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Identification of Antigenic Epitopes in a Surface Protein Antigen of *Streptococcus mutans* in Humans

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The reactivities of antibodies in human serum and saliva to a cell surface protein antigen (PAc) of *Streptococcus mutans* and synthetic peptides covering the PAc molecule were examined. Both an enzyme-linked immunosorbent assay (ELISA) and Western blotting (immunoblotting) showed that all the serum samples from five adult subjects harboring serotype c *S. mutans* in their oral cavity reacted with recombinant PAc (rPAc). On the other hand, the serum from a 4-month-old infant did not react with rPAc in ELISA. The immunoglobulin A (IgA) antibodies in saliva samples from the five adult subjects reacted with rPAc. However, in saliva samples from these subjects, the titers of IgA antibody to rPAc did not correlate with the titers of serum antibody to the antigen. To map continuous antigenic epitopes in the PAc molecule, we synthesized 153 decapeptides covering the entire mature PAc molecule, 121 overlapping decapeptides covering the alanine-rich repeating region (A-region) of the PAc molecule, and 21 overlapping decapeptides covering the middle region (residues 824 to 853) according to multiple pin-coupled peptide synthesis technology. Of 153 decapeptides covering the mature PAc, 27 decapeptides showed a strong reaction with the antibodies in serum from the adult subjects. The epitope-scanning patterns in the serum samples from these subjects were also very similar to each other. The antigenic epitope patterns in the saliva resembled those in the serum. However, the ELISA titers of salivary IgA antibodies to these decapeptides differed from the titers of the serum antibody. Of the 121 overlapping decapeptides covering the A-region, 27 decapeptides showed a positive reaction with the antibodies in serum from the adult subjects. All of these 27 decapeptides had either one or two of the five common sequences YQAXL, NADAKA, VQKAN, NNAKNA, and IKKRNA. Six decapeptides of the 21 overlapping decapeptides covering the middle region reacted strongly with the serum antibodies from a high PAc responder, and each of the six decapeptides had one of the two common sequences KVTKEKP and VKPTAPTK. These epitopes might therefore be relevant to the humoral responses against the PAc protein during natural infection with *S. mutans* in humans.

Streptococcus mutans has been strongly implicated as one of the causative agents of dental caries and is frequently isolated from human dental plaque (16, 29). *S. mutans* possesses various cell surface antigenic substances. Among these antigens, a 190-kDa cell surface protein antigen that has been variously designated antigen I/II (42), B (45), IF (20), P1 (12), SR (1), PAc (38, 39), and MSL-1 (10) was shown to be an effective dental caries vaccine in monkeys (28, 46). The application of mouse monoclonal antibodies against the antigen to tooth surfaces was demonstrated to inhibit either the subsequent implantation of *S. mutans* or the recolonization by indigenous mutans streptococci in monkeys and humans (27, 30). The surface protein antigen is considered to participate in attachment of the streptococcal cell to acquired pellicles on tooth surfaces (22, 26). The surface protein of *S. mutans* shows a serological cross-reactivity with a 210-kDa surface protein of *Streptococcus sobrinus* named SpaA (18) and PAg (37, 49, 51).

The complete nucleotide sequence of the gene for the 190-kDa protein antigen of *S. mutans* serotype c has already been determined by two groups (21, 39). The *pac* gene consists of 4,695 bp and codes for a 170,773-Da protein (39). The gene product contains a putative signal peptide consisting of 38 amino acids, resulting in a 166,817-Da mature protein. Two internal repeating amino acid sequences are present in the PAc: one repeating region (the A-region), located in the N-terminal region (Thr-219 to Lys-464), is rich in alanine, while the other (the P-region), located in the central region (Thr-851 to Glu-967), is rich in proline.

The nucleotide sequencing of the *pac* gene of *S. mutans* has led to the identification of functional domains and antigenic epitopes in the PAc molecule (9, 33–35, 40, 47). We previously showed that the intranasal immunization of BALB/c mice either with a synthetic peptide corresponding to residues 301 to 319 of the PAc protein, coupled to a cholera toxin B subunit, or with recombinant PAc (rPAc) and a free cholera toxin B subunit suppresses the colonization of murine tooth surfaces by *S. mutans* (48). Moreover, epitope scanning with anti-rPAc sera from various strains of mice and many decapeptides covering the mature PAc molecule suggested that the murine immune responses to the peptides might be either restricted or dominated by the major histocompatibility complex class II gene haplotypes (40, 47). Therefore, in order to apply the PAc as an anti-*S. mutans* vaccine to human beings, the immune responses to this protein in humans need to be elucidated. However, little is still known about the immune responses in humans to the PAc protein of *S. mutans*.

In this study, we examined the reactivities of antibodies in samples of serum and saliva from adult subjects harboring *S. mutans* in their oral cavity with the PAc by both an enzyme-

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TABLE 1. Amino acid sequences of 20-mer synthetic peptides containing antigenic epitopes of PAc

Peptide	Position[a]	Amino acid sequence[b]
PAc(96–114)	96–114	LDQAAKDAKSAGVNVVQDAC
PAc(331–349)	331–349	KATYEAAVAANNAKNAALTC
PAc(513–531)	513–531	EPNANLSLTTDGKFLKASAC
PAc(623–641)	623–641	SKIVYKYTVDPKSKFQGQKC
PAc(764–782)	764–782	PNSWYGAGAIKMSGPNNHVC
PAc(824–842)	824–842	AVNVPKVTKEKPTPPVKPTC
PAc(835–853)	835–853	PTPPVKPTAPTKPTYETEKC
NA(464–482)[c]		WFGKHRGQGGSNPKFENIA

[a] Position according to Okahashi et al. (39).
[b] The sequences are given in one-letter code. A cysteine was placed at the C terminus of each of these except the last.
[c] An irrelevant peptide derived from the amino acid sequence of Epstein-Barr virus nuclear antigen (2).

linked immunosorbent assay (ELISA) and Western blotting (immunoblotting). Furthermore, we synthesized 153 decapeptides covering the entire mature PAc molecule, 121 overlapping decapeptides covering the A-region of the PAc, and 21 overlapping decapeptides covering the middle region and determined the reactivities of the serum and salivary antibodies from the subjects with these decapeptides to identify the human continuous B-cell epitopes in the PAc protein.

MATERIALS AND METHODS

Human serum and saliva. Sera were obtained from 11 healthy donors between 25 and 43 years old and from a 4-month-old infant. Whole saliva was collected from five adult donors by paraffin stimulation in a chilled container and was clarified by centrifugation at 12,000 × *g* for 10 min at 4°C. The human leukocyte antigen types of subjects A to E, respectively, were as follows: A11, Aw33, B44, DRw6(w13), DRw8, DRw52, DQw1; A11, Aw33, B44, Cw7, DR2(w15), DRw6(w13), DRw52, DQw1(DQw5); A24, A11, Bw52, B7, Cw7, DR1, DR2(w15), DQw1(DQw5, DQw6); A2, A31, Bw48, Cw4, DR2(w15), DR9, DRw53, DQw1(DQw6); and A24, A31, Bw61, Bw59, Cw1, Cw3, DR1, DR9, DRw53, DQw1(DQw5), DQw9. The average number (mean ± standard deviation) of decayed, missing, or filled teeth in the 11 adult subjects was 11.0 ± 4.8. The numbers of decayed, missing, or filled teeth in subjects A to E were 3, 10, 9, 19, and 12, respectively.

Isolation of mutans streptococci. Swab samples, collected with a cotton applicator from the teeth or oral cavity, were serially diluted with 0.15 M NaCl and plated on a sucrose-bacitracin-supplemented mitis-salivarius (MS-SB) agar (15). The MS-SB plates were incubated at 37°C for 48 h. The colonies from each individual were purified by being streaked twice on the MS-SB agar and cultured in 5 ml of brain heart infusion (Difco Laboratories, Detroit, Mich.) broth at 37°C for 16 h. The identification of mutans streptococci and the serotyping of these isolates were performed as described by Hardie (17) and Masuda et al. (31), respectively.

Antigens. *S. mutans* MT8148 (serotype c) was grown at 37°C for 18 h in a diffusate medium of brain heart infusion broth. The cells were harvested by centrifugation, washed three times with distilled water, and lyophilized. rPAc was purified from the culture supernatants of transformant *S. mutans* TK18 by ammonium sulfate precipitation and chromatography on DEAE-cellulose (22). Seven 20-mer peptides corresponding to parts of the amino acid sequence of the PAc (39) and an irrelevant 19-mer peptide, NA(464–482), derived from the amino acid sequence of Epstein-Barr virus nuclear antigen (2) were synthesized by the solid-phase procedure (32) and purified by high-performance liquid chromatography (Table 1). A cysteine was placed at the carboxyl terminus of all 20-mer synthetic peptides. The purity of the peptides was assessed by high-performance liquid chromatography and amino acid analysis.

ELISA. For the ELISA, 96-well microtiter plates (Sumitomo Bakelite, Tokyo, Japan) were coated with 100 μl of whole cells of *S. mutans* MT8148 (100 μg/ml), rPAc (10 μg/ml), or peptide (1 μg/ml) in 50 mM carbonate-bicarbonate buffer (pH 9.6). After overnight incubation at 4°C, the plates were washed with phosphate-buffered saline (PBS) containing 0.1% (vol/vol) Tween 20 (PBST) and blocked with PBST containing 5% (wt/vol) skim milk. After the plates were washed three times with PBST, twofold serial dilutions of human serum or saliva were added (100 μl per well) and the wells were incubated for 1 h at 37°C. The bound antibodies were detected with either alkaline phosphatase-conjugated goat anti-human immunoglobulin A [IgA(α)] (Organon Teknica, Malvern, Pa.), anti-human IgM(μ) (Zymed Laboratories, South San Francisco, Calif.), anti-human IgG(γ) (Zymed), or anti-human IgG (heavy and light chains) (Zymed) serum followed by the addition of a *p*-nitrophenylphosphate substrate solution (1 mg/ml). After 1 h of incubation at 37°C with the substrate, the A_{405} was measured with a microplate reader (MPR A4; Tosoh, Tokyo, Japan). The ELISA antibody titer was expressed as the $\log_2$ of the reciprocal of the highest dilution giving an A_{405} of 0.1 above the conjugate control (no sample added) after 1 h of incubation with the substrate (48). To determine the amount of Igs specific for whole cells of *S. mutans* or rPAc, the calibration curves for IgA, IgM, and IgG were made. In brief, 96-well microtiter plates were coated with 1 μg of either goat anti-human IgA(α) (Organon), anti-human IgM(μ) (Organon), or anti-human IgG(γ) (Zymed) serum. After blocking with 5% (wt/vol) skim milk, twofold serial dilutions of 1 μg of affinity-purified human IgA, IgM, or IgG (Chemicon International Inc., Temecula, Calif.) per ml were added. The bound Igs were detected as described above. The calibration curves were obtained by using the equation of log-logit transformation, and the interpolations of the data for the serum and saliva samples were fitted to the linear portion of the curve with a correlation coefficient of higher than 96% (41). In the inhibition assay of the binding of human serum antibodies to rPAc, the ELISA plates were coated with rPAc (1 μg per well). A 1/100 dilution of the serum from subject A was allowed to react with various concentrations of synthetic peptides or rPAc overnight at room temperature. The reaction mixtures were added to rPAc-coated wells (100 μl/ml), and then the plates were incubated for 1 h at 37°C. The bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgG (heavy and light chains) serum, followed by the addition of the substrate. The percent inhibition of ELISA was calculated by the following formula: $100 \times [(A_{405}$ of human serum) − (A_{405} of human serum plus peptide or rPAc)]/(A_{405} of human serum).

Western blotting. Concentrated culture supernatants and cell extracts of *S. mutans* MT8148 were prepared as described by Ohta et al. (36). *S. mutans* MT8148 was grown at 37°C for 18 h in a diffusate medium of brain heart infusion broth. The cell-free culture supernatant was collected by centrifugation at 10,000 × *g* for 20 min at 4°C. Extracellular proteins were precipitated from the culture supernatant by addition of solid ammonium sulfate to 60% saturation. The precipitate was collected by centrifugation, dissolved in distilled water, dialyzed against distilled water, and lyophilized. The dried culture supernatant, rPAc, and whole cells (50 mg [dry weight] per ml)

were suspended separately in a solution containing 8 M urea, 10 mM Tris hydrochloride (pH 6.8), 1% (wt/vol) sodium dodecyl sulfate (SDS), and 1% (vol/vol) 2-mercaptoethanol and heated at 100°C for 3 min. The cell extract was then clarified by centrifugation. SDS-polyacrylamide slab gels were prepared as described by Laemmli (24). The culture supernatant (10 μg of protein), rPAc (2 μg of protein), and the cell extract (2.5 μl) were electrophoresed at 30 mA per gel for 90 min with a 7.5% (wt/vol) resolving and a 3% (wt/vol) stacking gel (90 by 80 by 1 mm) containing 0.1% (wt/vol) SDS and transferred to a nitrocellulose sheet by the Western blotting technique (5). Myosin (212 kDa), α-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamic dehydrogenase (53 kDa) were used as molecular mass markers. Nonspecific binding to the blots was blocked by incubation for 1 h at 20°C in TBS (20 mM Tris hydrochloride [pH 7.5], 500 mM NaCl) with 3% (wt/vol) gelatin. The blots were washed twice with TBST (TBS with 0.05% [vol/vol] Tween 20) and then incubated with human sera (1:20 in TBS–1% [wt/vol] gelatin) at 4°C overnight. After the blots were washed twice with TBST, they were incubated with goat anti-human IgG (heavy and light chains) conjugated to horseradish peroxidase (Nordic Immunological Laboratories, Tilburg, The Netherlands). The blots were washed twice with TBST and once with TBS, and then substrate (3,3′-diaminobenzidine-hydrogen peroxide) was added.

Epitope scanning. A set of 153 decapeptides serially covering the complete amino acid sequence of mature PAc (39), a set of 121 overlapping decapeptides covering the A-region of the PAc molecule (residues 219 to 468), and a set of 21 overlapping decapeptides covering the middle region of the PAc molecule (residues 824 to 853) were synthesized with an epitope scanning kit (Chiron Mimotopes, Clayton, Australia) as reported by Geysen et al. (13). The reactivities of peptides coupled to multiple pins with human saliva (1:10 dilution) and sera (1:800 or 1:1,600 dilution) were determined by ELISA according to the instructions of the manufacturer. The bound antibodies were detected with either alkaline phosphatase-conjugated goat anti-human IgA(α) or anti-human IgG (heavy and light chains) serum. The data were expressed as the A_{405}s after 1 h of incubation with the substrate.

RESULTS

Human antibody responses to *S. mutans* antigens. Antibodies to whole cells of *S. mutans* and rPAc in human serum samples were quantified by ELISA with class-specific Igs as references. The mean concentrations of IgG, IgA, and IgM antibodies to whole cells of *S. mutans* in the serum samples from 11 adults were 91.0 μg/ml (ranging from 13.4 to 372.6 μg/ml), 25.4 μg/ml (ranging from 3.5 to 113.0 μg/ml) and 10.2 μg/ml (ranging from 1.8 to 25.0 μg/ml), respectively. The concentrations of serum IgG, IgA, and IgM antibodies to whole cells of *S. mutans* in a 4-month-old infant were 0.07, 0.02, and 0.02 μg/ml, respectively. The mean concentrations of serum IgG, IgA, and IgM antibodies to rPAc were 16.6 μg/ml (ranging from 1.0 to 57.3 μg/ml), 6.3 μg/ml (ranging from 0.6 to 24.6 μg/ml) and 1.1 μg/ml (ranging from 0.2 to 4.1 μg/ml), respectively. The concentrations of the serum IgG, IgA, and IgM antibodies to rPAc in the infant were 0.04, 0.02, and 0.01 μg/ml, respectively. Total Ig concentrations in the sera from 11 adults averaged 14.8 ± 1.7 mg/ml (mean ± standard deviation), and the concentration in the infant's serum was 11.7 mg/ml. There was also a close correlation between the concentrations of serum class-specific antibodies to whole cells of *S. mutans* and those of antibodies to rPAc. The correlation coefficients of the IgG, IgA, and IgM antibody data were 0.961, 0.828, and 0.838, respectively.

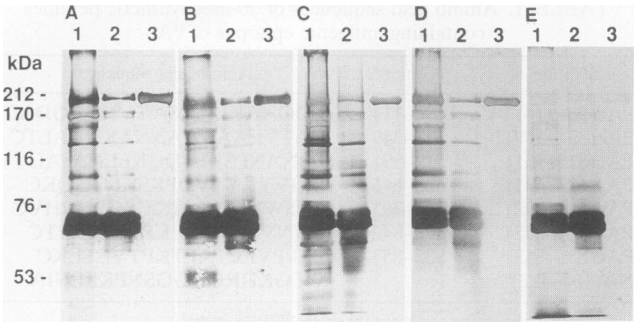

FIG. 1. Western blot analysis of both the cell extracts (lanes 1) and the culture supernatants (lanes 2) of *S. mutans* MT8148 and rPAc (lanes 3). The whole cells were suspended in 8 M urea–1% SDS–1% 2-mercaptoethanol and heated at 100°C for 3 min. The cell extracts were clarified by centrifugation. The culture supernatants were then concentrated by ammonium sulfate precipitation. rPAc was purified from the culture supernatants of transformant *S. mutans* TK18 by ammonium sulfate precipitation and chromatography on DEAE-cellulose. These antigens were electrophoresed on SDS-polyacrylamide slab gels and transferred onto a nitrocellulose sheet by an electrophoretic blotting procedure. The immobilized antigens were allowed to react with the sera (1:20) from five adult subjects (A to E). The antibodies which bound to the antigens were detected by solid-phase immunoassay with horseradish peroxidase-conjugated goat anti-human IgG (heavy and light chains).

On the basis of the titers of serum IgG antibody to rPAc, two high responders (subjects A and B), two medium responders (subjects C and D), and one low responder (subject E) were chosen from among the 11 adults. The reactivities of the sera from these subjects with cell extracts and culture supernatants of *S. mutans* and with rPAc were then analyzed by Western blotting with horseradish peroxidase-conjugated goat anti-human IgG (heavy and light chains) serum. The human serum antibodies reacted strongly with 190-, 70-, and 65-kDa antigens of *S. mutans* (Fig. 1). The reactivities of these sera with the 190-kDa PAc in Western blotting corresponded to those in ELISA (Fig. 2A). The titers of IgA antibody in saliva samples from these five subjects to rPAc did not correlate with the serum antibody responses to the protein antigen (Fig. 2B).

We examined whether these subjects were naturally infected with mutans streptococci. *S. mutans* was isolated from the samples of dental plaque from all the adult subjects, and all the *S. mutans* strains isolated in this study possessed the serotype c antigen described by Bratthall (4). Moreover, serotype g *S. sobrinus* as well as serotype c *S. mutans* was isolated from the dental plaque sample of subject D. Neither *S. mutans* nor *S. sobrinus* was recovered from the oral cavity of the 4-month-old infant.

Epitope scanning of the PAc molecule. To clarify the antigenic epitopes in the PAc molecule, we synthesized a set of 153 decapeptides covering the complete amino acid sequence of the mature PAc and used these decapeptides in the epitope-scanning analysis. Figure 3 shows the epitope-scanning patterns with serum samples from five adult donors infected naturally with serotype c *S. mutans* and from an infant. Serum (1:800 dilution) from subject A reacted with many decapeptides and showed positive reactions ($A_{405} > 1.0$) with the 27 decapeptides corresponding mostly to the N-terminal and the middle regions of the PAc (Fig. 3A). In particular, the serum gave positive reactions with five decapeptides corresponding to

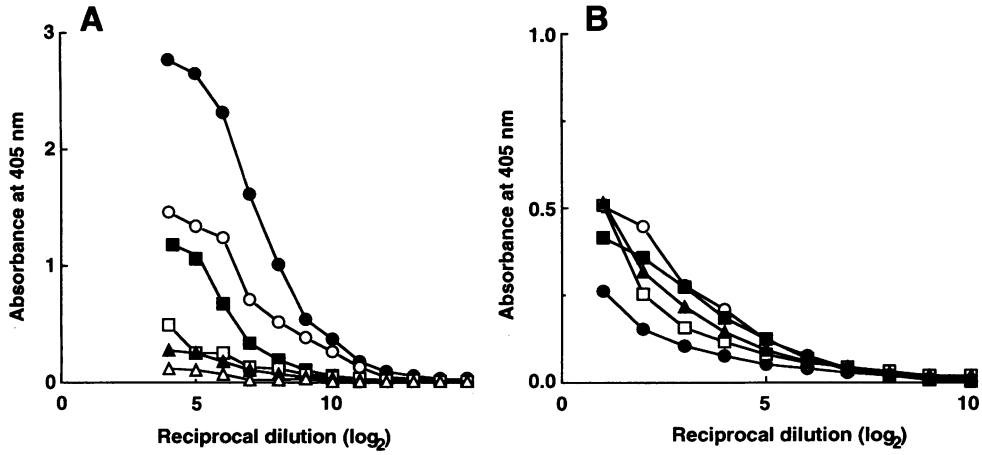

FIG. 2. Reactivities of rPAc with antibodies in serum (A) and saliva (B) samples from five adult subjects (●, subject A; ○, subject B; ■, subject C; □, subject D; ▲, subject E) and a 4-month-old infant (△). rPAc from transformant *S. mutans* TK18 was used as a coating antigen. Twofold serial dilutions of serum and saliva samples were assayed in triplicate by ELISA. The antibodies in the serum and saliva samples were detected with alkaline phosphatase-conjugated goat anti-human IgG (heavy and light chains) and alkaline phosphatase-conjugated goat anti-human IgA(α), respectively. The data are expressed as means for triplicate assays.

the A-region of the PAc (residues 269 to 278, 289 to 298, 319 to 328, 339 to 348, and 399 to 408) and four decapeptides corresponding to the middle region (residues 809 to 818, 819 to 828, 829 to 838, and 839 to 848). The epitope-scanning patterns in the serum samples from the other four adult donors were similar to the pattern in the serum sample from subject A, and the ELISA titers in the epitope scanning corresponded to the serum antibody titers to rPAc (Fig. 2A and 3). The serum from a 4-month-old infant hardly reacted with any decapeptides. Figure 4 shows the results of epitope scanning with the saliva samples (1:10 dilution) from five adult donors. The epitope-scanning patterns in the saliva samples from five adult donors were similar to those in serum samples from the same donors, that is, salivary antibodies reacted strongly with the decapeptides that the serum antibodies gave positive reactions to but reacted weakly with the decapeptides that the serum antibodies gave a negative reaction to. However, the ELISA titers of saliva antibodies to these decapeptides did not correspond to the ELISA titers of serum antibodies to them. For example, in the serum from subject A the ELISA titers of the antibodies to the decapeptides were generally high while in the saliva of the same subject the titers of the antibodies to them were low. Antibodies in the saliva sample (1:10 dilution) from subject A showed strong reactions ($A_{405} > 1.0$) with the 14 decapeptides (residues 999 to 1008, 1059 to 1068, 1079 to 1088, 1129 to 1138, 1199 to 1208, 1249 to 1258, 1259 to 1268, 1289 to 1298, 1299 to 1308, 1329 to 1338, 1409 to 1418, 1439 to 1448, 1459 to 1468, and 1556 to 1565) corresponding to the C-terminal region of the PAc, while antibodies in the saliva sample reacted weakly with decapeptides corresponding to the N-terminal and the middle regions of the PAc (Fig. 4A). On the other hand, in the saliva from subject E the ELISA titers of the antibodies to the decapeptides were generally high even though the antibodies in serum from the same subject reacted weakly with them.

Epitope scanning of the A-region and the middle region. As noted above, the epitope scanning of the PAc with human sera suggested that antigenic epitopes exist in the A-region and the middle region. Therefore, we synthesized a series of 121 overlapping decapeptides covering the A-region and a series of 21 overlapping decapeptides covering the middle region (residues 824 to 853) to determine the continuous antigenic epitopes in the regions. The serum (1:800 dilution) from subject A showed positive reactions ($A_{405} > 1.0$) with 27 decapeptides of the 121 overlapping decapeptides covering the A-region (Fig. 5). Among the five decapeptides in the A-region with which the serum showed positive reactions in the epitope scanning of the PAc, four decapeptides were recognized as antigenic epitopes in the epitope scanning of the A-region. Although residues 269 to 278 were not identified as an antigenic epitope in the epitope scanning of the A-region, in the serum from subject A the ELISA titer of antibody to the decapeptide was relatively high ($A_{405} = 0.74$) (Fig. 5A). All the decapeptides that showed positive reactions with human sera had either one or two of the five common sequences YQAXL, NADAKA, VQKAN, NNAKNA, and IKKRNA, where X is Asp, Glu, Lys, or Ala, suggesting that the antigenicity of the A-region might be determined by a few sequential B-cell epitopes. The epitope-scanning patterns with sera from other four adult donors and overlapping decapeptides corresponding to the A-region were similar to the pattern with the serum from subject A (Fig. 5). The serum from an infant did not react with any of the overlapping decapeptides corresponding to the A-region. The serum (1:1,600 dilution) from subject A reacted strongly ($A_{405} > 0.4$) with the 6 decapeptides of the 21 overlapping decapeptides covering the middle region of the PAc molecule (residues 824 to 853) (Fig. 6). These 6 decapeptides had one of the two common sequences KVTKEKP and VKPTAPTK.

Reactivity of human serum with purified 20-mer peptides. On the basis of the data in the epitope scanning of the PAc, we synthesized seven 20-mer peptides containing antigenic epitopes. All of the serum samples from five adult donors reacted with all the 20-mer peptides coated on the ELISA plates (Fig. 7). The serum from a 4-month-old infant did not react with any 20-mer peptides. To examine whether the antigenic activities of these peptides differ when they are tested as free peptides in solution or adsorbed to a layer of plastic, we used the inhibition assay for the binding of antibodies to rPAc. rPAc, PAc(96–114), PAc(331–349), PAc(513–531), PAc(623–641), PAc(824–842), and PAc(835–853) at 400 μg/ml resulted in 92.4% ± 3.6%, 23.6% ± 5.6%, 10.3% ± 2.0%, 23.3% ± 3.0%, 23.1% ± 0.9%, 31.8% ± 3.5%, and 19.7% ± 2.4%

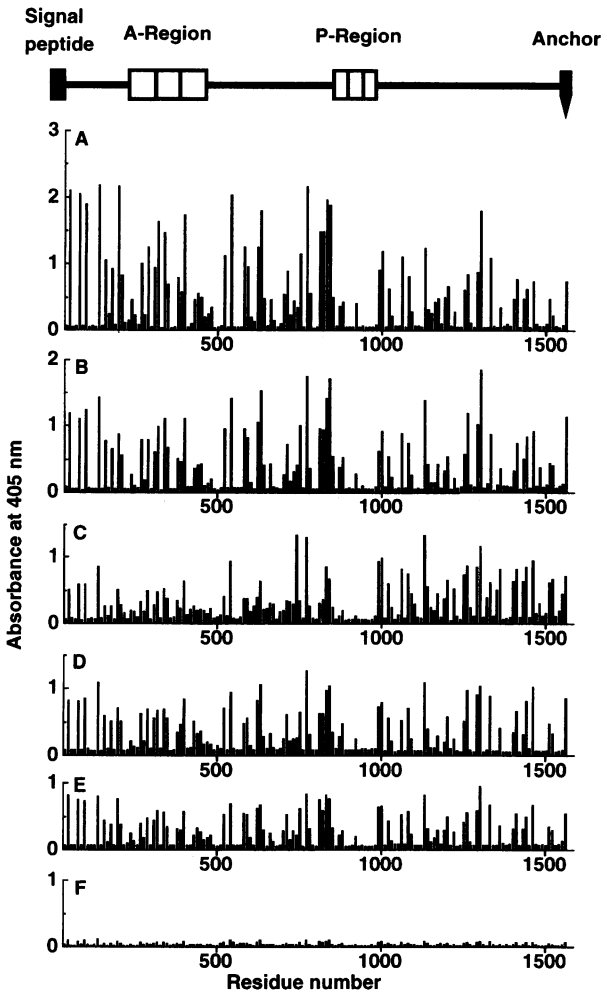

FIG. 3. Reactivities of human sera from five adult subjects (A to E) and a 4-month-old infant (F) with 153 decapeptides covering the complete amino acid sequence of the mature PAc. The reactivities of the peptides with human sera (1:800 dilution) were determined by ELISA as described in Materials and Methods. The bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgG (heavy and light chains). The data shown are means of A_{405}s after 1 h of incubation with the substrate for six assays. The proposed model of PAc is shown at the top. The residue number corresponds to the position of the amino-terminal residue of the indicated peptide in the PAc sequence (39).

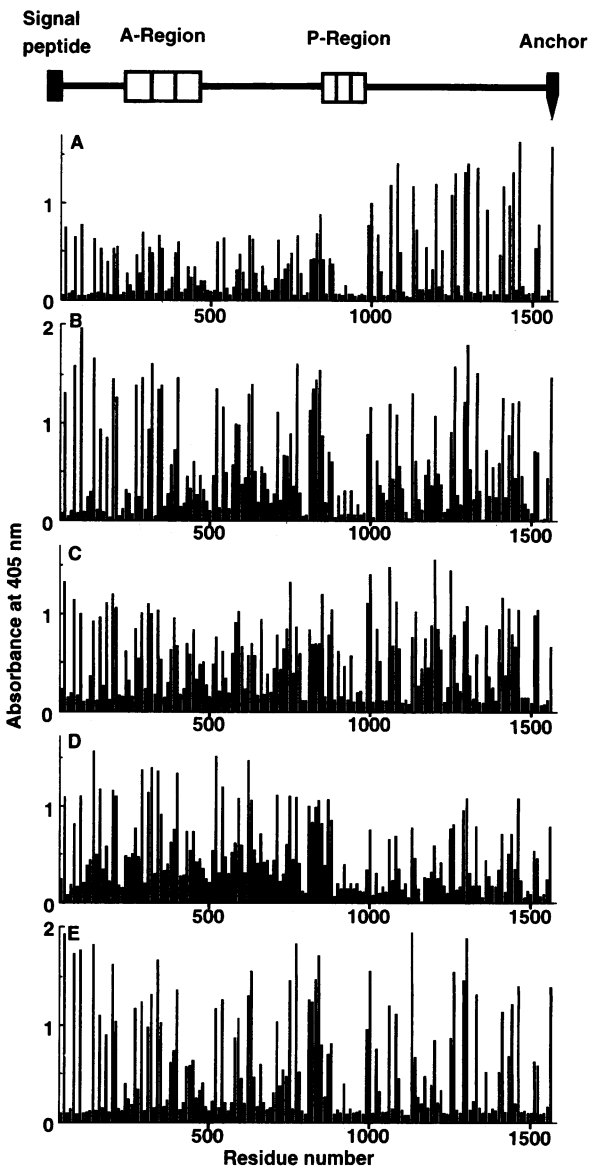

FIG. 4. Reactivities of human saliva from five adult subjects (A to E) with 153 decapeptides covering the complete amino acid sequence of the mature PAc. The reactivities of the peptides with human saliva (1:10 dilution) were determined by ELISA as described in Materials and Methods. The bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgA(α). The data shown are means of A_{405}s after 1 h of incubation with the substrate for six assays. The residue number corresponds to the position of the amino-terminal residue of the indicated peptide in the PAc sequence (39).

(mean ± standard deviation) inhibition, respectively, for the binding of antibodies to rPAc. However, PAc(764–782) and an irrelevant peptide, NA(464–482), at 400 μg/ml had no inhibitory effect. The discrepancy in PAc(764–782) might be ascribed to the aggregation of the peptide in solution or to the difference in conformation between the solid phase and the solution.

DISCUSSION

Using a complete set of decapeptides covering the mature PAc molecule, Takahashi et al. (47) mapped the antigenic sites of the protein antigen molecule in various strains of mice. They showed that the antigenic epitopes are scattered throughout the molecule and also that the antigenic epitope patterns differ in mice with different *H-2* haplotypes. To identify continuous antigenic epitopes in humans, we synthesized 153 decapeptides covering the mature PAc, 121 overlapping decapeptides covering the A-region of the PAc, and 21 overlapping decapeptides covering the middle region (residues 824 to 853) according to the multiple pin-coupled peptide technology. The antigenic epitope patterns that were determined by using these decapeptides and samples of serum and saliva from human subjects harboring *S. mutans* in their oral cavity differed considerably from those in mice. Despite the difference of the HLA type among these subjects, the epitope patterns in humans were similar to each other.

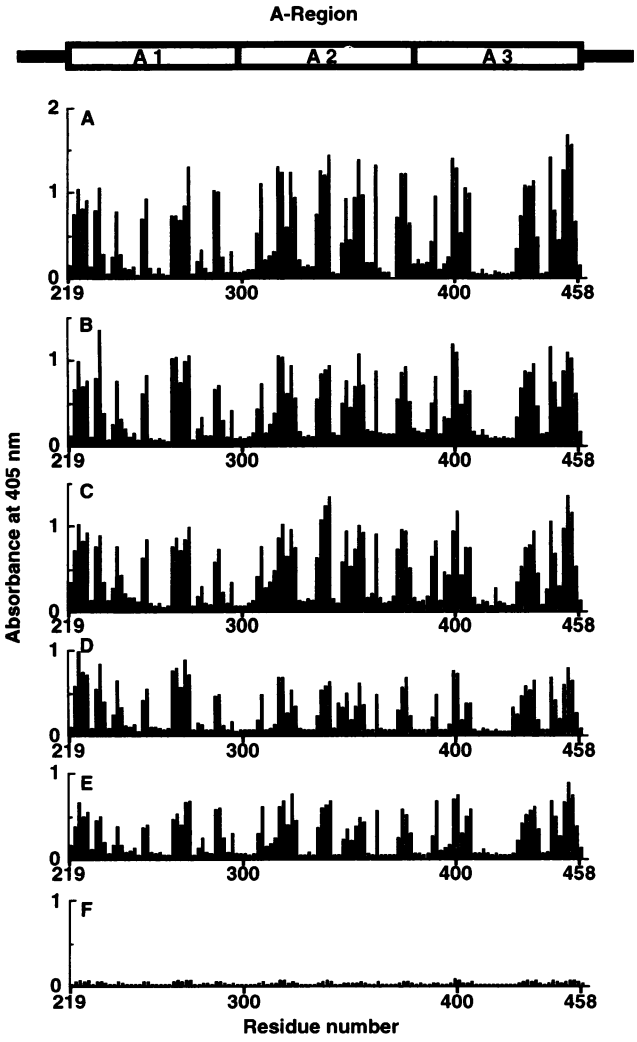

FIG. 5. Reactivities of human sera from five adult subjects (A to E) and a 4-month-old infant (F) with 121 overlapping decapeptides covering the A-region of the PAc protein. The reactivities of the peptides with human sera (1:800 dilution) were determined by ELISA as described in Materials and Methods. The bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgG (heavy and light chains). The data shown are means of A_{405}s after 1 h of incubation with the substrate for three assays. The residue number corresponds to the position of the amino-terminal residue of the indicated peptides in the PAc sequence (39).

In this study, 27 decapeptides of the 121 overlapping decapeptides covering the A-region showed positive reactions with human sera. All of these 27 decapeptides had one or two of five common sequences consisting of 4 to 6 residues, suggesting that the antigenicity of the A-region might be determined by a small number of B-cell epitopes. It is, however, difficult to rule out the presence to other B-cell epitopes, because only a limited number out of all possible epitopes were sampled in this study.

The PAc protein of *S. mutans* binds to human salivary agglutinin (3, 10, 43). The binding of the cell surface antigen to salivary agglutinin is considered to play an important role in the in vivo colonization of agglutinin-coated tooth surfaces by *S. mutans* (22, 26). Recently, several investigators have reported that a salivary agglutinin-binding domain exists in the A-region of the PAc (P1, SR) of *S. mutans* (9, 33, 35). Furthermore, it has been suggested that the A-region of the streptococcal cell surface antigen has antigenic and immunogenic epitopes in mice and rabbits (33, 40, 48). Takahashi et al.

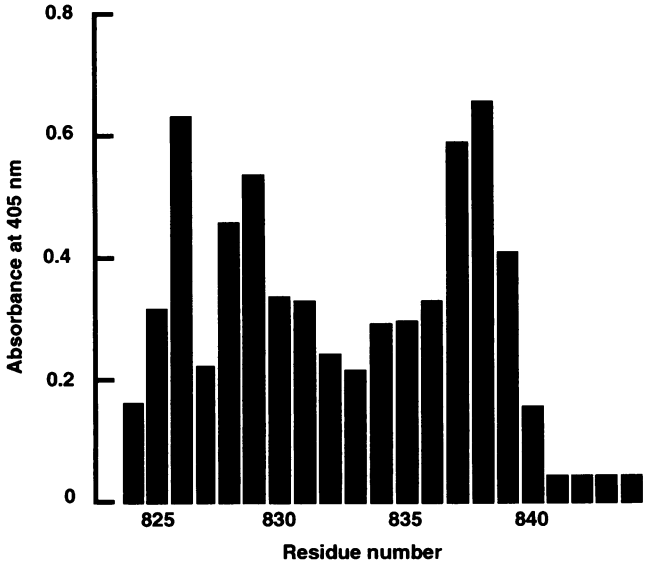

FIG. 6. Reactivities of human serum from subject A with 21 overlapping decapeptides covering the middle region of the PAc protein (residues 824 to 853). The reactivities of the peptides with human sera (1:1,600 dilution) were determined by ELISA as described in Materials and Methods. The bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgG (heavy and light chains). The data shown are means of A_{405}s after 1 h of incubation with the substrate for three assays. The residue number corresponds to the position of the amino-terminal residue of the indicated peptides in the PAc sequence (39).

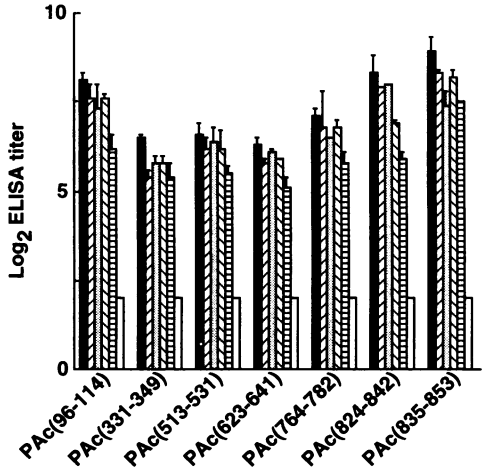

FIG. 7. Serum antibody responses to 20-mer synthetic peptides in five adult subjects (■, subject A; ▨, subject B; ▩, subject C; ▧, subject D; ▤, subject E) and a 4-month-old infant (□). The binding of the antibodies to synthetic peptides was detected by ELISA using alkaline phosphatase-conjugated goat anti-human IgG (heavy and light chains). The data shown are means ± standard deviations of the $\log_2$ ELISA antibody titers for triplicate assays. All experiments were performed three times, and similar results were obtained in each experiment.

(48) reported that the peptide ANAANEADYQAKLTAY QTE, corresponding to residues 301 to 319 of the PAc protein [PAc(301–319)], reacts with murine antisera to the PAc protein and induces protective immune responses in BALB/c mice. The peptide PAc(301–319) has one (YQAKL) of five amino acid sequences common among A-region-corresponding decapeptides that showed positive reactions with sera from human subjects harboring *S. mutans* in their oral cavity. Okahashi et al. (40) have identified antigenic epitopes in the A-region using antisera to rPAc from BALB/c, B10, B10.D2 and B10.DR mice and overlapping decapeptides covering the second repetitive unit of the A-region. They found that the peptide YEAALKQY (residues 366 to 373) is recognized by anti-rPAc sera from all of four strains of mice. In this study, the peptide KATYEAALKQ (residues 363 to 372) showed a positive reaction with human sera. Recently, Moisset et al. (33) have synthesized the peptide TELARVQKANADAKAAY (peptide 2), corresponding to the first repetitive unit of the A-region of the SR protein of serotype f *S. mutans*. Peptide 2 had two sequences, VQKAN and NADAKA, of the five antigenic common epitopes identified in this study. They demonstrated that peptide 2 reacts with anti-SR rabbit IgG and that the subcutaneous immunization of rabbits with the peptide conjugated to ovalbumin induces antibodies reactive with the peptide and SR protein. In addition, they showed that peptide 2 is able to inhibit the binding of human salivary glycoproteins to the SR protein. Taken together, these results suggest that the A-region of the PAc protein is a potentially important antigenic and functional domain.

PAc (I/II) of *S. mutans* is known to serologically cross-react with SpaA (PAg) of *S. sobrinus* (18, 42, 45, 49). The homology between the two protein antigens is 66% (25, 51). The sequences YEAKL and NAQAKA, which are similar to the antigenic common amino acid sequences in the A-region of PAc, are present in the amino acid sequence of the PAg (51). Recently, Okahashi et al. (40) demonstrated that residues 362 to 373 of the PAc protein and residues 365 to 376 of the PAg protein are one of the cross-reactive epitopes of both protein antigens in BALB/c mice. In this study, residues 363 to 372 of the PAc were identified as an antigenic epitope in humans. In addition, a saliva-binding protein (SSP-5) of *Streptococcus sanguis*, which is isolated most frequently among oral streptococci from human tooth surfaces, has a 59% homology with PAc of *S. mutans*, and the structure of the protein is very similar to that of PAc (11). The sequences NADAKA, VQ KAN, and NNAKNA exist in the amino acid sequence of the SSP-5 (11). Amino acid sequence homology research (National Biomedical Research Foundation-Protein Data Base, Washington, D.C.) showed that the antigenic common sequences YQAEL, YQADL, VQKAN, NADAKA, NNAKNA, and IKKRNA in antigenic epitopes in the A-region of PAc (I/II) exist in the amino acid sequences of 9, 7, 2, 32, 0, and 3 other proteins, respectively. It is possible that some of the antibodies to antigenic epitopes in the PAc might result from either cross-reactions or epitopes shared with other proteins if humans are exposed to them (50).

In the epitope scanning of the PAc protein with human saliva and serum samples, the reactivities against four decapeptides corresponding to the middle region of the PAc (residues 809 to 848) were as great as the reactivities against decapeptides corresponding to the A-region (Fig. 3 and 4). In addition, the reactivities of two 20-mer peptides, PAc(824–842) and PAc(835–853), with serum antibodies were greater than those of other 20-mer peptides (Fig. 7). Therefore, we synthesized 21 overlapping decapeptides covering the middle region (residues 824 to 853). Of the 21 decapeptides, 6 decapeptides reacted strongly with serum antibodies from subject A. Each of the six decapeptides had one of the two common sequences KVT KEKP and VKPTAPTK. The sequence KVTKEKP is present in PAg of *S. sobrinus* (residues 833 to 839) (51). In addition, residues 809 to 849 of the PAc adjacent to the P-region (residues 851 to 967) are identical to residues 813 to 843 of the PAg (39, 51). These findings suggest that the middle region adjacent to the P-region may be one of the immunologically cross-reactive domains of the *S. mutans* PAc protein and the *S. sobrinus* PAg protein.

Western blotting analysis with human sera demonstrated that the PAc protein of *S. mutans* was immunodominant in humans, in agreement with the results of other investigators (7, 44, 50). It has been reported that serum IgG antibodies to the immunodominant antigen I/II (PAc) are significantly greater in subjects with an experience of low caries than in subjects with high caries, thus suggesting that naturally induced serum IgG antibodies to the PAc are associated with protection against dental caries (7). Moreover, Challacombe (6) showed that there is an inverse relationship between the serum IgG antibodies to *S. mutans* and salivary IgA antibodies, and changes in the salivary antibody titer are negatively correlated with changes in the serum IgG antibody titer. These findings were also confirmed by the present study. Titers of salivary IgA to rPAc and synthetic peptides were not correlated with titers of serum antibody to them in this study. The inverse relationship between the serum IgG antibodies to *S. mutans* antigens and salivary IgA antibodies is therefore considered to be worthy of future study.

It has been thought that hydrophobic amino acids tend to be buried within the native structure of globular proteins, while hydrophilic side chains are on the exterior where they can interact with water (52). Hopp and Woods (19) showed that most hydrophilic segments of a protein tend to correspond to continuous antigenic epitopes. In this study, we predicted the hydrophilicity of the PAc molecule according to the procedure of Kyte and Doolittle (23). All the decapeptides that showed positive reactions with human sera had hydrophilic segments, but the chi-square statistical analysis showed that there was no correlation between antigenicity and hydrophilicity. Geysen et al. (14) also reported that there is little correlation between antigenicity and hydrophilicity in a myohemerythrin protein. Moreover, we predicted the secondary structure of the PAc molecule according to the procedure of Chou and Fasman (8). All the antigenic epitopes in the C-terminal three-fourths of the PAc protein contained beta-turn populations, but only three antigenic epitopes in the N-terminal quarter, which was predicted to be totally alpha-helical (39), contained beta-turn populations. These results suggest that antigenic epitopes may be located in the alpha-helixes at the surface of the protein as well as in the beta-turn populations.

In conclusion, we identified the continuous antigenic epitopes in the PAc molecule using 295 decapeptides and seven 20-mer synthetic peptides and human sera and saliva from subjects infected naturally with *S. mutans*. The identification of these immunodominant antigenic epitopes is considered to be a first step in the development either of specific diagnostic tests or of a vaccine against human dental caries.

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