Biochem. J. (1992) 287, 639–643 (Printed in Great Britain)

Structural features of the low-molecular-mass human salivary mucin

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The low-molecular-mass human salivary mucin has at least two isoforms, MG2a and MG2b, that differ primarily in their sialic acid and fucose content. In this study, we characterize further these isoforms, particularly their peptide moieties. Trypsin digests of MG2a and MG2b yielded high- and low-molecular-mass glycopeptides following gel filtration on Sephacryl S-300. The larger glycopeptides from MG2a and MG2b had similar amino acid compositions and identical *N*-terminal sequences, suggesting common structural features between their peptides. An oligonucleotide probe generated from the amino acid sequence of the smaller glycopeptide from MG2a was employed in Northern-blot analysis. This probe specifically hybridized to two mRNA species from human submandibular and sublingual glands. A cDNA clone selected from a human submandibular gland cDNA expression library with antibody generated against deglycosylated MG2a also hybridized to these two mRNA species. In both cases, the larger mRNA was polydisperse, and the hybridization signal was more intense in the sublingual gland. In addition, the *N*-terminal amino acid sequence of the larger glycopeptide was found to be part of one of the selected MG2 cDNA clones.

INTRODUCTION

Human submandibular–sublingual saliva (hSMSL) contains both high- and low-molecular-mass mucins which appear to be structurally and functionally distinct [1–5]. The low-molecular-mass mucin (MG2) functions, in part, to promote the clearance of various bacteria by masking their surface adhesins and thereby inhibiting colonization [3,6–10]. For example, the acidic trisaccharide of MG2 (NeuAcα2-3Galβ1-3GalNAc) specifically interacts with a sialic-acid-binding adhesin on *Streptococcus gordonii* and *Streptococcus oralis* [6–8], while the disaccharide (Galβ1-3GalNAc) may interact with *Streptococcus mutans* [9,10]. Recent immunocytochemical studies found that MG2 was produced in human submandibular and labial glands but not parotid or palatine glands [11]. In addition, two isoforms, MG2a and MG2b, have been identified which differ primarily in their content of the terminal sugars, sialic acid and fucose [5]. Little is known about the peptide moiety of MG2 isoforms except that it comprises about 20–25% of the mucin's composition and exists as a single peptide chain composed of 64–65% Ser, Thr, Pro and Ala. The purpose of the present study was to further examine the structural features of the peptide moiety of this mucin using biochemical and molecular-biological methods.

MATERIALS AND METHODS

Isolation of mucin isoforms

MG2a and MG2b were isolated from hSMSL as recently described [5]. As part of the purification protocol, reductive methylation with [^{14}C]formaldehyde and reduction and alkylation were employed to dissociate the ionic interactions between the MG2 and other salivary components [5].

Preparation of tryptic glycopeptides

^{14}C-labelled MG2a (12.0 mg) and ^{14}C-labelled MG2b (8.8 mg) were dissolved in ammonium bicarbonate (0.05 M, pH 7.6) at a concentration of 3 mg/ml and incubated with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-trypsin (3% w/w) for 24 h at 37 °C. The digest of ^{14}C-labelled MG2a was subjected to gel filtration on Sephacryl S-300 (1 cm × 45 cm) employing 0.02 M-acetic acid while the digest of ^{14}C-labelled MG2b was fractionated on Sephacryl S-300 using 0.1 M-Tris/HCl, pH 7.5, with 6 M-urea.

Chemical deglycosylation

MG2a (50 mg), phenylalanine (250 mg), and methionine (50 mg) were dried under vacuum at 60 °C for 3 days over P_2O_5. The sample was hydrolysed with HF at a concentration of 2.0 mg/ml for 3.5 h in a closed system [12]. Following removal of HF by distillation under vacuum, deglycosylated MG2a (dMG2a) was dissolved in 20 ml of water, dialysed against cold distilled water and lyophilized. Amino sugar analysis indicated that HF treatment of MG2a removed ~95% of the carbohydrate. This deglycosylated material was used to prepare an antiserum for subsequent screening of a human submandibular gland cDNA expression library for apo-MG2 cDNA clones.

Production of antibodies

Antibodies to dMG2a were produced in 6–8-week-old New Zealand White female rabbits. Approx. 0.2 mg of dMG2a in 0.2 ml of 0.154 M-NaCl was emulsified with 0.8 ml of Freund's complete adjuvant (Difco, Detroit, MI, U.S.A.) and injected subcutaneously. A booster injection was given subcutaneously after 3 weeks with 0.1 mg of antigen in 0.1 ml of 0.154 M-NaCl emulsified with 0.9 ml of Freund's incomplete adjuvant. Antibody titre was evaluated by Western-blot transfer.

Electrophoretic procedures

After SDS/PAGE [13], ^{14}C-labelled samples were visualized by fluorography whereby the gels were fixed in trichloroacetic acid (10% w/v) for 1–2 h at room temperature, and were then soaked in EN^{3}HANCE (New England Nuclear, Boston, MA, U.S.A.) for 1 h at room temperature [2]. For Western-blot

Abbreviations used: hSMSL, human submandibular–sublingual saliva; MG2, low-molecular-mass mucin; dMG2a, deglycosylated MG2a; TBS, Tris-buffered saline; HRP, horseradish peroxidase; pfu, plaque-forming unit.

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transfer [14,15], samples were run on SDS/PAGE and transferred to Immobilon-P membrane at room temperature for 30 min at 100 mA employing 0.1 M-Tris with 0.192 M-glycine and 20% (v/v) methanol as the transfer buffer. Following blocking with 5% (w/v) BSA in Tris-buffered saline (TBS) (0.01 M-Tris/HCl, pH 7.2, with 0.154 M-NaCl) for 1 h, the membrane was washed three times, for 10 min each wash, with 0.1% BSA/TBS and then incubated with anti-dMG2a (1:500) for 1 h. After washing with 0.1% BSA/TBS, three times for 10 min each, the membrane was incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:1000) (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The membrane was washed with 0.1% BSA/TBS as described above, and the immunoreactive components were identified by HRP colour-developing reagent (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Analytical procedures

Analyses of amino acids, amino sugars and neutral sugars were carried out as previously described [2,5]. *N*-Terminal amino acid sequencing was performed employing an Applied Biosystems Model 471A protein sequencer [5]. Samples (~ 1 nmol) were dissolved in water (30 μl) and dried on a glass fibre filter precycled with polybrene.

Screening of human submandibular gland cDNA library with anti-dMG2a antibody

A human submandibular gland cDNA library (constructed in the λgt11 Sfi-Not orientation-specific expression vector) [16] was plated onto *Escherichia coli* Y1090 at a density of 10000–20000 plaque-forming units (pfu) per 90 mm plate. Plaques were screened with the anti-dMG2a antibody (1:500) and the Protoblot Immunoscreening System following the manufacturer's instructions (Promega, Madison, WI, U.S.A.).

Northern-blot analysis

Human salivary gland tissues were obtained following prophylactic radial neck dissection as described earlier [16]. Rat submandibular gland RNA was a gift from Dr. G. Bedi, Department of Oral Biology, SUNY/Buffalo. CV-1 and CMT3 monkey kidney cells were obtained from Dr. M.-L. Hammarskjöld, Department of Microbiology, SUNY/Buffalo. Total RNA from all tissues was isolated by extraction with guanidinium isothiocyanate followed by centrifugation in a caesium chloride gradient as previously described [16]. RNA was denatured by a formaldehyde/formamide procedure and fractionated by 1.4% agarose/formaldehyde gel electrophoresis [17] and subsequently transferred to an Immobilon-N hydridization membrane as described by the manufacturer (Millipore Corp., Bedford, MA, U.S.A.).

Hybridization probes used for Northern blots were: (1) degenerate synthetic oligonucleotides (20-mer) corresponding to a region within the *N*-terminus of MG2a-T2 (amino acids 10–16, Fig. 2*d*) obtained from the DNA Factory (San Diego, CA, U.S.A.) and end-labelled with polynucleotide kinase and [γ^{32}P]ATP as recently described [17], and (2) a 0.4 kb cDNA clone (3-1) selected with antibody against dMG2a and labelled by the random primed DNA labelling method using a Genius Non-radioactive DNA Labelling Kit (Boehringer Mannheim, Indianapolis, IN, U.S.A.). For hybridization with the oligonucleotide probe, the RNA blot was prehybridized in 6 × SSC (1 × SSC = 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0), 5 × Denhardt's (1 × Denhardt's = 0.02% w/v each of Ficoll, polyvinylpyrrolidone and BSA), 0.05% sodium pyrophosphate, 0.5% SDS and 100 μg of boiled salmon sperm DNA/ml for 2 h at 42 °C, followed by hybridization at 42 °C for 18 h in the same buffer (without the SDS) containing the radioactive probe (2×10^6 c.p.m.). Hybridization was followed by low-stringency washes (twice for 10 min in 6 × SSC/0.05% sodium pyrophosphate at room temperature and once for 20 min at 42 °C) and autoradiography. For hybridization with the cDNA probe, the blot was prehybridized, hybridized and washed using the high-stringency hybridization. Briefly, the prehybridization mixture contained 50% (v/v) formamide, 5 × SSC, 5 × Denhardt's, 0.5% SDS, 0.1 M-sodium phosphate buffer and 100 μg of salmon sperm DNA/ml. Prehybridization was done for 2 h at 42 °C, followed by addition of the heat-denatured probe and hybridization for 18 h at 42 °C. The blot was washed twice for 15 min each in 2 × SSC/0.1% SDS at room temperature and then at 60 °C, followed by two washes, 15 min each, in 0.2 × SSC with 0.1% SDS. Finally, the blot was developed using the Genius Detection Kit as described by the manufacturer (Boehringer Mannheim).

RESULTS

Isolation of tryptic glycopeptides of MG2

SDS/PAGE-fluorography of the tryptic digests of the MG2a and MG2b (Figs. 1*c* and 1*d* and lanes 2 and 7) indicated the presence of two major (glyco)peptide components: a larger one of ~ 90 kDa and a smaller one of ~ 45 kDa. The tryptic glycopeptides of MG2a were separated by gel filtration on

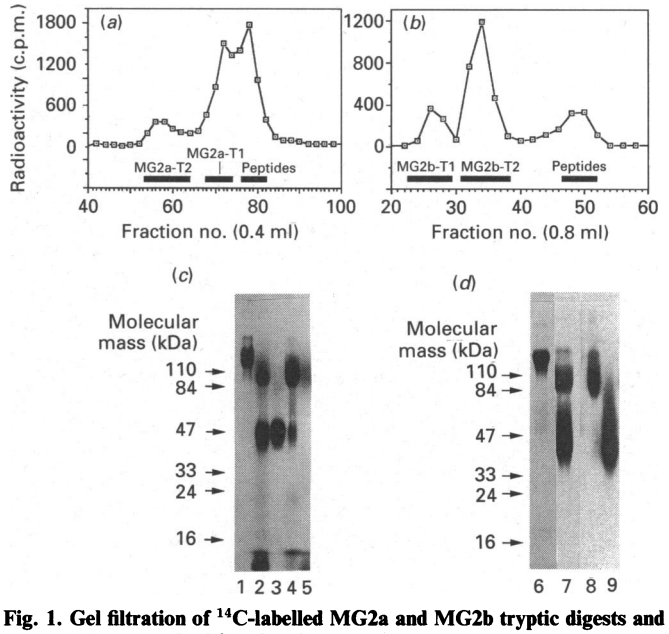

Fig. 1. Gel filtration of ^{14}C-labelled MG2a and MG2b tryptic digests and subsequent SDS/PAGE–fluorography

(*a*) Gel filtration of ^{14}C-labelled MG2a tryptic digest on Sephacryl S-300. Pools designated MG2a-T1 and MG2a-T2 were lyophilized. (*b*) Gel filtration of ^{14}C-labelled MG2b tryptic digest on Sephacryl S-300. Pools designated MG2b-T1 and MG2b-T2 were dialysed against cold distilled water and lyophilized. (*c* and *d*) SDS/PAGE–fluorography (10% gels). Lane 1, ^{14}C-labelled MG2a (5000) (values in parentheses represent c.p.m. run in each lane); lane 2, tryptic digest of ^{14}C-labelled MG2a (22000); lane 3, ^{14}C-labelled MG2a-T2 (6000); lane 4, ^{14}C-labelled MG2a-T1 (8000); lane 5, small peptides derived from MG2a (4000) and trypsin; lane 6, ^{14}C-labelled MG2b (5000); lane 7, tryptic digest of ^{14}C-labelled MG2b (32000); lane 8, ^{14}C-labelled MG2b-T1 (9000) and lane 9, ^{14}C-labelled MG2b-T2 (20000). Prestained molecular mass markers were: phosphorylase *b* (110 kDa), BSA (84 kDa), ovalbumin (47 kDa), carbonic anhydrase (33 kDa), soybean trypsin inhibitor (24 kDa) and lysozyme (16 kDa).

Table 1. Chemical composition of MG2 glycopeptides

MG2b-T2 contained at least two components when examined by *N*-terminal sequencing. Abbreviation: N.d., not determined.

	Residues/1000 amino acid residues					
	MG2a†	MG2b†	MG2a-T1	MG2b-T1	MG2a-T2	MG2b-T2
Aspartic acid	61	67	42	46	115	97
Threonine	186	171	204	215	146	103
Serine	105	113	135	129	103	78
Glutamic acid	71	75	75	79	52	114
Proline	230	223	244	244	230	114
Glycine	10	11	35	21	6	27
Alanine	133	129	179	183	49	82
Half-cysteine	14	14	0	0	0	0
Valine	34	36	24	23	66	67
Methionine	1	1	0	0	0	2
Isoleucine	21	23	10	9	42	37
Leucine	46	51	25	26	58	91
Tyrosine	5	6	0	0	0	13
Phenylalanine	17	18	0	0	41	47
Histidine	21	24	9	6	25	28
Lysine	23*	24*	18*	16*	42*	75*
Arginine	22	14	0	3	25	25
Fucose	421	221	258	94	230	84
Galactose	352	368	372	253	239	157
Mannose	21	34	76	97	93	116
N-Acetylglucosamine	225	243	123	240	283	116
N-Acetylgalactosamine	292	273	282	265	164	109
N-Acetylneuraminic acid	115	248	N.d.	N.d.	N.d.	N.d.

* As mono- and di-methyl-lysine.
† Amino acid data from Ramasubbu *et al.* [5].

Sephacryl S-300 using 0.02 M-acetic acid (Fig. 1*a*). Under these non-dissociating conditions, the smaller glycopeptide (MG2a-T2) eluted before the larger MG2a-T1, suggesting that MG2a-T2 may have eluted as an oligomer. A third pool containing predominantly small peptides and some residual MG2a-T1 was also obtained. This material could be fractionated into at least two additional pools on Sephadex G-25. *N*-Terminal sequencing indicated that these additional pools contained a mixture of several peptides, probably derived from both mucin and trypsin, and therefore they were not studied further. The tryptic glycopeptides of MG2b were separated by gel filtration on Sephacryl S-300 using 0.1 M Tris/HCl, pH 7.5, with 6 M-urea (Fig. 1*b*). Under these dissociating conditions, the larger glycopeptide (MG2b-T1) eluted before the smaller one.

Chemical composition of the mucin tryptic glycopeptides

The chemical compositions of the tryptic glycopeptides are presented in Table 1. The higher-molecular-mass glycopeptides (MG2a-T1 and MG2b-T1) had similar amino acid compositions which differed from those of the lower-molecular-mass glycopeptides, MG2a-T2 and MG2b-T2. In particular, the larger glycopeptides contained lower amounts of basic amino acids, valine, isoleucine and leucine, but higher amounts of alanine and proline. Most of the phenylalanine was present in the smaller glycopeptides. In contrast to the larger glycopeptides, the smaller glycopeptides (MG2a-T2 and MG2b-T2) differed in their amino acid compositions. These differences may be caused by heterogeneity in MG2b-T2 since this material appeared as a broad band on SDS/PAGE–fluorography (Fig. 1*d*). This heterogeneity was substantiated by *N*-terminal sequencing which indicated that the MG2b-T2 fraction was a mixture of at least two components. The larger glycopeptides showed some differences between their carbohydrate compositions. The lower content of fucose in MG2b-T1 as compared with MG2a-T1 was expected as the MG2b isoform contains less fucose than MG2a [5]. The similarity in *N*-acetylgalactosamine content suggests that the number of *O*-linked units is comparable between MG2a-T1 and MG2b-T1. However, differences in the content of galactose and *N*-acetylglucosamine may reflect variations in the composition of the larger *O*-linked units [18] or the *N*-linked units. Mannose, which is derived from *N*-linked units, was found in both the larger and smaller glycopeptides but was present in higher amounts in the latter.

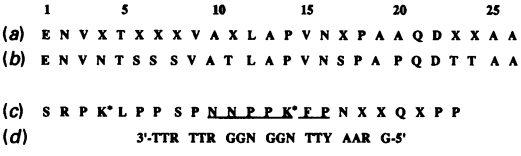

Fig. 2. (*a*) *N*-terminal amino acid sequence of MG2a-T1 and MG2b-T2, (*b*) amino acid sequence deduced from the nucleotide sequence of a region of the MG2 cDNA clone 6-1, (*c*) the *N*-terminal sequence of MG2a-T2 and (*d*) mixed oligonucleotide probes of the mRNA complementary strand prepared from the amino acid sequence of residues 10–16

*Lysine was present as dimethyl-lysine ($R_{proline}$0.91). N indicates all four dNTPs; R (A or G); Y (T or C).

N-Terminal sequencing of MG2 glycopeptides

Further assessment of the glycopeptides was done by *N*-terminal sequencing. The first 26 residues of the larger glycopeptides, MG2a-T1 and MG2b-T1, were the same with glutamic acid at the *N*-terminus (Fig. 2*a*). These results, as well as those obtained from amino acid analysis, suggested that the peptide moieties of MG2a and MG2b may share common structural features. MG2a-T2 also had a single *N*-terminus while MG2b-T2, as stated above, was a mixture of two (glyco)peptides.

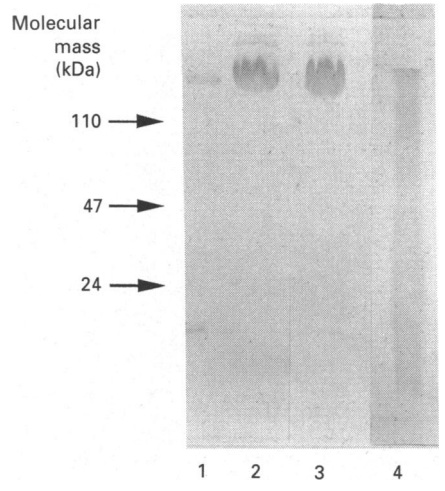

Fig. 3. SDS/PAGE and Western transfer using anti-dMG2a

Lane 1, hSMSL; lane 2, MG2a; lane 3, MG2b; lane 4, dMG2a. Gels of 7.5% were used. Prestained molecular mass markers were: phosphorylase *b* (110 kDa); ovalbumin (47 kDa) and soybean trypsin inhibitor (24 kDa).

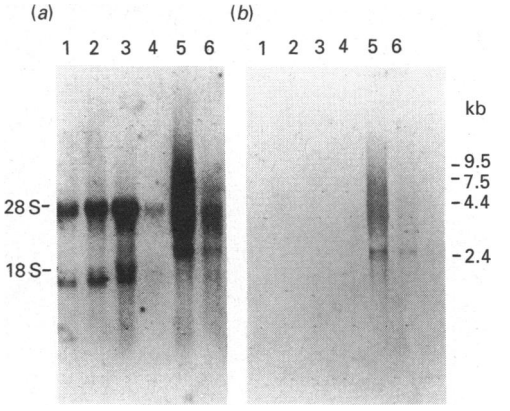

Fig. 4. Northern-blot analysis of total RNAs using a mixed oligonucleotide probe derived from the MG2a-T2 (*a*), or a 0.4 kb MG2 cDNA probe (*b*)

All lanes except lane 4 contained 10 μg of RNA; lane 4 contained 5 μg. In both panels, lane 1 indicates RNA from rat submandibular gland; lane 2, CV-1 monkey kidney cells; lane 3, CMT3 monkey kidney cells; lane 4, human submandibular gland; lane 5, human sublingual gland; lane 6, human submandibular gland.

The first 23 residues of MG2a-T2 are shown in Fig. 2(*c*); residues 10–16 were used to prepare a mixed oligonucleotide probe (Fig. 2*d*).

Specificity of anti-dMG2a

Antiserum against dMG2a reacted with purified mucin isoforms and with MG2 in native hSMSL (Fig. 3, lanes 1–3). This antiserum also reacted very strongly with dMG2a showing a broad smear (Fig. 3, lane 4). The broad smear indicated that cleavage of the MG2a protein core probably occurred during HF treatment, a phenomenon also noticed by others [12,19]. These observations suggested that the specificity of this antiserum was directed against naked peptide regions in MG2a and/or residual carbohydrate moieties. The subsequent identification of apo-MG2 cDNA clones with this antiserum indicated that it recognized peptide determinants. Four cDNA clones out of 10 possible positives identified in the first round of screening with this antiserum held positive throughout additional rounds of purification.

Preliminary characterization of MG2 cDNA clones

The four MG2 cDNA clones cross-reacted with each other at the DNA level, as determined by Southern-blot analysis, using the insert of the longest clone (6-1; 2.0 kb) as a hybridization probe. The insert of clone 6-1 has been subcloned into a pGem11Zf+ vector and a partial DNA sequence has been determined (L. A. Bobek, H. T. Tsai, M. S. Reddy, A. R. Biesbrock & M. J. Levine, unpublished work). As shown in Fig. 2(*b*), the deduced amino acid sequence of a region from this cDNA clone agreed (with one exception) with the *N*-terminal sequence of MG2a-T1 and MG2b-T1 (Fig. 2*a*). The residues marked with X at the amino acid level in Fig. 2*a* were deduced to be Thr or Ser from the nucleotide sequence (Fig. 2*b*). These results demonstrate that the clone 6-1 encodes the protein core of MG2 isoforms. In addition, the antiserum raised against dMG2a is specific for the mucin's peptide moiety.

Northern-blot analysis

The results of the RNA-blot analysis of total RNA from human and rat salivary tissues and monkey kidney cells are shown in Fig. 4. The mixed synthetic oligonucleotide derived from the partial amino acid sequence of the MG2a-T2 tryptic peptide was used as a probe in Fig. 4(*a*). This probe specifically hybridized to two distinct mRNA species present only in the human submandibular and sublingual glands (a sharp band of about 2.4 kb and a polydisperse band with an average size of about 5.0 kb, lanes 4–6). The intensity of the hybridization signal was much stronger in the RNA isolated from the sublingual gland. In addition, the mixed oligonucleotide non-specifically cross-hybridized with 18 S and 28 S rRNAs. No detectable hybridization signal other than a non-specific hybridization with the rRNAs was detected in all other analysed RNAs. The specificity of the oligonucleotide probe for the MG2 mRNAs was confirmed on a duplicate blot using an MG2 cDNA clone as a hybridization probe. The MG2 cDNA clone detected the same two mRNA species in the human salivary glands (Fig. 4*b*) and there was no cross-hybridization with the rRNAs due to higher specificity of this probe and higher hybridization and washing stringencies. The polydisperse nature of the signal is not from the degradation of the RNA preparations, since the ethidium bromide staining of the gel showed distinct rRNA bands (not shown here but in fact visible as intact bands in Fig. 4*a* due to a cross-hybridization with the MG2 mixed oligonucleotide probe). Similar mRNA polydispersity has been observed for other mucins, including human intestinal mucin [19], porcine submaxillary mucin [20] and a bovine submaxillary mucin-like protein [21].

DISCUSSION

For a clear understanding of the structural basis for the biological function of MG2, it is essential to determine the complete structure of this mucin. Such information includes the primary structure of the peptide backbone, oligosaccharide chains, distribution of oligosaccharides along the peptide backbone, and the mucin's macromolecular or supra-structure. The primary structures of the *O*-linked units of MG2 have been characterized [18]. The presence of mannose in both purified MG2a and MG2b [5] necessitates the characterization of this mucin's *N*-linked units. In general, the use of classical biochemical techniques for elucidating the complete amino acid sequences of mucins has been difficult because of their high molecular mass, high density of carbohydrate units, unique amino acid com-

position and physicochemical properties. Recently molecular biological techniques have been employed to deduce the amino acid sequences of human intestinal mucin [19], porcine submaxillary mucin [20,22], mucin-like protein from bovine submaxillary gland [21], human tracheal mucin [23], human breast mucin or episialin [24–26] and pancreatic tumour cells [27]. An interesting feature of the amino acid sequences of most of these mucins is the presence of tandem repeats. The size, number and composition of these tandem repeats can vary among different mucins. DNA sequencing of MG2 cDNA clone 6-1 also indicated the presence of tandem repeats. In this case, the repeats are 23 amino acid residues (69 bp) long.

This study was supported, in part, by USPHS grants DE07585, DE08240 and AI27401. We acknowledge Dr. A. R. Periathamby for his help in the deglycosylation of MG2a and Dr. A. Aguirre for help in the preparation of anti-dMG2a.

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Received 5 September 1991/8 March 1992; accepted 31 March 1992