Characterization of the *Streptococcus mutans* P1 Epitope Recognized by Immunomodulatory Monoclonal Antibody 6-11A

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Monoclonal antibody (MAb) 6-11A directed against *Streptococcus mutans* surface adhesin P1 was shown previously to influence the mucosal immunogenicity of this organism in BALB/c mice. The specificity of anti-P1 serum immunoglobulin G (IgG) and secretory IgA antibodies and the subclass distribution of anti-P1 serum IgG antibodies were altered, and the ability of elicited serum antibodies to inhibit *S. mutans* adherence in vitro was in certain cases increased. MAb 6-11A is known to recognize an epitope dependent on the presence of the proline-rich region of the protein, although it does not bind directly to the isolated P-region domain. In this report, we show that MAB 6-11A recognizes a complex discontinuous epitope that requires the simultaneous presence of the alanine-rich repeat domain (A-region) and the P-region. Formation of the core epitope requires the interaction of these segments whose binding depends on the presence of this domain (9). MAb 6-11A is known to recognize an epitope dependent on the presence of the proline-rich repeat domain (A-region) and the P-region. Formation of the core epitope requires the interaction of these segments whose binding depends on the presence of this domain (9).

* S. mutans has been implicated as a major etiologic agent of human dental caries (19, 34). The ~185,000-M, surface protein of *S. mutans* serotype c organisms is widely believed to mediate adherence to the salivary tooth pellicle and is variously referred to in the literature as antigen I/II (51), antigen B (52), P1 (15), and PAc (41). Data supporting a role for humoral immunity against human dental caries has been reported for many years. Immunization with P1 or parts thereof (18, 32, 54, 57, 67) or with *S. mutans* whole cells (8, 31) has been shown to prevent *S. mutans* adherence in vitro and colonization of the tooth surface and development of dental caries in animal models. Passive immunization studies with immunoglobulin G (IgG) antibodies against antigen I/II have also been shown to prevent caries in humans (34a) and nonhuman models (33).

As reviewed by Jenkinson and Demuth (23), the proteins of the antigen I/II family have all similar sizes (1,500 to 1,566 amino acids) and contain an amino-terminal signal sequence, a series of alanine-rich tandem repeats within the amino-terminal third of the molecule, a 150-residue variable region where most sequence variations between the P1 and PAc sequences are clustered (10), a series of proline-rich tandem repeats in the central portion of the molecule, and a carboxy-terminal sequence characteristic of wall- and membrane-spanning domains of streptococcal surface proteins, including the LPXTG motif involved in cell wall anchorage (53). A schematic representation of P1 is shown in Fig. 1. Members of the antigen I/II family are produced by most species of oral streptococci (23) and comprise multiple ligand binding sites (24). Discrete regions within these proteins are believed to interact with host tissue components, including salivary glycoproteins, calcium, collagen, laminin, keratin, fibronectin, and other microbial cells, and certain of these interactions appear to involve complex nonlinear structures (10, 17). A panel of MAbs was previously generated against P1 (5), and the binding sites of 11 unique MAbs were approximated based on reactivity with full-length and truncated P1 polypeptides (9, 10, 14, 49).

The immunomodulatory MAb 6-11A is one of four anti-P1 MAbs that do not bind directly to the isolated P-region but whose binding depends on the presence of this domain (9). The immunomodulatory effects of MAb 6-11A vary depending on the route of mucosal immunization and on the coating concentration of the antibody (12). Coating *S. mutans* with anti-P1 MAb 6-11A prior to mucosal immunization of mice results in notable changes in the specificity and subclass distribution of serum IgG antibodies. The specificity of the mucosal secretory IgA antibody response is similarly influenced by this MAb (50). Sera from mice immunized by gastric intubation with bacteria coated with MAb 6-11A are more inhibitory of *S. mutans* adherence to human salivary agglutinin than those
from mice immunized with bacteria alone, indicating that changes in the antibody response are associated with changes in potential biological activity.

Serum IgG antibodies against P1 from mice immunized with S. mutans and S. mutans were captured with MAb 6-11A, a segment necessary for the structural integrity, stability, and surface expression of the molecule (9). These sera are not reactive with the isolated P-region (12), again suggesting the involvement of this domain in the formation of complex epitopes not achieved within many partial recombinant P1 polypeptides (9, 50, 59).

The binding of MAb 6-11A to P1 on the surface of S. mutans alters P1’s susceptibility to proteolytic digestion in vitro (50). This suggests that a change of protein conformation and hence potential differences in antigen processing and presentation may contribute to the immunomodulatory effects of this MAb and influence the spectrum of antibodies elicited during a polyvalent response (29, 36, 38, 55). The location and physical character of the epitope for MAb 6-11A would be expected to yield information regarding the potential mechanism(s) of immunomodulation by MAb 6-11A. This work demonstrates that both the A- and P-regions are necessary to reconstitute the epitope recognized by MAb 6-11A. A segment of P1, consisting of amino acid residues 84 to 190, which is directly upstream of the A-region, was also demonstrated to contribute to the complex discontinuous epitope recognized by this MAb.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** Serotype c S. mutans strain NGS was used as previously described (50). Escherichia coli host strains DH5α, JM109, and TOP10 were grown aerobically at 37°C with vigorous shaking in Luria-Bertani broth (LB; 1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 1% [wt/vol] NaCl) supplemented with ampicillin (50 to 100 μg/ml). Plasmid pCR2.1-TOPO (Invitrogen Corp., San Diego, Calif.) was used as a cloning vector, and pMal-p (New England Biolabs, Inc., Beverly, Mass.) and pBAD (Invitrogen Corp.) were used as cloning and expression vectors.

**Anti-P1 monoclonal and polyclonal antibodies.** Anti-P1 MAb (5) were affinity purified from murine ascites fluid with a protein A cartridge and the BioLogic HR workstation (Bio-Rad, Hercules, Calif.), dialyzed against phosphate-buffered saline (PBS, pH 7.2) containing 0.3% sodium azide, aliquoted, and stored at −20°C. Rabbit polyclonal antisera 216 was made against P1 isolated by ion-exchange and gel filtration chromatography (11). Antiserum 220 was made against S. mutans NG8 whole cells (9) and rendered monospecific for P1 by exhaustive adsorption with the spadp-negative mutant PC3370 (13).

**PCR amplification and construction of spadp subclones.** Amplification and construction of P1 polypeptides MA3 (amino acids 819 to 1017), MA4 (amino acids 185 to 472), NR1 (amino acids 465 to 963), NR2 (amino acids 465 to 1218), NR3 (amino acids 816 to 1218), and NR4 (amino acids 465 to 1561) were previously described (9, 14, 50). These polypeptides were expressed as fusion proteins with maltose binding protein (MBP). Other sequences of interest within spadp were amplified by PCR with forward and reverse primers based on the published spadp sequence (28). The forward and reverse primers used to generate NR5 (amino acids 84 to 190) were 5′-CAAATGTTCAATCAGTGAAGTA CC-3′ and 5′-GGGTCTCAACCTCGGCTTT-3′, respectively. DNA encoding NR6 (amino acids 84 to 472) was amplified with the forward primer for NR5 and the reverse primer 5′-GGGAGATTCGCACTACTGAGATGC TGCTA-3′ (italics indicate an engineered EcoRI site).

The PCR for NR5 used pDC20 (9) as the template and was carried out for 30 cycles under the following conditions: (i) denaturation at 94°C for 30 s; (ii) primer annealing at 54°C for 1 min; and (iii) primer extension at 72°C for 20 s. The PCR for NR6 used NG8 chromosomal DNA as the template and was carried out for 35 cycles under the following conditions: (i) denaturation at 94°C for 30 s; (ii) primer annealing at 50°C for 1 min; and (iii) primer extension at 72°C for 1 min. Final primer extensions were carried out for an additional 7 min after the last cycle. Amplified PCR products were cloned into pBAD (Invitrogen Corp.) according to the manufacturer’s instructions. The sequence of each spadp subclone was confirmed by the University of Florida’s DNA Sequencing Core. A schematic representation of P1 and recombinant P1 polypeptides used in this study is shown in Fig. 1.

**SDS-PAGE and Western immunoblot analysis of recombinant P1 polypeptides.** Recombinant E. coli harboring vector alone or plasmids encoding P1-MBP fusion proteins NR1, NR2, NR3, and NR4 (50) were grown overnight in 5 ml of LB containing 50 μg of ampicillin per ml. Cultures were diluted 1:50 into fresh LB-ampicillin (2 ml) and grown to an optical density (600 nm) of 0.5. Cells were induced with IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 0.3 mM for 2 h. Cells were harvested by centrifugation at high speed for 10 min with a tabletop microcentrifuge and resuspended in 200 μl of sodium dodecyl sulfate (SDS) sample buffer. Samples were boiled for 5 min and centrifuged for 5 min prior to analysis by polyacrylamide gel electrophoresis (PAGE) and Western immunoblotting.
Each of the P1-MBP fusion polypeptides was separated on 7.5% polyacrylamide slab gels under nonreducing conditions and electroblotted onto nitrocellulose. A phosphate extract from *S. mutans* was extracted containing P1 (10) included a positive control on each gel. Replicate nitrocellulose blots were stained with anti-V5 antibodies (Diversified Biotech, Mass.) and with MAb 6-11A diluted 1:1,000, or with a cocktail of anti-P1 polyclonal rabbit antisera 216 and 220, each diluted 1:1,000. Expression of each fusion protein was confirmed with anti-MBP polyclonal rabbit antibody (New England Biolabs) diluted 1:10,000. Affinity-purified peroxidase-labeled goat anti-mouse immunoglobulin (ICN/Cappell ICN Biomedicals, Aurora, Ohio) diluted 1:2,000 and affinity-purified peroxidase-labeled goat anti-rabbit IgG (ICN/Cappell) diluted 1:1,000 were used as secondary reagents. Development was with 4-chloro-1-naphthol solution (7 ml of PBS, 1 ml of 4-chloro-1-naphthol [Sigma; 3 mg/ml in ice-cold methanol], and 8 µl of 30% hydrogen peroxide).

**Proteolytic treatment of MAb-coated *S. mutans* and recovery of liberated MAb and bound peptide.** *S. mutans* was harvested from 50 ml of an overnight culture by centrifugation and coated with MAb 6-11A as previously described (50). Bacteria were preincubated with MAb, washed free of unbound MAb, and treated with buffer alone or with 50 µg of endoprotease Arg-C (Sigma, St. Louis, Mo.) in 1 ml of 100 mM Tris-HCl–10 mM CaCl₂, pH 7.6, incubated for 60 h at 37°C. Bacteria were pelleted at high speed for 5 min in a tabletop microcentrifuge, and the supernatant was removed and boiled for 5 min in SDS sample buffer. Cell pellets were also resuspended and boiled in SDS sample buffer.

Products of digestion present in cell-free supernatants or associated with bacterial pellets were separated on 107 or 7.5% polyacrylamide slab gels under reducing and nonreducing conditions and electroblotted onto nitrocellulose. Replicate blots were stained with colloidal gold (Diversified Biotech) or reacted with affinity-purified peroxidase-labeled goat anti-mouse immunoglobulin (H and L chains) (ICN/Cappell) diluted 1:2,000 and developed with a 4-chloro-1-naphthol solution as described above.

Following proteolysis, goat anti-mouse immunoglobulin-agarose beads (Sigma) were used to recover MAb 6-11A from cell-free supernatants according to the manufacturer’s instructions. Beads were equilibrated with 0.01 M sodium phosphate buffer (PBS, pH 7.2) containing 0.5 M NaCl. One milliliter of the supernatant fraction of MAB 6-11A-coated *S. mutans* digested with endoprotease Arg-C was incubated with 250 µl of goat anti-mouse immunoglobulin-agarose beads for 1 h at room temperature. The beads were allowed to settle, and buffer containing unbound material was removed. Beads were washed three times with 1 ml of 0.01 M PBS, pH 7.2, containing 0.5 M NaCl. All washes were collected and analyzed to ensure that MAb 6-11A was not removed from the beads. The presumed immune complex consisting of MAb 6-11A and bound P1 fragments was eluted from the affinity beads with 200 µl of 0.5 M acetic acid–0.15 M NaCl, pH 2.4. This collection step was repeated four times. The presence of MAb in elution fractions was confirmed with SDS-gel electrophoresis and Western immunoblotting with peroxidase-conjugated anti-mouse immunoglobulin. As controls, untreated MAb 6-11A and MAb treated with endoprotease Arg-C were bound and eluted from the goat anti-mouse immunoglobulin-agarose beads as described above.

**MALDI-TOF mass spectrometry analysis of affinity-purified MAB and bound peptide.** Elution fractions from the goat anti-mouse immunoglobulin-agarose beads containing MAb 6-11A and any bound P1 peptide(s) were analyzed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) with the Voyager-SUPER DE STR instrument (Applied Biosystems, Framingham, Mass). MALDIMS analyses of samples were performed in the positive linear mode with an accelerating voltage of 25 kV, a grid voltage of 95%, and a delay time of 350 ns. All analyses in the positive-ion mode were performed with a saturated solution of α-cyano-4-hydroxycinnamic acid in water-ethanol-formic acid (4545:10, vol/vol) as the matrix. Control samples included untreated and protease endoproteinase Arg-C–treated MAB, bound and eluted from the goat anti-mouse-immunoglobulin–agarose beads at concentrations similar to that recovered from MAb-coated bacteria.

**ELISA analysis of P1 sequences contributing to recognition by MAB 6-11A.** *E. coli* harboring plasmids pMA3, pMA41, pNR1, pNR2, pNR3, pNR4, pNR5, or the pMal-p or pBAD vector-only controls were included as immunoglobulin with anti-V5 antibodies as appropriate. Beads were washed three times with 1 ml of 30% hydrogen peroxide. Wells coated with full-length P1 and with lysates from *E. coli* harboring vector only were included as positive and negative controls on each plate. Successful coating of comparable levels of individual recombinant P1 polypeptides was confirmed with anti-P1, anti-MBP, or anti-V5 antibodies as appropriate.

**RESULTS**

Reactivity of MAB 6-11A with defined recombinant P1 polypeptides. Using truncated and full-length recombinant P1 polypeptides, Brady et al. (10) previously localized a segment of P1 contributing to binding by MAB 6-11A to the central region of the molecule. To further characterize the epitope, reactivity with the products of *spaP* subclones spanning the central and central-carryo-terminal regions of P1, including NR1 (amino acids 465 to 963), NR2 (amino acids 465 to 1218), NR3 (amino acids 816 to 1218), and NR4 (amino acids 465 to 1561), was tested by Western immunoblot (Fig. 2). A schematic representation of the locations of these polypeptides is shown in Fig. 1. Each P1-MBP recombinant protein was reactive with anti-MBP polyclonal rabbit antisera (Fig. 2A) and with anti-P1 polyclonal antisera (Fig. 2B). Liberation of the P1 moiety from the MBP fusion partner by digestion with factor Va did not result in recognition by MAB 6-11A (data not shown). MAB 6-11A did not bind directly to any of the P1 polypeptides, including NR4, which encompasses the central-carryo-terminal two-thirds of the protein (Fig. 2C). This suggested that its epitope likely includes additional sequences not contained amino acids 465 to 1561.

**Copurification of MAB 6-11A and bound P1 fragment following proteolysis of MAB-coated *S. mutans.* When bound to P1 on the cell surface, immunomodulatory MAB 6-11A influences the susceptibility of the protein to numerous proteases in vitro (50). When *S. mutans* coated with MAB 6-11A was treated with endoproteinase Arg-C, it was observed that a high-molecular-weight band disappeared from the bacterial cell surface and appeared in the cell-free supernatant fractions (49). The band was not recognized by polyclonal anti-P1 or *S. mutans* antisera (data not shown) and was identified as immunoglobulin with affinity-purified goat anti-mouse IgG antibody (Fig. 3). A replicase gel and Western blot run under reducing conditions confirmed the presence of bands corresponding to the molecular size of IgG heavy and light chains (data not shown).

Because the MAb itself was resistant to proteolysis by endoproteinase Arg-C, it was reasoned that cleavage of P1 resulted in release of the antibody from the bacterial surface.
This suggested the utility of an epitope excision approach to further characterize the 6-11A epitope. In this method, a native antigen is bound by antibody prior to proteolytic digestion, thus enabling identification and characterization of linear as well as discontinuous epitopes (20). Therefore, to capture MAb 6-11A and any P1 fragment still bound to it, the cell-free supernatant fraction of endoproteinase Arg-C-treated MAb-coated bacteria was passed over goat anti-mouse immunoglobulin-agarose beads. SDS-PAGE and Western immunoblot analysis were used to monitor the elution of MAb 6-11A from the affinity beads (Fig. 4). MAb 6-11A was detected in the supernatant starting material and in elution fractions 1 through 4, but not in the flowthrough material or wash fractions (Fig. 4B). As a basis for comparison to ensure that the MAb itself was not altered during proteolysis, and as a positive control for affinity purification, MAb 6-11A alone and endoproteinase Arg-C-treated MAb were passed over the beads and eluted as described in Materials and Methods (data not shown).

**Identification of P1 peptide by MALDI/MS.** Elution fractions containing MAb 6-11A with a possible recovered P1 fragment and the MAb 6-11A control alone were analyzed by MALDI/MS. A MALDI/MS spectrum of MAb 6-11A alone passed over the goat anti-mouse immunoglobulin-agarose beads showed no peaks in the lower-molecular-weight range (data not shown). However, a relatively broad singly charged ion with an average observed \( M_c \) (\( M_{obs} \) of 11,780) was observed within the spectrum from the first elution fraction off of the goat anti-mouse immunoglobulin immunoaffinity beads (Fig. 5). The broad nature of the peak...
might potentially result from glycosylation, although P1 is not believed to be glycosylated. Alternatively, varying degrees of oxidation of the eluted peptide during handling or an effect of desorption of the peptide from the beads may account for the broad peak. Based on the known amino acid sequence of P1, the peptide size detected for this ion corresponds to a predicted cleavage fragment generated by endoproteinase Arg-C digestion of P1 and maps to amino acid residues 84 to 190 (Table 1). This amino acid sequence resides directly upstream of the alanine-rich tandem repeats. The ion corresponding to that with an $M_{\text{rots}}$ of 5,937 (calculated $M_r = 5,915$) was interpreted to represent the doubly charged ion of the putative peptide and the other ion at an $M_{\text{rots}}$ of 3,441 was

![Image](http://iai.asm.org/)

**FIG. 4.** Affinity purification of MAb 6-11A from cell-free supernatant following endoproteinase Arg-C treatment of *S. mutans* coated with MAb 6-11A. Proteins present in the cell-free supernatant starting material and in the flowthrough, wash, and elution fractions following passage over goat anti-mouse IgG-agarose beads were separated by SDS-PAGE under nonreducing conditions, electroblotted onto nitrocellulose, and stained with colloidal gold (A) or reacted with peroxidase-labeled goat anti-mouse immunoglobulin (H and L chains) (B).

![Image](http://iai.asm.org/)

**FIG. 5.** MALDI/MS spectrum of singly and doubly charged ions of material associated with affinity-purified MAb 6-11A released from antibody-coated *S. mutans* with endoproteinase Arg-C. The asterisk indicates an ion that could not be identified.

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<th>Predicted mol wt</th>
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*Boldface type indicates the P1 fragment putatively identified by MALDIMS analysis.*

**TABLE 1.** Predicted products of digestion following cleavage of P1 at arginine residues by endoproteinase Arg-C, listed in order of molecular weight

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interpreted to be due to interference. Corresponding elution fractions of MAb alone or supernatants from endoproteinase Arg-C-treated bacteria alone did not contain any peptide peaks in this mass range (data not shown).

**ELISA to detect contributions of P1 segments to binding by MAb 6-11A.** The dependency of MAb 6-11A on the P-region of P1 (9), its lack of reactivity with P1 polypeptides spanning the central- and carboxy-terminal regions of P1 (Fig. 2), and the MALDI/MS identification of a putative P1 peptide corresponding to amino acids 84 to 190 (Fig. 5 and Table 1) suggested that MAb 6-11A’s epitope may consist of multiple disconnected sequences of the protein. To test this hypothesis, additive ELISAs were performed with combinations of recombinant P1 polypeptides. In the first of these experiments, ELISA plate wells were coated with P-region-containing polypeptides NR4 and MA3. A-region-containing polypeptides MA4, NR6, and NR5, corresponding to the peptide identified by MALDI analysis, were reacted with the immobilized moieties prior to assessment of MAb 6-11A binding (Fig. 6A and B). As had been observed by Western immunoblotting, MAb 6-11A did not react directly with any of the individual P1 polypeptides by ELISA (data not shown). MAb 6-11A binding was detected when the polypeptide corresponding to the A-region, MA41, was overlaid on either of the P-region-containing fragments, NR4 (Fig. 6A) or MA3 (Fig. 6B). MAb 6-11A binding was greatest when NR6, which corresponds to the A-region and the upstream sequence identified by MALDI, was reacted with immobilized MA3 or NR4. MAb 6-11A did not bind when the NR5 peptide was reacted with MA3 or NR4. The addition of *E. coli* negative-control lysates harboring the pMal-p or pBAD vector to immobilized polypeptide MA3 or NR4 had no effect on MAb 6-11A reactivity.

**FIG. 6.** Restoration of MAb 6-11A binding by additive ELISA. *E. coli* cell lysates containing recombinant P1 polypeptides NR4 (A) or MA3 (B) were immobilized on ELISA plate wells and subsequently incubated with serial dilutions (beginning at 100 ng of protein/well) of *E. coli* cell lysates containing recombinant P1 polypeptide NR5, NR6, or MA41 or the pMal-p or pBAD vector as negative controls. The degree of restoration of binding of MAb 6-11A was detected with peroxidase-conjugated goat anti-mouse IgG. Panels C and D illustrate MAb 6-11A binding when the order of addition of the P1 polypeptides was reversed. *E. coli* cell lysates containing recombinant polypeptide NR5, NR6, or MA41 or vector-only negative controls were immobilized and incubated with serial dilutions of *E. coli* cell lysates containing NR4 (C) or MA3 (D).
ELISAs in which MA41, NR6, or NR5 was immobilized and MA3 or NR4 was overlaid prior to assessment of MAb 6-11A binding were also performed (Fig. 6C and D). Again, no reactivity was observed with the individual immobilized polypeptides. The addition of NR5 or vector-only controls had no effect on MAb 6-11A reactivity. The addition of either NR4 (Fig. 6C) or MA3 (Fig. 6D) to immobilized MA41 or NR6 resulted in detectable binding by 6-11A, but reactivity was not restored to the same extent as when these P-region-containing fragments were used to coat the wells (Figs. 6A and B). This suggests that the majority of contact residues may lie near or within the A-region. Hence, these may be more accessible when either MA41 or NR6 is added to immobilized NR4 or MA3 and masked when either MA3 or NR4 is added to immobilized MA41 or NR6. Addition of NR6 to immobilized MA3 and NR4 also resulted in higher binding of MAb 6-11A than did addition of MA41, but the effect was not as pronounced as when NR4 and MA3 were immobilized.

The detection of MAb 6-11A reactivity following interaction of the isolated A- (MA41) and P-regions (MA3) indicates that such an interaction is required for the formation of the core epitope recognized by this antibody. The higher binding of MAb 6-11A following interaction with P-region-containing polypeptides of NR6, which spans both the peptide identified by MALDI/MS and the A-region (amino acids 84 to 472), compared to the A-region alone (amino acids 185 to 472), suggests that the complete epitope recognized by this MAb also involves amino acids contained within residues 84 to 190 of P1. The positional effect observed when amino-terminal versus central-carboxy-terminal polypeptides were immobilized is also consistent with MAb 6-11A binding predominantly in proximity to the A-region.

**DISCUSSION**

Murine MAb 6-11A against the surface protein P1 of *S. mutans* (5, 11) modulates the humoral immune response to *S. mutans* when live *S. mutans* bacteria coated with the MAb are used as the immunogen (12). Compared to *S. mutans* alone, MAAb-coated *S. mutans* triggered an in vivo humoral immune response which was more inhibitory of *S. mutans* adherence to salivary agglutinin and differed in epitope specificity against P1 (12, 50). There are two major mechanisms through which antibodies contained in immune complexes can affect an adaptive immune response against antigen in the complex. The first involves the Fc portion of the antibody molecule and Fc receptors on antigen-presenting cells and can be independent of epitope specificity (2-4, 37, 46). The second depends on epitope specificity and may result from blockage of specific antigenic sites on the antigen, steric hindrance, changes in accessibility of antigenic determinants by bound antibody, and/or conformational changes in the three-dimensional structure of the antigen induced by antibody binding (4, 36, 61). Changes in susceptibility of a protein to proteolytic degradation are an indication of an alteration in its conformation (21, 27, 60, 63). The facts that MAb 6-11A-mediated immunomodulation results in the production of antibodies that are more inhibitory of *S. mutans* adherence to salivary agglutinin and influences the protease digestion of P1 (50) suggest that its binding to P1 may increase the response against cryptic or nondominant epitopes. In light of the effects of MAb 6-11A on the immunogenicity of P1, characterization of its epitope would be helpful in elucidating the potential mechanism of immunomodulation and in gaining an understanding of the tertiary structure of P1.

Experiments were undertaken to evaluate MAb 6-11A reactivity with recombinant partial P1 polypeptides and combinations thereof. In addition, a P1 peptide was copurified with MAb 6-11A after protease digestion of antibody-coated bacteria and identified by MALDI/MS. The data derived from these experiments indicate that the epitope recognized by MAb 6-11A is complex and discontinuous in nature. It is contributed to by segments of P1 encompassing the A-region, the sequence amino-terminal to the A-region, and the P-region, suggesting that these discontinuous sequences are in close proximity to one another in the native protein. MAb 6-11A does not bind to A- or P-region fragments alone (9, 14) or to recombinant P1 with either of these regions deleted (9, 14, 54), indicating that both segments are required simultaneously to establish the appropriate epitope configuration.

An epitope recognized by 6-11A was reconstituted by interactions of A- and P-region polypeptides corresponding to amino acids 185 to 472 and 84 to 190. Residues contained within amino acids 84 to 190 immediately upstream of the A-region also contributed to recognition by the MAb. In addition, increased restoration of MAb 6-11A binding when A-containing fragments were overlaid on immobilized P-region-containing fragments suggests the presence of important contact residues within the A-region. The binding of two discontinuous amino acid segments of a P1 homolog, SpaA of *Streptococcus sobrinus*, to form an immunodominant conformational epitope similar if not identical to that on the native protein was first described by Goldschmidt et al. (16). More recently, it was also reported that the anti-antigen I/Ii MAb Guy’s 13 binds to a determinant dependent on the interaction between two discontinuous A- and P-region-containing segments (59). However, in ELISA experiments described by these investigators, recognition by Guy’s 13 did not appear to be influenced by which fragment was immobilized on the plate.

The suggested close proximity of the A- and P-regions within the native molecule is supported by crystallography of the intervening variable region. Troffer-Charlier et al. (58) published the crystal structure of a segment of antigen I/I of *S. mutans* serotype f spanning the last eight amino acids of the A-region through the first 18 amino acids of the P-region. They describe the topology of this domain as a distorted β-sandwich made up of two sheets of eight anti-parallel β-strands each and report that the solvent-exposed arm of the N-terminal helix (A-region) and the extended C-terminal peptide (P-region) lie on the same side of the β-sandwich. This information supports an A- and P-region interaction within the native molecule that would achieve the tertiary structure of the epitope recognized by MAb 6-11A. However, additive ELISA experiments described above indicate that the intervening variable sequence between the A- and P-regions is not necessary for these domains to interact in a configuration compatible with MAb 6-11A binding.

The fortuitous finding that intact MAb 6-11A was released from the surface of *S. mutans* by endoprotease Arg-C digestion (50) suggested the utility of epitope excision (20) to identify portions of P1 in contact with the MAb. Since an antibody itself is...
relatively resistant to limited protease digestion (22, 56), polypeptide sequences comprising or in close proximity to contact residues are protected from proteolysis. Such a footprinting approach has been used in combination with MALDI/MS to characterize both linear (26, 30, 42–44, 64, 65, 68) and discontinuous (20) epitopes of various antigens. The peptide that copurified with MAb 6-11A after protease cleavage of P1 from antibody-coated bacteria apparently contributes to a discontinuous epitope in which the A-region and P-region of P1 are absolutely required for this MAb binding. Whereas deletion of the A- or P-region completely eliminated MAb 6-11A reactivity, deletion of residues 90 to 186 did not (data not shown). However, the contribution of P1 residues 84 to 190 to an optimal epitope structure is supported by additive ELISA experiments.

Capture of peptide comprising amino acids 84 to 190 with MAb 6-11A after protease cleavage of cell-associated P1 illustrates the ability of the antigen binding site within an antigen to body retain a portion of a more complete epitope following proteolysis. Conversely, monoclonal antibodies are sometimes capable of binding individual peptide fragments that contribute to discontinuous conformational epitopes. As reviewed by Mumey et al. (40), this is the basis behind an approach, in which the antigen of interest is first digested with protein footprinting, MALDI/MS, and additive ELISA experiments clearly demonstrate the contribution of the pre-A-region sequence to MAb 6-11A binding. That this isolated portion of the epitope was not recognized out of context underscores the benefit in certain cases of epitope excision compared to the more traditional epitope extraction approach, in which the antigen of interest is first digested enzymatically before being reacted with immobilized antibody for capture of reactive fragments (20).

The identification and mapping of discontinuous epitopes on a protein such as S. mutans adhesion P1 provides insight into the structure of the molecule. Since P1 is a promising target of immunomodulation by MAb 6-11A results in altered antigenicity and lack of surface expression of the Streptococcus mutans P1 adhesin molecule. Infect. Immun. 66:4274–4282.

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REFERENCES


