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Alterations in Immunodominance of *Streptococcus mutans* AgI/II: Lessons Learned from Immunomodulatory Antibodies

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Abstract

Streptococcus mutans Antigen I/II (AgI/II) has been widely studied as a candidate vaccine antigen against human dental caries. In this report we follow up on prior studies that indicated that anti-AgI/II immunomodulatory monoclonal antibodies (MAbs) exerted their effects by destabilizing the native protein structure and exposing cryptic epitopes. We show here that similar results can be obtained by immunizing mice with truncated polypeptides out of the context of an intra-molecular interaction that occurs within the full-length molecule and that appears to dampen the functional response against at least two important target epitopes. Putative T cell epitopes that influenced antibody specificity were identified immediately upstream of the alanine-rich repeat domain. Adherence inhibiting antibodies could be induced against two discrete domains of the protein, one corresponding to the central portion of the molecule and the other corresponding to the C-terminus.

Keywords

Streptococcus; Monoclonal antibody; immune complex; immunodominance; vaccine design

1. Introduction

Due to its etiological association with dental caries [1], multiple antigens of *Streptococcus mutans* have been studied as vaccine candidates [2–6]. One such protein is the cell-surface localized Antigen I/II adhesin [7], also called P1 [8], Antigen B [9], or PAc [10]. AgI/II family members mediate interactions with host salivary constituents, cell matrix proteins, and other bacteria (reviewed in [11]). Until recently, a lack of high-resolution structural information hindered the design and interpretation of immunological studies. As deduced from the primary sequence, AgI/II has discontinuous alanine (A)- and proline (P)-rich tandem repeats that flank a variable (V) region where strain differences are clustered [10, 12, 13]. Recently, an unusual tertiary structure was discovered in which the A-repeats form an α-helix that intertwines with the polyproline II (PPII) P-region helix to form a long narrow stalk [14]. The intervening segment including the V-region comprises a β sandwich arranged in two sheets [15]. The crystal structure of the C-terminus also revealed β sheet structure with three consecutive domains adopting a DE-variant IgG fold [16]. Hence, two

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globular regions lie on either end of an extended stalk. A high affinity intra-molecular interaction between the N-terminus, which has not been crystalized, and the C-terminus increases stability of AgI/II and enhances adhesive function [17]. The primary and modeled tertiary structures of AgI/II are illustrated (Figure 1).

AgI/II's interaction with salivary components is complex and involves two distinct adherence sites [16, 18]. The interaction differs depending on whether the major physiologic receptor, salivary agglutinin (SAG), is immobilized or is in fluid-phase. Monoclonal antibodies differ in their ability to inhibit adherence to SAG compared to SAG-mediated bacterial aggregation indicating that the determinants that mediate these two processes are not identical [19]. SAG is an oligomeric protein complex consisting primarily of the scavenger receptor glycoprotein gp340, and also containing amylase, sIgA and an 80 kDa protein [20, 21]. Different regions of both gp340 [22] and AgI/II [19] contribute to the different interactions. *S. mutans* adherence *in vivo* involves binding of AgI/II to immobilized SAG within the salivary pellicle coating the tooth surface [23]. Disruption of this interaction by antibodies is the focus of preventative therapeutic protocols. In contrast, interaction of fluid-phase SAG with cell surface AgI/II represents an innate host defense mechanism [24, 25], whereby aggregated are removed by swallowing. Hence it is desirable to elicit antibodies that disrupt SAG-mediated adherence, but not aggregation.

Numerous studies have demonstrated the relevance of an antibody response against AgI/II in protection against *S. mutans* colonization and cariogenicity (reviewed in [3, 11, 26, 27]). Both salivary and serum antibodies, that enter the oral cavity via transudation through the gingival crevice, have been reported to be protective [6, 28–33], or in some instances non-protective [34–36]. Subtle and potentially unapparent differences among immune responses can be crucial in determining the outcome of a host pathogen interaction. Naturally dominant epitopes are often not optimal for protection and pathogens can persist in the face of an immune response [37]. Therefore, it is fine specificity and functional activity, more so than total antibody amount, which likely determines whether colonization and cariogenicity is sufficiently inhibited to prevent disease by *S. mutans*.

Our laboratory has evaluated seven different anti-AI/II (P1) MAbs for immunomodulatory properties using an active immunization approach that incorporated them as part of immune complexes (IC) with whole bacterial cells [38–44]. Their approximate binding sites are illustrated in Figure 1B and were deduced based on reactivity with internal deletion constructs and combinations of truncated polypeptides [44–47]. MAbs 1–6F and 4–9D are influenced by overall conformation and bind within the region intervening the A- and P-repeats. 4–10A recognizes a repeated epitope formed by interacting A- and P-region sequences. Guy's 13 also binds an epitope formed by interacting of A- and P-region sequences, but on a different part of the stalk. 6–11A, 5–5D, 3–10E bind epitopes that depend on the A-P interaction, but also involve a pre-A-post-P-region interaction. 3–10E's binding is almost completely eliminated when this interaction is disrupted. MAbs 1–6F, 4–9D, and 4–10A inhibit bacterial adherence, while 6–11A, 5–5D, 3–10E, and Guy's 13 do not [41, 44].

Previous studies showed that when incorporated within ICs, MAbs 6–11A, 5–5D, 3–10E, 4–10A, and Guy's 13 redirected the adaptive immune response toward one of increased efficacy with regard to inhibition of bacterial adherence to SAG [38–44]. The presence of the MAbs within ICs altered the fine specificity and isotype composition of the elicited antibody response. These effects appeared to stem from a structural perturbation of the cell surface adhesin resulting in increased exposure of at least one normally cryptic or subdominant epitope, with the epitope recognized by 1–6F, an adherence-inhibiting MAb, shown to be affected [43, 44]. In the current study we sought to determine whether the

effects of anti-Ag I/II MAbs could be mimicked by immunization with truncated and internal deletion variants of AgI/II in which important target epitopes might be better exposed. As a result, novel putative T helper cell and C-terminal epitopes were identified.

2. Materials and Methods

2.1 Bacterial strains, plasmids, expression and protein purification

S. mutans NG8 was grown aerobically for 16 hr in Todd-Hewitt broth with 0.3 % yeast extract (BBL, Cockeysville, MD). *E. coli* strains were grown aerobically at 37°C in Luria-Bertani broth (1 % [wt/vol] tryptone, 0.5 % [wt/vol] yeast extract, 1 % [wt/vol] NaCl) supplemented with ampicillin (50–100 μg/mL) or kanamycin (25–50 μg/mL). Construction of the CK1 and RR2 [45], NA1, P3C, and NR7 [17], and NR21 [43] polypeptides has been described. Recombinant proteins were purified on amylose or nickel resin. An additional in-frame deletion construct of AgI/II lacking two putative T-cell epitope sequences (aa 164–193) was generated by circle PCR-mutagenesis using primers 5'-GCTGCTCATGAGGCAGCTGCAAATGCTGC and 5'-GCAGCATTTGCAGCTGCCTCATGAGCAGC with pCG14 [48] as template. Amplified DNA was self-ligated using Quick T4 ligase (New England Biolabs) and transformed into *E. coli* Top10. Plasmid DNA [17] with the confirmed deletion, pPC303, was transformed into *E. coli* M15 (pRep4). The histidine-tagged T-delete protein was purified on nickel resin following induction of mid-exponential phase cells with 1 mM IPTG for 2–4 hours at 37 °C.

2.2 Mice

Six-eight week old female BALB/c mice were purchased from Charles River (Laboratories, Wilmington, MA) and housed in biosafety level 2 facilities under infectious disease conditions and fed a standard diet.

2.3 Source of antibodies

MAb 3–10E was obtained from a previously established hybridoma [49]. IgG was purified from murine ascites fluid using an ImmunoPure (A Plus) IgG Purification Kit (Pierce, Rockford, IL). Peroxidase-labeled and unlabeled secondary reagents were obtained from Southern Biotech (Birmingham, AL).

2.4 Immunizations and sample collections

AgI/II demonstrates numerous SDS-resistant discontinuous conformational epitopes that are preserved in Western blots [45]—Groups of five mice were immunized intraperitoneally with emulsified polyacrylamide gel slices containing recombinant full-length AgI/II (CG14), or recombinant AgI/II polypeptides NR7, NR21, CK2, RR2, T-delete, NA1, PC3, or a mixture of NA1 and P3C. Sham immunized groups received polyacrylamide only. Protein samples were loaded onto 7.5 % SDS-polyacrylamide preparative gels, negatively stained with 0.3 M cupric chloride for 5 min and rinsed with water. Negative stained bands of the correct size (∼1 □g total protein) were excised and de-stained with three 10 min washes with 0.25 M EDTA and 0.25 M Tris (pH 9) at room temperature with a final exchange into PBS. Gel slices were emulsified into 1 ml of PBS, and 100 μl was used to immunize individual mice. Mice were pre-bled 1 week before the first inoculation, immunized on days 0, 14, 21, and 35, and exsanguinated on day 50. Experiments were approved by the University of Florida Institutional Animal Care and Use Committee (Protocol # 201105486).

2.5 *S. mutans* adherence by BIAcore

Adherence of *S. mutans* to SAG immobilized on a CM5 sensor chip (GE Healthcare, Piscataway, NJ**)** was evaluated using the BIAcore 3000 machine (BIAcore AB, Uppsala, Sweden) [40]. SAG was prepared by adsorption to and desorption from *S. mutans* NG8 as described [22, 50]. Sera from mice within each group were pooled. Adherence of *S. mutans* reacted with sera (diluted 1:50) from mice immunized with full-length protein, versus those from mice immunized with truncated polypeptides, were compared. Each sample was run at least three times. Values were normalized to the delta RU of an *S. mutans* control sample run for each experiment in the absence of sera.

2.6 Biotin-labeling of MAb 3–10E and competition ELISA

Approximately 1 mg of purified 3–10E was biotinylated using EZ-Link™ Biotin-LC-Hydrazide (Pierce, Rockford, IL). ELISA plate wells were coated with *S. mutans* ($\sim 10^7$ cfu/well) [49] in carbonate-bicarbonate buffer, pH 9.6. Pooled antisera from each group were serially diluted two-fold in PBS containing 0.03% Tween-20 beginning at 1:25, 100 µl were added to the wells followed immediately by biotinylated 3–10E and incubated at 37 °C for two hours. Plates were washed and avidin-HRP conjugate (Pierce, IL) was applied to the wells for 30 minutes at room temperature. Plates were washed again and developed with 0.1 M o-phenylenediamine dihydrochloride containing 0.012 % hydrogen peroxide in 0.01 M phosphate citrate buffer. Percent inhibition was calculated as [OD_{450} direct binding of biotin-labeled 3–10E - OD_{450} experimental well / OD_{450} direct binding of biotin-labeled 3–10E] × 100. Positive and negative control wells contained unlabeled 3–10E, no inhibitor, or avidin-HRP only.

2.7 Quantitative subclass ELISA

ELISA plate wells were coated with *S. mutans* whole cells as described above or with 100 ng/well of purified recombinant NR21. Pooled sera from each group were serially diluted three-fold beginning at 1:50 and 100 µl were to the wells. Antibody reactivity was detected using affinity-purified peroxidase-labeled goat anti-mouse peroxidase conjugated IgG1, IgG2a, or IgG2b subclass specific antibodies (Southern Biotech) at a 1:2000 dilution. Plates were developed as above. Concentrations of anti-NR21 subclass antibodies were calculated by interpolation on standard curves generated using purified mouse subclass reagents (Southern Biotech, Birmingham, AL).

2.8 Prediction of T cell epitopes

We utilized RANKPEP (http://bio.dfci.harvard.edu/RANKPEP) to identify AgI/II peptides likely to interact with the class II I-A^d and I-E^d MHC alleles from Balb/c mice. The 1522 amino acid sequence representing the mature polypeptide was input and the 5 peptides predicted to bind the I-A^d and I-E^d molecules with the highest affinities, respectively, were identified. These are listed in order in Table 1 including their locations and the truncated polypeptides that contain them.

2.9 Statistics

Statistically significant differences were determined by one- or two-way analysis of variance (ANOVA) using Graph Pad Prism 4.0. A *p*-value of less than 0.05 was considered significant. Tukey's Multiple Comparison Test determined differences among the groups.

3. Results

3.1. P1 polypeptides vary in eliciting adherence-inhibiting antibodies

To evaluate the immunogenicity of truncated P1 polypeptides (Figure 1a), mice were immunized with full-length recombinant AgI/II (CG14), compared to RR2, CK2, and NR7. These were chosen because of their increased reactivity with MAb 1–6F [45], a strong inhibitor of *S. mutans* adherence [22, 41]. Inhibition of *S. mutans* adherence to immobilized SAG correlates with the level of 1–6F–like antibodies in the sera of mice immunized with IC of *S. mutans* and immunomodulatory MAbs [43]. As previously observed [43], pooled sera from mice that received CK2 was better able to inhibit adherence compared to that from mice that received full-length AgI/II (Figure 2A). This was also true of pooled sera from mice that received RR2. Surprisingly though, pooled sera from mice that received the internally-deleted NR7 polypeptide was no better at inhibiting adherence than pooled sera from mice immunized with CG14, despite the change in antigenicity and improved recognition of NR7 by MAb 1–6F [45]. Comparable levels of anti-*S. mutans* IgG were measured in all groups of mice that received any of the AgI/II constructs (Figure 2B), with no statistically significant differences detected among the groups.

3.2. Predicted helper T cell epitopes affect antibody specificity and isotype

The top five I-A^d and five I-E^d AgI/II T helper cell epitopes in Balb/c mice were predicted using RANKPEP (Table 1). Since I-E^d peptide 5 and I-A^d peptide 4 were contiguous and contained within the segment that had been deleted from NR7 (aa 84–190), we constructed a more defined in-frame deletion (aa 164–192). This construct was no more effective than CG14 at eliciting an adherence-inhibiting response (Figure 2A). Next, we determined whether elimination of the two putative T cell epitopes affected formation of 1–6F–like antibodies, particularly those of the IgG2a and IgG2b isotypes, since these had correlated with improved adherence inhibition [41, 43]. We utilized polypeptide NR21 that corresponds to the central region of AgI/II and has an in-frame deletion of the P-region. It is recognized by MAb 1–6F, but no others in our panel; hence, it is a useful tool to evaluate 1–6F–like antibodies of particular isotypes contained within polyclonal sera. The levels of anti-NR21 antibodies in the sera of mice that had been immunized with the T-delete polypeptide, particularly the IgG2a and IgG2b isotypes, were significantly decreased compared to the full-length recombinant AgI/II group (Figure 3).

3.3 Relevant C-terminal epitopes are masked within AgI/II

To evaluate potential masking of protective epitopes within the native protein resulting from the pre-A/post-P-region interaction additional murine immunizations using NA1 and P3C were performed (Figure 1A). These fragments form a stable complex that reconstitutes multiple discontinuous epitopes, including that recognized by MAb 3–10E. MAb 3–10E does not itself inhibit adherence [41], but serves as a marker for achievement of native structure [17]. To evaluate the ability of NA1 and P3C to maintain an interaction *in vivo*, a competition ELISA was employed in which the ability of pooled immune sera to inhibit binding of biotinylated 3–10E to *S. mutans* was tested. Pooled sera from the full-length CG14 group, followed by the NA1+P3C group, contained antibodies able to compete for MAb 3–10E binding, while pooled sera from P3C-, and particularly NA1-immunized mice, were less able to compete (Figure 4A). Pooled sera from mice immunized with P3C also were increased in their ability to inhibit *S. mutans* adherence to immobilized SAG compared to pooled sera from CG14 ($p<0.05$) or sham immunized ($p<0.0001$) groups (Figure 4B). This effect was not observed for pooled sera from the NA1 or NA1+P3C groups. Of note, total anti-*S. mutans* IgG measured in pooled sera from the P3C immunized mice was significantly less than that measured from mice immunized with CG14 and comparable to that from mice immunized with the NA1/P3C mixture (Figure 4C).

4. Discussion

In proteins with complex conformations, formation of functional antibodies may be hampered by inaccessibility of cryptic, but protective, target epitopes. Binding of an antibody to its cognate antigen can induce a conformational change in that antigen [51–67]. Importantly, these conformational changes can lead to exposure of functional subdominant or neoepitopes [54, 60, 65, 68–87], and such determinants can represent important targets of functional or neutralizing antibodies [62, 68, 88–97]. This is most evident in viral systems in which the availability of well-resolved crystal structures has outpaced that of bacterial molecules.

A tertiary model for *S. mutans* AgI/II has now allowed the approximation of SAG binding domains [14, 16], and MAb binding sites [44]. In this study we capitalized on information from previous work that suggested destabilization of AgI/II structure was the basis of immunomodulatory effects by several MAbs [43]. Destabilization of protein by antibody can identify key internal segments that are integral to overall tertiary structure [63]. We had discovered that several anti-AgI/II MAbs recognize epitopes contributed to by aa 84–190 immediately upstream of the A-region [39, 98]. Circular dichroism, surface plasmon resonance, and differential scanning calorimetry confirmed this segment contributes to folding and function of the adhesin, and increases stability of its elongated hybrid helical stalk [17]. The NR7 polypeptide lacking this sequence demonstrates increased exposure of the 1–6F epitope [45]; therefore, we speculated that NR7 would represent a superior immunogen. Surprisingly, it did not. Therefore, we evaluated whether elimination of two putative T cell epitopes within the segment deleted from NR7 affected immunogenicity against the 1–6F epitope. Compared to unaltered AgI/II, the T-delete polypeptide resulted in the formation of significantly less antibody reactive with the NR21 polypeptide that is used as a tool for measurement of 1-6F-like antibodies. Unlike NR7, the T-delete polypeptide did not lose its ability to interact with MAb 3–10E or gain in reactivity with MAb 1–6F (data not shown), hence it appears to be structurally similar to the unaltered full-length adhesin. This suggests that its altered immunogenicity was a consequence of the elimination of the two putative T cell epitopes identified by RANKPEP rather than a structural alteration.

Two other constructs also demonstrate increased reactivity with MAb 1–6F compared to full-length AgI/II [45]. The longer, RR2, lacks a major portion of the C-terminus but retains an ability to form the 3–10E epitope [99]. RR2 was superior to CG14 in its ability to elicit an efficacious adherence-inhibiting response. The second shorter construct, CK2, lacks the entire C-terminus, and was also a more efficacious immunogen than full-length CG14. The interaction of AgI/II with SAG is multivalent and contains two distinct binding sites [18]. These have been localized within two discrete fragments. A_3VP_1, corresponds to the third alanine-rich repeat through the third proline-rich repeat, a segment contained within both RR2 and CK2. The second binding site is contained within the C-terminus [16]. Based on our current results, it appears that relevant target epitopes are associated with each of the binding sites, but are obscured within the context of the complete native structure. P3C, but not its intra-molecular binding partner NA1, was significantly better able to elicit adherence-inhibiting antibodies compared to the full-length adhesin or a mixture of P3C and NA1. The A_3VP_1-containing RR2/CK2 polypeptides, as well the P3C polypeptide, therefore retain important functional epitopes, yet expose them in such a way that a more desirable host response is achieved. It will be important in future studies to test whether co-immunization with a mixture of CK2 and P3C has an additive effect. Unlike RR2, CK2 lacks the entire N-terminus of AgI/II so would not be expected to bind to or influence the immunogenicity of the C-terminus.

While dental caries is associated with acidogenic and aciduric organisms such as *S. mutans*, oral health is associated with other oral streptococci that also express AgI/II family molecules [100–102]. Cross-reactivity of immune sera from this study was observed against *S. oralis* and to a lesser extent *S. mitis*; however, these organisms did not adhere to the SAG prepared using *S. mutans* (data not shown). Since the binding specificities of AgI/II homologs among species are known to differ [103], the cross-reactive antibodies should not be problematic. Minimal cross-reactivity was observed against *S. salivarius* and *S. sanguinis*. In a previous study [45] we found that anti-AgI/II monoclonal antibodies cross-reactive with *S. gordonii* were directed against epitopes that were subsequently mapped to the hybrid helical stalk rather than to the globular adherence domains lying at either end of it [44]. This is consistent with our current study in which more cross-reactivity was detected in sera from animals immunized with polypeptides that included the stalk. Unlike *S. oralis* and *S. mitis*, *S. gordonii* demonstrated substantial adherence to SAG, but this binding was not blocked by the anti-P3C sera that significantly inhibited *S. mutans* adherence (data not shown). Taken together, our results suggest that AgI/II-derived vaccine immunogens can be designed to selectively interfere with adherence of *S. mutans*. Ag I/II-mediated adhesion represents a sucrose-independent mechanism. Thus in conjunction with immunogens such as glycosyltransferase and glucan binding proteins that target sucrose-dependent adhesion [2] truncated AgI/II polypeptides such as CK2 and/or P3C would be useful adjuncts in an integrated immunoprophylactic approach.

The three-dimensional structure of an antigen clearly impacts immunogenicity against conformational epitopes, but stimulation of particular T cell subsets can also depend on minor alterations within an Ag [104]. Despite the presence of the newly identified predicted T cell epitopes in full-length CG14 (Table 1), its folded structure still appears to interfere with responses against important protective epitopes. Our results reiterate that in any system, optimal immunogenicity is a balance between structure, epitope exposure and availability of T cell epitopes following antigenic processing of any given conformer, and are consistent with an accumulating body of literature that points to an ability to capitalize on information regarding antibody-mediated changes in an antigen and to apply that information to mimic antibody-mediated destabilization of protein structure. These data will facilitate future studies regarding *S. mutans* AgI/II as a vaccine candidate. In addition, this approach can serve as a model for improvement of protective responses against pathogenic microbes for which the natural immunodominance of candidate antigens is less than optimal.

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Highlights

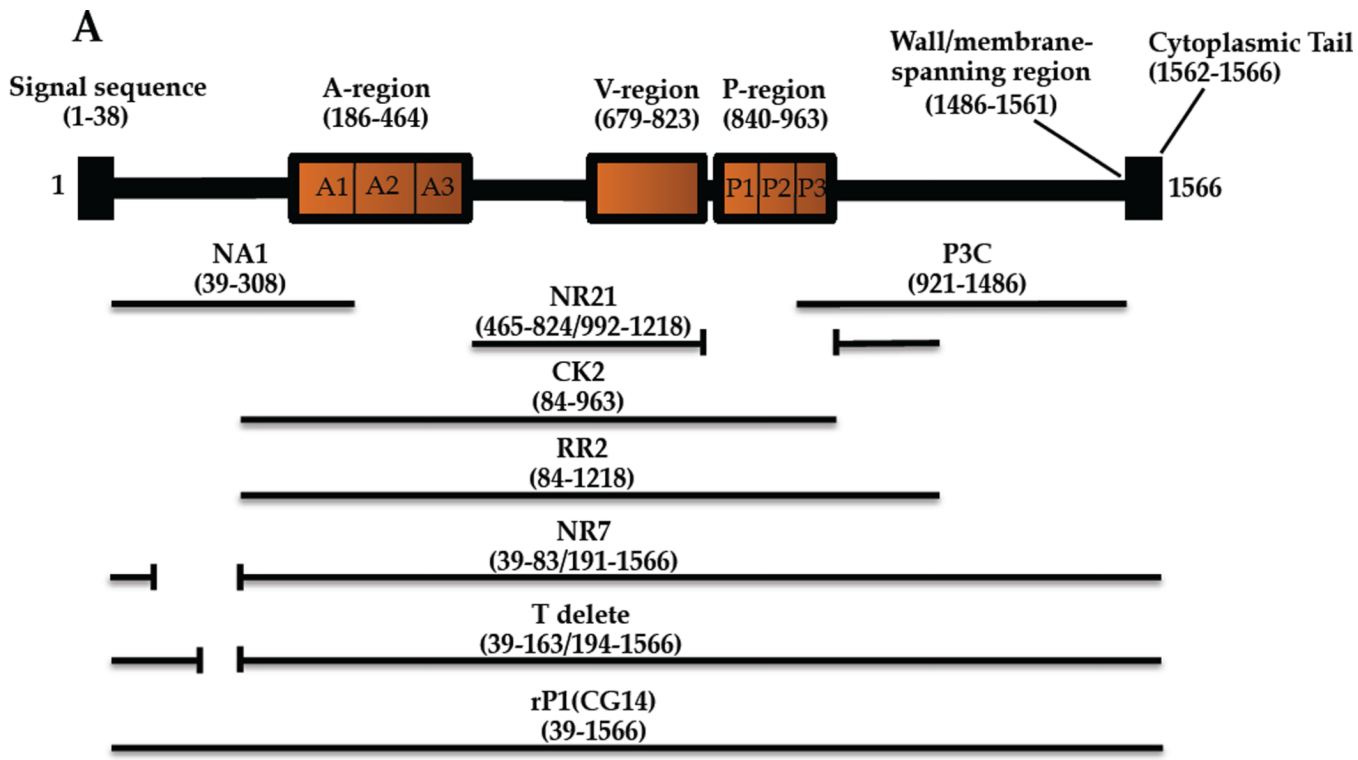
Streptococcus mutans adhesin AgI/II elicits adherence-inhibiting antibodies.

We utilize truncated proteins to identify targets of relevant antibodies.

Putative amino-terminal T cell epitopes impact formation of adherence-inhibiting antibodies.

The C-terminus was identified as a new target of adherence-inhibiting antibodies.

An intramolecular interaction appears to dampen the adherence-inhibiting immune response.



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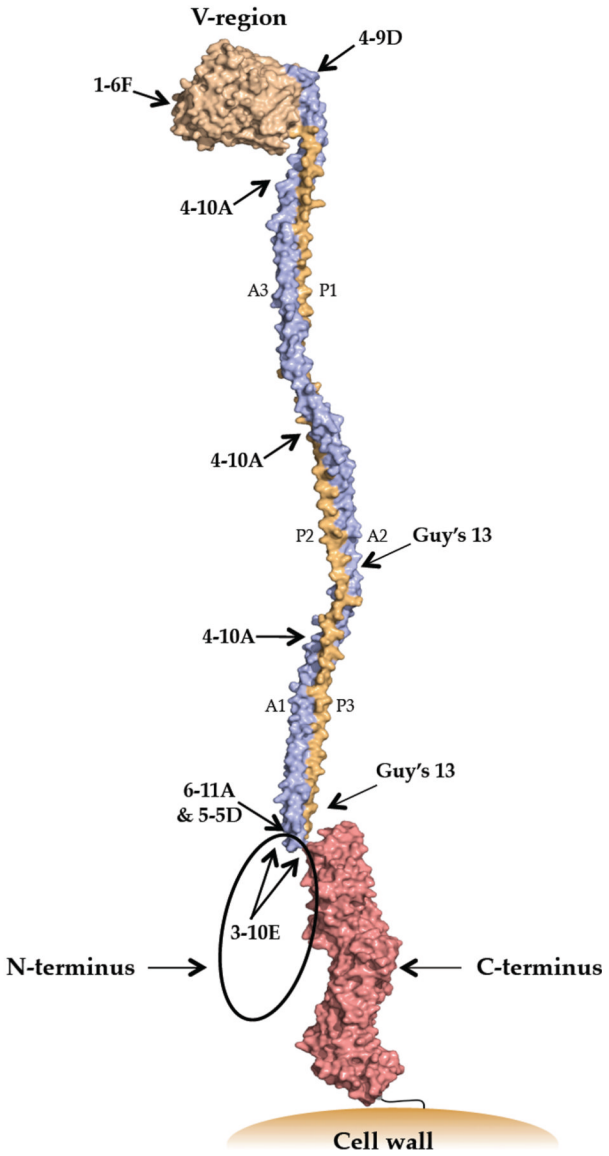

Figure 1.

Schematic representations of *S. mutans* Antigen I/II illustrating location of putative T cell epitopes and approximate antibody binding sites. (**A**) A representation of the primary structure of AgI/II and the recombinant polypeptides used in this study. (**B**) A three-dimensional model of Ag I/II. Approximate binding sites of monoclonal antibodies are indicated.

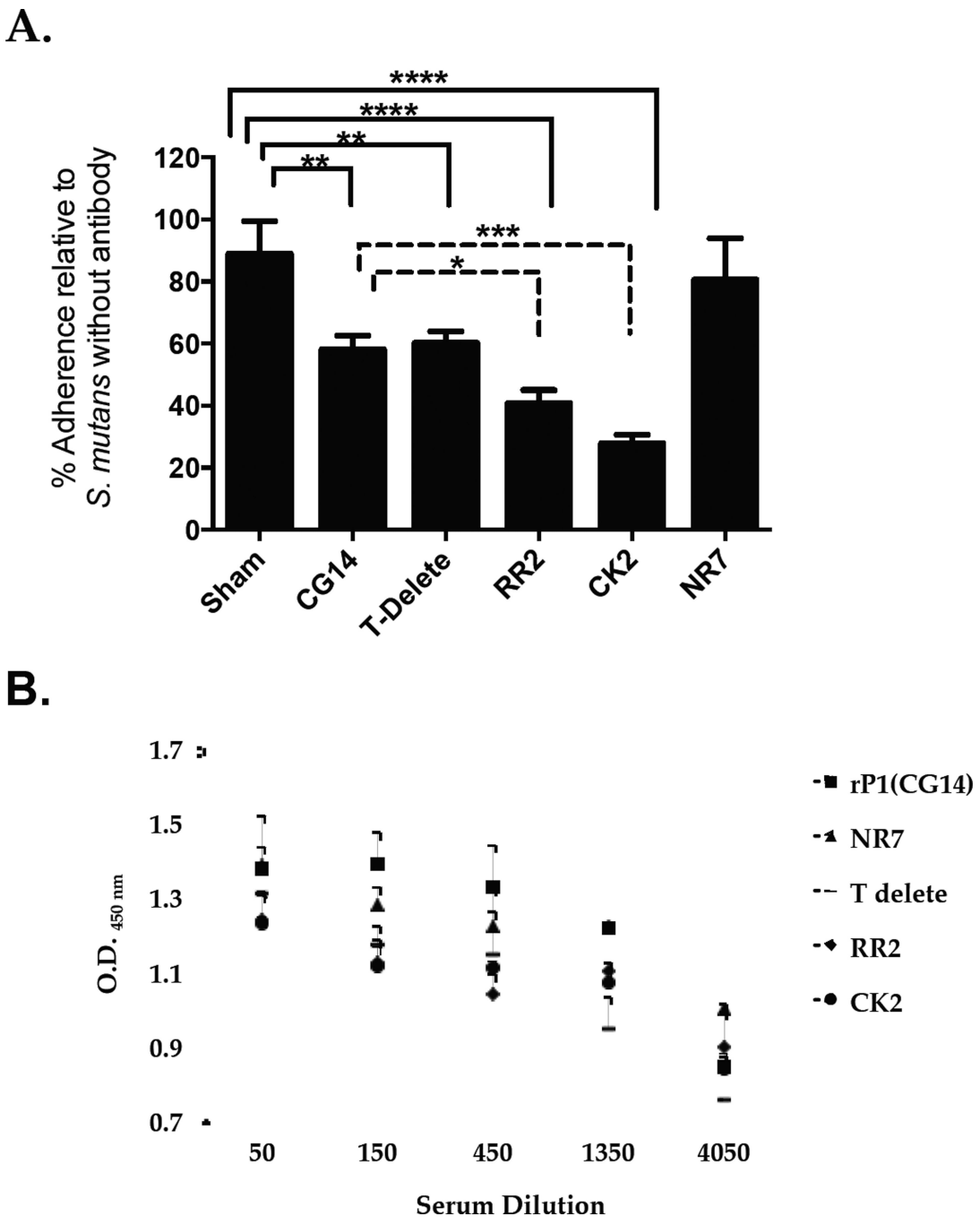

Figure 2.

Evaluation of *S. mutans* adherence to SAG and detection of anti-*S. mutans* IgG. (**A**) BIAcore SPR analysis was used to evaluate bacterial adherence following incubation of *S. mutans* with sera collected from mice immunized with full-length P1 (CG14) and truncated derivatives of P1 (NA1, P3C). Values were normalized to the change in resonance units (ΔRU) for *S. mutans* NG8, in the absence of added serum, detected following the 60-second injection cycle. Immunogens are indicated on the X-axis. Tukey's Multiple Comparison Test determined differences among the groups; *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 (**B**) The presence of anti-*S. mutans* IgG in the sera of mice immunized with P1 versus

truncated P1 polypeptides was evaluated by serial dilution and standard ELISA. Data are representative of at least three independent experiments. Statistical significance between groups was evaluated by two-way ANOVA and no significant differences were observed.

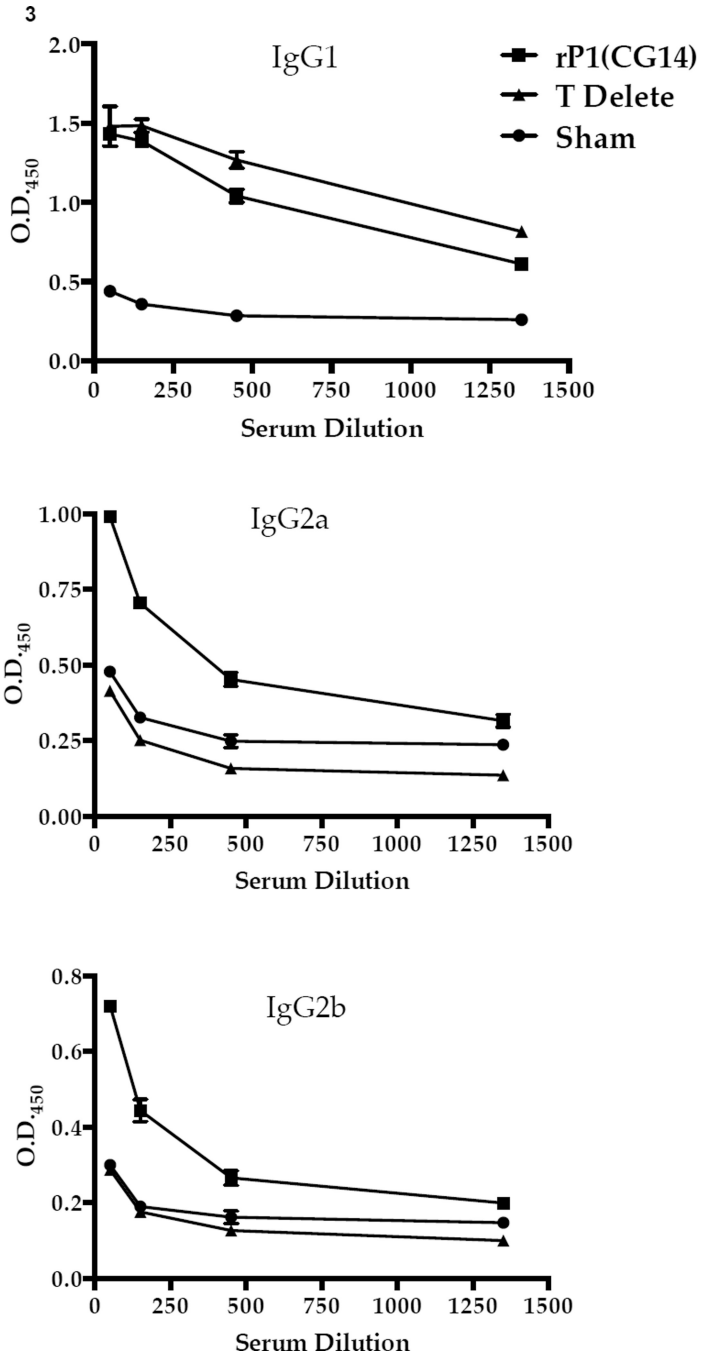

Figure 3.

Evaluation of the specificity and isotype of serum antibody responses by ELISA. The level of anti-NR21-specific IgG1, IgG2a, and IgG2b subclass antibody in the sera collected from sham-immunized mice, mice immunized with full-length P1, or the truncated protein lacking 2 putative T cell epitopes was evaluated by ELISA. All results are expressed as mean ± SEM and data are representative of at least three independent experiments. Statistically significant differences of the T-delete compared to CG14 were determined by two-way ANOVA. $p \leq 0.05$ (IgG1) , $p \leq 0.0001$ (IgG2a), $p \leq 0.01$ (IgG2b)

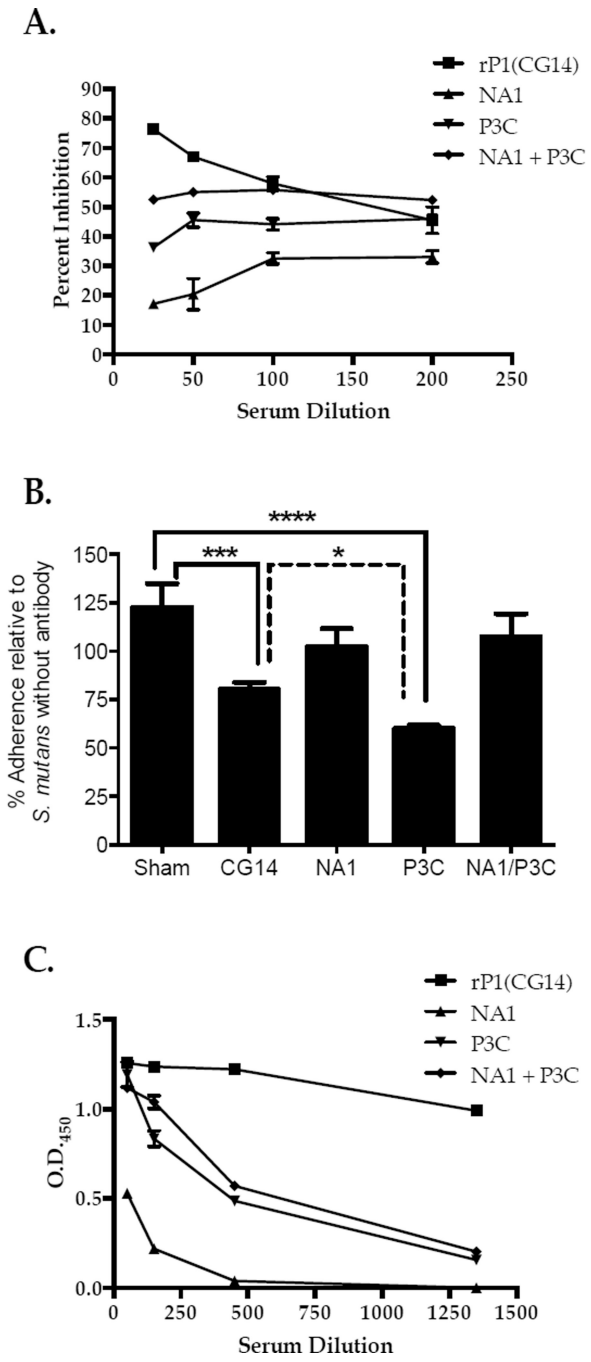

Figure 4.

Functional C-terminal epitopes are masked by intra-molecular interactions. (**A**) Sera from mice immunized with full-length P1 (CG14), NA1, P3C, or a mixture of NA1 and P3C were used in a competition ELISA to evaluate inhibition of binding of biotin-labeled MAb 3–10E to *S. mutans*. Serum dilution is indicated on the x-axis. (**B**) BIAcore SPR analysis was used to evaluate bacterial adherence following incubation of *S. mutans* with sera collected from immunized mice. Values were normalized to the change in resonance units (ΔRU) for *S. mutans* NG8, in the absence of added serum, detected following the 60-second injection cycle. Immunogens are indicated on the X-axis. Tukey's Multiple Comparison Test

determined differences among the groups; * p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001. (**C**) The level of anti-*S. mutans* IgG in the sera from P1 versus truncated P1 -immunized mice was evaluated by serial dilution and standard ELISA. Data are representative of at least three independent experiments. Statistically significant differences compared to the CG14 group were determined by two-way ANOVA. p≤0.01 (P3C, NA1+P3C), p≤0.0001 (NA1).

Table 1

MHC class II P1 helper T cell epitopes predicted by RANKPEP*

I-A^d peptides	**Residue numbers**	**Polypeptides containing predicted epitopes**
LLERGQSATATYTNL	599–613	CK1, CK2, RR2, NR21, NR7
AYQKALAAYQAELKR	227–241	CK1, CK2, RR2, NR7
NLPEAQGSASKEAEQ	62–76	NR7
EKDMAAHKAEVERIN	179–193	CK2, RR2
RVQEANAAAKAAYDT	241–255	CK1, CK2, RR2, NR7
I-E^d peptides	Residue numbers	Polypeptides containing predicted epitopes
DRTLVAKQSVVKFQL	1017–1031	RR2, NR7
QKALAAYQAELKRVQ	229–243	CK1, CK2, RR2, NR7
NEEIRKRNATAKAEY	271–285	CK1, CK2, RR2, NR7
ANEEIRKRNATAKAE	270–284	CK1, CK2, RR2, NR7
VAKIKAKNQATKEQY	164–178	CK2

* The rankpep prediction program (http://bio.dfci.harvard.edu/RANKPEP) was used to identify the top 5 potential peptides likely to be displayed to helper T cells by MHC Class II I-A^d and I-E^d molecules in BALB/c mice and their distribution among the polypeptide immunogens was tabulated.