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Identification and Characterization of a *Candida albicans*-Binding Proteoglycan Secreted from Rat Submandibular Salivary Glands

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A previously identified *Candida albicans*-binding glycoprotein secreted from rat submandibular glands (RSMG) has been further purified from an aqueous RSMG extract by ion-exchange chromatography and gel filtration. Biochemical analysis of the glycoprotein revealed high levels of uronic acid and sulfate, suggesting that it was a proteoglycan. Its amino acid and carbohydrate compositions were similar to those observed for other proteoglycans and differed significantly from those of RSMG mucin, the major secretory glycoprotein of RSMG. In addition, the apparent molecular weight of the glycoprotein was reduced following treatment with either chondroitinase ABC or heparitinase, demonstrating the presence of chondroitin sulfate and heparan sulfate. On the basis of its structure and anatomical source, the glycoprotein is referred to as submandibular gland secreted proteoglycan 1 (SGSP1). SGSP1 also binds monoclonal antibody 1F9, which recognizes the human blood group A carbohydrate epitope found on RSMG mucin. Hence, SGSP1 appears to be a hybrid molecule with carbohydrate structures found in both proteoglycans and RSMG mucin. Enzymatic digestion of SGSP1, followed by its interaction with a radiolabelled *C. albicans* strain in a filter-binding assay, demonstrated that binding to this strain appears to be mediated primarily via the heparan sulfate side chains of SGSP1 and not via the blood group A oligosaccharide.

The fungus *Candida albicans* is carried as a commensal in the oral cavity of approximately one-third of the population (42, 46). Alteration of host defense mechanisms caused by underlying disease or disease-related therapeutic regimens may result in increased levels of *C. albicans* in the oral cavity, leading to the onset of overt oral candidiasis (10, 16, 18, 19, 56). Salivary secretion is a primary host defense mechanism in the oral cavity (32, 35). Saliva modulates microbial populations in the oral cavity, either by facilitating the clearance of microorganisms or by promoting microbial adhesion to mucosal and solid surfaces (6, 26, 32, 35, 51, 52, 54). The modulatory effect of saliva is mediated through the specific binding of salivary constituents to ligands on the microbial surface (6, 17, 33, 39, 41, 50). To identify molecules in saliva that may modulate colonization of the fungus in the oral cavity, we investigated the binding of two *C. albicans* strains (23) to electrophoretically separated salivary constituents using a filter-binding assay (40) and metabolically radiolabelled fungal cells.

By using the filter-binding assay, a constituent present in whole rat saliva, rat submandibular gland (RSMG) ductal saliva and an aqueous extract of RSMG to which two strains of *C. albicans* bound was identified (23). The *C. albicans*-binding glycoprotein was partially purified from the RSMG extract, and initial chromatographic analysis revealed that the glycoprotein was highly acidic. The acidic glycoprotein reacted with a monoclonal antibody, MAb 1F9, specific for the human blood group A oligosaccharide present on RSMG mucin (38, 53). On the basis of the apparent size of the *Candida*-binding glycoprotein, its anatomic source, and the MAb-binding activity, our initial conclusion was that we had identified an acidic, low-molecular-weight salivary mucin capable of binding the fungus (23).

In the present study, we report the further purification and biochemical characterization of the *Candida*-binding RSMG glycoprotein. Biochemical analysis of the purified glycoprotein revealed that it contained both chondroitin sulfate and heparan sulfate side chains, in addition to the human blood group A oligosaccharide similar to that found on RSMG mucin. Throughout the purification process, we have been unable to dissociate the presence of either glycosaminoglycans (GAG) or human blood group A oligosaccharide from the glycoprotein's cell-binding activity. This suggests that the *Candida*-binding glycoprotein is a hybrid molecule possessing carbohydrate structures found in proteoglycans and mucins, two closely related molecular species. However, the amino acid composition of the protein core of the *Candida*-binding glycoprotein more closely resembled that found in proteoglycans (3, 4, 20) than those described for rat salivary mucins (1). Therefore, we propose that the molecule be referred to as RSMG secreted proteoglycan 1 (SGSP1). Enzymatic and chemical treatment of rat SGSP1, followed by its interaction with *C. albicans* in the solid-phase filter-binding assay, demonstrated that cell binding was mediated primarily via the heparan sulfate side chains.

Several reports document the synthesis of proteoglycans in submandibular glands (13, 14, 22), parotid gland secretory vesicles (5, 8), and parotid saliva (24). Our findings corroborate these studies demonstrating that proteoglycans are secreted in saliva. Moreover, on the basis of the interaction of SGSP1 with the *C. albicans* strain used in this study, we propose that at least one biological function of the proteoglycan secreted from RSMG may be to modulate levels of the fungus in the oral cavity via specific binding to the surface of the organism.

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MATERIALS AND METHODS

Cells and culture medium. *C. albicans* 613m1BK, the strain used in these studies, has been described previously (23, 36, 54). On yeast extract-peptone-dextrose agar, *C. albicans* 613m1BK produced rough colonies, with an irregular perimeter and wavy prominences radially distributed from the center of the colony. Microscopically, 613m1BK appeared in aggregated blastoconidia of 20 to 50 closely packed spheroid cells, with occasional pseudohyphae. *C. albicans* was grown to stationary phase in yeast nitrogen base-galactose (YNB-gal) broth (0.67% yeast nitrogen base [Difco, Detroit, Mich.] and 277 mM galactose [J. T. Baker, Inc., Phillipsburg, N.J.]), and aliquots were frozen at −70°C until use. For each experiment, a 300-μl aliquot of cells was used to inoculate a flask containing 30 ml of YNB-gal medium and 6 mCi of L-[^{35}S]methionine per ml (10 mCi/ml; >1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) and grown at 37°C until log phase (approximately 10^7 cells per ml). In our previous cell-binding studies, we also examined the adhesion of two previously described clinical isolates of *C. albicans*, strain 613p (23), the parental strain from which 613m1BK was derived, and strain 228 (54). Both clinical isolates adhered to the glycoprotein, whose characteristics are described below (23; data not shown). However, *C. albicans* strain 613m1BK was used in the cell-binding studies described herein because of its increased radiolabel uptake and ease of detection by autoradiography (40).

Purification and iodination of SGSP1. Aqueous extracts of RSMG derived from male Wistar rats (3 to 4 months old) were prepared as previously described (53). However, the fractionation protocol for the gland extract was modified from that in the initial report. The gland extract was resuspended at a concentration of 25 mg/ml in 0.1 M Tris buffer (pH 7.5) with 6 M urea (Tris-urea), applied to a column (2.5 by 20 cm) of DE-52 cellulose anion-exchange resin (Whatman, Maidstone, England) equilibrated with Tris-urea buffer, and eluted with a linear NaCl gradient (0 to 1.0 M). The column was then washed with Tris-urea buffer with 1.5 M NaCl (high-salt wash). Fractions (7 ml) were monitored for protein at A_{280} and for reactivity to MAb 1F9 (38), which was raised against RSMG mucin and possesses specificity for human blood group A carbohydrate determinants found on RSMG mucin. Peak fractions were pooled on the basis of their reactivity to MAb 1F9 in an enzyme-linked immunosorbent assay (ELISA), dialyzed against water, and lyophilized. The lyophilized peak fractions were also analyzed in a radiolabelled cell adhesion assay to determine the fraction bound by *C. albicans* (23). The *C. albicans*-binding fraction was then separated by S-200 Sephacryl gel filtration (Pharmacia LKB Biotechnology, Piscataway, N.J.). One milligram (dry weight) was resuspended in Tris-urea buffer and applied to a column (1 by 95 cm) equilibrated with the same buffer, and 1-ml fractions were collected. The protein concentration at A_{280}, reactivity to MAb 1F9 by ELISA, and ability to bind *C. albicans* in a radiolabelled-cell adhesion assay were determined for each fraction. The *C. albicans*-binding fractions were pooled, dialyzed, and lyophilized. Further separation of closely associated proteins from SGSP1 in the *C. albicans*-binding fraction was achieved by repeating the S-200 Sephacryl gel filtration with the addition of 1% Triton X-100 and 1 M NaCl in the Tris-urea buffer. Both iodinated SGSP1 and unlabelled SGSP1 were separated by S-200 chromatography in the buffer containing detergent. SGSP1 was iodinated by the method of Bolton and Hunter (9). A total of 30 μg (dry weight) was resuspended in 10 μl of PBS (pH 8.2) and iodinated with 250 μCi of ^{125}I-Bolton-Hunter reagent (NEN Research Products, Boston, Mass.) as described by the manufacturer. The radiolabelled material was separated from unincorporated radiolabel by using a 10-ml G-50 column equilibrated in Tris-urea buffer with 1 M NaCl and 0.1% Triton X-100. The radiolabelled material was pooled, concentrated in a Centricon 30 (Amicon, Danvers, Mass.), resuspended in Tris-urea–0.1% Triton X-100–NaCl buffer, and applied to a Sephacryl S-200 column (1 by 95 cm); and 850-μl fractions were collected. Aliquots of the column fractions were assayed for radioactivity in a gamma counter (Gamma 4000; Beckman, Palo Alto, Calif.). Column fractions were also analyzed by 3 to 20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography of the dried gels. The column fractions containing radiolabelled SGSP1 were pooled; detergent and salts were removed by precipitation of SGSP1 with 4 volumes of acetone at −20°C for 18 h. The radiolabelled SGSP1 precipitate was washed twice in 80% ethanol and resuspended in distilled H_2O. Unlabelled SGSP1 was also separated by S-200 chromatography in detergent containing buffer, and *C. albicans*-binding activity was determined for each column fraction by using the radiolabelled cell adhesion assay. The column fractions containing cell-binding activity were pooled; detergent and salts were removed by precipitation as described above. The SGSP1 pellet was washed twice in 80% ethanol and resuspended in distilled H_2O.

***Candida* cell adhesion assay.** In the adhesion assay (23) to assess cell binding to SGSP1 and RSMG extract fractions during the purification of SGSP1, a dot blot format (Minifold Dot Blot apparatus; Schleicher & Schuell, Inc., Keene, N.H.) was used. To analyze cell binding to the chromatographically separated samples, a 100-μl aliquot of each of the column fractions was applied directly to a polyvinylidene difluoride (PVDF) membrane filter (Millipore, Bedford, Mass.) in the dot blot apparatus. The filters were blocked in 5% skim milk in phosphate-buffered saline (PBS) with 0.01% sodium azide for 30 min at 4°C. The cell-binding mixtures contained sonicated cells suspended in 5% skim milk in PBS containing 0.01% sodium azide. Sodium azide did not affect the cell-binding pattern observed in the cell adhesion assay and was included as an antibacterial agent. Substitution of a solution of 1% bovine serum albumin (BSA) for skim milk as a blocking reagent gave identical results in the filter-binding assay. However, use of BSA resulted in a higher level of background in the assay than with skim milk. The radiolabelled cell-binding mixture was incubated with the filters for 18 h at 4°C with gentle rocking. The filters were then washed three times in PBS, dried, and exposed to radiographic film. Reduction of the incubation time from 18 to 3 h gave identical results. However, for convenience, an overnight incubation of 18 h was routinely used. Detectable release of radiolabelled material by the cells during the 18-h incubation was not observed.

ELISA. Chromatography column fractions were also analyzed for reactivity to MAb 1F9 by ELISA. With 96-well flat-bottom ELISA plates (Corning, Corning, N.Y.), 50 μl of each column fraction and 50 μl of borate-buffered saline (25 mM sodium borate, 0.1 M boric acid, 9% NaCl) were added to the wells and incubated overnight at 4°C. The plates were washed three times with BBS, blocked with 1% BSA–0.05% Tween 20 in borate-buffered saline for 30 min, washed, and probed with MAb 1F9 in 1% BSA–0.05% Tween 20 in borate-buffered saline for 4 h. The plates were washed, blocked again for 10 min, and probed with a goat anti-mouse antibody conjugated to alkaline phosphatase in the blocking buffer for 2 h. After being washed, the plates were developed with phosphatase substrate (Sigma Chemicals Co., St. Louis, Mo.) for 1

h. The reaction was measured at A_{410} in an automated microtiter plate reader (Dynatech, Chantilly, Va.).

Analyses of composition of SGSP1. Amino acid and hexosamine analysis was performed after vapor hydrolysis in 6 N HCl for 24 h with an Amino Quant II amino acid analyzer (Hewlett-Packard Co., Palo Alto, Calif.). Sialic acid composition was determined after hydrolysis in 0.5 N H_2SO_4 for 1 h by using the thiobarbituric acid assay (55). Uronic acid content served as an indicator of GAG content and was measured by using the modified carbazole assay as described by Bitter and Muir (7), with chondroitin sulfate A as a standard. Sulfate content was determined by the benzidine method described by Spencer (48), with potassium sulfate as a standard.

Enzymatic treatment of radiolabelled SGSP1. ^{125}I-SGSP1 precipitated from the S-200 column fractions was resuspended in distilled H_2O and digested with 0.05 U of protease-free chondroitinase ABC (Seikagaku America, Inc., Rockville, Md.) in 0.1 M Tris-HCl–0.03 M sodium acetate (pH 8.0) at 37°C for 4 h. All glycosidase digests contained a mixture of protease inhibitors as recommended by the enzyme manufacturer, which included 10 mM EDTA, 5 mM phenylmethylsulfonyl fluoride, 10 mM *N*-ethylmaleimide, 0.36 mM pepstatin A, and 10 μg of trypsin inhibitor (Sigma Chemical Co.) per ml. ^{125}I-SGSP1 was digested with 0.05 U of chondroitinase ACII (Seikagaku America, Inc.) in 0.01 M calcium acetate–0.1 M sodium acetate containing protease inhibitors (pH 6.0) at 37°C for 4 h. ^{125}I-SGSP1 was digested with 0.005 U of heparitinase (Seikagaku America, Inc.) in 0.01 M calcium acetate–0.1 M sodium acetate containing protease inhibitors (pH 7.0) at 43°C for 4 h. Double enzyme digests involved digestion with heparitinase as described above; the pH was then adjusted to either 6.0 or 8.0; 0.05 U of either protease-free chondroitinase ABC or chondroitinase ACII, respectively, was added; and digestion was continued for 4 h. at 37°C. Pronase (Sigma Chemical Co.) digests involved incubating ^{125}I-SGSP1 with 20 μg of pronase in 0.05 M Tris-HCl–1 mM $CaCl_2$ at pH 7.0 at 65°C for 18 h. The change in size of ^{125}I-SGSP1 after enzymatic digestion was determined by S-200 gel filtration. After digestion, the sample was loaded onto a column (1 by 95 cm) equilibrated with Tris-urea–NaCl–0.1% Triton X-100 buffer as described above. Column fractions were assayed for radioactivity to locate ^{125}I-SGSP1.

Cell-binding assay and immunoblot to enzymatically and chemically modified unlabelled SGSP1. Unlabelled SGSP1 isolated by S-200 gel filtration in detergent containing buffer as described above was precipitated from the column fractions with acetone and digested with the enzymes as described above. Chemical modification of the SGSP1 involved alkaline borohydride cleavage of the oligosaccharides in 0.8 M $NaBH_4$–0.1 N NaOH at 37°C for 24 h. Mild acid hydrolysis was used to remove sialic acid by treatment with 0.05 N H_2SO_4 at 80°C for 1 h. Intact SGSP1, as well as enzymatically and chemically modified SGSP1, was then immobilized on a PVDF filter in a dot blot format. *C. albicans* cell binding was then assayed in a solid-phase adhesion assay with metabolically radiolabelled cells as described above. After the cell adhesion assay, the same PVDF filter was rehydrated, blocked with 5% skim milk, and incubated with MAb HepSS-1 (Seikagaku America, Inc.), which recognizes heparan sulfate. A secondary antibody conjugated to alkaline phosphatase was used to detect HepSS-1 binding.

SDS-PAGE and Western blot analysis of rat saliva, RSMG extract, and SGSP1. Rat saliva and RSMG extract were precipitated in 4 volumes of acetone overnight at −20°C, washed two times in 80% ethanol, resuspended in SDS-PAGE sample buffer, and separated with an SDS–10% polyacrylamide gel containing 0.5% cross-linker by the SDS-PAGE system described by Giulian et al. (21). SGSP1 was digested with both chondroitinase ABC and heparitinase as described above and then resuspended in SDS-sample buffer. A sample containing the enzymes alone was used as a negative control for antibody binding. After electrophoresis, the proteins were electrotransferred to a PVDF membrane in Tris-glycine buffer containing 10% methanol for 6 h. The PVDF filter was blocked with 5% skim milk. For Western blot (immunoblot) analysis, both MAbs 1F9 and HepSS-1 were used individually to probe the electrotransferred proteins on separate filters. *C. albicans* cell binding was assayed by incubating the filter strips with metabolically radiolabelled cells as described above.

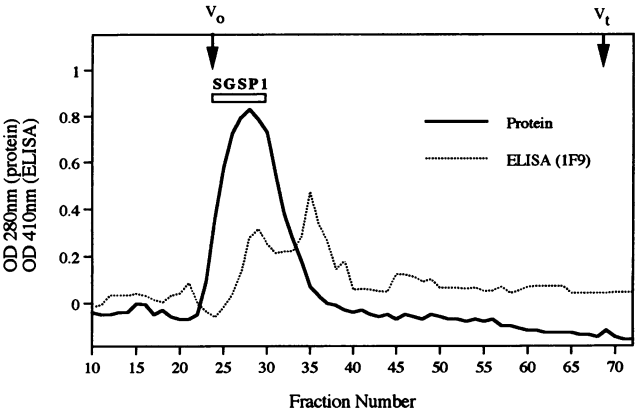

FIG. 1. Elution profile of previously identified (23) *Candida*-binding fraction separated by S-200 gel filtration in buffer containing 6 M urea–0.1 M Tris-HCl (pH 7.5). Each column fraction was assayed for MAb 1F9 binding by ELISA and for protein at A_{280}. *Candida* cell binding was determined by using a radiolabelled cell adhesion assay. Fractions bound by *C. albicans* are labelled SGSP1. V_0, void volume; OD, optical density.

RESULTS

Purification and iodination of rat SGSP1. The separation of aqueous RSMG extract by anion-exchange chromatography was performed as described previously (23). The *C. albicans*-binding fraction of the aqueous RSMG extract was then further separated by gel filtration with Sephacryl S-200. For clarity of discussion, and on the basis of the results presented below, the *Candida*-binding glycoprotein will hereafter be referred to as SGSP1. Initially, Tris-urea buffer was used to elute SGSP1 from the Sephacryl S-200 column, and column fractions were assayed spectrophotometrically for protein content at A_{280}. A single large peak containing SGSP1 (Fig. 1) eluted from the column in fractions 22 to 31, with a slight protein shoulder visible in fractions 32 to 38. When column fractions were assayed by ELISA using MAb 1F9, two peaks were resolved (Fig. 1). Column fractions 22 to 31 and fractions 34 to 38 were then pooled, dialyzed, and lyophilized. One milligram (dry weight) of material was recovered from fractions 22 to 31; however, insufficient material was recovered from fractions 34 to 38 to permit further analysis. Thirty micrograms of the material recovered from fractions 22 to 31 was then iodinated with Bolton-Hunter reagent (9) and applied to an S-200 column with 1% Triton X-100 and 1 M NaCl included in the Tris-urea buffer. In this buffer system, the shoulder peak from Fig. 1 was resolved from the major peak containing SGSP1. Figure 2 shows a profile of 2 μl of each

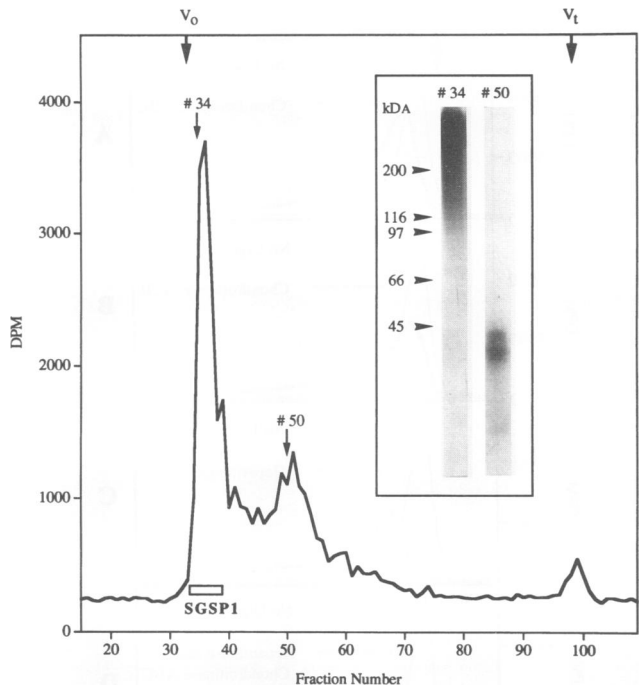

FIG. 2. Separation of radiolabelled SGSP1 by S-200 gel filtration and SDS-PAGE analysis. An elution profile of SGSP1 separated by S-200 gel filtration in buffer containing 6 M urea, 0.1 M Tris-HCl, 1 M NaCl, and 1% Triton X-100 is shown. The insert shows an autoradiogram of 3 to 20% gradient SDS-PAGE analysis of column fractions 34 and 50. V_0, void volume.

column fraction assayed for radioactivity. SDS-PAGE analysis of the radiolabelled column fractions on a 3 to 20% gradient gel showed that the major peak appeared as a high-molecular-weight polydisperse smear (Fig. 2, insert). The minor peak appeared as a triplet of bands with an apparent molecular mass of approximately 43 kDa, which was similar in appearance to the salivary glutamine/glutamic acid-rich proteins (GRP) reported by Mirels et al. (37). The glutamine/glutamic acid-rich proteins are abundant proteins secreted by RSMG which often associate and copurify with RSMG mucin. However, from experiments using unlabelled material, *C. albicans* cell binding was associated with the column fractions containing SGSP1. The fractions containing ^{125}I-SGSP1 were pooled, the glycoprotein was precipitated with acetone, and the precipitate used for further analyses.

Compositional analyses. Because of the colocalization of *C. albicans*-binding activity with reactivity to MAb 1F9, we initially concluded that we had isolated an acidic isoform of RSMG mucin (23). However, after comparison of the amino acid and carbohydrate compositions of SGSP1 with those of the HPLC-purified neutral mucin secreted from the RSMG (Table 1), it was apparent from the strikingly different profiles that the glycoprotein which we had identified was not an isoform of RSMG mucin. The amino acid analysis of SGSP1 showed high levels of glycine (238 residues per 1,000 amino acid residues compared with 61/1,000 amino acid residues in RSMG mucin) and low levels of threonine and proline (61 and 90 residues, respectively, per 1,000 amino acid residues in SGSP1 compared with 251 and 201 residues, respectively, per 1,000 amino acid residues in RSMG mucin). The amino acid composition suggested that SGSP1 was not a mucin isoform but perhaps a proteoglycan.

TABLE 1. Amino acid composition of SGSP1 and RSMG mucin

Amino acid	No. of residues/1,000 amino acid residues	
	SGSP1	RSMG mucin[a]
Aspartic acid/asparagine	116	60
Glutamic acid/glutamine	83	53
Serine	113	92
Histidine	ND[b]	9
Glycine	238	61
Threonine	61	251
Alanine	103	110
Arginine	69	7
Tyrosine	ND	6
Valine	33	20
Methionine	ND	3
Tryptophan	ND	1
Phenylalanine	16	13
Isoleucine	22	32
Leucine	55	25
Lysine	Trace	51
Proline	90	201

[a] Data courtesy of L. A. Tabak (1).
[b] ND, not detected.

The uronic acid and sulfate analyses further supported our hypothesis that SGSP1 was a proteoglycan. In both of these assays, SGSP1 was compared with the major neutral mucin peak that eluted as flowthrough when RSMG extract was separated by DE-52 anion-exchange chromatography (23). To assay GAG, the uronic acid-carbazole reaction described by Bitter and Muir (7) was used, with chondroitin sulfate A as a standard. SGSP1 contained 554 residues of uronic acid per 1,000 amino acid residues compared with background levels in the uronic acid assay of 10 residues per 1,000 amino acid residues for RSMG mucin (Table 2).

Inorganic sulfate was determined by the ultramicrobenzidine method of Spencer (48). Sulfate analysis indicated that SGSP1 was more sulfated (2,834 residues per 1,000 amino acid residues) than RSMG mucin, which again contained background levels of 107 residues per 1,000 amino acid residues in the sulfate assay (Table 2).

The GalNAc and GlcNAc composition of SGSP1 compared with that of high-pressure liquid chromatography (HPLC)-purified RSMG mucin is presented in Table 2. These data also supported the conclusion that SGSP1 was a proteoglycan and contained carbohydrate structures different from those of RSMG mucin. The high levels of GalNAc (479 residues per 1,000 amino acid residues) and GlcNAc (856 residues per 1,000 amino acid residues) for SGSP1 were again in contrast to those in RSMG mucin, which contained 396 residues of GalNAc per 1,000 amino acid residues and 29 residues of GlcNAc per 1,000 amino acid residues. Also, the ratio of GalNAc to GlcNAc indicated a difference in carbohydrate composition between SGSP1 and RSMG mucin. Of the total GalNAc and GlcNAc present in RSMG mucin, GalNAc accounts for 93% and GlcNAc accounts for only 7%; however, in SGSP1 36% of the total is GalNAc and 64% is GlcNAc.

The results of sialic acid determination by using the thiobarbituric acid assay (55) are expressed as the number of residues of *N*-acetylneuraminic acid per 1,000 amino acid residues (Table 2) and indicated that SGSP1 (296 residues per 1,000 amino acid residues) contained more sialic acid than HPLC-purified RSMG mucin (162 residues per 1,000 amino acid residues). The ratio of *N*-acetylneuraminic acid to protein was 0.66 for SGSP1 and 0.50 for RSMG mucin.

Table 2. Carbohydrate composition of SGSP1 and RSMG mucin

Analyte	No. of residues/1,000 amino acid residues		Mol%	
	SGSP1	RSMG mucin[a]	SGSP1	RSMG mucin
N-acetylgalactosamine	479	396	41	93
N-acetylglucosamine	856	29	59	7
N-acetylneuraminic acid	296	162		
Uronic acid[b]	554	0[d]		
Sulfate[c]	2,834	0[d]		

[a] Data courtesy of L. A. Tabak (1).
[b] Uronic acid content was determined with chondroitin sulfate A as the standard.
[c] Sulfate content was determined with potassium sulfate as the standard.
[d] Determined with the major neutral mucin peak that eluted as flowthrough when RSMG extract was separated by DE-52 anion-exchange chromatography (23). In RSMG mucin, the uronic acid (10/1,000) and sulfate (107/1,000) levels were equivalent to background levels in the assays.

Identification of GAG present on SGSP1. The finding that SGSP1 contained uronic acid led us to identify the GAG present by digesting radiolabelled SGSP1 and detecting either a shift in elution profile on a gel filtration column or a shift in apparent molecular weight by SDS-PAGE analysis. After the samples were digested, they were fractionated by gel filtration, and the fractions were monitored for radioactivity. The results (Fig. 3A to C) show that digestion of SGSP1 with either chondroitinase ABC, chondroitinase ACII, or heparitinase resulted in a shift of the peak to the right, indicating a reduction in the size of SGSP1. A double-enzyme digest with both heparitinase and one of the chondroitinases resulted in a more dramatic shift in the gel filtration profile (Fig. 3D). Digestion with pronase resulted in a complete degradation of SGSP1, with all the radioactivity appearing near the total volume (V_t) at column fraction 108 (data not shown) (Fig. 3E). These data indicated that SGSP1 contained both chondroitin sulfate and heparan sulfate and was sensitive to digestion with pronase.

SDS-PAGE analysis of enzyme digests of radiolabelled SGSP1, followed by autoradiography, is shown in Fig. 4A. Undigested radiolabelled SGSP1 (lane 1) appeared as a high-molecular-weight smear on the gel. In lane 3, SGSP1 has been digested with chondroitinase ABC, which resulted in the partial disappearance of the high-molecular-weight smear and the appearance of two bands, one at ~150 kDa and the other at ~130 kDa. Digestion of SGSP1 with chondroitinase ACII resulted in a gel profile identical to that seen with chondroitinase ABC digestion (data not shown). Chondroitinase ABC was used for the double digestion of SGSP1 with heparitinase because it is commercially available in a highly purified and protease-free form. Digestion with heparitinase alone (lane 2) resulted in a decrease in the size and intensity of the high-molecular-weight material at the origin of the resolving gel. However, only a double digest with both chondroitinase ABC and heparitinase resulted in the disappearance of the high-molecular-weight smear (lane 4). The double digest also resulted in the more prominent appearance of the 130 and 150 kDa doublet that is faintly visible with a single digest with chondroitinase ABC. In Fig. 4B, unlabelled SGSP1 was digested with both chondroitinase ABC and heparitinase, separated by SDS-PAGE, and transferred to a PVDF membrane. Lane 5 shows a Western blot of the double digest with MAb 1F9, which binds to the blood group A oligosaccharide found on SGSP1. This oligosaccharide would not be affected by

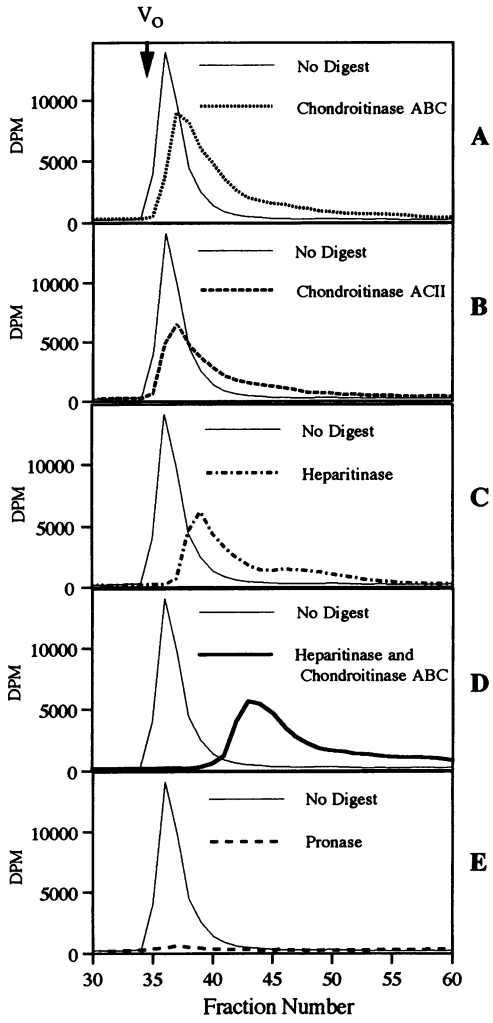

FIG. 3. S-200 gel filtration analyses of enzymatically treated ^{125}I-SGSP1. Untreated SGSP1 elutes as a single peak. (A to E) Enzyme treatment of ^{125}I-SGSP1, as indicated in each panel. The V_t at fraction 108 is not shown. V_0, void volume.

either of the two enzymes used to digest the GAG; therefore, MAb 1F9 should bind to the blood group A oligosaccharide still attached to the proteoglycan core protein. The MAb 1F9 bound only to the 150-kDa band and did not bind the 130-kDa band.

Figure 4, lane 6, shows a Western blot of the same double-enzyme digest with MAb HepSS-1, which binds to heparan sulfate and should bind to any undigested heparan sulfate remaining on the proteoglycan protein core, along with any undigested or partially digested SGSP1. Because of its substrate specificity (20), enzymatic digestion with heparitinase does not completely remove heparan sulfate from the protein core of proteoglycans. Therefore, some reactivity with HepSS-1 was still expected after heparitinase treatment. A Coomassie blue stain of the gel profile of the double digest of SGSP1 is shown in Fig. 4, lane 7, and a stained control lane with enzymes only is shown in lane 8. The 150-kDa band was more prominent than the 130-kDa band in the double digest (lane 7). The most prominently staining species was BSA, which was added to the heparitinase as a stabilizer. Heparitinase has an expected molecular mass of 70 kDa, and chon-

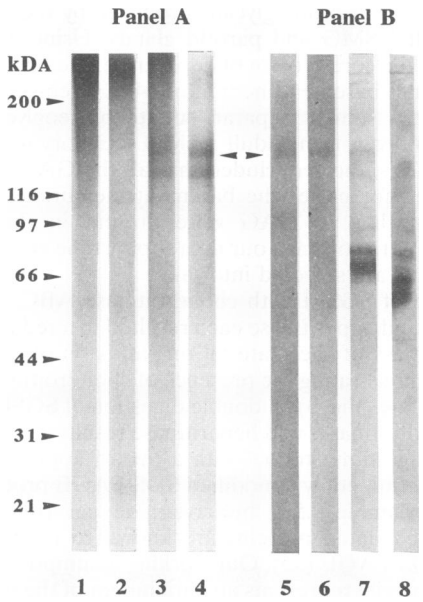

FIG. 4. SDS-PAGE analysis of enzymatically treated SGSP1. (A) Autoradiographic detection of untreated and enzymatically treated ^{125}I-SGSP1 analyzed by SDS-PAGE. Lanes: 1, untreated ^{125}I-SGSP1; 2, heparitinase-treated ^{125}I-SGSP1; 3, chondroitinase ABC-treated ^{125}I-SGSP1; 4, heparitinase and chondroitinase-treated ^{125}I-SGSP1. (B) Immunochemical detection and Coomassie blue staining of unlabelled SGSP1 treated with heparitinase and chondroitinase ABC, separated by SDS-PAGE and transferred to PVDF membrane. Lanes: 5, Western blot probed with MAb 1F9, with specificity for the human blood group A carbohydrate epitope found on RSMG mucin; 6, Western blot probed with MAb HepSS-1, with specificity for heparan sulfate; 7, SDS-PAGE of enzyme and SGSP1 digestion products stained with Coomassie blue; 8, SDS-PAGE of heparitinase and chondroitinase enzymes alone stained with Coomassie blue.

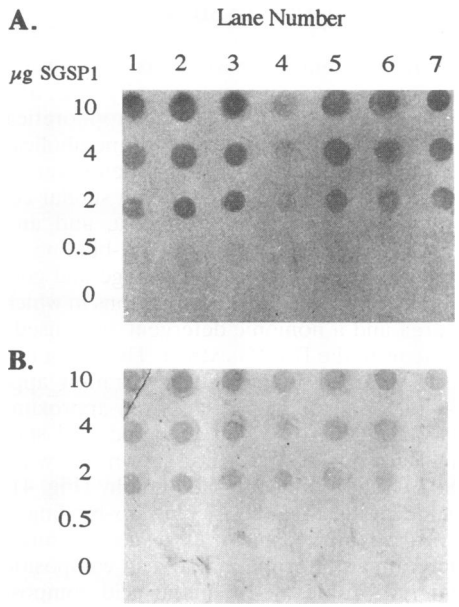

FIG. 5. Assay of radiolabelled *C. albicans* cell adhesion to enzymatically and chemically treated unlabelled SGSP1, with subsequent immunochemical detection with MAb HepSS-1. (A) Autoradiograph of metabolically radiolabelled *C. albicans* cells bound to enzymatically and chemically treated unlabelled SGSP1. Lanes: 1, untreated SGSP1; 2, chondroitinase ABC-treated SGSP1; 3, chondroitinase ACII-treated SGSP1; 4, heparitinase-treated SGSP1; 5, pronase-treated SGSP1; 6, alkaline sodium borohydride-treated SGSP1; 7, sialic acid removed from SGSP1 by treatment with mild acid. (B) The same filter from panel A was used in an immunodot blot with MAb HepSS-1, with specificity for heparan sulfate.

droitinase ABC has a molecular mass of between 120 and 145 kDa as determined by gel filtration. Therefore, the possibility exists that the 130-kDa band that appears in lane 4 may be an artifact caused by trapping of free radiolabel by chondroitinase ABC in the radiolabelled SGSP1 digestion. Evidence that the 130-kDa band was not a digestion product of SGSP1 was provided by the Western blot data, which showed that the 130-kDa band did not react with MAb 1F9 or MAb HepSS-1 (Fig. 4B, lanes 5 and 6, respectively). In further support of this conclusion, in the enzyme-only lane, chondroitinase ABC was present as a diffuse Coomassie blue-staining band at ~130 kDa. However, as can be seen by comparing lanes 7 and 8, all the proteins migrated with slightly larger apparent molecular masses when SGSP1 was present. The proteins in lanes 7 and 8 were run on the same gel and transferred to the same filter. Nonetheless, even the BSA present in the double digest (lane 7) appeared to run at a larger molecular mass than the BSA in lane 8.

Binding of metabolically radiolabelled *C. albicans* to enzymatically and chemically modified SGSP1. Unlabelled SGSP1 was enzymatically and chemically modified to determine the structure involved in binding *C. albicans*. Four dilutions of either enzymatically or chemically modified SGSP1 were spotted onto membrane filters and incubated with metabolically radiolabelled *C. albicans* cells. In Fig. 5A, the autoradiograph of the filter shows that cell binding is significantly decreased in SGSP1 treated with heparitinase (lane 4), indicating that the predominant structure bound by the cells was heparan sulfate. Binding of the cells was not significantly decreased with digestion with chondroitinase ABC (lane 2) or chondroitinase ACII (lane 3), which degrades chondroitin sulfate. Pronase treatment for 24 h at 65°C digested the protein core of SGSP1 but did not affect cell binding (lane 5). Pronase digestion of SGSP1 was also assessed by gel filtration in Fig. 3E, in which it was shown that SGSP1 was susceptible to complete digestion by pronase. Alkaline sodium borohydride treatment (lane 6) of SGSP1 released the oligosaccharides and degraded the protein core but did not reduce cell binding. The affect of alkaline sodium borohydride treatment on radiolabelled SGSP1 was assessed by SDS-PAGE analysis and subsequent autoradiography, which resulted in all the radioactivity migrating with the dye front, indicating that the protein core had been completely degraded by this treatment (data not shown). Mild acid hydrolysis (0.05 N H_2SO_4 at 80°C for 1 h) is expected to remove sialic acid from SGSP1. Although we did not quantitate the amount of sialic acid removed, the mild acid hydrolysis did not reduce cell binding (lane 7). Chemical and enzyme reaction mixtures alone were spotted on the filter as negative controls but showed no cell binding (data not shown).

After the cell-binding assay, the same filter was incubated with MAb HepSS-1. The immunodot blot in Fig. 5B shows that the antibody recognized SGSP1. After heparitinase digestion, there was a visible decrease in antibody binding (lane 4). However, as expected, heparitinase treatment did not completely remove the heparan sulfate. The binding of MAb HepSS-1 to SGSP1 that has been treated with heparitinase indicated that some undigested heparan sulfate remains on the molecule (lane 4).

DISCUSSION

In an effort to identify constituents in saliva that may modulate the colonization of the oral cavity by *C. albicans*, we used a filter-binding assay in which electrophoretically separated salivary proteins were probed with metabolically radiolabelled cells to determine which constituents were bound by *C. albicans*. The binding assay identified a similar constituent in whole rat saliva, ductal RSMG saliva, and an aqueous extract of RSMG (23). The *C. albicans*-binding moiety in RSMG extract was purified by ion-exchange and gel filtration chromatography under denaturing conditions in which a buffer containing urea and a nonionic detergent were used to dissociate components in the RSMG extract. The major *C. albicans*-binding peak obtained following gel filtration appeared as polydisperse material ranging in size from approximately 130 to 300 kDa on a SDS–10% polyacrylamide gel stained with Alcian blue-silver nitrate (data not shown) or with radiolabelled SGSP1 visualized by autoradiography (Fig. 4).

Our first indication that the *C. albicans*-binding moiety in RSMG extract was not a mucin, as suspected initially, came from the determination of the amino acid composition of the purified material (Table 1). Its amino acid composition differed significantly from that of the major neutral mucin isoform found in RSMG extracts. The most obvious difference was the high glycine content accompanied by low levels of threonine and proline in the *Candida*-binding fraction, compared with the levels of these amino acids observed in neutral mucin. The difference in the amino acid composition compared with that of RSMG mucin, its highly negative charge, and its polydispersity in SDS-polyacrylamide gels suggested to us that the moiety may be a secreted proteoglycan. This hypothesis was supported by the presence of uronic acid and sulfate in the purified *Candida*-binding fraction (Table 2). Furthermore, an MAb to the GAG heparan sulfate bound to the purified *Candida*-binding fraction, as well as to a moiety in whole rat saliva and RSMG extract that colocalized with *C. albicans*-binding activity. Because of its biochemical nature and anatomical source, we refer to the *Candida*-binding material as SGSP1.

Proteoglycans are present in gingival and periodontal tissues, as well as in gingival crevicular fluid (3, 4, 47). Recently, the partial characterization of proteoglycans synthesized by gingival epithelial cells indicated that these cells secrete heparan sulfate principally, and, to a lesser extent, chondroitin sulfate and dermatan sulfate proteoglycans (44). However, there is little information concerning the presence of proteoglycans in saliva. Iversen et al. (24) identified chondroitin sulfate in the secretory granules of the rat parotid gland and in parotid saliva; they also partially characterized the sulfated species. It was hypothesized that since intragranular GAG were secreted from the parotid gland, they may play some functional role in the oral cavity by interacting with other salivary macromolecules and microorganisms (24). Using a gland slice system, they later showed that the chondroitin sulfate produced was part of a proteoglycan molecule (45).

Other reports of proteoglycans in salivary gland secretory vesicles have focused on the role that they play in secretory granule packaging and maturation (8, 11). The role of proteoglycans in salivary gland development and morphogenesis has been well documented (reviewed in reference 15). During development, the GAG profile of RSMG undergoing differentiation changes from one that is rich in hyaluronate and chondroitin sulfate to one that is rich in heparan sulfate with little chondroitin sulfate (13, 14). Data from the laboratory of Beeman and Cutler (5) indicated that heparan sulfate was the principle GAG in proteoglycans produced by the acinar units of the adult RSMG and parotid glands. Using a polyclonal antibody to the core protein of heparan sulfate proteoglycan in rat glomerular basement membrane (49), Gremski and Cutler (22) localized similar heparan sulfate proteoglycans in the RSMG. However, using adult RSMG secretory units to study GAG synthesis, they concluded that all the GAG synthesized was destined for either the basement membrane or the cell surface and that no GAG were present in the secretory material (13). In contrast, our data support the conclusion that proteoglycans are secreted into saliva.

Digestion of SGSP1 with chondroitinase ABC, chondroitinase ACII, and heparitinase each resulted in a reduction of the molecular mass of the material on the basis of gel filtration profiles, demonstrating the presence of chondroitin sulfate and heparan sulfate (Fig. 3). A double digestion of SGSP1 with one of the chondroitinases and heparitinase resulted in the appearance of a digestion product with a size of approximately 150 kDa, as determined by a modified SDS-PAGE procedure with a 10% acrylamide–0.5% bisacrylamide gel (pH 9.3) (21). Heavily glycosylated proteins are known to migrate anomalously in SDS-PAGE (53). Our working assumption is that the 150-kDa material represents an enrichment of the protein core of the proteoglycan. However, the 150-kDa protein core still contains some heparan sulfate, as determined by its continued reactivity to MAb HepSS-1 (Fig. 4, lane 5), and retains the human blood group A carbohydrate, as evidenced by reactivity to MAb 1F9 (Fig. 4, lane 6). Therefore, the molecular weight of the completely deglycosylated core has not yet been determined. Because the species enriched for the protein core migrates as a fairly sharp band on SDS-polyacrylamide gels, the polydispersity observed in the undigested material is likely to reflect heterogeneity in the lengths of the chondroitin sulfate and/or heparan sulfate chains of the proteoglycan.

We next sought to determine the functional relationship between SGSP1 structure and its ability to bind *C. albicans*. We used specific enzyme treatments of SGSP1 to identify the portion(s) of the molecule involved in binding *C. albicans*. Pretreatment of SGSP1 with either chondroitinase ABC, chondroitinase ACII, or pronase did not reduce its cell-binding capacity compared with untreated SGSP1. Treatment of SGSP1 with heparitinase alone, however, significantly decreased the *Candida*-binding function of SGSP1 (Fig. 5, lane 4). Cell binding was unaffected by either pronase or alkaline sodium borohydride treatment. Both treatments digested the protein core of ^{125}I-SGSP1 so that the radioactivity migrated near the dye front on SDS-polyacrylamide gels and the radioactivity eluted near V_t during S-200 gel filtration. Taken together, these results suggest that the protein core of SGSP1 is not directly involved in cell binding.

Heparan sulfate binding has been reported for a variety of microorganisms, including *Streptococcus pyogenes* (57), *Streptococcus mutans* (12), *Staphylococcus aureus* (34), *Helicobacter pylori* (2), *Chlamydia trachomatis* (25), and *Trypanosoma cruzi* (43). However, a heparan sulfate-binding adhesin from *C. albicans* has not been reported. Our observation that heparan sulfate, and not the protein core of SGSP1, mediated cell binding appears at variance with the report by Klotz (30) that *C. albicans* does not bind heparan sulfate. In addition, it was shown that the binding of *C. albicans* to extracellular matrix components, in which proteoglycans are abundant, was mediated primarily by binding to arg-gly-asp and related peptide sequences, possibly by an integrin-like structure on the fungal surface (27–29, 31).

There are several possible explanations for the apparent differences in our observations. First, the most likely explana-

tion is related to inherent differences in the individual *C. albicans* strains used and the different binding assays used, which would each influence the assay's outcome. We have observed a marked difference in the ability of different *C. albicans* strains to bind individual salivary constituents, as well as in their ability to adhere to acquired whole-saliva pellicle on denture acrylic (23, 54). In our current study, we focused on the interaction of SGSP1 and one strain of *C. albicans*, 613m1BK. Strain 613m1BK adhered avidly to SGSP1, and this interaction proved useful during the identification and purification of SGSP1. Second, because heparan sulfate is the most complex and heterogeneous of the GAG (20), the structure of the proteoglycans in the subendothelial extracellular matrix may differ from the structure of SGSP1 in a way that influences the binding of the organism. Third, the culture medium and carbon source used to cultivate *C. albicans* and growth stage of the organisms were different and may have influenced the binding characteristics of the *C. albicans* strains. We have observed that cells grown to log phase bind most effectively in the filter-binding assay that we employ. Cells grown to stationary phase are metabolically radiolabelled to a higher specific activity but adhere to SGSP1 considerably less well than mid-log-phase cells. Finally, the filter-binding assay format that we used may also affect the way that the target is presented for binding. Any or all of these considerations could account for the differences in our observations.

The role of SGSP1 in maintaining the health of the oral cavity remains unclear. It is unknown whether SGSP1 functions in the fluid phase of saliva to mediate the clearance of *C. albicans* or whether it facilitates its adhesion to mucosal surfaces by participating in pellicle formation. It is also unknown whether SGSP1 binds strongly to other microorganisms found in the oral cavity, in addition to *C. albicans*. Furthermore, in order to assess the potential importance of SGSP1 in the modulation of *C. albicans* populations in the oral cavity, the ability of other clinical isolates of *C. albicans* to bind SGSP1 needs to be evaluated in addition to the two clinical isolates examined thus far. Aside from its potential role in modulating populations of oral microorganisms, SGSP1 may play a role in maintenance of the health and integrity of the oral mucosal surface as a part of an acquired pellicle. Alternatively, SGSP1 may be involved in the packaging and release of other secreted products, as has been suggested for the proteoglycans found in the secretory granules of the parotid salivary gland. Further investigation will be required to elucidate both the complete structure of SGSP1 and its function in oral health.

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