Clin Oral Invest (2012) 16:761–766
DOI 10.1007/s00784-011-0556-5

ORIGINAL ARTICLE

An unusual glycoform of human salivary mucin MG2

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Received: 17 December 2010 / Accepted: 14 April 2011 / Published online: 3 May 2011
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Abstract Since in a previous study we encountered a subject with an unusual split MG2 banding pattern, the aim of this study was to investigate the molecular basis of this observation. Submandibular/sublingual secretion was collected under resting and stimulated conditions and examined on Western blots probed with anti-MG2 antibodies or on gels stained with periodic acid-Schiff reagent. Genomic DNA was isolated and the N-, tandem repeat (TR), and C-terminal regions of MUC7 were amplified by PCR since MG2 is known to display a genetic polymorphism. Although the typical appearance of MG2 on blots and gels is a single 180 kDa band, salivary secretions from the subject exhibited doublet immunoreactive bands of approximately 180 and 125 kDa. Additionally, under resting conditions the 180 kDa band was predominant whereas upon stimulation the 125 kDa band became predominant. Genomic DNA analysis showed that MUC7 in the individual with split MG2 bands was not truncated and that the MUC7 genotype in this individual was (6/6) where both alleles encoded six TRs. The MG2 split banding pattern observed in this subject was not derived from proteolytic degradation of this salivary mucin in whole saliva or from genetic polymorphism. The expression of two isoforms of MG2 could in principle improve or reduce the activity of this key component of the oral host defense system.

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Keywords Saliva · Mucin · MG2 · Innate immunity · Genetic polymorphism

Introduction

Human salivary mucin MG2 is encoded in the single-copy MUC7 gene that spans 10.0 kb and contains three exons and two introns and has been localized to 4q13–4q32 [1, 2]. The entire coding sequence for the secreted mucin is contained with Exon 3. MG2 is a unique member of the mucin gene family because it is a small, soluble, and monomeric molecule [3, 4]. MG2 is secreted by submandibular, sublingual, and minor salivary glands although there is controversy with respect to which cell type expresses MG2 in these glands. Immunochemical and in situ hybridization studies have localized MG2 to serous acini in submandibular, sublingual, and minor salivary glands [5, 6] whereas another group using the same procedures localized MG2 to a subset of mucous acini in submandibular and labial salivary glands [7, 8]. On the other hand, by using immunogold electron microscopy, we found that MG2 could be localized in both mucous and serous acini of submandibular and sublingual glands, but not in parotid glands [9]. Together, the information above indicates that the MUC7 gene for MG2 has complex expression patterns in the major salivary glands.

Secreted MG2 contains 357 amino acids organized into five distinct regions represented by a basic N-terminal domain (residues 1–51) containing a histatin-like sequence and a leucine zipper-like segment, a moderately glycoslyated domain (residues 52–93), a heavily glycolysated domain (residues 94–138) containing six tandem repeats (TR; each 23 amino acids), a second heavily glycosylated domain (residues 139–334) and a C-terminal domain (residues 335–357)

containing a leucine zipper-like segment [10]. The theoretical mass of MG2 apomucin is 37,009.14 Da and the calculated p*I* is 9.28 (ExPASy, Swiss Institute of Bioinformatics), and earlier compositional studies indicated that MG2 is composed of 30.4% protein, 68% carbohydrate, and 1.6% sulfate [11]. The carbohydrate side chains in MG2 range in length from two to seven residues and the majority of the monosaccharides are *N*-acetylglucosamine, *N*-acetylgalactosamine, galactose, fucose, and sialic acid [12]. Two isoforms of MG2, which vary in their sialic acid to fucose ratios, were isolated from submandibular/sublingual (SMSL) secretion by ion exchange chromatography and were named MG2a and MG2b [13]. The chemical composition of MG2a and MG2b was similar in different individuals.

MG2 is present in the biofilm that covers oral surfaces preventing desiccation of soft tissues and aiding in mastication, swallowing, and speech [14]. This salivary mucin was reported to have affinity for hydroxyapatite [15], to be present in short-term (10–60 min) pellicle formed on cementum [16], and is also a component of the 2-h acquired enamel pellicle [17]. MG2 has been shown to exhibit candidacidal activity [18, 19] and to kill the periodontal pathogen, *Agregatibacter actinomycetemcomitans* [20]. MG2 interacts with a diverse number of oral microbes indicating that this mucin is an important component of the innate immune system [19–23]. In a recent study on the interaction of MG2 with *A. actinomycetemcomitans*, we examined whole saliva (WS) from 60 subjects on Western blots probed with our anti-MG2 antibody directed against a synthetic peptide corresponding to residues 47–63 in the N-terminal region of the apomucin [20]. Surprisingly, one subject was found who exhibited a unique electrophoretic pattern where MG2 appeared as two immunoreactive bands of approximately 125 and 180 kDa rather than one band of 180 kDa.

In the present investigation, comparison of MG2 in WS and glandular secretions indicated that 125 kDa band was not derived from the 180 kDa band due to proteolytic processing. Physiological studies with unstimulated and stimulated SMSL suggested the possibility that the upper and lower MG2 bands originate in different salivary glands or in different cell types. Analyses by polymerase chain reaction with genomic DNA showed that the MUC7 genetic polymorphism could not explain the split MG2 bands because both MUC7 alleles in this subject contained six TRs (6/6).

Materials and methods

Saliva collection

Resting and stimulated SMSL secretion was collected from two subjects (one male and one female; 35 and 25 years of age, respectively) between 10:00 a.m. and 11:00 a.m. Subjects were asked not to eat for 2 h prior to collection. SMSL was collected with a custom-fitted device as described previously [24]. Resting SMSL was collected for 10 min and the first 4 min of the collections were discarded. Subsequently, three consecutive 2-min samples were collected in separate graduated 1.5-ml centrifuge tubes followed by ten consecutive 1-min samples that were collected in 15-ml Falcon screw cap tubes. Gustatory stimulation was induced by placing fruit flavored candies on the tongue (Jolly Rancher, Hershey, PA). All samples were kept on ice during the collection procedure. This study was approved by the Institutional Review Board at Boston University Medical Center, informed consent was obtained from all subjects prior to their participation and subjects' rights were protected at all times.

Gel electrophoresis

Samples of resting and stimulated SMSL were thawed on ice and made 5 mM with respect to EDTA in order to promote solubilization of mucins and glycoproteins [25]. For each subject, equal volumes (50 μl) of resting and stimulated SMSL were lyophilized, taken up in 10 μl of sample buffer [26], heated at 95°C for 5 min, and subjected to sodium dodecylsulfafe polyacrylamide gel electrophoresis on 10% gels.

Western blots

Proteins in gels were electrophoretically transferred to nitrocellulose membranes (Protran, Schleicher and Schuell, Keene, NH) in 25 mM Tris–HCl, pH 8.3, containing 192 mM glycine and 20% methanol at 100 V for 1 h at room temperature. Blots were probed with a rabbit polyclonal antibody directed against a synthetic peptide corresponding amino acid residues 47–63 in the N-terminal region of secreted MG2 [27]. Briefly, blots were equilibrated in 10 mM Tris–HCl, pH 8.0 containing 150 mM NaCl and 0.1% Tween 20 (TBST) for 15 min and blocked with 5% non-fat, dried milk in TBST overnight at room temperature. After blocking, blots were washed three times with TBST for 15 min and incubated with anti-MG2 antibody diluted 1:1,000 in 1% milk/TBST for 1 h at room temperature. After washing, blots were incubated with the second antibody which was anti-rabbit IgG coupled to alkaline phosphatase (Promega, Madison, WI) diluted 1:7,500 in 1% milk/TBST for 1 h at room temperature. Blots were washed three times in TBST for 5 min and color development was carried out with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium according to manufacturer's instructions.

PAS staining

Proteins in gels were stained with periodic acid-Schiff (PAS) as described [28]. Briefly, after electrophoresis gels were fixed in a solution of 40% ethanol and 5% acetic acid for 24 h. Gels were oxidized in an aqueous solution containing 30 mM periodic acid and 5% acetic acid for 2 h, reduced in an aqueous solution containing 10 mM sodium metabisulfite and 5% acetic acid for 90 min at room temperature, and stained with freshly prepared Schiff reagent.

Genomic DNA

DNA was extracted from 10 ml of whole blood obtained from three subjects using a QIAamp DNA Blood Maxi Kit (Quiagen). Blood was drawn into heparinized tubes and DNA was prepared according to manufacturer's instructions. The concentration of DNA in samples was estimated spectrophotometrically at 260 nm using a specific absorption coefficient of 20 (i.e., A_{260}=20 for a solution with a concentration of 1 μg/μl).

Amplification of MUC7 fragments

Taq polymerase and other PCR reagents were purchased from Promega. PCR was performed in 50 μl reactions containing 5 μl of 10× magnesium-free buffer (1× buffer=10 mM Tris–HCl, pH 9, 50 mM KCl, and 0.1% Triton X-100), 3 μl of 25 mM magnesium chloride solution, 1 μl of 10 mM dNTPs, 0.5 μl of Taq polymerase (5 u/μl), and 38 μl of water. The template was 50 ng of genomic DNA. Finally, 1 μl of each specific sense (10 pmol) and antisense primers (10 pmol), designed to amplify three different regions of MG2 (Table 1), were added to separate reactions. The PCR parameters were: 95°C for 5 min, 35 cycles of 95°C for 45 s, 64°C for 45 s, and 72°C for 1 min with a final extension step at 72°C for 5 min.

Agarose gel electrophoresis

PCR products were electrophoresed on 2% agarose/Tris–acetate–EDTA (TAE) gels (1× TAE=40 mM Tris–acetate, pH 8.5, and 2 mM EDTA), containing 0.5 μg of ethidium per 10 ml of agarose gel and examined on a Transilluminator (model TFM; UVP, Upland, CA).

Table 1 MUC7 primers

N-Terminal forward 5′ GAAGGTCGAGAAAGGGATCATG 3′
N-Terminal reverse 5′ GTCTTGTGGAGCTGGGGAAT 3′
TR forward 5′ GGTCAACCCTACCTTAGTG 3′
TR reverse 5′ TTGCTCCACCATGTCGTCAA 3′
C-Terminal forward 5′ GACCACAGCTGCCCCAATTACC 3′
C-Terminal reverse 5′ TTGCTCCACCATGTCGTCAA 3′

Results

Characterization of MG2 in SMSL

In a typical subject, MG2 in resting SMSL appeared as a single diffuse band of approximately 180 kDa on both Western blots probed with anti-MG2 antibodies (Fig.1, lane 1) and on PAS-stained gels (Fig. 1, lane 3). In the subject that exhibited split MG2 bands, MG2 appeared as two bands of approximately 180 and 125 kDa on Western blots probed with the same antibody (Fig. 1, lane 2) and on PAS-stained gels (Fig. 1, lane 4). The upper band had the typical diffuse appearance reflecting microheterogeneity in the O- and N-linked glycans whereas the lower band was much more compact which likely reflects reduced glycosylation and reduced microheterogeneity among glycans.

Secretion patterns

Previously, we have conducted studies on the physiology of mucin secretion in SMSL [29, 30]. Therefore, we asked the question whether the secretory pattern of the split MG2 bands would be influenced by gustatory stimulation and duration of the stimulus. SMSL samples were collected under resting and stimulated conditions from a subject with the typical MG2 banding pattern and from the subject with split MG2 bands. Samples were electrophoresed and gels were used for Western blotting or stained with PAS. The pattern of MG2 from a typical subject under resting and stimulated conditions on Western blots (Fig. 2, A) and on PAS-stained gels (Fig. 2, C) showed that the quantity of MG2 increased immediately after stimulation and gradually decreased as a function of the duration of stimulation. A somewhat different pattern was observed with the subject having split MG2 bands. The Western blot (Fig. 2, B) and PAS-stained gel (Fig. 2, D) showed that after stimulation there was a significant decrease in the intensity of the upper 180 kDa band and an increase in the intensity of the lower

Fig. 1 Electrophoretic pattern of MG2 in resting SMSL. Stimulated SMSL was electrophoresed in multiple lanes (50 ml per lane). Western blot using specific antibodies against MG2 (*lane 1*, typical appearance and *lane 2*, atypical appearance) and PAS-stained gel (*lane 3*, typical appearance and *lane 4*, atypical appearance)

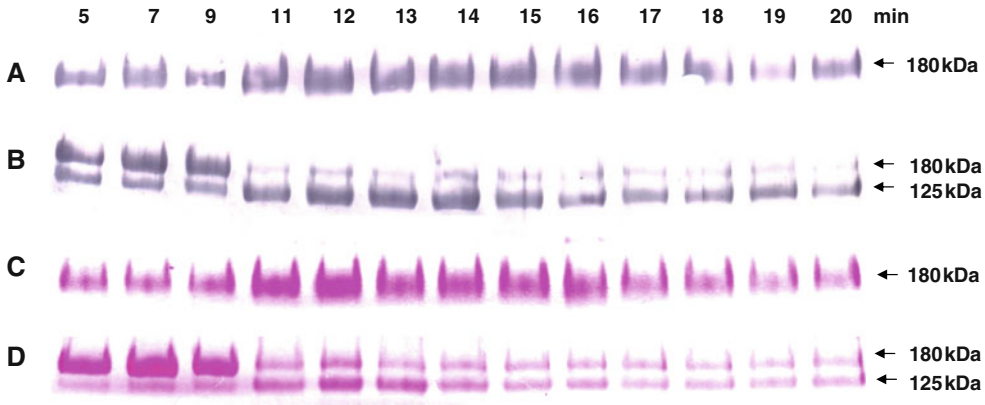

Fig. 2 Secretion pattern of MG2 under resting and stimulated conditions. Typical appearance of MG2 in a Western blot (*A*) and in a PAS-stained gel (*C*). Atypical appearance of MG2 in a Western blot (*B*) and in a PAS-stained gel (*D*)

125 kDa band. Again, the intensity of both bands decreased with as a function of the duration of stimulation.

Analysis of MUC7 gene

In order to determine whether the split MG2 bands could be attributed to truncation of one MUC7 allele, or to a previously unrecognized genetic polymorphism, a PCR strategy was planned for use with genomic DNA. This was possible because the entire coding region of secreted MG2 is contained within exon 3 of MUC7 [2]. PCR primers (Table 1) were designed to amplify sequences encoding the N-terminal, TR and C-terminal regions of MG2 (i.e., MUC7). The N-terminal primers amplify a 435 bp fragment coding for residues 1 to 144. The TR primers amplify a 639 bp fragment coding for residues 85 to 354 corresponding to a portion of the N-terminal region, the entire TR domain and the C-terminal region. The C-terminal primers amplify a 219 bp fragment coding for residues 282 to 354 of the C-terminal region.

Genomic DNA was isolated from whole blood from the subject with the split MG2 bands and from two other subjects. These DNAs were amplified using the primers described above and PCR products were electrophoresed on 1% agarose TAE gels. The size of amplified fragments corresponding to the N- and C-terminal regions was identical in all three subjects (Fig. 3, A and C). The fragments corresponding to the TR and flanking regions for subject 1 (with split MG2 bands) and subject 3 were identical with sizes consistent with MUC7 alleles containing six TRs (6/6) (Fig. 3, B (lanes 1 and 3)). Subject 2 was heterozygous (5/6) for the TR genetic polymorphism and fragments of 639 bp and 570 bp were observed (Fig. 3, B (lane 2)).

Discussion

We have examined MG2 from subjects on Western blots probed with anti-MG2 antibodies [31–33] and typically, MG2 appeared as a single polydisperse band of approximately 180 kDa. One exception was a subject where MG2 appeared as a doublet with immunoreactive bands of approximately 180 kDa and 125 kDa. We asked the question whether the 125 kDa band could be derived from the typical 180 kDa band due to the presence of a previously unrecognized mucin degrading enzyme in the oral cavity. To address this question, a custom fitted device was prepared for this subject, SMSL was collected and was

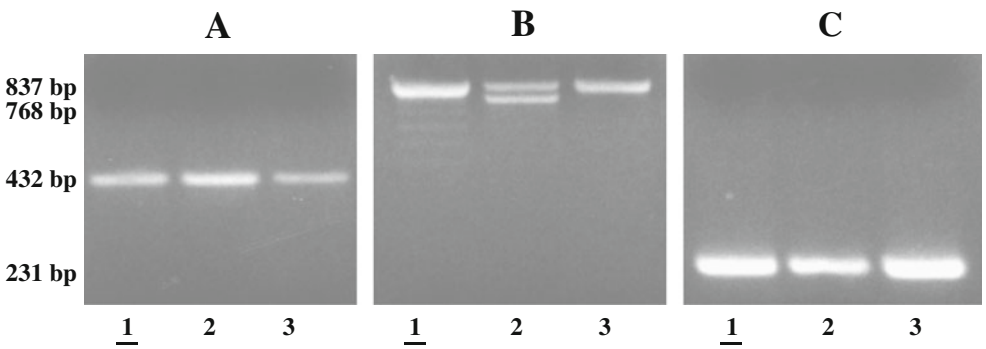

Fig. 3 MUC7 PCR of genomic DNA. Amplified products from the N-terminal (*A*), TR (*B*), and C-terminal (*C*) can be observed. Samples on *lane 1* were collected from the individual that express the atypical MG2. Samples on *lanes 2* and *3* from individuals that express MG2 with the typical appearance on Western blots and PAS-stained gels

analyzed by Western blotting and on PAS-stained gels. The results showed that the split bands were also present in SMSL (Fig. 1) suggesting that these bands are unlikely to be derived from proteolytic degradation of MG2 in WS.

Two isoforms of MG2 named MG2a and MG2b have been previously described [13]. In principle, one or both isoforms could be related to the unusual split MG2 banding pattern described in the present study. Since MG2a and MG2b differ primarily in their content of sialic acid and fucose, that are terminal sugars, these isoforms display a similar electrophoretic mobility on polyacrylamide gels [13] that does not explain the 55 kDa difference reported in our study, ruling out the relation between MG2a and MG2b and the origin of the split bands.

Since it is known that the MUC7 gene exhibits a genetic polymorphism, we considered the possibility that the split MG2 bands could result from a truncated MUC7 allele or from alleles containing different numbers of TRs. The original MUC7 cDNA clone encoded an open reading frame for MG2 apomucin with six TRs [1]. Later, a study conducted with 14 subjects revealed a genetic polymorphism where MUC7 alleles contained either five or six TRs [34]. In this study, ten of 14 subjects (71.4%) were homozygous for six TRs (6/6) and four subjects (28.5%) were heterozygous (5/6) where one allele contained five TRs and the other contained six TRs. More recently, MUC7 polymorphisms were examined in a cohort of 375 subjects of various ethnic origins [35]. In this study, three subjects (0.8%) were homozygous five of five, 80 subjects (21.3%) were heterozygous 5/6, and 291 subjects (77.6%) were homozygous 6/6. Interestingly, one subject (0.26%) exhibited a polymorphism (6/8) where one allele contained six and the other allele contained eight TRs.

How would the presence five, six, or eight TRs affect the electrophortetic mobility of MG2? Each 23 residue repeat contributes approximately 2,300 Da (100 Da/amino acid× 23 residues/TR=2,300 Da) to the molecular mass of the apomucin. Therefore, a TR domain containing five repeats contributes 11,500 Da, one containing six TRs contributes 12,800 Da and one containing eight TRs contributes 18,400 Da to the molecular mass. Therefore, the maximum difference in the molecular weight of secreted MG2 (180 kDa) containing five or eight TRs is 6,900 Da. This relatively small value could not explain the 55 kDa difference between the MG2 bands of 125 and 180 kDa in the subject described here.

In contrast, much larger differences would be predicted if the carbohydrate content of TRs is taken into account. Assuming that all nine *O*-glycosylation sites are occupied and that the average molecular weight of a glycan is approximately 1,300 Da [12], then carbohydrate contribution to a TR would be approximately 11,700 (9×1,300) Da. Thus, the polypeptide and glycan contribution of a TR would contribute 14,000 Da (2,300+11,700) to the overall weight of MG2. Could the presence of additional TRs explain the 55 kDa difference between the 125 and 180 kDa MG2 bands described above? In principle, a subject with a MUC7 phenotype of 5/9 could produce MG2 glycoforms with a difference of 56 kDa (i.e., 4 TRs× 14,000 Da=56,000 Da) which is nearly identical to that observed in the subject with split MG2 bands. Consequently, we examined this possibility using a PCR strategy with genomic DNA to determine the MUC7 genotype of the subject in question. It was found that this subject was homozygous for the 6/6 MUC7 polymorphism and this appeared to rule out that the split banding pattern was the result of a genetic polymorphism.

Another explanation for the unusual MG2 banding pattern could be that the polypeptide backbone of the two MG2 glycoforms was identical and that the large and small MG2 species originate due to differences in the extent of glycosylation in the submandibular and sublingual glands. This is attractive because it is known that the contribution of the submandibular gland decreases upon stimulation and that the contribution of the sublingual gland remains constant [36]. In this scenario, the 180 kDa band could be derived primarily from the submandibular gland and the 125 kDa band could be derived primarily from the sublingual gland. This is consistent with the observed secretion pattern where the 180 kDa band was more abundant under resting conditions and the 125 kDa band became more abundant after gustatory stimulation (Fig. 2). Alternatively, the split MG2 bands could arise by expression of different glycoforms in mucous or serous acini. This is consistent with immunogold electron microscopy experiments showing that both mucous and serous acini produce MG2 [9].

In summary, the split MG2 bands observed in this study could be derived from differential expression of MG2 glycoforms in salivary glands, from differential synthesis in serous or mucous acini or from modified glycosylation patterns in these glands and cell types. More studies are required to fully understand the origin of the split MG2 bands in WS and SMSL from this subject.

Acknowledgments This study is supported in part by Fundação de Amparo à Pesquisa do Estado de Minas Gerais grant no. 261-08.

Conflicts of interest The authors declare that they have no conflicts of interest.

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