Cloning of the Saliva-Interacting Protein Gene from Streptococcus mutans

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Genomic libraries from Streptococcus mutans OMZ175 were constructed in bacteriophage vectors. DNA fragments 1 to 2 kilobases in length were cloned in expression vector λgt11. S. mutans DNA fragments 15 to 20 kilobases in length were inserted in the BamHI site of phage EMBL3. Rabbit antiserum raised against an S. mutans saliva-interacting protein with a molecular weight of 74,000, designated 74K SR, was used to screen the λgt11 library. A recombinant phage carrying an S. mutans DNA sequence of 1.45 kilobases, λSmAD2, was detected and isolated. This fragment, named SmAD2, was used to construct the recombinant expression plasmid pSmAD2-4 which encoded for the expression of a 60,000-molecular-weight protein controlled by the β-galactosidase promoter from plasmid pUC8. The SmAD2 fragment and polyclonal anti-74K SR antibodies were used to screen the EMBL3 library. A total coincidence between the screening with antibodies and the DNA probe was observed, and two phages, λSmAD9 and λSmAD10, were isolated. They contained a common S. mutans DNA sequence of about 11.8 kilobases and coded for a protein with a molecular weight of about 195,000, which comigrated with a protein of an S. mutans cell wall extract. The expressed protein was purified, and a very strong relationship with the S. mutans 74K SR protein was found by competitive enzyme-linked immunosorbent assay. Thus, cloning of the 74K SR gene allowed us to demonstrate that the saliva receptor appears to be a part of an S. mutans precursor molecule with a molecular mass of 195,000 daltons.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and maintenance. S. mutans OMZ175 serotype f, previously described by Schöller et al. (33), was grown in brain heart infusion broth (Difco Laboratories). Escherichia coli Y1088, Y1089, and Y1090 and phage λgt11 were obtained from Young and Davis (37). E. coli Q358 and Q359 and phage EMBl3 were obtained from Frischauf et al. (9). Plasmids pUC8, pUC8-1, and pUC8-2 were given by Hana et al. (11), and E. coli JM103 was given by Messing and Vieira (21). E. coli BHB2688 and BHB2690 have been described by Hohn (12). E. coli C600-5K has been described by Hubacek and Glover (15), and plasmid pBR322 has been described by Bolivar et al. (3). E. coli Y1088, Y1089, and Y1090 were grown in L broth (22) supplemented with ampicillin (50 µg/ml). The medium for E. coli Q358, BHB2688, BHB2690, and C600-5K was L broth, while strain Q359 was grown on BBL broth (9).

Chemicals. DNase I and RNase A from bovine pancreas, lysozyme from chicken egg white, bovine serum albumin, Triton X-100, isopropyl-β-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galactoside, tetracycline, and ampicillin were purchased from Sigma Chemical Co. Restriction endonucleases were obtained from Appligene SA and Bethesda Research Laboratories, Inc. Yeast tRNA, glycogen, and agarose were obtained from Appligene. The Nick Translation System, biotin-7-dATP, T4 DNA ligase, bacterial alkaline phosphatase, cesium chloride, and phenol were obtained from Bethesda Research Laboratories. Proteinase K was provided by Boehringer Mannheim Biochemicals. Nitrocellulose and nylon sheets and disks (Hybond C and N), [αS32P]dATP, and 125I-protein A were purchased from Amersham Corp. Goat alkaline phosphatase (GAP)-labeled

In recent years, considerable progress has been made in understanding the interactions involved in Streptococcus mutans adherence to teeth surfaces. Salivary glycoproteins adsorbed onto the hydroxyapatite of the tooth enamel seem to play a major role in the initial attachment of S. mutans (4). Recent data suggest the existence of nonspecific (10, 38) and specific (24, 28) interactions between salivary glycoproteins and streptococcal cell wall components. Several lines of evidence suggest that the cell wall protein of S. mutans serotype f named 74K SR (2), the glucan-binding protein (30), SpaA, and SpaA-related antigens (6) like antigens I/II (29) and B (31) play a role in the specific interactions of S. mutans with salivary glycoproteins. Antigen I/II has been shown to interact preferentially with salivary amylase by Western blotting analysis (28). Polyclonal antibodies against SpaA can partially block the adherence of S. mutans to saliva-coated hydroxyapatite beads (8). Purified 74K SR protein has the ability to inhibit the interactions between S. mutans cells and salivary glycoproteins (2). In a previous study, we described monoclonal antibodies against the S. mutans serotype f 74K SR protein. They blocked saliva binding to S. mutans cells (24) and recognized common epitopes on proteins present in cell wall extracts (CWEs) from strains representative of different S. mutans serotypes (1). These monoclonal antibodies were also able to recognize epitopes on a higher-molecular-weight protein in the CWE from S. mutans serotype f (1). Therefore, to study the correlation between the proteins in the S. mutans CWE, one approach is to clone the gene of one of the proteins and analyze its expression products.

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anti-rabbit immunoglobulin G (IgG) antibodies and anti-rat IgG antibodies were obtained from Miles Laboratories, Inc.

**Antiserum preparation.** Rabbit polyclonal antibodies and rat monoclonal antibodies raised against the *S. mutans* OMZ175 74K SR protein were obtained as described by Ackermans et al. (1, 2). Rabbit anti-*S. mutans* lactate dehydrogenase and anti-CWE antibodies were prepared as previously described (33, 34). Preimmune sera were obtained from rabbits and rats. Anti-*E. coli* antibodies were removed from all antibodies as described previously (37).

*S. mutans* DNA, phage, and plasmid DNA preparations. Total *S. mutans* DNA was isolated by the method of Robeson et al. (27). Phages were grown as liquid lysates in L broth or NZCYM medium (20) for *Agt11* and in L broth or BBL medium for EMBL3. After lysis, the phages were purified from bacterial debris as described by Frischauf et al. (9), and the DNA was obtained after treatment of the phage particles with 50% (vol/vol) formamide in 200 mM Tris hydrochloride (pH 7.5)-20 mM EDTA, followed by two dialyses against 50 mM Tris hydrochloride (pH 7.5)-10 mM EDTA-100 mM NaCl. Small quantities of partially purified phage DNA were obtained after RNase A (50 μg/ml) and DNase I (50 μg/ml) treatments of phage lysates and precipitation with 10% (wt/vol) polyethylene glycol in 5 mM Tris hydrochloride (pH 8.0)-1 M NaCl-5 mM MgSO4. The DNA was extracted after destabilization of phage particles with 0.1% (vol/vol) sodium dodecyl sulfate (SDS) in 5 mM EDTA. Large-scale plasmid preparations were obtained as described by Clewell and Helinski (5), and the plasmids were purified on CsCl-ethidium bromide gradients. Small-scale plasmid preparations were obtained by the method of Hymes and Quigley (13).

**Construction of recombinant phages.** A genomic library was constructed in *Agt11* phage by the method of Young and Davis (37). Chromosomal *S. mutans* DNA was partially digested with *AluI*, and a DNA fraction enriched in 1- to 2-kilobase-pair (kbp) fragments was obtained after centrifugation of the digested DNA through a 15 to 40% (wt/vol) sucrose gradient. Synthetic *EcoRI* linkers (New England BioLabs, Inc.) were ligated to these fragments with T4 DNA ligase. After digestion with *EcoRI*, the free linkers were eliminated on a Bio-Gel A-50M column (Bio-Rad Laboratories). The DNA fragments were inserted into *EcoRI*-cleaved and phosphatase-treated *Agt11* DNA, with a 1:50 mass ratio of *S. mutans* DNA/vector DNA. After ligation with T4 DNA ligase, the phages were in vitro packaged (12), and the bank was titrated and amplified on *E. coli* Y1088.

Another genomic library was constructed in phage EMBL3 by the method of Frischauf et al. (9). The chromosomal DNA was partially digested with *Sau3A*, and a fraction enriched in 15- to 20-kbp fragments was obtained after centrifugation through a 15 to 40% sucrose gradient. These fragments were inserted into *BamHI*-cleaved and phosphatase-treated EMBL3 DNA with a 1:40 mass ratio of *S. mutans* DNA/vector DNA. After in vitro packaging, the phages were amplified on *E. coli* Q359.

**Screening of phage libraries.** The *Agt11* library was plated on *E. coli* Y1090 to give approximately 20,000 plaques per plate (85-mm diameter) and screened with rabbit antisera raised against the *S. mutans* 74K SR protein (diluted 1:500). Immune complexes were detected with 32P-protein A (37). Subsequent screening was repeated until all plaques were positive. The clones were finally amplified on *E. coli* Y1090.

Positive clones were also isolated from the EMBL3 library by using a similar immunological procedure. However, since *E. coli* Q359 was used as the host (9), the nitrocellulose disks were not impregnated with IPTG, and the transfer of phages was achieved over 17 h at 37°C. Screening by in situ hybridization of EMBL3 recombinants was also performed with 32P-labeled probes (20).

In all cases, the selected clones were amplified and their antigenic specificity and DNA complementarity with defined DNA probes were checked by dot phase blotting (25).

**Construction of recombinant plasmids.** The recombinant DNA of *Agt11* positive clones was partially purified as described above and digested with *EcoRI* in the presence of RNase A under standard conditions (20). Samples were mixed with 400 ng of *EcoRI*-digested and phosphatase-treated pBR322 by using a 3:1 mass ratio of DNA/vector and ligated with T4 DNA ligase. Transfection of competent *E. coli* C600-5K was done essentially as described by Maniatis et al. (20). Tetracycline-resistant recombinants were selected, and their DNAs were prepared as described above. The appropriate insert was further digested with *EcoRI* and purified on a 5 to 20% (wt/vol) sucrose gradient. Subcloning of this insert in *EcoRI*-digested and phosphatase-treated plasmids pUC8, pUC8-1, and pUC8-2 was achieved by using a 3:1 mass ratio of DNA/vector (20).

**Hybridization.** Total *S. mutans* OMZ175 DNA (20 μg) or plasmid DNA (5 μg) was digested with one or two restriction endonucleases, electrophoresed for 17 h in an 0.8%/wt/vol) agarose gel, and transferred to nylon membranes (Hybond N; Amersham). DNA fragments used as probes were labeled with the Nick Translation System of Bethesda Research Laboratories by using [α-32P]dATP or biotin-7-dATP. Southern blotting (35) was performed essentially by the method of Reed and Mann (26) for the radioactive probe and by the procedure described by Bethesda Research Laboratories when a biotinylated probe was used.

**Preparation of protein fractions.** CWE was used as an antigenic reference that was obtained from washed *S. mutans* OMZ175 cells (33). The 74K SR protein was purified from this extract as described by Ackermans et al. (2).

The production of fusion proteins in *Agt11* was obtained in the lysogenic Y1089 cells (16, 18). The expression of inserted *S. mutans* DNA was also checked in the expression plasmids pUC8, pUC8-1, and pUC8-2 (11). Transformed cells (JM103) grown in L broth supplemented with 10 mM IPTG were harvested by centrifugation and suspended in 20 mM Tris hydrochloride (pH 8.0). Lysozyme (1 μg/ml of culture) was added for 1 h at 4°C, and the cells were sonicated at maximum power for 2 min. After treatment with DNase I and RNase A (10 μg of each enzyme per ml, 15 min, 4°C), the soluble fraction was separated from insoluble material by centrifugation (12,000 × g, 20 min, 4°C) and dialyzed against 20 mM Tris hydrochloride (pH 8.0). For some rapid verifications, JM103 pellets were directly suspended in the electrophoresis dissociation buffer (18). The lysozyme-RNase-DNase treatment was also used to analyze the expression products of recombinant EMBL3 in infected cells. Further purification of expression products of recombinant EMBL3 was achieved by ammonium sulfate precipitation at 70% (wt/vol) saturation. After centrifugation (25,000 × g, 30 min, 4°C), the precipitated proteins were suspended and dialyzed against 10 mM Tris hydrochloride (pH 7.4) containing 100 mM NaCl and chromatographed on a Sephacryl S-300 column (200 by 1.6 cm; Pharmacia Fine Chemicals, Inc.) at a flow rate of 15 ml/h in the same buffer. Fractions of 3.5 ml were collected and concentrated by lyophilization, suspended in 5 mM phosphate buffer (pH 7.4)-150 mM NaCl, and conserved at 4°C for further investigations.
Protein determination. Protein concentrations were measured by the method of Lowry et al. (19).

Electrophoresis and Western blot (immunoblot) analysis. Protein fractions were electrophoresed in 10% (wt/vol) polyacrylamide slab gels by the method of Laemmli (18). Antigens were either stained with 0.1% (wt/vol) Coomassie blue or transferred to nitrocellulose sheets (pore size, 0.42 μm; Millipore Corp.) by the procedure of Towbin et al. (36). The sheets were incubated with rabbit antiserum (diluted 1 : 100) raised against the S. mutans 74K SR protein, which had been incubated previously with E. coli extracts adsorbed onto nitrocellulose sheets. GAP anti-rabbit IgG antibody (1 : 2,500) was used to detect immune complexes on the sheets (1, 2).

Solid-phase immun assay. The antibody recognition of expressed proteins was checked by enzyme-linked immunosorbent assay (ELISA), sandwich ELISA, or competitive ELISA. In the first procedure, microtiter plates (Dynatech Laboratories, Inc.) were coated with 200 μl of proteins. Serial dilutions of rat monoclonal or rabbit polyclonal anti-74K SR antibodies were added to the wells. Antibody binding was detected with GAP anti-rabbit or anti-rat antibodies (1). Sandwich ELISA was performed by coating the rabbit anti-74K SR antibodies (15 μg per well). Serial dilutions of the tested antigens were then added, and the immune complexes were detected with rat monoclonal anti-74K SR antibodies, followed by GAP anti-rat antibodies, as described above.

In the competitive ELISA procedure, 50 μl of a predetermined saturating amount of polyclonal anti-S. mutans 74K SR antibodies was incubated for 2 h at 37°C with 50 μl of serial dilutions of the fractions under study. Samples were then transferred to microtiter plates coated with S. mutans CWE (1.5 μg per well), and antibody binding was determined as described above. The results are expressed as percent inhibition of antibody binding to the homologous antigen. Percent inhibition was calculated as 100 x [(OD of the control - OD of the competitor)/OD of the control], where OD is the optical density (1, 2).

RESULTS

Detection of phage recombinants in the λgt11 library. A genomic library of S. mutans DNA was prepared by shotgun cloning in the expression vector λgt11, giving 1.6 x 10⁶ recombinant phages. Plaques (2 x 10⁵) were screened with polyclonal antibodies directed against the S. mutans 74K SR protein. Strong antibody recognition was detected for only six plaques, and two clones (named λSmAD1 and λSmAD2) were isolated by further screening and amplified on E. coli Y1090 until titers reached at least 10⁸ phages per ml. The lysates were then probed by dot blotting with two unrelated rabbit antibodies (preimmune and anti-S. mutans lactate dehydrogenase antibodies) and anti-S. mutans 74K SR protein. Only the λSmAD2 clone behaved unambiguously and was used to produce a fusion protein. Lysogens of λgt11 and λSmAD2 were prepared and induced with IPTG. Expressed proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting (Fig. 1). A protein with a molecular weight of about 165,000 was recognized by anti-S. mutans 74K SR antibodies in lysate infected with λSmAD2 (Fig. 1, lane c) but was not present in the λgt11 lysate (Fig. 1, lane d). Concomitantly, a protein with a mass of about 115,000 daltons, which was expected to be β-galactosidase, was observed in the λgt11-infected bacteria (Fig. 1, lane a) but was not observed in the λSmAD2-infected cells (Fig. 1, lane b). Thus, the 165,000-dalton protein corresponded to a fusion protein that harbored antigenic determinants of the S. mutans 74K SR protein on a peptide with a mass of more than 50,000 daltons fused to the β-galactosidase fragment encoded by λgt11. This peptide corresponded to the coding capacity of the 1.45-kbp EcoRI insert of λSmAD2 which was obtained from a small-scale preparation of the recombinant phage DNA.

Construction of chimeric plasmids containing the S. mutans EcoRI DNA fragment SmAD2 of λSmAD2. The SmAD2 1.45-kbp DNA fragment was ligated to the EcoRI-digested pBR322 vector, and competent E. coli C600-5K cells were transformed with the resulting chimeric plasmids called pSAD2. Restriction endonuclease analysis of SmAD2 revealed single sites for BamHI, EcoRV, HindIII, AccI, and PstI and no sites for KpnI, Sall, EcoRI, or NarI (Fig. 2a). The S. mutans SmAD2 fragment was further inserted into expression plasmid vectors to examine the possible translation products. The 1.45-kbp fragment was purified from pSAD2 after digestion with EcoRI and inserted in the EcoRI site of plasmids pUC8, pUC8-1, and pUC8-2. Six chimeric plasmids were then constructed, which resulted in two orientations of SmAD2 in each vector. Orientation 1 was such that the small EcoRI-BamHI fragment was proximal to the lac promoter (Fig. 2b). This orientation was found for plasmids pSAD2-1, pSAD2-2, and pSAD2-3, which were derived from pUC8, pUC8-1, and pUC8-2, respectively. The

FIG. 1. Immunological identification of chimeric proteins after SDS-PAGE of E. coli Y1090 cell extracts infected by λgt11 (lanes a and d) or by λSmAD2 (lanes b and c). The proteins were either stained with Coomassie brilliant blue (lanes a and b) or transferred to nitrocellulose sheets (lanes c and d) and then probed with anti-S. mutans 74K SR antibodies adsorbed with λgt11-infected Y1090 extract. The following protein markers, with sizes indicated in kilodaltons, were used: macroglobulin, 170; β-galactosidase, 116; phosphorylase b, 94; bovine serum albumin, 67; and ovalbumin, 43. The arrow on the right indicates the position of the fusion protein expressed by λSmAD2.
BamHI site with HindIII (H), and PstI (P). The thick arrow indicates the reading frame orientation determined in λSmAD2. The thick line represents SmAD2 DNA, and the broken line represents pBR322 DNA. Symbol: \( \wedge \), positions of EcoRI linkers. (b and c) Construction of expression plasmids. EcoRI-digested SmAD2 fragments were inserted into plasmids pUC8, pUC8-1, and pUC8-2, and the insert orientation was checked by restriction analyses with BamHI (B) and PstI (P). Orientation I (panel b) corresponds to the position of the BamHI site near the lac promoter of pUC plasmids. The plasmids in this orientation were designated pSAD2-1, pSAD2-2, and pSAD2-3 and derived from plasmids pUC8, pUC8-1, and pUC8-2, respectively. Orientation II (panel c) corresponds to the position of the PstI site near the lac promoter, and the resulting plasmids were named pSAD2-4, pSAD2-5, and pSAD2-6.

Small EcoRI-PstI fragment was proximal to the lac promoter in orientation II (Fig. 2c) and was found for pSAD2-4, pSAD2-5, and pSAD2-6.

**Characterization of SmAD2 by expression.** The orientation of the SmAD2 fragment in phage λSmAD2 was first determined by restriction analysis with BamHI and HindIII, as shown in Fig. 2a. Construction of chimeric plasmids in pUC8, pUC8-1, and pUC8-2 allowed us to obtain the insertion of SmAD2 in all three reading frames under the control of the lac promoter (11). The expression of a fusion protein was detected only in IPTG-induced JM103 cells containing pSAD2-4, in which the reading frame was as in λSmAD2. These cells synthesized a fusion peptide with a molecular weight of about 60,000 (Fig. 3, lane b) which was recognized by anti-*S. mutans* 74K SR antibodies (Fig. 3, lane e). Other products with slightly lower molecular weights were also detected by antibodies (Fig. 3, lane e) and might be degradation products. No fusion peptide was made by uninduced cells (data not shown) or by transformants carrying plasmid pUC8 (Fig. 3, lanes a and f) and the other chimeric plasmids with SmAD2 in orientation I (data not shown) and II (Fig. 3, lanes c and d). The molecular weight of the fusion peptide was consistent with the expression of all or almost all of the cloned sequence. However, a molecular weight of about 60,000 was far from 74,000, which is the molecular weight of the *S. mutans* 74K SR protein (2), and a true correlation between both antigens required the determination of common epitopes. Six rat monoclonal antibodies (one IgG and a mixture of five IgM antibodies) raised against the *S. mutans* 74K SR peptide were able to detect such common products.

**FIG. 2.** Restriction map of SmAD2 and construction of expression plasmids. (a) The physical map of SmAD2 was constructed with restriction enzymes AccI (A), BamHI (B), EcoRI (E), EcoRV (R), HindIII (H), and PstI (P). The thick arrow indicates the reading frame orientation determined in λSmAD2. The thick line represents SmAD2 DNA, and the broken line represents pBR322 DNA. Symbol: \( \wedge \), positions of EcoRI linkers. (b and c) Construction of expression plasmids. EcoRI-digested SmAD2 fragments were inserted into plasmids pUC8, pUC8-1, and pUC8-2, and the insert orientation was checked by restriction analyses with BamHI (B) and PstI (P). Orientation I (panel b) corresponds to the position of the BamHI site near the lac promoter of pUC plasmids. The plasmids in this orientation were designated pSAD2-1, pSAD2-2, and pSAD2-3 and derived from plasmids pUC8, pUC8-1, and pUC8-2, respectively. Orientation II (panel c) corresponds to the position of the PstI site near the lac promoter, and the resulting plasmids were named pSAD2-4, pSAD2-5, and pSAD2-6.

**FIG. 3.** Expression of recombinant pUC plasmids. Whole-cell extracts of *E. coli* JM103 carrying the pUC8 plasmid (lanes a and f) and the recombinant plasmids pSAD2-4 (lanes b and e), pSAD2-5 (lane c), and pSAD2-6 (lane d) were analyzed by SDS-PAGE and Western blotting. *S. mutans* CWE was submitted to the same treatment (lane g). Proteins were stained with Coomassie brilliant blue (lanes a to d) or transferred to nitrocellulose sheets (lanes e to g) and revealed with anti-*S. mutans* 74K SR antibodies adsorbed with pUC8-infected JM103 cell extract. The arrow on the left indicates the position of the fusion peptide expressed by pSAD2-4. The following protein markers are indicated on the figure, with sizes indicated in kilodaltons: phosphorylase b, 94; bovine serum albumin, 67; ovalbumin, 43; and carbonic anhydrase, 30.

**FIG. 4.** Characterization of expression products from recombinant pUC8 plasmids by ELISA. Rabbit polyclonal antibodies raised against the *S. mutans* 74K SR protein were coated on polystyrene plates (15 μg per well) and allowed to react for 1 h at 37°C with increasing amounts of protein extracts. The results of incubation of extracts from IPTG-induced cells carrying pSAD2-4 (● and ×) or pUC8 (▲ and ●) are shown. *S. mutans* CWE was used as a reference (■). A mixture of five rat IgM monoclonal antibodies (■, ●, and ▲) or one IgG monoclonal antibody (× and ●) directed against the *S. mutans* 74K SR protein was then added at 50 μg/ml and incubated for 1 h at 37°C. The complexes were finally detected with GAP anti-rat antibodies. The absorbance values were measured after incubation with substrate for 45 min at 37°C. O.D., Optical density.
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BamHI (B), EcoRI (E), EcoRV (R), HindIII (H), PstI (P), and some of the enzymes indicated above and probed with nick-translated SmAD2. The EMBL3 DNA is indicated by the broken line. (c) Genomic DNA mapping. S. mutans chromosomal DNA (20 µg) was digested with BamHI (B), EcoRI (E), EcoRV (R), HindIII (H), KpnI (K), and PstI (P) and probed with SmAD2 by Southern hybridization. The thick line corresponds to the SmAD2 sequence. The thin line represents S. mutans genomic DNA.

determinants on the fusion peptide synthesized in cells containing pSAD2-4 in ELISA experiments (Fig. 4). Extracts from noninduced cells (data not shown) and cells harboring the pUC8 plasmid (Fig. 4) and the other pSAD plasmids (data not shown) did not show any reaction.

Screening of the EMBL3 library. The SmAD2 fragment was labeled and used as a probe to screen the EMBL3 library. Among 4,000 recombinant phages screened, 30 phages were able to hybridize with SmAD2, but only six clones gave a strong signal and were further isolated. Direct immunological screening of the EMBL3 library with the anti-74K SR antibodies gave almost the same rate of positive clones. Isolated positive recombinant clones were tested for the ability to produce antigens expressed under the control of their own promoters and recognizable by anti-S. mutans 74K SR antibodies. In all cases, positive signals were obtained. The recombinants which exhibited the strongest reactions, λSmAD9 and λSmAD10, were further analyzed.

Restriction map of EMBL3 recombinants. The restriction endonuclease maps of λSmAD9 and λSmAD10 were realized by using the restriction endonucleases BamHI, EcoRI, KpnI, NruI, SalI, and XbaI. The whole sequence of λSmAD9 and λSmAD10 inserts (called SmAD9 and SmAD10) obtained after digestion with SalI (Fig. 5a and b) was estimated to about 13.5 and 12.5 kbp, respectively, with a common region of 11.8 kbp. The SmAD2 fragment was shown to hybridize roughly in the central part of both SmAD9 and SmAD10 (Fig. 5a and b). Southern blotting experiments (Fig. 5c) for S. mutans chromosomal DNA showed the same map as for SmAD9 and SmAD10 fragments.

Expression of λSmAD9 and λSmAD10. Protein synthesis directed by λSmAD9 and λSmAD10 was studied with E. coli Q358 and Q359. Protein fractions were submitted to SDS-PAGE and tested by Western blotting with polyclonal anti-S. mutans 74K SR antibodies. Figure 6 shows the production of a protein with a molecular weight of about 195,000 in cells infected with λSmAD9 and λSmAD10 (Fig. 6, lanes b, c, g, and h), which migrated just like one of the proteins detected in the CWE from S. mutans OMZ175 (Fig. 6, lanes e and i) and was the sole subunit which was recognizable in E. coli-infected extracts by anti-74K SR antibodies. Furthermore, when the 195,000-molecular-weight subunit was purified on Sephacryl S-300 from λSmAD9-infected Q358 cells (Fig. 6, lane a), only fractions containing this subunit reacted with polyclonal anti-74K SR antibodies (Fig. 7A). Uninfected cells, as well as cells infected with wild-type EMBL3 (data not shown) and nonrelated EMBL3 recombinants (Fig. 6, lanes d and f), did not produce any protein of the same size. Production of a subunit of 74,000 daltons corresponding to the 74K SR protein purified from S. mutans and recognizable by the antibodies was never found. However, a strong antigenic relationship between the 195,000-molecular-weight protein and the 74K SR protein could be demonstrated by ELISA: monoclonal antibodies were able to recognize antigenic determinants on the 195,000-molecular-weight protein (data not shown), and 85% inhibition of recognition of the S. mutans 74K SR protein by polyclonal anti-74K SR antibodies was obtained when the 195,000-molecular-weight protein was added before the homologous 74K SR (Fig. 7B).

DISCUSSION

We described the cloning in E. coli of the gene of the 74K SR protein, which has been implicated in S. mutans sucrose-independent adhesion (2). When produced in recombinant E. coli, the protein has a molecular weight of 195,000. The relationship between the 195,000-dalton protein expressed in recombinant E. coli and the 74K SR protein was demonstrated by the homology of the antigenic behavior of the two proteins. The cloned gene is strongly expressed in E. coli under the control of its own promoter. The use of the EMBL3 vector enabled the expressed protein to be directly detected by antibody within lysis plaques but did not allow

![FIG. 5. Restriction maps of phages λSmAD9 (a) and λSmAD10 (b). Maps were obtained by using BamHI (B), EcoRI (E), KpnI (K), NruI (N), SalI (S), and XbaI (X). The localization of SmAD2 on the phages was done by Southern hybridization. The phage DNA was digested with EcoRV (R), HindIII (H), PstI (P), and some of the enzymes indicated above and probed with nick-translated SmAD2. The EMBL3 DNA is indicated by the broken line. (c) Genomic DNA mapping. S. mutans chromosomal DNA (20 ng) was digested with BamHI (B), EcoRI (E), EcoRV (R), HindIII (H), KpnI (K), and PstI (P) and probed with SmAD2 by Southern hybridization. The thick line corresponds to the SmAD2 sequence. The thin line represents S. mutans genomic DNA.](http://jb.asm.org/)

![FIG. 6. Expression of EMBL3 recombinants. Crude cell extracts of E. coli Q358 infected by λSmAD9 (lanes b and h), λSmAD10 (lanes c and g), or a nonrelated EMBL3 recombinant (lanes d and f), as well as the Sephacryl S-300-purified 195,000-molecular-weight protein encoded by λSmAD9 (lane a) were analyzed by SDS-PAGE (lanes a to d) and Western blot (lanes f to h) and compared with an S. mutans CWE (lanes e and i). Proteins were stained with Coomasie brilliant blue or transferred to nitrocellulose sheets and revealed with rabbit anti-S. mutans 74K SR antibodies (lanes f to h) or anti-CWE antibodies (lane i). The arrows indicate (in kilodaltons) the positions of the following protein markers: myosin, 200; β-galactosidase, 116; phosphorylase b, 94; bovine serum albumin, 67; ovalbumin, 43; and carbonic anhydrase, 30.](http://jb.asm.org/)
us to study the eventual translocation of the expressed protein to the surface of the E. coli host.

Genes of several other S. mutans surface proteins implicated in the adherence process have already been cloned. The 74,000-molecular-weight glucan-binding protein from S. mutans serotype c, which has been described by Russell (30), was compared to the 74K SR protein. No immunological cross-reaction was observed between these proteins (R. R. B. Russell, personal communication), and the cloning of the glucan-binding protein gene in E. coli resulted in the expression of a protein with a molecular weight of 74,000 (32). This latter point indicates that the two proteins are expressed differently in E. coli. The SpaA gene, cloned in E. coli, expressed a protein with a molecular weight of 160,000 to 170,000 (14), which is different from the molecular weight of 210,000 of the SpaA characterized in S. mutans serotype g. Comparison of the restriction maps of the genes of the SpaA protein (14) and the 74K SR protein (Fig. 5) did not show any correlation between the positions of the restriction sites of BamHI, EcoRI, PstI, and HindIII. This result suggests that there is not a close relationship between the two proteins, and at least a great part of their sequences should be different. This point may be related to the unequal epitope distribution observed for several cross-reacting proteins from S. mutans serotypes, previously determined with monoclonal antibodies (1), and may be related particularly to the weak recognition between cell wall-associated proteins from serotypes g and f.

The molecular weight of the expression product of the SpaA gene in E. coli indicated that a maturation step was probably not realized in E. coli (6). A similar phenomenon may explain the difference in molecular weights between the 74K SR protein characterized in S. mutans OMZ175 serotype f and the 195,000-dalton protein expressed in recombinant E. coli. The 195,000-dalton protein could be a precursor of the 74K SR protein, which could be the result of a maturation process in S. mutans cells. Besides, breakdown fragments of SpaA-related antigens have been reported (29, 31), and cross-reactions have been observed between the 74K SR protein and a cell wall protein with a molecular weight of about 125,000, recently reevaluated at 150,000 (1). The presence of saliva-binding proteins with similar molecular weights in CWEs from all the strains tested in our laboratory (23) suggests that this maturation process may be an essential and general step in the interactions between salivary components and S. mutans cells.

We are now interested in comparing the proteolytic pathways of different S. mutans strains and the effect of the mode of antigen preparation on the resulting digestion pattern. The cloning of protease genes from S. mutans (17) will be of great help in these studies. Another approach in understanding the maturation process is to study the sequential breakdown of the expression products of the cloned genes and analyze the maturation steps and the function of these proteins by using directed mutagenesis of the cloned genes.

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LITERATURE CITED


CLONING OF AN S. MUTANS SALIVA RECEPTOR