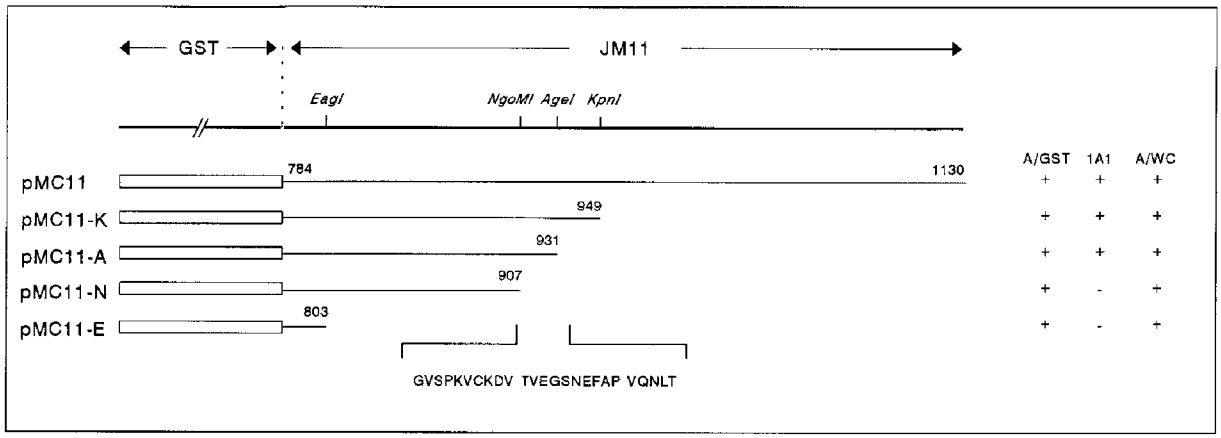

FIG. 3. Immunoreactivity of progressive truncations of an internal region of the ArgI β component expressed as a GST fusion protein. The bold horizontal line represents the restriction map of the internal fragment of *prpR1* coding for residues D-784 to V-1130 (JM11) in pGEX-3X showing unique sites used to generate progressive truncations. Numbers refer to PrpRI residues. The table to the right shows the presence (+) or absence (−) of immunoreactivity with anti-GST antiserum (A/GST), MAb 1A1, and A/WC.

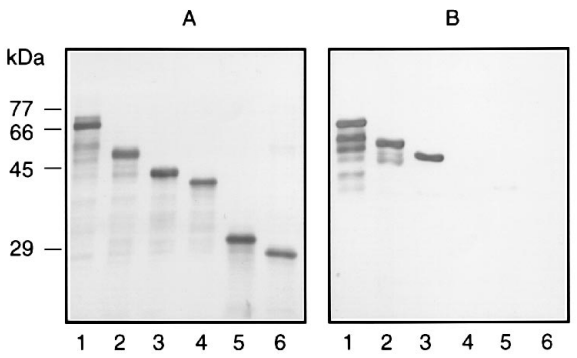

FIG. 4. Western blot analysis of truncations of internal regions of the ArgI β component expressed as a GST fusion protein. (A) Rabbit anti-GST antiserum. (B) MAb 1A1. Lanes 1 to 6 contain lysates of *E. coli* XL-1 Blue cells harboring the following: 1, pMC11; 2, pMC11-K; 3, pMC11-A; 4, pMC11-N; 5, pMC11-E; and 6, pGEX-3X.

PrpRI D-865 to T-949, thereby incorporating the peptide repeat.

Hemagglutination inhibition by MAbs. On the basis of the primary sequence of the β chain, we have proposed that this polypeptide may have a role in attachment and binding processes, particularly to human erythrocyte surfaces. Therefore, the ability of these MAbs to block hemagglutination mediated by *P. gingivalis* cells and culture supernatants was examined. MAb 1A1 was found to be inhibitory at low concentrations. With culture supernatant at four times the minimum hemagglutinating dose, the MICs were 1 μg/ml for MAb 1A1, 16 μg/ml for 2B/H9, and 35 μg/ml for 7D5 and 3B1. No inhibition of hemagglutination by the control IgG MAb was observed in the same dose range.

Immunochemical analysis of overlapping synthetic peptides. The location of the binding site for MAb 1A1 was investigated further by synthesis of a series of overlapping peptides which spanned the internal region of the ArgI β component corresponding to the reactive recombinant protein expressed by JM11 (PrpRI D-784 to V-1130). Two series of peptides either 8 or 12 amino acid residues in length were prepared, and each series was offset by 2 or 3 residues respectively. MAb 1A1 failed to bind any of the synthetic peptides, and hence we were unable to localize the binding site for this antibody by this method.

Localization of the MAb 1A1 binding site. The binding site was further investigated by 3′ truncation of the pJM11 insert and analysis of recombinant protein immunoreactivity. In order to provide a readily detectable marker of protein expression, the pJM11 insert was first transferred into pGEX-3X to generate MC11, which produced a fusion protein of JM11 with GST at the N terminus. The experiments described above with subclones of *prpR1* and the *tla* gene had mapped the 1A1 binding site to PrpRI residues D-865 to T-949. Consequently, derivatives of the GST-JM11 fusion protein which spanned this region were prepared by deletion of fragments (with sizes of 543, 597, 669, and 803 bp) from the 3′ end of the insert by using the unique sites for *Kpn*I, *Age*I, *Ngo*MI, and *Eag*I, respectively, and the *Eco*RI site in the residual multiple cloning site. The resulting proteins fused to GST at the N terminus corresponded to PrpRI residues D-784 to T-949, D-784 to T-931, D-784 to G-907, and D-784 to P-803 (Fig. 3).

Western blot analysis with anti-GST antiserum of the resultant clones confirmed the predicted protein size. Immunoreactivity with MAb 1A1 was preserved in the deletions up to and including the *Age*I derivative (D-784 to T-931) but lost after truncation to the *Ngo*MI site (D-784 to G-907) (Fig. 4). These data therefore indicated that the determinant for this antibody was located within the amino acid sequence GVSPKVCKDV TVEGSNEFAP VQNLT (PrpRI G-907 to T-931).

Immunochemical analysis of *P. gingivalis* whole-cell proteins. The finding that MAb 1A1 was able to block the hemagglutinating activity of whole-culture supernatants and cell sonicates led us to examine whether other *P. gingivalis* proteins, in addition to the ArgI β component, are recognized by this antibody. Whole-cell sonicates of commonly used laboratory strains were analyzed by Western blotting. In all cases, numerous proteins in the molecular weight range 20,000 to 120,000 were reactive with MAb 1A1 (Fig. 5). To exclude the possibility that this complex banding pattern reflected proteolytic degradation of immunoreactive protein(s) during the sample preparation, we examined the same *P. gingivalis* cell sonicates via Western blotting with MAbs specific for two other unrelated *P. gingivalis* proteins: a 55-kDa outer membrane antigen and a 47-kDa cytosolic protein (22). In both cases, only a single immunoreactive species corresponding to the intact protein was detected (Fig. 5, lanes 1 to 3), indicating that proteolytic processing during sample preparation was not a significant factor. (Only the data for *P. gingivalis* W50 are shown.)

Southern blot hybridization of *P. gingivalis* chromosomal DNA digests with an oligonucleotide probe specific for the MAb 1A1 epitope. Further evidence that the binding site for the MAb 1A1 is present on multiple gene products of *P. gingivalis* was obtained via Southern blot analysis of chromosomal DNA of the strains used in the Western blotting. *Sma*I digests of genomic DNA were probed with an oligonucleotide (oligo 1A1C) corresponding to the coding sequence for a region of the PrpRI β component which contains the determinant for MAb 1A1. The mapping studies presented earlier had indicated that the MAb 1A1 epitope is located within PrpRI G-907 to T-931, and oligo 1A1C was designed to be specific for an internal region within this sequence at T-917 to N-929. As anticipated, oligo 1A1C hybridized to a 3.2-kb *Sma*I fragment in all strains corresponding to the coding region for the α and β components of PrpRI (Fig. 6). In addition, two further hybridizing fragments in the size range 8 to 10 kb were identified

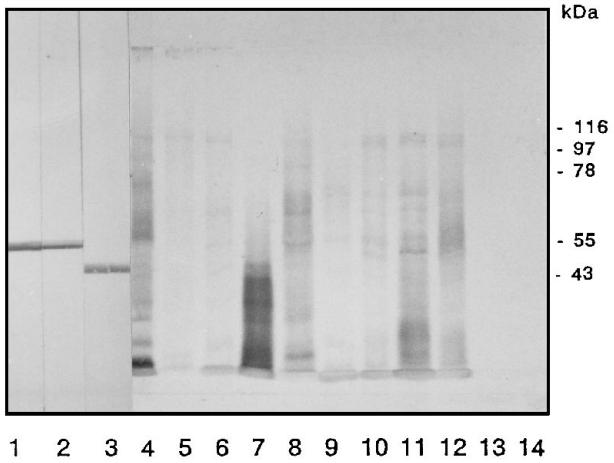

FIG. 5. Western blot analysis of whole-cell sonicates of *P. gingivalis* with MAb 1A1. Cells from 16-h cultures were heat inactivated at 60°C in EDTA for 30 min prior to sonication and electrophoresis of 1 mg of total protein per lane. Lanes: 1 to 4, *P. gingivalis* W50; 5, W50Be1; 6, W50Br1; 7, W83; 8, LB13D3; 9, A7436; 10, 381; 11, 33277; 12, 11834; 13, *P. asaccharolytica* ATCC 25260; 14, *E. coli* XL-1 Blue. Lanes 1 to 3 were developed with control MAbs specific for unrelated *P. gingivalis* proteins (see text).

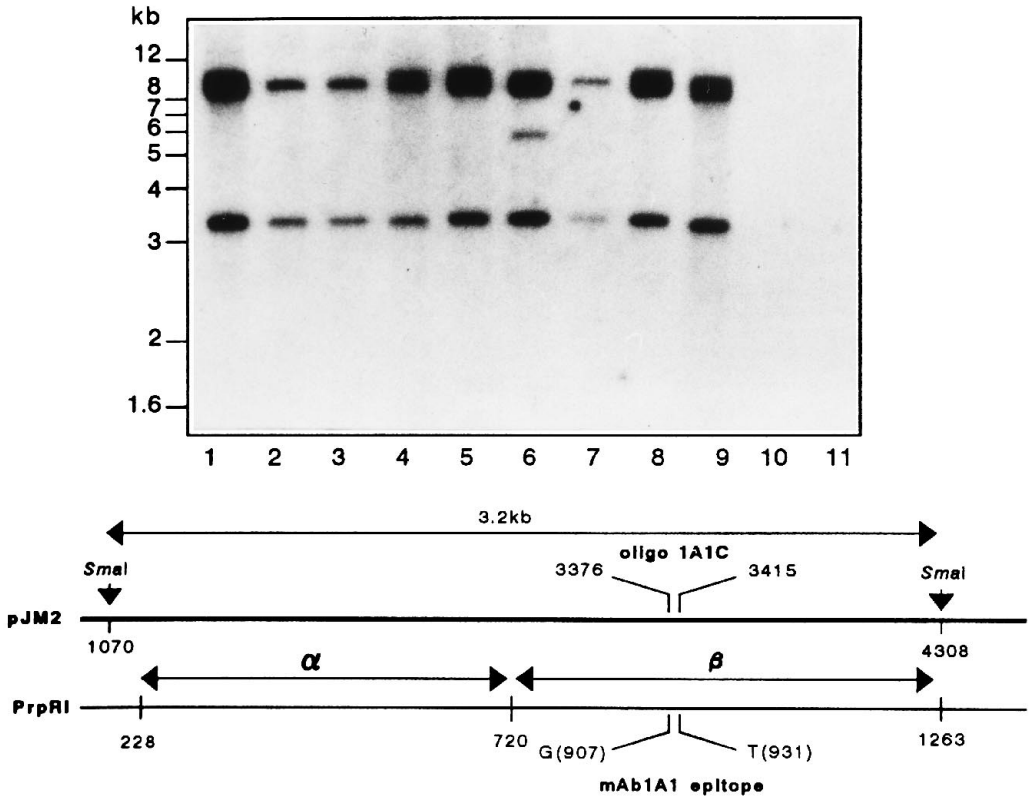

FIG. 6. Southern blot hybridization analysis of *P. gingivalis* chromosomal DNA with oligonucleotide 1A1C. Bacterial DNA was digested with *Sma*I, resolved by agarose gel electrophoresis, and probed with ^{32}P-oligo 1A1C. The region of PrpRI containing the MAb 1A1 (mAb1A1) epitope is indicated on the lower line. The corresponding nucleotide sequence on the pJM2 insert which was used to generate 1A1C is shown on the upper, bold line. Lanes 1 to 11 are the same as lanes 4 to 14 in Fig. 5. Numbers to the left of the figure refer to the positions of standards.

in *P. gingivalis* W50, W83, LB13D3, A7436, 33277, and 11834. In strains W50Be1, W50Br1, and 381, a single homologous band in this size range was detected. The distribution of these loci in *P. gingivalis* W50 was examined in greater detail by restriction of the DNA with a variety of endonucleases (Fig. 7). In all digests, a minimum of three separate hybridizing fragments were detected, confirming that the coding sequence for the MAb 1A1 epitope occurs at multiple discrete loci on the *P. gingivalis* chromosome.

DISCUSSION

The aim of this investigation was to determine the identity and functional properties of the antigen(s) reactive with a panel of MAbs specific for *P. gingivalis*, whose binding sites are also targets of the host immune response in periodontal disease. Our preliminary characterization studies had indicated that the epitopes were present on an extracellular antigen(s) which was retained on arginine affinity chromatography resins and which displayed proteolytic activity against arginine-containing peptide bonds (5). We have recently demonstrated that the extracellular Arg-X protease activity of *P. gingivalis* comprises three interrelated enzymes: ArgI, ArgIA, and ArgIB. ArgI is the only form which binds to arginine affinity resins and is a heterodimer containing an α component which bears the protease active site and a β component which shows some primary sequence similarity to adhesins and/or hemagglutinins from other microorganisms. The ability to resolve the three forms of enzyme together with the recent cloning and sequencing of the gene for ArgI (*prpR1*) facilitated the identification of the determinants of these antibodies. Thus, by examination of the immunoreactivity of ArgI, ArgIA, and ArgIB, purified from *P. gingivalis* culture supernatants, and of recombinant proteins derived from expression of different regions of the *prpR1* gene in *E. coli* cells, we have established that binding sites for all four MAbs are located on the β subunit of the ArgI dimer.

The physiological functions of this component of the ArgI

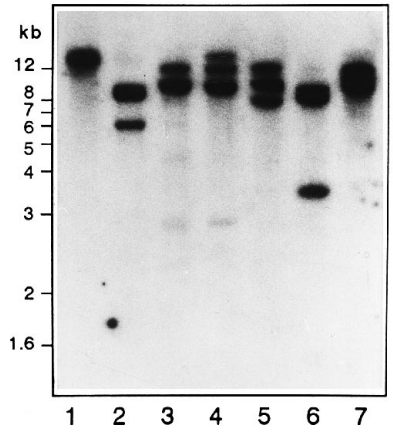

FIG. 7. Southern hybridization analysis of *P. gingivalis* W50 chromosomal DNA probed with oligo 1A1C. The bacterial DNA was digested to completion with *Bgl*II (lane 1), *Bss*HII (lane 2), *Bst*EII (lane 3), *Eco*RV (lane 4), *Hin*dIII (lane 5), *Sma*I (lane 6), and *Sph*I (lane 7).

protease have not been established. However, the aforementioned sequence similarity to other microbial adhesins and the frequently reported association of proteases of this organism in the hemagglutination reaction suggest a role in binding processes which may thereby target the action of the catalytic subunit of the enzyme. The identities of the natural ligands for this binding are still under investigation. However, estimation of the ability of these MAbs to block hemagglutination by *P. gingivalis* afforded a means of assessing their effects on the overall adherence properties of this polypeptide and related gene products. The finding that MAb 1A1 was able to block hemagglutination by whole-culture supernatants of *P. gingivalis* suggests that the epitope recognized by this MAb or an overlapping or adjacent region in the native protein is a critical binding determinant. Consequently, natural antibody with this specificity in periodontal patients colonized by *P. gingivalis* may be functionally important in the control of this organism in vivo.

On the basis of the immunoreactivity of recombinant PrpRI protein, we mapped the binding site for MAb 1A1 to PrpRI G-907 to T-931. The first 12 residues of this sequence, G-907 to V-918, form part of a major peptide repeat in PrpRI (D-865 to V-918 and D-1323 to V-1375), and repeat sequences in microbial proteins are frequently implicated in binding processes, particularly in binding to extracellular matrix proteins (39). We were unable to map the binding site of MAb 1A1 beyond these coordinates, since overlapping synthetic peptides corresponding to the sequence of the ArgI β component were not reactive with MAb 1A1. Furthermore, a synthetic peptide, PrpRI (G-907 to T-931), did not block the binding of MAb 1A1 to *P. gingivalis* whole cells, nor was it able to interfere with hemagglutination by *P. gingivalis* culture supernatants (data not shown). There may be a number of explanations for these observations. It is possible that the binding site for MAb 1A1 is a discontinuous epitope which includes another region(s) of the β component: the sequence G-907 to T-931 contains a half-cystine residue at C-913 which may be involved in a disulfide bond with another half-cystine elsewhere in the molecule. Alternatively, MAb 1A1 may bind to a sequence determinant within G-907 to T-931, the conformation of which is critically dependent on other regions of the polypeptide chain and which is probably distorted in the linear peptides used in this investigation.

A number of proteins with hemagglutinating properties from *P. gingivalis* have been described. These include an outer membrane hemagglutinating activity (3), culture supernatant hemagglutinins devoid of proteolytic activity (13, 27) or with proteolytic activity (6, 25, 29), and a hemagglutinating adhesin from *P. gingivalis* ATCC 33277 (23) which forms a complex with the surface fimbriae of this strain (4). In addition, Progulske-Fox et al. have cloned and expressed two separate hemagglutinins, HagA (30) and HagB (31), from *P. gingivalis* 381. On the basis of these reports, it appears that agglutination of erythrocytes by *P. gingivalis* may be a complex multifactorial process involving multiple bacterial components. Conversely, in the present investigation, the ability of low concentrations of MAb 1A1 to inhibit hemagglutination by *P. gingivalis* whole-culture supernatants suggested that the determinant of this antibody on the ArgI β component represents a critical determinant of the whole process. We considered two possible explanations for this apparent contradiction. First, the molar concentration of the ArgI protease may be far higher than that of the other bacterial products with hemagglutinating potential in the *P. gingivalis* cultures used in this study. Interference in the binding of the ArgI β component to the erythrocyte surface would thereby have a large effect on the entire hemagglutination reaction. Second, the epitope recognized by MAb 1A1 may be a common binding determinant of different hemagglutinins or gene products of this organism. Data from both Western blotting of whole-cell sonicates and Southern hybridization with an oligonucleotide probe to an internal region of the MAb 1A1 epitope supported the latter proposal. These observations are therefore consistent with previous Southern blot hybridization analyses of *P. gingivalis* genomic DNA digests which demonstrated that elements of the coding region for the ArgI β component are present at multiple loci on the bacterial chromosome. The TonB-linked adhesin cloned from *P. gingivalis* W50 (2), which was used in the mapping studies in the present investigation, represents one of these distinct products. Two other recently cloned and sequenced genes whose products are involved in hemagglutination also share this sequence: the lysine-specific protease Lys-gingipain from *P. gingivalis* H66, which has a molecular organization similar to that of the arginine-specific enzyme with a catalytic domain in association with a hemagglutinin domain (29, 29a), and HagA of *P. gingivalis* 381, which contains four tandem repeats of a hemagglutinin domain, all of which contain the G-907-to-T-931 PrpRI motif (29b).

These data lend further support to the proposal that the proteases and related gene products of *P. gingivalis* may act as a family of adhesins distinct from other surface components of this organism with more specialized adherence function. *P. gingivalis* fimbriae appear to mediate adherence to oral epithelial cells and salivary pellicle-coated tooth surfaces. For example, peptides derived from the deduced sequence of the fimbrillin monomer (20), a structural subunit of the fimbriae, and monoclonal antibodies specific for fimbriae (14) are able to inhibit adherence of *P. gingivalis* to human buccal epithelial cells. However, since not all clinical *P. gingivalis* isolates are fimbriated (36, 37), it is likely that other attachment strategies are employed. Whether adherence phenomena associated with the β component of the PrpRI and related proteins are complementary to fimbria-mediated attachment or fulfill a separate role in, for example, nutrient acquisition, or whether there is a temporal separation in the expression of these adherence mechanisms in vivo during the course of initial colonization and subsequent maintenance of the infection in the colonized host requires further investigation.

Our earlier investigations had indicated that the determinants for these MAbs may also be targets for the host's immune response in adult periodontitis. In the case of the binding site for MAb 1A1, the current studies suggest that an effective host immune response to this determinant would be predicted to have a significant effect on the binding and/or attachment processes of the whole organism. Further characterization of the structural determinants involved and the physiological targets of this abundant binding domain should help clarify this aspect of the host-parasite interaction in periodontal disease associated with *P. gingivalis.*

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