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Monoclonal Antibodies to the Extracellular Glucosyltransferases from *Streptococcus sobrinus* 6715

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Murine monoclonal antibodies (MAbs) were raised against the glucosyltransferases (GTFs) of *Streptococcus sobrinus* 6715. The antibody panels included MAbs raised against the primer-independent, soluble product enzyme (GTF-Si) which did not cross-react with other GTFs, as well as MAbs raised against the primer-dependent, soluble product enzyme (GTF-Sd) which recognized both GTF-Si and GTF-Sd, thus indicating that these catalytically distinct enzymes share epitopes. MAbs raised against GTF-I recognized several forms of GTF-I and did not cross-react with the GTF-S enzymes. None of the MAbs recognized the major glucan-binding protein of *S. sobrinus*. Two MAbs inhibited glucan synthesis, one blocking primer synthesis by GTF-Si by 89% and the second inhibiting that by GTF-I by 92%.

Extracellular water-insoluble glucans synthesized from sucrose are necessary for the formation of cariogenic dental plaque by *Streptococcus sobrinus* (7). The synthesis of these complex polysaccharides generally is attributed to the synergistic action of two types of extracellular glucosyltransferase (GTF) produced by *S. sobrinus*: GTF-I, which synthesizes a water-insoluble, essentially linear 1,3-α-D-glucan, and GTF-S, which produces a water-soluble glucan having abundant 1,3,6-α-D-glucose branch points, as well as predominant 1,6-α-D-linked sequences (21). The water-soluble product of GTF-S has long been considered a logical source of primer for the synthesis of water-insoluble glucan by GTF-I (4), which is primer dependent (i.e., it requires a pre-existing glucan to rapidly synthesize water-insoluble glucan). Most GTF-S enzymes described are also primer dependent when they are in pure form; however, a primer-independent GTF-S (GTF-Si) was recently described, and its characteristics suggested that it might be the in vivo source of the primer required by other GTFs (11, 14, 18).

Immunological comparisons of *S. sobrinus* GTFs by using polyclonal antibodies indicated that GTF-I is immunologically (and therefore structurally) distinct from GTF-S (see reference 19 for an example). Similar immunological comparisons have not been reported for the various isozymes of either GTF-S or GTF-I detected among the mutants and serotypes of *S. sobrinus* (14, 20), nor have the cell surface complements of GTF isozymes been determined in the various strains and serotypes of *S. sobrinus*. Such studies would be facilitated by panels of monoclonal antibodies (MAbs) to the GTF isozymes. MAbs capable of specifically inhibiting GTF isozymes would be powerful tools for dissecting the pathway of glucan synthesis in *S. sobrinus*. Furuta et al. (5) raised several MAbs specific for GTF-I. One of these antibodies partially inhibited glucan synthesis by GTF-I. This report describes the preparation of panels of MAbs specific for isozymes of the several GTFs found in *S. sobrinus* 6715 (14) and identification of inhibitory antibodies in those panels.

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MATERIALS AND METHODS

Cell growth and preparation of proteins. *S. sobrinus* 6715-13 wild type and mutant 27 were obtained from Michael Freedman, University of Connecticut Health Center, Farmington, Conn. Mutant 27 is a noncariogenic, nonadhering strain which synthesizes abnormally high levels of water-soluble glucan (3). Cells were grown, as described previously (15), in Trypticase (BBL Microbiology Systems, Cockeysville, Md.)-yeast extract medium containing 1% glucose and supplemented with cysteine hydrochloride (0.05 mg/ml). Preparation of crude proteins from cell-free culture liquors has been previously described, as have procedures for isolating GTFs by affinity chromatography on cross-linked dextran beads (Sephadex G50; 13). Enzymes isolated by affinity chromatography were further purified by DEAE ion-exchange chromatography (11, 14). The preparations of GTF enzymes used as antigens here were free of heterologous GTFs (e.g., GTF-I was free of GTF-Sd and GTF-Si) but were not homogeneous and clearly were contaminated by high-molecular-weight glucan-binding proteins, as can be seen from the data presented below.

MAb preparation. Kennett's modification (9) of the method of Kohler and Milstein (10) was used to raise MAbs in 6-week-old female BALB/c mice. The antigens used were pure primer-dependent GTF-S, partially pure primer-independent GTF-S (free of other GTFs), partially pure GTF-I (free of other GTFs), or a mixture of the GTF enzymes isolated from mutant 27 by affinity chromatography on Sephadex G50. The mice were immunized with intraperitoneal injections of antigen (0.2 mg, 0.2 ml) in Freund complete adjuvant. Two to four intraperitoneal boosts were administered at 3-week intervals. Three weeks after the final intraperitoneal boost, 0.1 mg (0.1 ml) of antigen in phosphate-buffered saline (0.01 M, pH 7.2 sodium phosphate containing 0.15% NaCl) was injected intravenously. Mice were sacrificed 3 days after the intravenous boost, and spleen cells were fused with Sp2/0Ag14 murine myeloma cells. Hybridomas producing antibodies to GTFs were detected by enzyme-linked immunosorbent microassay with antigen immobilized in 96-well microtiter plates. Positive hybridomas were cloned by limiting dilution. Antibody

subtypes were determined with an enzyme-linked immunosorbent microassay-based kit from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Undiluted clone culture supernatants served as sources of MAbs. Immunoblot assays were used to determine the antigen specificity of each MAb (below). The specificities of the MAbs reported here (see Fig. 3 to 5 and Table 1) are representative of the spectrum of specificities observed in the anti-GTF-I and anti-GTF-S panels.

SDS-PAGE, IEF, and protein transfer. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (22) was carried out in 6% polyacrylamide gels (5% cross-linking) cast in a slab format (0.075 by 12.5 by 14 cm). Gels were run for 6 h at 100 mA per gel and 20°C. Isoelectric focusing (IEF) was conducted in similar polyacrylamide gel slabs (5% cross-linking) containing the appropriate ampholyte, pH 4.0 to 6.0 or 3.0 to 10.0, at a concentration of 5% (vol/vol). Gels were prefocused for 30 min with limits of 1,200 V, 30 W, and 50 mA and cooling to 4°C. Samples then were applied directly to the gel surface and focused under the same conditions for 60 min. Electrophoresis chemicals were obtained from Bio-Rad Laboratories (Richmond, Calif.), and Serva (Heidelberg, Federal Republic of Germany) ampholytes were purchased from Accurate Chemical and Scientific Corp. (Westbury, N.Y.).

Proteins were transferred to nitrocellulose paper from SDS-PAGE gels as described by Burnette (1) or from IEF gels as described by Reinhart and Malamud (16). Proteins transferred to nitrocellulose paper were probed with MAbs. Clone culture supernatants, diluted 1/10 in 0.01 M, pH 7.4 Tris hydrochloride containing 0.9% NaCl and 3% bovine serum albumin (16) served as sources of MAbs. The MAb-antigen complexes were stained by a modification of the procedures described by Reinhart and Malamud (16). Peroxidase-conjugated goat anti-mouse immunoglobulin G (immunoglobulin G fraction; Cooper Biomedical, Inc., West Chester, Pa.), diluted 1/1,000, was used as a second antibody. The peroxidase substrate was 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, Mo.) dissolved at 60 mg/20 ml of methanol before use and then diluted in 100 ml of the above buffer containing 0.012% peroxide.

Gels were stained for protein with Coomassie brillant blue R250 or silver. GTFs were detected by incubating unfixed gels at room temperature in 1% sucrose buffered with 0.1 M, pH 6.0 sodium phosphate or in 1% sucrose plus 0.1% primer dextran in the same buffer (the primer dextran was 60,000-molecular-weight clinical dextran; ICN Nutritional Biochemicals, Richmond, Calif.). The nonionic detergent Tween 80 (1%; Sigma) was added for assays of GTFs in SDS gels to reverse SDS inhibition of GTFs (17). Zones of glucan synthesis in the gels usually were indicated by the presence of either opaque zones of water-insoluble glucan or transparent, raised zones of water-soluble glucan (2). These visible zones of glucan synthesis and those too diffuse to be seen were readily detected after staining by the periodic acid-Schiff technique of Zacharius et al. (23).

Assay for inhibition of GTF activity by MAbs. Antibodies capable of inhibiting glucan synthesis by GTFs were sought. The diluted hybridoma clone culture supernatants described above were used as MAb sources. Enzyme (0.025 ml of GTF-I, GTF-Sd, or GTF-Si adjusted to an activity of 0.25 IU of GTF/ml) was mixed with MAb (0.1 ml) in phosphate-buffered saline and incubated overnight at 0°C. Controls comprised incubation of GTFs with either culture supernatants of clones producing irrelevant MAbs or culture supernatants of negative hybridomas. A 0.05-ml portion of the enzyme-MAb mixture was then added to an equal volume of buffered [U-14C]sucrose (1.67 mCi; 33.33 mg of purified sucrose in 1 ml of 0.5 M, pH 6.0 sodium phosphate buffer) containing 0.25 mg of clinical dextran per ml and incubated for 15 min at 37°C. Glucan synthesis was detected by assay of 0.05-ml portions of reaction mixture for alcohol-insoluble radioactivity (15).

GTF nomenclature. Reports from this laboratory have described multiple GTFs (11, 14). We identified each isozyme according to its product (GTF-I, producing water-insoluble glucan; GTF-S, producing water-soluble glucan) and the order of migration toward the anode during electrophoresis (GTF-S1 being the most rapidly migrating GTF-S isozyme, followed by isozyme GTF-S2, etc.). We are abandoning this electrophoresis-based nomenclature and hereafter will identify each GTF according to its glucan product and its requirement for primer dextran. Thus, the *S. sobrinus* 6715 GTF-S isozymes GTF-S1 and GTF-S2 (11, 14), which produce a highly branched water-soluble glucan and require a dextran primer (11, 18), are identified here as GTF-Sd (d indicating a primer-dependent GTF-S), while GTF-S4 (11), which produces a nearly linear water-soluble glucan and does not require a dextran primer (11, 18), will be referred to as GTF-Si (i indicating a primer-independent GTF-S). GTF-I isozymes I1 through I4 (14) will be grouped and identified as GTF-I in this report.

RESULTS

IEF and SDS-PAGE. The proteins eluted from the affinity chromatography column were resolved into several zones by focusing in pH 3 to 10 gels (Fig. 1; see reference 18 also). GTF-I focused in one zone at pI 4.5 and a cluster of zones at pI 5.4 to 5.9, GTF-Sd focused at pI 4.0, and GTF-Si focused in a series of zones ranging from pI 6.5 to 7.5. A few minor GTF-Si zones, extending to pI 8.0, were occasionally observed but were detectable only by activity staining. Several unidentified proteins with no GTF activity were present in the acidic regions of the focusing gels, interspersed among the GTF zones.

SDS-PAGE of various GTF preparations yielded closely grouped GTF zones with molecular weights of 150,000 to 175,000 (Fig. 2). Although Fig. 2 indicates that only the major proteins at 165,000 and 175,000 possessed GTF-I activity, traces of water-insoluble glucan were synthesized by proteins as small as 150,000 but were not stained by periodic acid-Schiff reagent and are not visible in the photograph. GTF-Sd activity resided in one zone at 170,000, whereas GTF-Si activity was present in zones at 150,000 and 160,000. SDS-PAGE also resolved several unidentified proteins which lacked GTF activity. Most of these latter proteins had molecular weights ranging from 35,000 to 157,000 (not shown in Fig. 2), but a protein at 175,000 was also observed. The 175,000-molecular-weight protein was found in GTF-Si preparations stained with Coomassie blue (Fig. 2, lane 2), whereas the lower-molecular-weight proteins (35,000 to 157,000) were trace proteins not observed on protein-stained gels but detected in immunoassays of blots of SDS-PAGE gels of affinity-isolated proteins from either mutant 27 (data not shown) or the wild type (see Fig. 5, lanes 3 and 4).

MAb characterization. The discrete patterns of specificity for the various GTF isozymes and the unidentified proteins observed in blots of IEF and SDS-PAGE gels were used to separate MAbs into panels. Pure antigen preparations were used when searching for reactivity of MAbs with unrelated antigens. Negative results are reported below, but the neg-

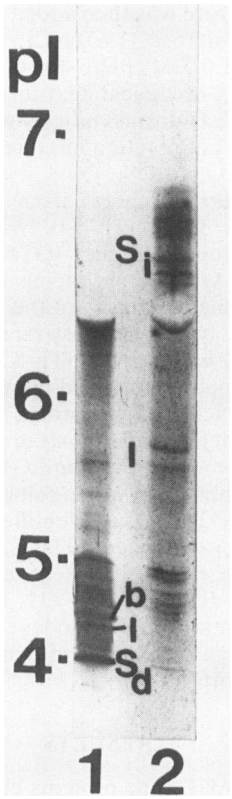

FIG. 1. IEF of affinity-isolated proteins from the wild type and mutant 27 on a pH 3 to 10 gel. The gel was silver stained. Lanes: 1, proteins from the wild type; 2, proteins from mutant 27. The locations of the major glucan-binding protein (b), GTF-Sd (S_d), GTF-Si (S_i), and GTF-I (I) are indicated.

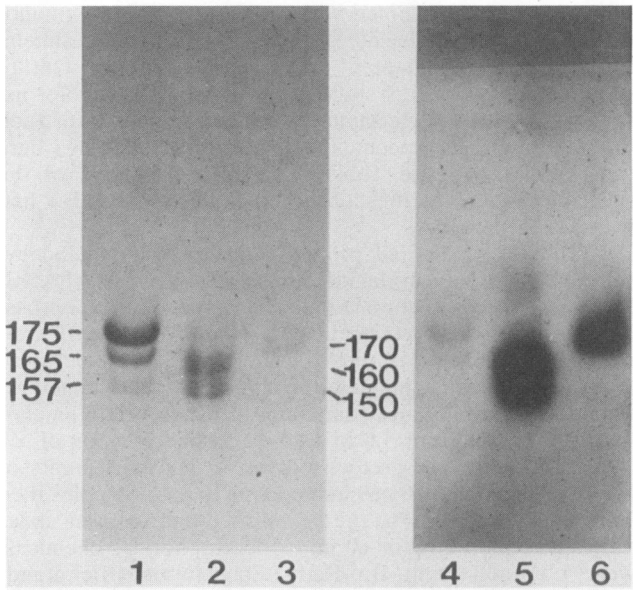

FIG. 2. SDS-PAGE of GTF preparations. Lanes: 1 and 4, GTF-I; 2 and 5, GTF-Si; 3 and 6, GTF-Sd. Lanes 1 to 3 were stained for protein with Coomassie blue R250. Lanes 4 to 6 were assayed for GTF activity in the presence of primer dextran, and the glucan synthesized was stained with periodic acid-Schiff reagent. The molecular weights of proteins, ranging from 150,000 (150) through 175,000 (175) are shown in the margins of lanes 1 and 3.

ative blots are not presented. The results presented in Fig. 3 through 5 and Table 1 are from MAbs selected as representative of the GTF specificities of each panel. Specific immunoglobulin subtypes are presented in Table 1 for each of the MAbs discussed here.

MAbs 3B1 and 3C4, which were derived by immunization with pure GTF-I, recognized discrete determinants on the GTF-I isozymes. Blots of GTF-I resolved in an IEF gel (Fig. 3, lane 1; Table 1) revealed that MAb 3B1 bound to all zones of GTF-I (pI 4.5 and 5.4 to 5.9) and to several proteins lacking GTF activity (pI 4.7 to 5). Antibody 3C4, in contrast, reacted only with the GTF-I focusing at pI 5.7 to 5.9. Blots of SDS-PAGE gels (Fig. 5) indicated that MAb 3B1 (lane 1) bound to all of the detectable GTF-I zones, ranging from the trace GTF-I zone at 150,000 to the major GTF-I zones at 165,000 and 175,000, whereas MAb 3C4 (lane 2) bound only to the major GTF-I zones at 165,000 and 175,000. Results obtained with the affinity-isolated proteins from both the wild type and mutant 27, preparations which include several lower-molecular-weight unidentified proteins, as well as other GTFs, also indicated that no lower-molecular-weight proteins were bound by 3B1 or 3C4 (data not shown). The absence of binding to low-molecular-weight proteins suggested that these anti-GTF-I MAbs did not recognize the major glucan-binding protein elaborated by *S. sobrinus* (13). This protein has a subunit molecular weight of 7,000 and a pI of 4.6. Blots of homogeneous preparations of the major glucan-binding protein also failed to elicit recognition by

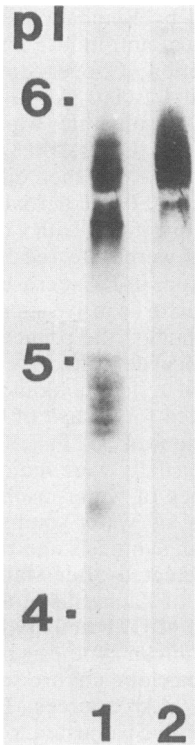

FIG. 3. Blot of GTF-I electrofocused in a pH 4 to 6 IEF gel. The blot was cut into lanes, and each lane was probed with a single anti-GTF-I MAb. Both lanes contained the preparation of GTF-I seen in Fig. 2, lanes 1 and 4. Lane 1 was probed with MAb 3B1, and lane 2 was probed with MAb 3C4.

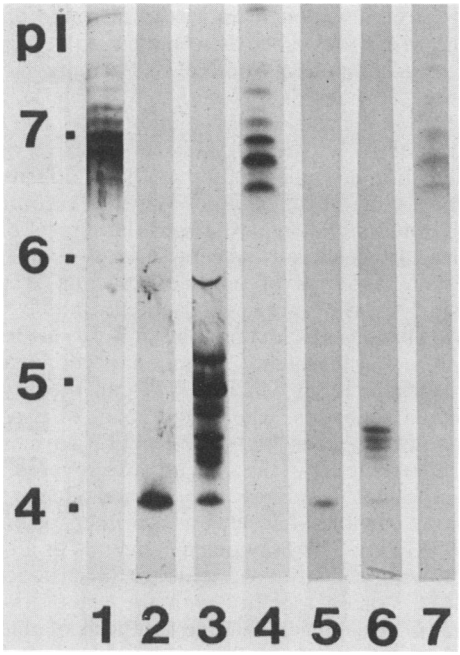

FIG. 4. Blot of GTF-Sd, GTF-Si, and affinity-isolated proteins electrofocused in a pH 3 to 10 IEF gel. The blot was cut into individual lanes, and each lane was probed with anti-GTF-Sd or anti-GTF-Si MAb. Lanes: 1, 4, and 7, GTF-Si; 2 and 5, GTF-Sd; 3 and 6, affinity-isolated wild-type proteins as in lane 1 of Fig. 1, including GTF-I, GTF-Sd, the major glucan-binding protein, and several unidentified proteins, but not GTF-Si. Lane 1 was probed with MAb 5G4, lanes 2 through 4 were probed with MAb 27F11, and lanes 5 through 7 were probed with MAb 27H4.

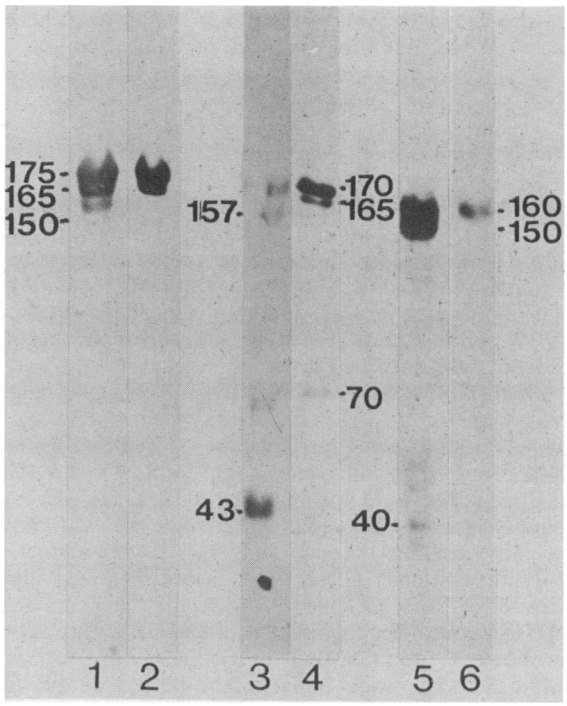

FIG. 5. Blot of GTF-I, GTF-Si, and affinity-isolated proteins resolved in an SDS-PAGE gel. The blot was cut into lanes, and each was probed with anti-GTF-I, anti-GTF-Sd, or anti-GTF-Si MAb. Lanes 1 and 2 contain GTF-I and were probed with MAbs 3B1 and 3C4, respectively. Lanes 3 and 4 contain affinity-isolated proteins as in Fig. 4 (lanes 3 and 6) and were probed with MAbs 27F11 and 27H4, respectively. Lanes 5 and 6 contain GTF-Si and were probed with MAbs EA4 and 5G4, respectively. Molecular weights are indicated for selected proteins at 40,000 (40) through 175,000 (175).

these MAbs, as did blots of preparations of GTF-Sd and GTF-Si (data not shown).

MAb 5G4, derived by immunization with affinity-isolated proteins from mutant 27, and MAb EA4, derived by immunization with pure GTF-Si, bound all of the GTF-Si zones in blots of IEF gels (Fig. 4, lane 1; Table 1). Blots of SDS-PAGE gels showed that MAb EA4 bound both of the GTF-Si zones and several small unidentified proteins with molecular weights as low as 35,000, whereas 5G4 bound only the larger of the two GTF-Si zones (Fig. 5, lanes 5 and 6, respectively; Table 1). Neither of these MAbs reacted with other GTFs or with the major glucan-binding protein. These MAbs often detected traces of GTF-Si in preparations of mutant 27 GTF-I and, on a few occasions, in affinity-purified proteins from the wild type.

MAbs 27F11 and 27H4 were obtained by immunization with pure GTF-Sd. These MAbs bound to GTF-Sd in blots of IEF gels, producing a single band of staining at the location of the two closely spaced zones of this enzyme (Fig. 4, lanes 2 and 5; Table 1). They also bound to several unidentified proteins and to GTF-Si (Fig. 4, lanes 3, 4, 6, and 7). Staining patterns against the unidentified proteins observed in blots of IEF gels suggested that discrete determinants were recognized by each of these MAbs, which appears to be confirmed by the results of blots of SDS-PAGE gels of affinity-isolated wild-type proteins (Fig. 5, lanes 3 and 4). Thus, although both 27F11 and 27H4 bound to the single GTF-Sd zone at 170,000, 27F11 recognized several unidentified proteins ranging in size from 43,000 to 157,000, whereas 27H4 bound only two unidentified proteins at 165,000 and 70,000. Both MAbs bound to the 150,000 and 160,000 zones of GTF-Si (Table 1; data not shown in Fig. 5). Neither MAb bound to GTF-I or the major glucan-binding protein. Although the unidentified 165,000 and 157,000 proteins bound by MAbs 27H4 and 27F11 in blots of SDS-PAGE gels of affinity-isolated proteins from the wild type had molecular weights similar to known zones of GTF-I, the observation that neither MAb bound to proteins in preparations of pure GTF-I suggests that the former proteins are not GTF-I.

Inhibition of GTF activity by MAbs. Among 61 MAbs assayed, 2 were found to inhibit glucan synthesis. MAb 4B11 (Table 1) inhibited glucan synthesis by purified GTF-I by

TABLE 1. Molecular weights and isoelectric points of GTFs bound by MAbs

Clone no.	Isotype	GTF		
		Type[a]	pI	Mol wt, 10^3
3B1, 4B11	IgG1(κ)	I	4.5, 5.4–5.9	150, 157, 165, 175
3C4	IgG1(κ)	I	5.7–5.9	165, 175
5G4	IgG2a(κ)	Si	6.5–7.5	160
EA4	IgG1(κ)	Si	6.5–7.5	150, 160
27F11	IgG1(κ)	Sd	4.0	170
		Si	6.5–7.5	150, 160
27H4	IgG1(λ)	Sd	4.0	170
		Si	6.5–7.5	150, 160

[a] The type of GTF was determined by the glucan synthesized and the requirement for primer: I, water-insoluble glucan, S, water-soluble glucan; d, primer-dependent, i, primer independent.

92%, whereas MAb 5G4 inhibited that by purified GTF-Si by 89%. No MAbs inhibiting GTF-Sd were detected.

DISCUSSION

Grahame and Mayer (6) reported that multiple forms of *Streptococcus sanguis* dextransucrase (GTF-S) were generated by proteolysis. We have not directly observed a similar phenomenon with *S. sobrinus* GTFs, but the results of the blots of IEF gels and SDS-PAGE gels reported here suggest that some of the GTFs and unidentified proteins were derived in this manner. The unidentified proteins detected by MAb 3B1 among the GTF-I zones in blots of IEF gels (Fig. 3) did not appear to yield low-molecular-weight antigens (less than 150,000) in blots of SDS-PAGE gels (Fig. 5), perhaps because they have molecular weights similar to that of GTF-I and thus comigrate with the GTF in SDS-PAGE or because the denaturation associated with SDS-PAGE disrupts the epitopes held in common with the GTF. The former possibility is more likely since no components with molecular weights smaller than that of GTF-I were detected in GTF-I preparations. These large, acidic proteins may be inactive GTF-I or unrelated glucan-binding proteins bearing the GTF-I epitope recognized by MAb 3B1. Explanations aside, however, the fact that the GTF-I epitope recognized by MAb 3C4 is present only on the largest of the GTF-I proteins (165,000 and 175,000), whereas the epitope recognized by MAb 3B1 was present on all GTF-I forms, including the smallest (150,000), suggests that the smaller GTF-I forms recognized by MAb 3B1 were derived from the antigens recognized by 3C4.

The unidentified acidic proteins bearing GTF-Sd epitopes detected by MAbs 27F11 and 27H4 in blots of IEF gels of affinity-isolated proteins (Fig. 4). may yield the lower-molecular-weight proteins detected by these MAbs in blots of SDS-PAGE gels (Fig. 5). These proteins and the low-molecular-weight proteins bound by MAb EA4 in blots of SDS-PAGE gels of GTF-Si (Fig. 5), all of which are isolated, along with the GTF, by virtue of their propensity to bind dextran, may contain the dextran-binding domains of GTF-Sd and GTF-Si.

The anti-GTF-Sd MAbs 27F4 and 27F11 recognized epitopes which were common to GTF-Si as well as GTF-Sd and thus will not be useful for specific detection and quantitation of GTF-Sd in the presence of GTF-Si. This cross-reactivity of anti-GTF-Sd MAbs with GTF-Si was not accompanied by a reciprocal cross-reactivity of anti-GTF-Si MAbs with GTF-Sd. Anti-GTF-Sd MAbs with appropriate affinities can be used for purification of GTF-Sd provided that anti-GTF-Si MAbs are useful as a means of depleting contaminating GTF-Si. We are preparing additional MAbs to GTF-Si and GTF-Sd to further explore the intriguing antigenic relationships of these two GTFs, which differ markedly in the nature of their products and their catalytic characteristics.

Our intent is to construct diverse panels of MAbs useful for structural comparisons of the GTFs and the other extracellular proteins implicated in the synthesis of glucan by *S. sobrinus*, as probes of cell surface protein complements, for selective inhibition of protein functions, and for isolation of individual proteins, especially the GTF isozymes. The low yield of inhibiting antibodies from this round of antibody preparation is not surprising, considering the nature of MAbs. The high levels of inhibition observed for the two inhibiting MAbs, however, suggest that each will be very useful for specific inhibition of its antigen. Additional inhibiting MAbs are being sought in a current round of fusions. Two of the MAbs described here appear to recognize discrete isozyme components of the GTF complement of *S. sobrinus*, i.e., 3C4 and 5G4 (Fig. 3 to 5; Table 1), and bind only the high-molecular-weight components of GTF-I and GTF-Si, respectively. These MAbs may prove useful for specific detection and quantitation of their antigens.

The process of glucan synthesis by *S. sobrinus* is incompletely understood, regardless of the efforts of several laboratories. The generally accepted view of this process suggests that synthesis of complex glucans is primarily accomplished by the synergistic actions of two GTF enzymes, GTF-I and GTF-Sd, upon the sucrose substrate, with GTF-Sd providing the highly branched, predominantly 1,6-α-D-linked primer upon which GTF-I can then synthesize 1,3-α-D-linked segments. The requirement of a primer for GTF-Sd, however, calls for a third GTF. This third GTF may be represented by the recently discovered GTF-Si, which synthesizes a largely unbranched glucan (11, 18) demonstrably capable of serving as a primer for both GTF-Sd and GTF-I (11). The previous discoveries of a branching enzyme (12) and a dextranase inhibitor (8) produced by *S. sobrinus* 6715, taken together with the elaboration of the above three GTFs, suggest that the synthesis of glucan by *S. sobrinus* is not only a complex undertaking but is tightly controlled by a network of extracellular proteins which influence the course of glucan synthesis by modulating synthetic and hydrolytic activities. We expect the panels of MAbs being prepared here to be instrumental for the analysis of the roles of these several proteins in the process of glucan synthesis.

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